METHYLENE BLUE + LIGHT-INDUCED DNA & CELL DAMAGE IN CHO CELLS

THE ROLE OF NUCLEOTIDE EXCISION REPAIR GENES IN THE REPAIR OF METHYLENE BLUE PLUS VISIBLE LIGHT-INDUCED DNA AND CELLULAR DAMAGE IN CHINESE HAMSTER OVARY CELLS

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Abstract

Base excision repair (BER) is a DNA repair mechanism that involves the removal of single damaged bases from DNA and their excision as free bases. Another DNA repair mechanism known as nucleotide excision repair (NER) involves the removal of bulky lesions from DNA. Previous published reports have suggested a role for certain NER proteins in BER. Methylene blue (MB) is a type II photosensitizer, which upon excitation by visible light (VL) produces singlet oxygen that reacts with DNA to form 8-hydroxyguanine (8-oxoG) lesions. The BER mechanism has been shown to preferentially remove 8-oxoG lesions from DNA. In the current work, the role of NER proteins in the BER of [MB+VL]-induced DNA damage was examined using a reporter gene assay. AdMCMV*lacZ* and AdHCMV*lacZ* are non-replicating adenoviruses that express the bacterial β-galactosidase (β-gal) reporter gene under the control of the murine or human cytomegalovirus immediate early promoter, respectively. Host cell reactivation (HCR) of β-gal activity for a [MB+VL]-treated AdMCMV*lacZ* was examined in two repair-proficient normal Chinese hamster ovary (CHO) cell lines, NER-deficient mutants from rodent complementation groups (RCGs) 1 through 6, and the BER-deficient cell line EM9. Results show a decreased HCR capacity of [MB+VL]-induced DNA damage in the UV61 (RCG-6; CSB) cell line when compared to the repair-proficient normal AA8. This suggests an involvement of CSB in BER of [MB+VL]-induced damage. In contrast, the *XRCC1*-deficient EM9 showed an increased HCR capacity when compared to the parental AA8. This suggests a beneficial role for an *XRCC1* deficiency or for the

specific gain-of-function gene mutation in the *XRCC1* gene for the repair of [MB+VL]-induced damage. Similarly, the *ERCC1*-deficient UV20 (RCG-1) showed an increased HCR capacity when compared to the parental AA8. In contrast, another *ERCC1*-deficient cell line, 30PV, and the *ERCC1* knock-out cell line, CHO-7-27, showed no significant increase in β-gal activity when compared to the parental CHO-K1. The ability to induce BER of [MB+VL]-induced DNA damage was also examined. HCR of β-gal activity for [MB+VL]-treated AdHCMV*lacZ* or AdMCMV*lacZ* was examined in several CHO cell lines that were either untreated or treated with low levels of UVC or MB plus VL. Pre-treatment of NER-deficient UV61 and repair-proficient AA8 cells with UVC resulted in an enhanced HCR for [MB+VL]-treated AdHCMV*lacZ*, although the results were only significant for UV61. Similarly, an enhanced HCR of [MB+VL]-treated AdMCMV*lacZ* was observed in AA8 cells, suggesting that the repair of the [MB+VL]-treated reporter gene is inducible by UVC. A significant enhancement in HCR of [MB+VL]-treated AdMCMV*lacZ* was not observed in AA8 cells following pre-treatment with MB, VL, or both, indicating that the detection of any induction in the repair of DNA damage from MB plus VL could not be made with the conditions of the HCR assay employed. As investigations into the effects of MB plus VL on whole cells have been limited, clonogenic survival of BER- and NER-deficient CHO cell lines was also observed following treatment with MB alone or MB plus VL. Results showed that the sensitivities of the NER-deficient CHO cell lines from RCGs 2, 4 and 5 and the BER-deficient EM9 cell line to MB alone were within the range obtained for the two repair-proficient CHO normal cell lines, AA8 and K1. In contrast, the UV20 cell line was more

sensitive to MB alone compared to the normal cell lines. Additionally, both UV24 (RCG-3; XPB) and UV61 showed a decreased sensitivity to MB alone when compared to the normal AA8 cell line. The sensitivity of cells to MB plus VL was greater in the UV24, UV135 (RCG-5; XPG) and the *XRCC1*-deficient EM9 cell lines compared to that of the range obtained for the two repair-proficient normal cell lines, AA8 and K1. Taken together with the results from the HCR assays, these results suggest that [MB+VL]-induced DNA damage to cells and its repair do not play a major role in the survival of cells following treatment with MB plus VL. It appears likely that damage to cellular components other than DNA, such as protein, lipid and biological membranes, play a more important role in cell killing by MB plus VL.

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List of Abbreviations

$^1O_2$	singlet oxygen
8-oxoG	8-hydroxyguanine
α-MEM	α-minimum essential media
β-gal	β-galactosidase
Ad	adenovirus
ADP	adenosine diphosphate
AP	apurinic / apyrimidinic
ATP	adenosine triphosphate
BER	base excision repair
CHO	Chinese hamster ovary
CPD	cyclobutane pyrimidine dimer
CPRG	chlorophenol red-β-D-galactopyranoside
CS	Cockayne syndrome
D_{37}	dose that gives a survival fraction of 0.37 (e^{-1})
D_{50}	dose that gives a survival fraction of 0.50
DNA	deoxyribonucleic acid
DSB	double-stranded break
E1	early 1
EDTA	ethylenediamine tetra-acetic acid
EMS	ethyl methanesulfonate
ERCC	excision repair cross-complementing
FBS	fœtal bovine serum
FEN1	flap endonuclease
FPG	formamidopyrimidine-DNA glycosylase
GGR	global genome repair
H_2O_2	hydrogen peroxide
HCG	human complementation group
HCMV	human cytomegalovirus
HCR	host cell reactivation
hMSH	human MutS homologue
hHR23B	human homologue of Rad23 B
HR	homologous recombination
IE	immediate early
k/o	knock-out
lacZ	gene that codes for the enzyme β-galactosidase
MB	methylene blue
MCMV	murine cytomegalovirus
MMR	mismatch repair
MOI	multiplicity of infection
NER	nucleotide excision repair
NF-κB	nuclear factor-κB

NHEJ	non-homologous end-joining
$O_2^{\bullet -}$	superoxide anion
OGG1	8-oxoG DNA glycosylase
$OH^{\bullet}$	hydroxyl radical
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
pfu	plaque-forming unit
pol β	DNA polymerase β
pol η	DNA polymerase η
RCG	rodent complementation group
RNA	ribonucleic acid
RNAPII	RNA polymerase II
ROS	reactive oxygen species
RPA	replication protein A
SE	standard error
SSB	single-stranded break
TCR	transcription-coupled repair
TFIIH	transcription factor-II H
Tg	thymine glycol
TTD	trichothiodystrophy
UV	ultraviolet
UV-DDB	UV-damaged DNA binding protein
VL	visible light
XP	xeroderma pigmentosum
XRCC	X-ray repair cross-complementing

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CHAPTER 1

Introduction

1.0 Genetic Instability and DNA Repair

Genetic stability is required for proper propagation of the species over successive generations. However, errors in the genome often arise as a result of DNA replication errors or from DNA-damaging agents such as ultraviolet (UV) light, reactive oxygen species (ROS), ionizing radiation and various chemicals. Damage to DNA can occur in any of its components, namely the purine and pyrimidine bases, the phosphate groups or the sugar moieties, and can result in such processes as oxidation and methylation as well as DNA strand breakage (as reviewed by Reddy and Vasquez, 2005). Although certain mutations within the germ line are responsible for natural biological evolution, mutations arising in somatic cells are often detrimental to the organism, and can give rise to such diseases as cancer (as reviewed by Friedberg, 2001). In order to contend with the variety of DNA lesions or base mispairings that occur within the genome, cells have devised a number of repair mechanisms. The variety of repair mechanisms have been studied in great detail in both prokaryotic and eukaryotic cells, as well as in humans and model organisms where several repair-deficient syndromes have been identified. Many DNA-damaging agents and experiments have been employed to better understand the manner in which DNA is both damaged and repaired, in the hopes of advancing our collective knowledge of diseases such as cancer and ultimately to developing improved disease treatments.

2.0 DNA Damage

2.1 Oxidative Damage

Damage to DNA as a result of oxidative stress is a significant concern for cellular systems as such damage can lead to mutations or even cell death. Consequently, organisms have devised strategies to cope with both endogenous and exogenous sources of oxidative damage. Oxidative damage includes single- and double-stranded breaks (SSBs and DSBs, respectively), as well as damage to bases and the sugar-phosphate backbones in DNA (as reviewed by Slupphaug *et al.*, 2003). Upon replication, SSBs may become DSBs, which are lethal unless repaired (as reviewed by Pfeiffer *et al.*, 2000), whereas base damage can be mutagenic, cytotoxic or both (as reviewed by Slupphaug *et al.*, 2003). As its name suggests, oxidative damage is formed in the presence of oxygen (as reviewed by Fang *et al.*, 2002). The most important endogenous reactive oxygen species (ROS) are hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), the superoxide anion ($O_2^{\cdot -}$), and singlet oxygen ($^1O_2$) (as reviewed by Foote *et al.*, 1984 & Fang *et al.*, 2002). Cells have devised several methods of counteracting the amount of ROS, including the use of enzymes such as superoxide dismutase and catalase. Superoxide dismutase, as its name suggests, acts on superoxide anions, converting two superoxide radicals and two protons into the less reactive hydrogen peroxide and oxygen (as reviewed by Slupphaug *et al.*, 2003). Hydrogen peroxide is then in turn converted to water and oxygen by catalase (as reviewed by Slupphaug *et al.*, 2003). Additionally, cells use other factors to eliminate ROS, such as antioxidant amino acids, vitamins and

enzyme-bound minerals (as reviewed by Fang *et al.*, 2002). The generation of ROS and their subsequent deactivation are summarised in Table 1.1.

ROS can damage base pairs in DNA. To date, more than twenty types of base damage caused by oxidative stress have been identified, with the most common purine and pyrimidine lesions being 8-hydroxyguanine (8-oxoG) and thymine glycol (Tg), respectively (as reviewed by Slupphaug *et al.*, 2003). Both 8-oxoG and Tg are represented diagrammatically in Figure 1.1. Superoxide anions have been shown to efficiently produce genotoxic effects in the presence of transition metals owing to its rapid decomposition to highly reactive hydroxyl radicals (as reviewed by Epe *et al.*, 1988). Hydroxyl radicals, for instance, can react with guanine to form a C8-OH adduct radical; the subsequent loss of an electron and proton yields 8-oxoG (as reviewed by Slupphaug *et al.*, 2003). Singlet oxygen is also quite reactive, reacting with many organic molecules; however, it is selective and will not react with molecules that are not electron rich (as reviewed by Foote *et al.*, 1984). Singlet oxygen can be produced from lipid peroxidation, superoxide anions, or from exogenous sources such as photosensitization (Epe *et al.*, 1988). Guanine is the only base that significantly reacts with singlet oxygen (as reviewed by Cadet *et al.*, 2003) and almost exclusively results in the formation of 8-oxoG (Devasagayam *et al.*, 1991).

2.2 Ultraviolet Radiation Damage

Sunlight comprises a continuous spectrum of electromagnetic radiation that spans the wavelengths of ultraviolet (UV) light, visible light and infrared (as reviewed by

Matsumura and Ananthaswamy, 2002). UV radiation comprises the wavelengths from 200 to 400 nm and can be further divided into three sections with distinct biological effects: UVA (320 – 400 nm), UVB (280 – 320 nm), and UVC (200 – 280 nm) (as reviewed by Kim *et al.*, 2002). The shorter wavelength UVC is absorbed by the ozone layer, and is therefore not clinically relevant (as reviewed by Wang *et al.*, 1998). However, UVC exposure can arise from artificial sources such as germicidal lamps (as reviewed by Matsumura and Ananthaswamy, 2002). Both UVC and UVB radiation produce primarily bulky lesions, such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts, by direct excitation of the DNA molecule (as reviewed by Kim *et al.*, 2002). In contrast, the long-wavelength UVA indirectly damages cells by creating reactive oxygen species (ROS) and inducing oxidative stress, as well as cell membrane damage and other types of DNA damage such as single-strand breaks (as reviewed by Wang *et al.*, 1998). Base damage arising from UVA radiation can include both purine and pyrimidine damage, such as 8-hydroxyguanine or thymine glycol, respectively (as reviewed by Slupphaug *et al.*, 2003). UVB radiation has also been shown to produce lesions via oxidative stress, although in far lesser quantities than UVA (as reviewed by Wang *et al.*, 1998).

3.0 Repair Pathways for DNA Damage

3.1 Overview

Cellular systems have devised mechanisms to protect themselves from DNA-damaging agents and replication errors to ensure survivability and consistency of their

genome. In humans, there are four major DNA repair pathways, which repair specific types of lesions or breaks within the DNA. These repair mechanisms are nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and recombination repair that consists of homologous recombination (HR) and non-homologous end-joining (NHEJ) (as reviewed by Krokan *et al.*, 2004). NER has been described as the most flexible of these repair mechanisms, as it is able to remove a vast array of structurally unrelated lesions from the DNA (de Boer and Hoeijmakers, 2000). MMR is involved in the recognition and repair of incorrectly incorporated bases in DNA (as reviewed by Friedberg, 2001). MMR proteins have been associated with a range of functions, including regulation of mitotic and meiotic recombination, transcription-coupled repair, and possibly apoptosis (as reviewed by Krokan *et al.*, 2004). Both HR and NHEJ involve the repair of double-strand breaks (DSBs) and interstrand crosslinks in DNA. Although NHEJ is considered more important than HR in mammalian cells due to the high volume of DSBs repaired (as reviewed by Krokan *et al.*, 2004), HR is still an essential mechanism that repairs both endogenous and exogenous DNA damage (as reviewed by Thompson and Schild, 1999). Deficiencies in HR or NHEJ may result in such diseases as ataxia teleangiectasia or early onset breast cancer (as reviewed by Slupphaug *et al.*, 2003). All of these repair mechanisms are highly conserved, having been discovered in prokaryotes as well as both lower and higher eukaryotes. The four major DNA repair pathways are summarised in Figure 1.2.

3.2 Nucleotide Excision Repair

Nucleotide excision repair (NER) involves the removal of damaged bases (in the form of bulky lesions) from the genome and its release as part of an oligonucleotide fragment. The mechanism of NER was first characterised in *Escherichia coli* and served as a model for the discovery of homologous pathways in higher organisms (as reviewed by de Boer and Hoeijmakers, 2000). Although NER is a highly conserved repair mechanism, the proteins involved in prokaryotic systems are quite different from those of eukaryotic systems (as reviewed by Hoeijmakers, 1993). Additionally, mammalian cells tend to have a more complex system than other eukaryotes (as reviewed by de Boer and Hoeijmakers, 2000). In humans, the importance of NER is evident by the existence of the three known autosomal recessive syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). In these syndromes, a defect in one of the NER proteins can have a drastic effect, leading to an increase in sun-sensitivity, neurodevelopmental abnormalities, early death, and in the case of XP patients, an increased risk of skin cancer (as reviewed by Krokan *et al.*, 2004).

In general, NER consists of five steps that are true for all organisms: damage recognition; incision of the damaged strand on either side of the lesion; excision of the oligonucleotide containing the lesion; synthesis of a new, undamaged strand; and ligation of the new strand with the undamaged strand (as reviewed by Hoeijmakers, 1993). The rate at which NER performs these tasks depends both on the type of lesion and the organism itself. For instance, it has been shown that when cells from different species are administered an equivalent dose of ultraviolet radiation, the cells will respond with

different rates and extents of repair (as reviewed by Hanawalt, 2002). Additionally, efficiency of repair by the NER pathway depends upon the degree of structural disruption to the normal DNA helix (as reviewed by Wood, 1996). In eukaryotes, UVC-induced (6-4) photoproducts are removed predominantly from the genome five to ten times more efficiently than the similarly produced yet less distorting cyclobutane pyrimidine dimers (CPDs) (as reviewed by Wood, 1996).

Two distinct subpathways of NER have been identified: transcription-coupled repair (TCR) and global genome repair (GGR). TCR involves the removal of DNA lesions from transcribed strands in active genes, whereas GGR involves the removal of lesions throughout the genome, including those found in non-transcribed strands of active genes (as reviewed by Hanawalt, 2002). A number of genes involved in mammalian NER have been discovered. These include genes from seven complementation groups (XPA→XPG) isolated from human patients suffering from XP, as well as *CSA* and *CSB*, isolated from patients with CS (as reviewed by Friedberg, 2001). An accepted model of the mammalian NER pathway, including the differences between TCR and GGR, has been established (Figure 1.3).

TCR and GGR differ in the way lesions are detected. In GGR, damage is recognised by the XPC protein, which forms a complex with hHR23B that in turn recruits the entire repair protein apparatus to the lesion (as reviewed by Krokan *et al.*, 2004). The involvement of XPC in GGR is evident from experiments that showed its absence still allowed for successful TCR of pyrimidine dimers, yet unsuccessful removal of those lesions in untranscribed sequences (as reviewed by Vermeulen *et al.*, 1997).

Additionally, the XPE protein may play a role in damage recognition of CPDs (as reviewed by de Boer and Hoeijmakers, 2000). The XPE protein is a heterodimer consisting of two UV-damaged DNA binding proteins (UV-DDB), DDB1 and DDB2. Its role in NER has not yet been accurately determined, as there is conflicting evidence about the involvement of XPE in any form of repair (as reviewed by Kulaksiz *et al.*, 2005).

In contrast, for TCR, damage recognition begins during transcription when RNA polymerase II (RNAPII) stalls at a bulky lesion in the DNA (as reviewed by Vermeulen *et al.*, 1997). Subsequently, the CSA and CSB proteins are recruited to the site and may allow access to the lesion by dissociating the RNAPII or inducing retraction (as reviewed by van Gool *et al.*, 1997).

Following the recognition step, in both TCR and GGR, the repair process continues in the same manner. XPA and the single-stranded binding complex replication protein A (RPA) are recruited and bind to the DNA (as reviewed by Friedberg, 2001). These proteins may participate in lesion verification as well as the recruitment of a subcomplex known as the TFIIH basal transcription factor (as reviewed by Hanawalt, 2002 & Friedberg, 2001). TFIIH is a multi-protein complex, which includes XPB and XPD. The ATP-dependent XPB and XPD components facilitate the unwinding of the DNA surrounding the lesion in a 3'→5' and 5'→3' direction, respectively (as reviewed by de Boer and Hoeijmakers, 2000). The next step, incision, requires the presence of RPA, and it is believed RPA and XPA may help to stabilize the unwinding DNA (as reviewed by Vermeulen *et al.*, 1997).

The lesion-containing oligonucleotide is subsequently incised from the DNA using the separate structure-specific endonuclease activity of XPF and XPG. The XPG protein makes a 3' incision prior to and independently of the 5' incision catalysed by an XPF-ERCC1 complex (as reviewed by Stary and Sarasin, 2002). Although it is possible for both XPG and the XPF-ERCC1 complex to incise the undamaged strand, in practice this is avoided by means of coordination from RPA and TFIIH (as reviewed by de Boer and Hoeijmakers, 2000). The completion of both incisions releases an oligonucleotide fragment of approximately thirty bases in length (as reviewed by Friedberg, 2001). The resulting gap is filled by DNA polymerase δ or ε, with the assistance of the proliferating cell nuclear antigen (PCNA), RPA and replication factor C (as reviewed by Vermeulen *et al.*, 1997). Finally, the nicks are sealed by DNA ligase I (as reviewed by Krokan *et al.*, 2004).

3.3 Base Excision Repair

Base excision repair (BER), as its name suggests, involves the removal of single damaged bases in DNA and their excision as free bases, leaving apurinic or apyrimidinic (AP) sites in the DNA (as reviewed by Friedberg, 2001). BER is responsible for a majority of endogenous DNA lesions that cause minor helix distortions, as well as the removal of base damage caused by many environmental agents (as reviewed by Slupphaug *et al.*, 2003). Endogenous sources of damage can include oxidation by reactive oxygen species, hydrolytic processes and endogenous alkylating agents, whereas environmental sources of damage can include ionizing radiation and alkylation from

nitosamines (as reviewed by Slupphaug *et al.*, 2003). Unlike NER, inherited deficiencies in BER have not yet been linked to any human genetic disorder, which may be attributed to the functional redundancy between many proteins involved in BER and other repair pathways (as reviewed by Krokan *et al.*, 2004). The mechanism of BER has been studied in some detail. Two distinct pathways for BER have been identified in mammalian cells that involve different subsets of proteins: short- and long-patch BER (Figure 1.4). In short-patch BER, damaged DNA results in a single-nucleotide repair patch, whereas repair events in long-patch BER result in a two to eight-nucleotide-long repair patch.

Both pathways for BER are initiated when DNA glycosylases bind to a target lesion in the DNA and hydrolyse the N-glycosylic bond, which releases the inappropriate base leaving an AP site in the DNA (as reviewed by Dianov *et al.*, 2001). DNA glycosylases are specific for a certain type of lesion or group of related lesions (as reviewed by Wood, 1996), and unlike NER, the lesions recognised by DNA glycosylases in BER do not generally cause major helix distortions (as reviewed by Krokan *et al.*, 2004). To date, at least twelve different DNA glycosylases have been identified in humans (as reviewed by Slupphaug *et al.*, 2003).

Following the excision of the lesion, the AP site is recognised by the AP endonuclease (APE1), which introduces a strand break in the phosphodiester bond immediately 5' to the AP site (as reviewed by Dianov *et al.*, 2001). Short-patch BER then proceeds with DNA polymerase β (pol β), which removes the 5'-sugar-phosphate residue and fills the AP site with a single nucleotide (as reviewed by Slupphaug *et al.*, 2003). Finally, DNA ligase I, or a complex of XRCC1 and DNA ligase IIIα seals the

nick (as reviewed by Boiteux and Le Page, 2001). XRCC1 also interacts with DNA pol β (Kubota *et al.*, 1996) and poly(ADP-ribose) polymerase (PARP) (Masson *et al.*, 1998).

In long-patch repair, after the actions of the DNA glycosylase and APE1, DNA polymerase (δ/ε or β) adds several nucleotides to the 3'-end of the nick (as reviewed by Dianov *et al.*, 2001). Subsequently, a flap endonuclease (FEN1) excises the flap-like structure produced by the polymerase and the DNA is ligated by DNA ligase (as reviewed by Dianov *et al.*, 2001). Long-patch repair also requires proliferating cell nuclear antigen (PCNA), which interacts directly with DNA ligase I, and may utilise replication protein A (RPA) to complete repair (as reviewed by Slupphaug *et al.*, 2003).

3.4 Role of Nucleotide Excision Repair Proteins in Base Excision Repair

To date, approximately 150 human DNA repair genes have been identified (as reviewed by Wood *et al.*, 2005). A number of these genes encode proteins that function in multiple repair pathways. For example, the replication binding protein A (RPA), which is involved in both the incision and resynthesis stages of nucleotide excision repair (NER), as well as the recruitment of the TFIIH basal transcription factor, has also been identified as playing a role in base excision repair (BER) and the repair of double-strand breaks (as reviewed by Krokan *et al.*, 2004). Similarly, the XPG protein, which is involved in the 3' incision of lesion-containing oligonucleotide fragments in NER, has been implicated in BER. It is believed that the XPG protein aids in recruitment of a DNA glycosylase to sites of oxidized pyrimidines, such as thymine glycol and cytosine glycol (as reviewed by Lindahl and Wood, 1999 & Clarkson, 2003). In addition, the NER

proteins hHR23A and hHR23B have been shown to interact with the 3-methyladenine-DNA glycolsylase, which is involved in the removal of a number of DNA lesions by BER, such as N-methylpurines (Miao *et al.*, 2000). An XPC-HR23B complex was also shown to associate with thymine DNA glycosylase in yeast, which is responsible for initiating BER of guanine-thymine mismatches (Shimizu *et al.*, 2003). The CSB protein has also been associated with BER, in the repair of 8-hydroxyguanine (8-oxoG) lesions (as reviewed by Pastoriza Gallego and Sarasin, 2003). Similar to its role in NER, a role in a proposed transcription-coupled repair (TCR) pathway of BER has been proposed for CSB (as reviewed by Boiteux and Le Page, 2001).

4.0 Repair-Deficient Syndromes

4.1 Nucleotide Excision Repair-Deficient Syndromes

Three recessively inherited syndromes associated with a deficiency in nucleotide excision repair (NER) have been identified in humans: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD). Seven complementation groups have been identified within XP, designated XPA to XPG, along with one variant form of the disease, XPV (which is not caused by a deficiency in NER) (as reviewed by Meira *et al.*, 2001). Additionally, two complementation groups have been isolated from patients with CS, designated CSA and CSB, and three have been identified in TTD, named XPB, XPD and TTDA. There are also three groups where patients have a combined XP and CS condition: XPB/CS, XPD/CS and XPG/CS. These different outcomes have been identified in patients despite the same gene being affected,

indicating that different mutations in the *XPB*, *XPD*, and *XPG* genes are associated with specific phenotypes (as reviewed by de Boer and Hoeijmakers, 2000). For example, it has been hypothesised that decreased XPD helicase activity, without major structural changes to the XPD protein, will result in XP, whereas structural alterations in combination with impaired NER activity will result in XPD/CS (Winkler and Hoeijmakers, 1998). Moreover, severe mutations that affect the protein stability of XPD may cause TTD (as reviewed by Winkler and Hoeijmakers, 1998).

4.2 Xeroderma Pigmentosum

Xeroderma pigmentosum (XP) is a rare autosomal recessive disorder characterised by hypersensitivity to sunlight, specifically ultraviolet (UV) light, and an increased risk of skin cancer (as reviewed by Tian *et al.*, 2004). Its name derives from the prominent features of XP patients: parchment skin (xeroderma) and freckles (pigmentosum) (as reviewed by Clarkson, 2003). The average age of onset of such visible symptoms in XP patients is two years (as reviewed by Matsumura and Ananthaswamy, 2002). XP arises from a deficiency in the nucleotide excision repair (NER) pathway, and involves one of seven genes (*XPA* to *XPG*). XP is the only NER-deficient syndrome that leads to an increased risk of skin cancer. Specifically, XP is characterised by a more than 1000-fold increased risk of developing skin cancer, and basal cell carcinomas, squamous cell carcinomas and melanomas develop almost exclusively in areas exposed to sunlight, such as the face, neck and head (as reviewed by Matsumura and Ananthaswamy, 2002). The mean age of onset of the first skin neoplasm

in XP patients is eight years, and many XP patients succumb to neoplasia, reducing their life span by approximately thirty years (as reviewed by Friedberg, 2001). Additionally, XP also leads to a 10- to 20-fold increased risk of developing certain internal cancers before the age of twenty years (as reviewed by de Boer and Hoeijmakers, 2000). Although cancer has also been identified in patients with combined XP and Cockayne syndrome (CS), only about a dozen of such individuals have been identified worldwide and definitive conclusions about cancer proneness cannot be made (as reviewed by Friedberg, 2001).

There are varying degrees of severity among XP individuals, which correspond to the severity of the NER deficiency. Severe cases of XP develop progressive neurologic abnormalities where neuronal death occurs due to a lack of repair of endogenous lesions in nerve cell DNA (as reviewed by de Boer and Hoeijmakers, 2000). In total, approximately 18% of all XP patients display such abnormalities (as reviewed by de Boer and Hoeijmakers, 2000). In North America and Europe, the most common form of XP is that of the XPC genetic complementation group (as reviewed by Meira *et al.*, 2001). Due to XPC's involvement in the recognition step of global genome repair (GGR), XPC individuals are proficient in the repair of DNA damage in actively transcribed genes, but deficient in the repair of the rest of the genome. XPC individuals therefore only have a partial NER deficiency, much like XPE patients, and therefore are less likely to be afflicted neurologically (as reviewed by Friedberg, 2001). However, many XPA, XPB, XPD and XPG individuals exhibit a severe NER defect, as those proteins are highly

involved in both transcription-coupled repair (TCR) and GGR (as reviewed by de Boer and Hoeijmakers, 2000).

In humans, viable mutations in the TFIIH complex have only been found in the *XPB* and *XPD* genes (as reviewed by Winkler and Hoeijmakers, 1998). Only five XPB patients have been identified, bearing three mutant alleles, whereas more than twenty mutant alleles have been identified in XPD patients worldwide (as reviewed by Winkler and Hoeijmakers, 1998). Indeed, the type of mutation an individual contains can correspond to the severity of their syndrome. For instance, patients with a missense mutation in the *XPG* gene will likely only develop XP, while patients with a large truncation in the XPG protein will often develop features of both XP and CS (as reviewed by Tian *et al.*, 2004). This can be attributed to XPG's involvement in other biological roles such as stimulating base excision repair or aiding in transcription: large deletions of the XPG protein will affect multiple pathways, whereas a missense mutation may only affect NER (as reviewed by Tian *et al.*, 2004).

XP can also result from a mutation in the gene encoding DNA polymerase η (pol η), and is known as xeroderma pigmentosum variant (XPV) (as reviewed by Masutani *et al.*, 1999). Although not a deficiency in NER, pol η catalyzes the incorporation of nucleotides opposite thymine dimers, and shows that XP can result both from an NER deficiency and a deficiency in error-free synthesis across thymine dimer lesions (as reviewed by Meira *et al.*, 2001).

4.3 Cockayne Syndrome

Cockayne syndrome (CS) is characterised by increased photosensitivity, but does not result in the pigmentation or predisposition to skin cancer associated with xeroderma pigmentosum (XP) (as reviewed by Friedberg, 2001). Its name derives from the London physician that first characterised the symptoms in 1936, Edward A. Cockayne (as reviewed by Clarkson, 2003). A deficiency in the transcription-coupled repair (TCR) pathway of nucleotide excision repair (NER) is responsible for the development of CS, namely a defect in either the *CSA* or *CSB* gene. In addition to an increased photosensitivity, a vast array of traits have been attributed to CS including severe postnatal growth retardation, skeletal abnormalities (such as bird-like face, dental caries and curvature of the spinal cord), microcephaly, thin hairs, cataracts, sensorineural hearing loss, delayed psychomotor development, and neurologic dysfunction (as reviewed by de Boer and Hoeijmakers, 2000 & Özdirim *et al.*, 1996). The average age of death for patients with CS is 12.5 years, generally resulting from such ailments as pneumonia and respiratory infections, due to the poor health of the individual (as reviewed by de Boer and Hoeijmakers, 2000).

Many attempts have been made to explain the devastating effects observed in CS patients, as such individuals are only lacking in the TCR pathway of NER, while a majority of such symptoms are not observed in patients that are completely lacking the NER pathway, as is the case with XPA patients. The deleterious effects of a mutant *CSA* or *CSB* gene suggest that a transcription deficiency is partly responsible (as reviewed by de Boer and Hoeijmakers, 2000). Support for this theory originates from the implication

that CSB is involved in transcription based on its association with RNA polymerase II (as reviewed by van Gool *et al.*, 1997).

4.4 Trichothiodystrophy

A third nucleotide excision repair (NER)-deficient syndrome is known as trichothiodystrophy (TTD). Like its counterparts, TTD is associated with an increased sensitivity to sunlight in most, but not all, patients (as reviewed by de Boer and Hoeijmakers, 2000). However, TTD individuals do not develop the severe skin problems attributed to xeroderma pigmentosum (XP), nor do they exhibit the disfiguring traits of Cockayne syndrome (CS) (as reviewed by Friedberg, 2001). The primary phenotype of TTD patients is brittle hair and nails, which arise due to a deficiency of sulphur-rich proteins found in those tissues (as reviewed by Friedberg, 2001). Additionally, TTD can also be characterised by scaling of the skin (known as ichthyosis), as well as mental and physical retardation (as reviewed by de Boer and Hoeijmakers, 2000).

The genes involved in TTD are *XPB*, *XPD* and *TTDA*. Both XPB and XPD have previously been shown to be involved in NER as components of the TFIIH complex. Likewise, it is currently believed that TTDA is also a subunit of the TFIIH complex. This theory is supported by experiments that show the repair mechanism in TTDA-deficient cells can be rescued by the introduction of purified TFIIH complex (as reviewed by de Boer and Hoeijmakers, 2000).

5.0 Chinese Hamster Ovary Cell Lines

5.1 Derivation

While xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) patients are deficient in DNA repair, the rarity of the disorders cause difficulties with respect to scientific research. Consequently, model organisms (particularly rodents) have been identified that exhibit some of the properties of the human DNA repair pathways. Chinese hamster ovary (CHO) cell lines that are sensitive to mutagens such as ultraviolet (UV) light, ionizing radiation, and various chemicals have been isolated.

The largest collection of CHO cell lines has been derived from the AA8 strain (as reviewed by Friedberg *et al.*, 1995). UV-sensitive mutant cell lines were isolated using various screening methods, including both semi-automated and non-automated techniques. Thompson *et al.* developed a method whereby cells were plated in Petri dishes and exposed to UV light or ethyl methanesulfonate (EMS) to develop mutagenized cells (Thompson *et al.*, 1980). Subsequently, mutagenized cells were plated in Petri dishes and allowed to form small colonies of approximately 500 cells (Thompson *et al.*, 1980). Colonies were then treated with a low dose of UV light and observed for cell killing to isolate cells that are hypersensitive to UV exposure (Thompson *et al.*, 1980). Busch *et al.* also carried out such a screening method using a semi-automatic process involving comparisons between post-treatment photographs. In their method, cells were mutagenized using EMS, plated and allowed to form colonies, and finally exposed to UV

light (Busch *et al.*, 1980). Photographs of the treated colonies were taken at two time intervals after UV exposure. The colonies that exhibited little or no growth between the two photographs were isolated as potential UV-hypersensitive mutants (Busch *et al.*, 1980). These methods are able to isolate UV-sensitive mutants at an approximate rate of 1 in every 15 000 colonies (Thompson *et al.*, 1980; Busch *et al.*, 1980). In addition to UV-sensitive mutants, several cells were isolated that are sensitive to chemicals like EMS or the chemotherapy drug, mitomycin C (Thompson *et al.*, 1980). Since the development of these procedures, additional CHO mutants have been isolated and characterised.

After successfully isolating hypersensitive mutants, it was important to investigate the cause of the phenotype and determine whether the sensitivity was due to a deficiency in DNA repair. It was shown that sensitivity to a challenge dose of UV in one mutant line could be compensated for by fusing the mutant with another mutant line, thereby increasing the survivability after UV exposure by 25 to 50 times (Thompson *et al.*, 1981). Following repeated fusion analysis, the mutant lines could be placed into several complementation groups. After the isolation of a number of mutant lines, including some derived from mouse cells, the number of rodent complementation groups (RCGs) continued to increase (Thompson *et al.*, 1988). To date, eleven RCGs have been identified, named RCG-1 to RCG-11 (as reviewed by Friedberg *et al.*, 1995).

Following the assignment of RCGs, it became possible to transfect the mutant CHO lines with genomic human DNA, thereby correcting the phenotype of UV-sensitivity in the rodent cells (as reviewed by Friedberg *et al.*, 1995). Such a complementation led to the discovery of specific human genes that efficiently corrected

the CHO mutants and corresponded to the various RCGs. The first two human genes that were found to correct the UV-sensitivity in RCG-1 and RCG-2 were termed *ERCC1* and *ERCC2*, respectively, where *ERCC* represents excision repair cross-complementing (Thompson *et al.*, 1988). Similarly, human genes that were later shown to correct RCG-3 to RCG-6 were named *ERCC3* to *ERCC6* (as reviewed by Friedberg *et al.*, 1995). Following evidence that an *ERCC* gene corresponded to a gene deficiency in XP, CS or TTD, the gene was renamed to *XP-*, *CS-*, or *TTD-*, respectively (as reviewed by Lehmann *et al.*, 1994). Although the *ERCC1* gene has not been conclusively linked to any of the nucleotide excision repair (NER) disorders, *ERCC2* to *ERCC6* have been correspondingly renamed to *XPD*, *XPB*, *XPF*, *XPG* and *CSB* (as reviewed by Friedberg *et al.*, 1995). The RCGs and their corresponding human genes are summarised in Table 1.2.

Another AA8-derived CHO mutant, EM9, was isolated based on its hypersensitivity to EMS and has been complimented by the X-ray repair cross-complementing (XRCC) gene, *XRCC1*, which is involved in base excision repair and not NER (Thompson, 1991).

Other CHO cell lines have also been used to isolate UV-sensitive mutants. For example, the parental CHO-K1 was used to generate mutants in a similar manner as AA8, using EMS and selecting UV-hypersensitive cells (as reviewed by Rolig *et al.*, 1997). CHO-30PV is an example of an *ERCC1* mutant (RCG-1) isolated from the parental K1 (Stefanini *et al.*, 1989). A positive-negative selection method has also been employed with CHO-K1 to generate an *ERCC1* knock-out cell line, CHO-7-27 (Rolig *et al.*, 1997).

5.2 Properties of Cell Lines

The Chinese hamster ovary (CHO) cell lines derived from the wild-type AA8 and isolated by Thompson *et al.*, have been shown to have a similar degree of sensitivity to ultraviolet (UV) light (Thompson *et al.*, 1980). Specifically, both UV5 (RCG-2; XPD) and UV20 (RCG-1; *ERCC1*) were seven times more UV-sensitive than the normal, AA8 (Thompson *et al.*, 1980). Both UV5 and UV20 had a D_{37} value (dose required to reduce survival to 37%) of 1 J/m^2 of UV, whereas AA8 had a D_{37} value of 7 J/m^2 (Thompson *et al.*, 1980). However, there is variability amongst the other established cell lines with respect to their UV-sensitivity. UV24 (RCG- 3; XPB) is only five times as sensitive to UV as the parental, AA8 (Busch *et al.*, 1980) and UV61 (RCG-6; CSB) has been shown to be less sensitive than the other mutants (Thompson *et al.*, 1988). CHO-K1-derived mutants can also be important in experimentation, as they are less sensitive to UV light as their AA8-derived counterparts (Stefanini *et al.*, 1989) and could therefore be used for comparative purposes. For example, CHO-30PV (RCG-1; *ERCC1*) is only twice as sensitive to UV exposure as its parental strain (Stefanini *et al.*, 1989). The *ERCC1* knock-out, CHO-7-27, was also shown to be highly sensitive to UV exposure, yet not to the same degree as UV20 (Rolig *et al.*, 1997). Additionally, these cell lines have been shown to be abnormal in their response to other challenges, such as the cytotoxic protein pirerisin-1 (Shiotani *et al.*, 2005), mitomycin C (Thompson *et al.*, 1980), or X-rays (Busch *et al.*, 1980).

The variability in UV-sensitivity amongst the mutant cell lines can be attributed in part to the role their affected genes play in DNA repair. For example, UV61, which

efficiently removes pyrimidine-(6-4)-pyrimidone UV photoproducts but not cyclobutane dimers, is deficient in transcription-coupled repair (TCR) of nucleotide excision repair (NER), but remains proficient in global genome repair (GGR) (Thompson *et al.*, 1988). This is due to the involvement of the hamster homologue of the CSB protein in the early stages of TCR. Similarly, 30PV, 7-27, UV5, UV20, UV24, UV41 (RCG-4; XPF) and UV135 (RCG-5; XPG) are deficient in both the TCR and GGR pathways of NER. The repair properties of each of these cell lines are summarised in Table 1.3.

The *XRCC1* gene, which compliments the deficiency in EM9 (namely an increased sensitivity to agents that induce DNA base damage), has been linked to the short-patch repair pathway of base excision repair, and is therefore less capable of removing certain damaged bases. EM9 has been shown to have a sensitivity to ethyl methanesulfonate that is 10-fold that of the parental AA8 (Thompson *et al.*, 1982).

5.3 Chinese Hamster Ovary Cell Lines as Model Organisms

Certain advantages and disadvantages present themselves when using Chinese hamster ovary (CHO) cell lines as a model for DNA repair in mammalian cells. Although much can be learned from patients with nucleotide excision repair (NER)-deficient syndromes such as xeroderma pigmentosum (XP) and Cockayne syndrome, the rarity of such disorders limits the number of mutations that can be obtained. For example, as previously mentioned, only three mutant alleles have been identified in the *XPB* gene from patients with a NER-deficient syndrome (as reviewed by Winkler and Hoeijmakers, 1998). Additionally, the cell lines obtained from human patients may not

exhibit all the possible types or degrees of deficiency in DNA repair. For instance, the NER gene *ERCC1* has not been shown to be defective in any XP patients, and defective copies in rodents produce runted or inviable animals, yet cultures can be obtained for analysis (as reviewed by Friedberg, 2001). Conversely, CHO cell lines deficient in *XPA* or *XPC* have not yet been identified (as reviewed by Thompson, 1998) despite the fact the most common mutations in XP patients involve *XPC* (as reviewed by Meira *et al.*, 2001). It is possible that these genes lie in regions of the hamster chromosome that are resistant to deletion (Thompson, 1998), as *XPC* mutants have been isolated in mice (Cheo *et al.*, 1997). Therefore, using CHO cells presents an advantage over using only human cell lines as different mutations can be obtained; yet there are also certain limitations with respect to mutated genes, as is the case with the lack of a suitable *XPC* mutant.

CHO cells are also practical in that they can be easily manipulated in a quantitative manner and have a short doubling time of approximately 13 to 16 hours (Thompson *et al.*, 1981). CHO cells are also mammalian, and therefore represent the human repair pathways well, with nearly identical repair mechanisms.

6.0 Methylene Blue

6.1 Interaction with DNA

Methylene blue (MB) is a thiazine dye also known by its formal chemical name, 3,7-bis(dimethylamino)phenothiazine-5-ium chloride (as reviewed by Floyd *et al.*, 2004), which is derived from its structure (Figure 1.5). MB is a type II photosensitizer that produces singlet oxygen ($^1O_2$) when excited by visible light (VL) in the presence of

oxygen (Slameňová *et al.*, 2002), and has been shown to produce 8-hydroxyguanine (8-oxoG) lesions in DNA (Floyd *et al.*, 1989) with minimal strand breaks (Epe *et al.*, 1993). MB has been used for a variety of applications since its isolation as a new chemical in 1876, including its use as an antiseptic, disinfectant, antidote for nitrate poisoning, and recently as a treatment to inactivate viruses in the blood (as reviewed by Floyd *et al.*, 2004).

The specific types of damage that MB introduces in DNA, as well as the mechanism involved in producing that damage, has been studied at length. Epe *et al.* showed that MB, upon excitation by VL, produced predominantly lesions that are sensitive to the repair endonuclease formamidopyrimidine-DNA glycosylase (FPG protein) (Epe *et al.*, 1993). The FPG protein has been demonstrated to recognise 8-oxoG lesions, as well as ring-opened purines and apurinic or apyrimidinic (AP) sites (Epe *et al.*, 1993). Epe *et al.* also showed that few strand breaks or other DNA modifications such as 5,6-dihydropyrimidine derivatives or AP sites are generated (a second repair endonuclease, endonuclease III, was employed to rule out AP site formation) (Epe *et al.*, 1993). Floyd *et al.* also showed that guanine residues became hydroxylated at the C8 position in the presence of MB (Floyd *et al.*, 1989), and it has been accepted that MB causes a large number of 8-oxoG lesions. The amount of 8-oxoG formed in DNA is dependent on the length of VL exposure (Floyd *et al.*, 1989) and as much as 4% of the total guanines can be converted to 8-oxoG (Floyd *et al.*, 2004). In DNA, singlet oxygen appears to only affect guanine residues with any significance (Devasagayam *et al.*, 1991).

As previously stated, MB is a type II photosensitizer, meaning the excited sensitizer interacts with DNA indirectly via singlet oxygen, as opposed to a type I photosensitizer whereby excited photosenisizer molecules react directly with the DNA (as reviewed by Slameňová *et al.*, 2002). MB produces singlet oxygen in a process requiring both VL and aeration. Ground state MB absorbs light and becomes excited to a singlet state (^{1}MB) intermediate, which then has an equal probability of reverting back to a ground state or becoming an excited triplet state (^{3}MB) molecule (Floyd *et al.*, 1989). Excited triplet state MB will decay slowly to a ground state, or react with DNA in either a type I or type II reaction (as reviewed by Floyd *et al.*, 2004). In a type I reaction, ^{3}MB reacts with a substrate or solvent to produce an electron transfer, whereas in the type II reaction, ^{3}MB can produce either singlet oxygen or superoxide, although the most rapid reaction produces singlet oxygen (as reviewed by Floyd *et al.*, 2004). The entire process is permitted because oxygen naturally exists as a triplet state molecule; and also, the lifetime of an unprotonated ^{3}MB is 90 μs, which is relatively quite long (as reviewed by Tuite and Kelly, 1993). The reactions are summarised in Figure 1.6.

Evidence for the involvement of singlet oxygen is supported by the increase in the lifetime of ^{3}MB in an anoxic environment, consistent with the notion of energy transfer to oxygen forming singlet oxygen (as reviewed by Floyd *et al.*, 2004). Additionally, when D_2O was substituted for H_2O in the buffer, increased damage formation was observed in DNA following excitation of MB with VL (Epe *et al.*, 1993). In D_2O, the lifetime of singlet oxygen is approximately 10-fold longer than it is in H_2O, and therefore increased damage is expected if singlet oxygen is involved (Epe *et al.*, 1993). Singlet oxygen has

also been shown to produce 8-oxoG lesions independently of MB (Devasagayam *et al.*, 1991).

6.2 Repair of 8-Hydroxyguanine Lesions

The removal of 8-hydroxyguanine (also known as 7,8-dihydro-8-oxoguanine or 8-oxoG) lesions in DNA is important, as they can mispair with adenine, giving rise to G:C to T:A transversions (Epe *et al.*, 1993). Although 8-oxoG lesions can also pair with cytosine, it has been shown in both bacterial and mammalian cells to almost exclusively bind to adenine, and is therefore considered highly mutagenic, as mutations may activate oncogenes or inactivate tumour suppressor genes (as reviewed by Boiteux and Radicella, 1999). Human nucleotide excision repair (NER) proteins *in vitro* have been reported to recognise and remove 8-oxoG lesions as part of an oligonucleotide fragment (as reviewed by Dianov *et al.*, 2001). Additionally, in *Saccharomyces cerevisiae*, the mismatch repair (MMR) genes *MSH2* and *MSH6* have been shown to be involved in the removal of misincorporated adenines opposite 8-oxoG lesions (Ni *et al.*, 1999). Similarly, the human MutS homologues, hMSH2/hMSH6, have also been shown to bind to 8-oxoG lesions that have mispaired with adenine (as reviewed by Bai *et al.*, 2005). However, the repair of 8-oxoG lesions is almost exclusively conducted via the base excision repair (BER) pathway, and the short-patch repair mechanism has been shown to preferentially repair such lesions (as reviewed by Dianov *et al.*, 2001 & Boiteux and Le Page, 2001). The removal of 8-oxoG lesions from DNA can also be performed by the long-patch repair mechanism of BER, although with a much lower efficiency than short-patch repair

(Fortini *et al.*, 1999). Additionally, it has been shown that the repair of 8-oxoG in rodent cells is coupled to transcription, suggesting a transcription-coupled repair (TCR) pathway for BER (as reviewed by Boiteux and Le Page, 2001).

A number of genes have been associated with the prevention of mutagenesis from incorporated 8-oxoG in the DNA. As is the case with both short-patch and long-patch BER, the mechanism begins when a glycosylase specific to the lesion is bound to the DNA. In *Escherichia coli*, the *fpg* and *mut*Y genes code for two glycosylases: the FPG protein (or MutM) and the MutY protein, respectively. It has been shown that the FPG protein excises 8-oxoG from DNA, whereas the MutY protein is able to excise the adenine that has improperly paired with 8-oxoG (as reviewed by Boiteux and Radicella, 1999). Additionally, a third protein, MutT, degrades 8-oxo-dGTP before it can be incorporated into DNA (as reviewed by Arai *et al.*, 1997). However, in eukaryotic cells, the genes involved vary somewhat. There has been no *mut*Y homologue found in *S. cerevisiae*, yet a homologue has been discovered in other yeasts and in humans (as reviewed by Boiteux and Radicella, 1999). The human MutY homologue (hMYH) has also been shown to remove adenines that have been misincorporated into DNA opposite 8-oxoG lesions and have been shown to interact with the MMR protein hMSH6 (Bai *et al.*, 2005). Clearly the mechanism for preventing G:C to T:A transversions following 8-oxoG lesions is highly conserved and includes a number of different repair pathways and proteins. However, the glycosylase that has specifically been identified with removing 8-oxoG lesions paired with cytosine is the 8-oxoG DNA glycosylase (OGG1).

In *S. cerevisiae*, the *OGG1* gene was found to encode a DNA glycosylase that catalyses the removal of 8-oxoG lesions from DNA (as reviewed by Boiteux and Le Page, 2001). Using the yeast OGG1 sequence as a template, the cDNA sequence for human OGG1 (hOGG1) was discovered (Arai *et al.*, 1997). The *hOGG1* gene encodes two isoforms, α-hOGG1 and β-hOGG1, which are 37 kDa and 44 kDa, respectively (as reviewed by Boiteux and Le Page, 2001). The two isoforms arise as a result of alternative splicing; the *α-hOGG1* transcribed region encodes seven exons, whereas the *β-hOGG1* transcribed region shares the first six exons with the α-isoform, but replaces the final exon with an alternate exon (Arai *et al.*, 1997; Boiteux and Radicella, 1999). The result is that the α-isoform is targeted to the nucleus while the β-isoform is targeted to the mitochondria (Takao *et al.*, 1998). A homologous gene was also discovered in mice (*mOGG1*) that is 84% identical to the human nuclear α-hOGG1, although a gene has not yet been identified in mice to correspond to the β-isoform (as reviewed by Boiteux and Radicella, 1999).

Additionally, a second glycosylase, hOGG2, has been identified in humans and is also capable of removing 8-oxoG lesions from DNA. While the hOGG1 proteins are capable of removing 8-oxoG lesions that are paired with cytosine, it appears that the hOGG2 protein specifically removes 8-oxoG residues that are paired with guanine or adenine (Hazra *et al.*, 1998).

6.3 Effect of Methylene Blue plus Visible Light Treatment on Cells

There are many published reports concerning the effects of exposure to methylene blue (MB) plus visible light (VL) on plasmids (Seah and Burgoyne, 2001), naked DNA (Floyd *et al.*, 1989) or viruses (Floyd *et al.*, 2004). In contrast, reports concerning the effects of MB plus VL to whole cells have been limited. Previous colony-forming assays have shown a toxic effect of MB plus VL on cells at a concentration of 5 μmol/L of MB and exposure to a VL-emitting laser for 30 to 40 seconds (Schmidt *et al.*, 1991). Even in the absence of VL, MB was shown to have a toxic effect on cultured human HeLa tumour cells (Schmidt *et al.*, 1991). In rat epithelial cells, MB was found to localise in the cytoplasm prior to VL exposure by microspectrofluorimetry (as reviewed by Tuite and Kelly, 1993). However, MB was observed in the cell nucleus following exposure to light (as reviewed by Tuite and Kelly, 1993). It was also shown that MB enters mouse and human fibroblasts upon irradiation with light, suggesting that MB is initially exogenous, but is able to enter cells during irradiation as a result of photodynamic membrane damage (as reviewed by Tuite and Kelly, 1993). Indeed, singlet oxygen, which is produced by MB, has been implicated in protein, lipid and biological membrane damage (as reviewed by Tuite and Kelly, 1993).

A statistically significant number of formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites have been shown to be formed in hamster V79 and human colon cancer cells following exposure to MB plus VL (Slameňová *et al.*, 2002), which had previously only been shown to be formed in cell-free systems (Epe *et al.*, 1993). This confirms the ability of MB to form lesions that may include any or all of 8-oxoG, ring-

opened purines, or abasic sites in the DNA of cells. In the current work, the effects of MB plus VL on colony survival were investigated in nucleotide excision repair (NER)- and base excision repair (BER)-deficient Chinese hamster ovary (CHO) cell lines.

7.0 Host Cell Reactivation

7.1 Overview

A consequence of the investigation of constitutive DNA repair is that the cells are often damaged in some manner, making conclusive determinations about the repair mechanisms difficult as the cells themselves may have become activated by the damaging agent (as reviewed by Francis and Rainbow, 1999). To circumvent this dilemma, a technique known as a host cell reactivation (HCR) assay is sometimes employed. The HCR assay employs a shuttle vector carrying a reporter gene that is treated with a DNA-damaging agent to introduce lesions into the reporter gene. The shuttle vector is subsequently introduced into cells of interest to measure the activity of the reporter gene (as reviewed by Ganesan *et al.*, 1999). The resulting relative level of expression of the reporter gene is considered to be reflective of the repair capacity of the cell (as reviewed by Ganesan *et al.*, 1999), as transcription is expected to only occur from a lesion-free gene. HCR assays employ plasmids (Merkle *et al.*, 2004) or viruses (Lee *et al.*, 2004) as shuttle vectors.

7.2 Viruses as Probes for DNA Repair

Adenovirus vectors have been shown to be very efficient at delivering foreign genes into mammalian cells both *in vitro* and *in vivo* (as reviewed by Zacal *et al.*, 2005). Adenoviruses consist of double-stranded DNA packaged in a non-enveloped icosahedral protein capsid (as reviewed by Verma and Weitzman, 2005). In humans, infection with an adenovirus typically results in minimal disease pathology, however some symptoms may include cold-like symptoms, pneumonia and conjunctivitis (as reviewed by Amalfitano, 2004). There are at least fifty different adenovirus serotypes that have been identified, and typical adenovirus vectors are derived from the type 5 serotype (Ad5) (as reviewed by Amalfitano, 2004); however, adenovirus vectors have also been created from other serotypes, including Ad2, Ad4 and Ad7 (as reviewed by Verma and Weitzman, 2005). Non-replicating adenoviruses are typically constructed by inserting foreign DNA into the deleted early 1 (E1) region (Bett *et al.*, 1994). The E1 region codes for proteins that are crucial in viral replication, and adenovirus constructs with a deleted E1 region are therefore replication-defective in most cell lines (as reviewed by Verma and Weitzman, 2005). However, cells that provide the E1 gene products, such as the human 293 cells, will permit viral replication (as reviewed by Bett *et al.*, 1994).

The shuttle vectors employed in the host cell reactivation (HCR) assays of the current work are non-replicating adenovirus constructs expressing the bacterial *lacZ* β-galactosidase (β-gal) reporter gene under the control of the murine or human cytomegalovirus (MCMV or HCMV, respectively) immediate early (IE) promoter (Addison *et al.*, 1997). The *Escherichia coli* reporter gene was inserted into the deleted

E1 region of the Ad5 genome, making the viruses non-replicable (Addison *et al.*, 1997). The two viruses are referred to as AdMCMV*lacZ* or AdHCMV*lacZ*, or alternatively as AdCA35 or AdCA17, respectively. The HCMV IE promoter is capable of inducing expression in a large variety of cell types, including human and murine cells, although levels of expression are much lower in non-human cells (Addison *et al.*, 1997). The MCMV IE promoter has been shown to consistently express the gene of interest as well as, or better than, the HCMV IE promoter in both murine and human cells (Addison *et al.*, 1997). A schematic representation of the AdMCMV*lacZ* adenoviral vector is shown in Figure 1.7.

7.3 Host Cell Reactivation of a Methylene Blue plus Visible Light-Treated Reporter Gene

Methylene blue (MB) plus visible light (VL) is known to produce high levels of 8-hydroxyguanine (8-oxoG) lesions in DNA (Floyd *et al.*, 1989). In the current work, [MB+VL]-treated AdMCMV*lacZ* was infected into Chinese hamster ovary (CHO) cell lines deficient in the base excision repair (BER) or nucleotide excision repair (NER) pathways. Increasing exposure to the DNA-damaging agent is expected to produce an increasing number of lesions in the viral genome. Proper β-galactosidase (β-gal) expression in mammalian cells is expected to only occur when lesions in the reporter gene are absent. Therefore, a comparison of host cell reactivation (HCR) of β-gal activity from a [MB+VL]-treated reporter gene can be made between a repair-proficient normal cell line and a cell line with a known gene deficiency as a measure of DNA repair in the reporter gene. HCR of the reporter gene is thought to be reflective of the cell line's

lesion-specific constitutive repair mechanism (as reviewed by McKay *et al.*, 1999). The HCR assay is represented diagrammatically in Figure 1.8.

The HCR assay can also be used to investigate whether the repair of DNA lesions is inducible by stimulating the cells with a DNA-damaging agent prior to infection (Francis and Rainbow, 1999). If cells are exposed to a small amount of UVC or MB plus VL, repair mechanisms may be activated prior to the introduction of a damaged reporter gene, resulting in a higher β-gal activity.

8.0 Project Introduction

UVA radiation is known to cause 8-hydroxyguanine (8-oxoG) lesions in DNA: the most common form of an oxidized purine (as reviewed by Slupphaug *et al.*, 2003). However, UVA also creates other base modifications such as thymine glycols and uracil derivatives (Wang *et al.*, 1998). Methylene blue (MB), upon excitation with visible light (VL), has been shown to create large amounts of 8-oxoG lesions in DNA almost exclusively when in the presence of oxygen (Epe *et al.*, 1993). Although the formation of 8-oxoG lesions in DNA has been studied in some detail, the repair mechanism of such lesions has not been entirely discerned. The short-patch repair mechanism of base excision repair (BER) has been implicated in the repair of 8-oxoG lesions (as reviewed by Dianov *et al.*, 2001 & Boiteux and Le Page, 2001), and there is evidence for a transcription-coupled pathway for the removal of 8-oxoG (as reviewed by Boiteux and Le Page, 2001 & Pastoriza Gallego and Sarasin, 2003). Nucleotide excision repair (NER) proteins have previously been implicated in some aspects of BER (as reviewed by

Lindahl and Wood, 1999), and it is possible that NER proteins play a role in the BER of 8-oxoG. The primary lesion formed by MB plus VL is 8-oxoG, and MB is therefore a useful tool for investigating the repair of 8-oxoG specifically compared to UVA, which results in a spectrum of lesions including cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts.

In the current work, the role of NER proteins in the BER of oxidative damage caused by MB plus VL has been investigated. Cell survival following treatment with MB plus VL and host cell reactivation (HCR) of a recombinant adenovirus-encoded [MB+VL]-damaged reporter gene was examined in various BER- and NER-deficient Chinese hamster ovary (CHO) cell lines. In addition, the ability to induce BER of [MB+VL]-induced DNA damage by pre-exposing cells to MB plus VL or UVC radiation was investigated in select NER-deficient CHO cell lines using HCR assays.

Table 1.1 — Generation and deactivation of reactive oxygen species (ROS). ROS, such as hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$), and the superoxide anion ($O_2^{\bullet -}$) can arise spontaneously, or from endogenous and exogenous sources within the cell. To cope with ROS, superoxide dismutase converts superoxide anions to hydrogen peroxide and catalase converts hydrogen peroxide to water (H_2O) and oxygen (O_2). (Adapted from Slupphaug *et al.*, *Mutat. Res.* 2003; 531: 231-251.)

Generation of ROS
$H_2O \rightarrow H_2O^+ + e^-$
$H_2O+ + H_2O \rightarrow OH^{\bullet} + H_3O$ (hydroxyl radical)
$OH^{\bullet} + OH^{\bullet} \rightarrow H_2O_2$ (hydrogen peroxide)
$e_{aq} + O_2 \rightarrow O_2^{\bullet -}$ (superoxide anion)
$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$
Protection against ROS
$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$ (superoxide dismutase)
$2H_2O_2 \rightarrow 2H_2O + O_2$ (catalase)

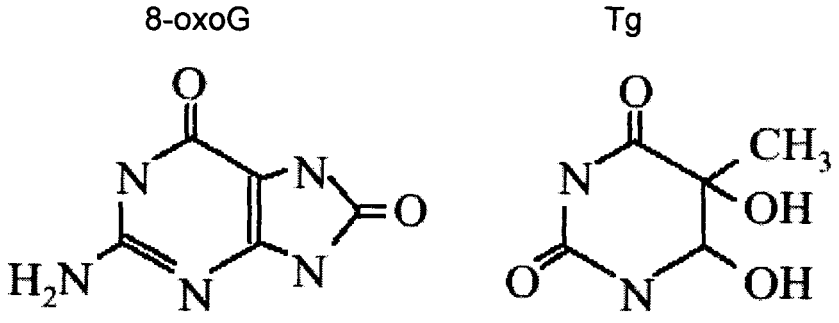

Figure 1.1 — Chemical structures of 8-hydroxyguanine (8-oxoG) and thymine glycol (Tg), the most common purine and pyrimidine base modifications in DNA, respectively. (Adapted from Slupphaug *et al.*, *Mutat. Res.* 2003; 531: 231-251.)

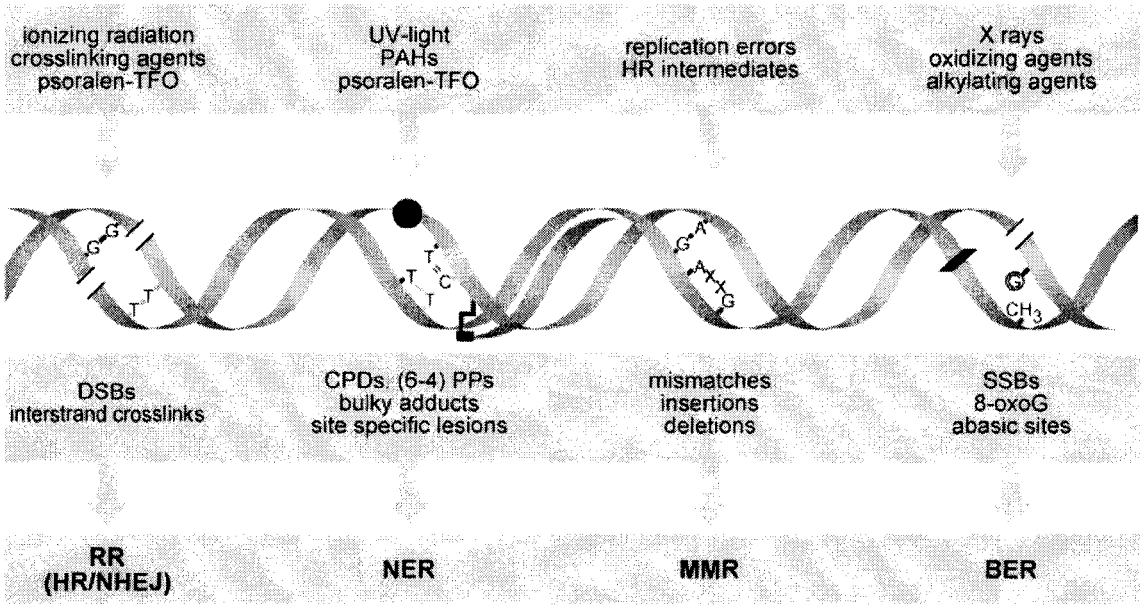

Figure 1.2 — Types of lesions induced by various DNA-damaging agents and their corresponding repair mechanisms. The four repair mechanisms are recombination repair (RR), which consists of homologous recombination (HR) and non-homologous end-joining (NHEJ), nucleotide excision repair (NER), mismatch repair (MMR) and base excision repair (BER). Other abbreviations used include: triplex-forming oligonucleotide (TFO), ultraviolet (UV), polycyclic aromatic hydrocarbons (PAHs), double-strand breaks (DSBs), cyclobutane pyrimidine dimers (CPDs), photoproducts (PPs), single-strand breaks (SSBs) and 8-hydroxyguanine (8-oxoG). (Adapted from Reddy and Vasquez, *Radiat. Res.* 2005; 164: 345-356.)

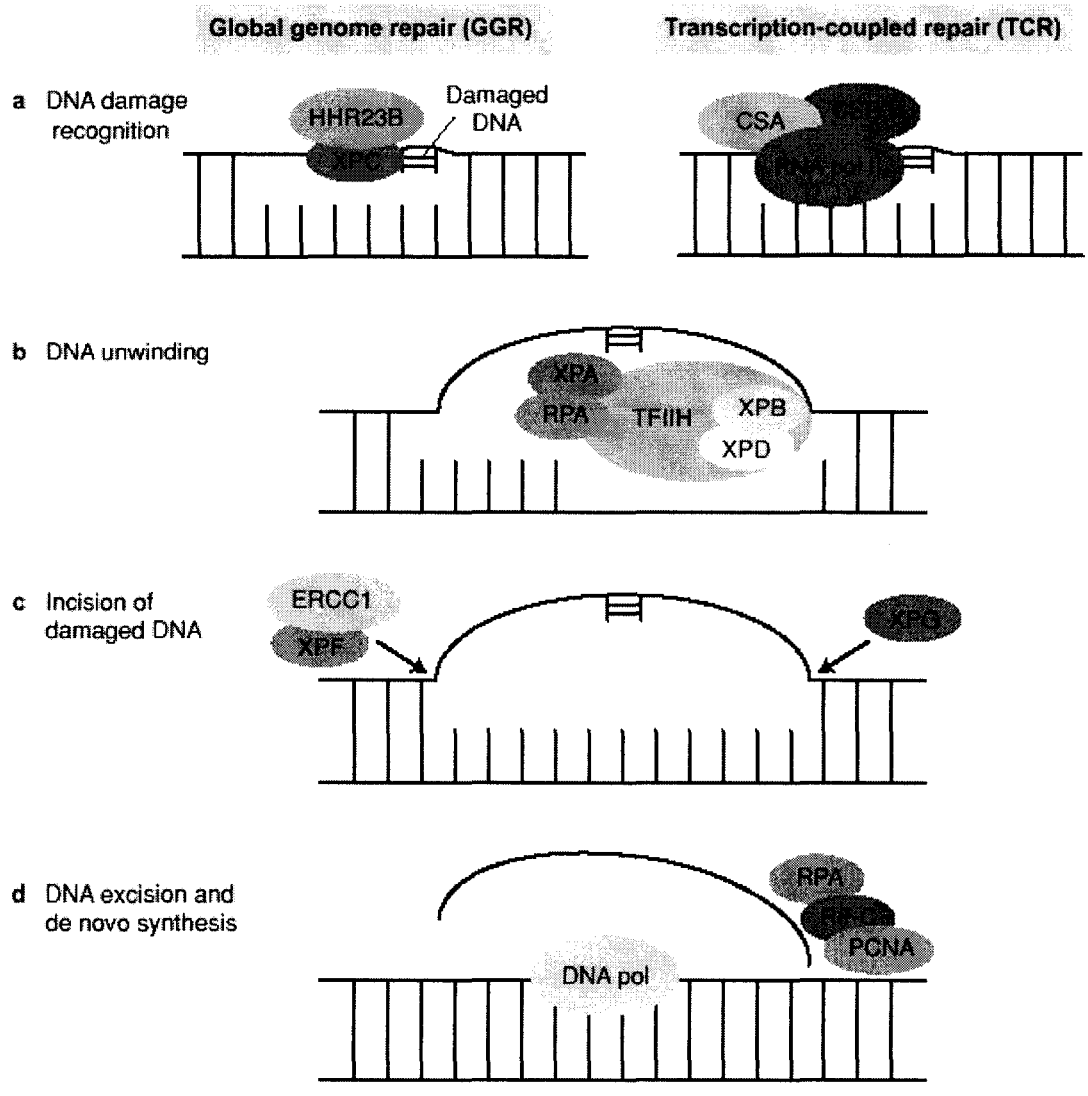

Figure 1.3 — Nucleotide excision repair. Global genome repair (GGR) and transcription-coupled repair (TCR) differ in their DNA damage recognition step. In GGR, an XPC-hHR23B complex or XPE is responsible for recognising damage, whereas in TCR, a stalled RNA polymerase II recognises the lesion and recruits CSA and CSB. Following recognition, XPA and the replication binding protein A (RPA) bind to the DNA and participate in recruitment of the TFIIH basal transcription factor. The XPB and XPD subunits of TFIIH facilitate unwinding of the DNA so that XPG and an XPF-ERCC1 complex can incise the lesion-containing oligonucleotide at the 3' and 5' end, respectively. A DNA polymerase fills in the resulting gap with the aid of RPA, replication factor C (RF-C) and the proliferating cell nuclear antigen (PCNA). The nicks are sealed by DNA ligase I. (Matsumura and Ananthaswamy, *Expert. Rev. Mol. Med.* 2002; 4(26): 1-22.)

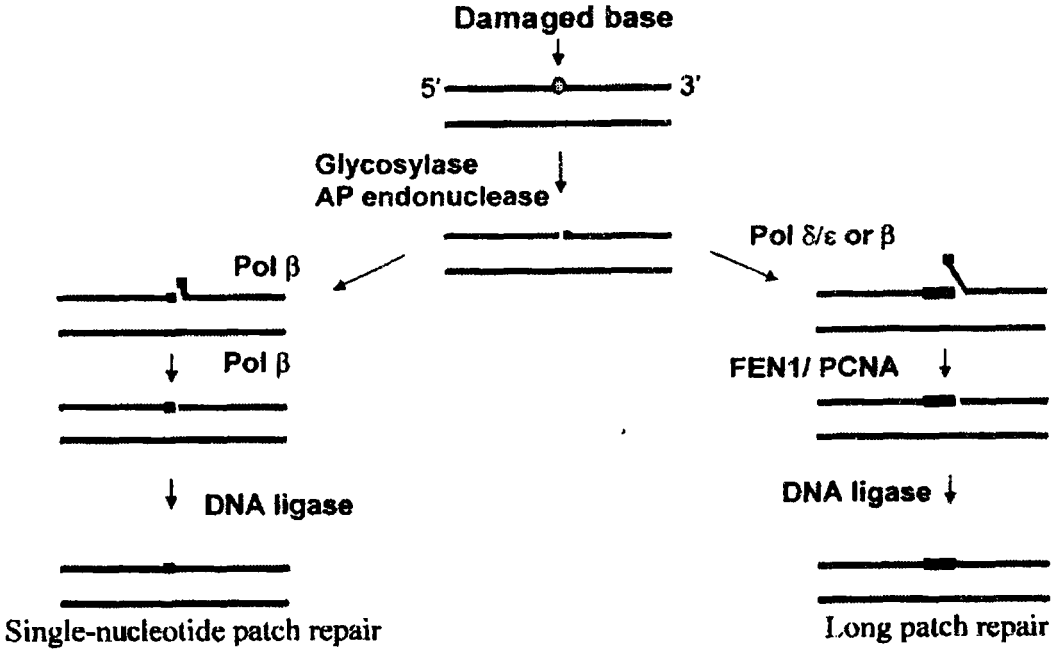

Figure 1.4 — Base excision repair (BER). A glycosylase, specific for the damaged base, recognises and removes the lesion as a free base before an AP endonuclease introduces a strand break immediately 5' to the abasic site. Short-patch BER involves DNA polymerase β (pol β), which fills the abasic site with a single nucleotide. DNA ligase completes the pathway. In long-patch BER, DNA polymerases (pol δ/ε or β) extend the 3' end of the abasic site. A flap endonuclease (FEN1) removes the flap-like region produced by the DNA polymerases and DNA ligase seals the nick. Proliferating cell nuclear antigen (PCNA) is also involved in long-patch BER. (Adapted from Dianov *et al.*, *Prog. Nucleic Acid Res. Mol. Biol.* 2001; 68: 285-297.)

Table 1.2 — Rodent complementation groups (RCG) and their corresponding human complementation groups (HCG) for nucleotide excision repair (NER). Where applicable, representative mutants are listed for RCG-1 to RCG-11 as well as corresponding human genes. In some instances, human genes or complementation groups have been discovered that do not yet have a corresponding RCG, and *visa versa*. This is not a comprehensive list of all NER genes. (Adapted from Friedberg *et al.*, DNA Repair and Mutagenesis. pp. 318-319. ASM Press, Washington, D.C., 1995 & Friedberg, *Nat. Rev. Cancer.* 2001; 1: 22-33.)

RCG	Representative mutant	HCG	Human Gene
1	UV20		*ERCC1*
2	UV5	XPD	*XPD*
3	UV24	XPB	*XPB*
4	UV41	XPF	*XPF*
5	UV135	XPG	*XPG*
6	UV61	CSB	*CSB*
7	VB11 (V79 cells)		
8	US31 (mouse lymphoma)		
9	CHO4PV		
10	CHO7PV		
11	UVS1		
		XPA	*XPA*
		XPC	*XPC*
		XPE	
		XPV	
		CSA	*CSA*
		TTDA	

Table 1.3 — Repair properties of Chinese hamster ovary (CHO) cell lines with respect to nucleotide excision repair (NER). Cell lines are listed with their rodent complementation group (RCG) and their corresponding human counterpart. Additionally, proficiency (+) or deficiency (-) in transcription-coupled repair (TCR) and global genome repair (GGR) of NER is indicated for each cell line.

Cell line	Group	TCR	GGR
AA8	Normal	+	+
UV5	RCG-2 ; XPD	-	-
UV20	RCG-1; *ERCC1*	-	-
UV24	RCG-3; XPB	-	-
UV41	RCG-4; XPF	-	-
UV61	RCG-6; CSB	-	+
UV135	RCG-5; XPG	-	-
K1	Normal	+	+
30PV	RCG-1; *ERCC1*	-	-
7-27	RCG-1; *ERCC1* k/o	-	-

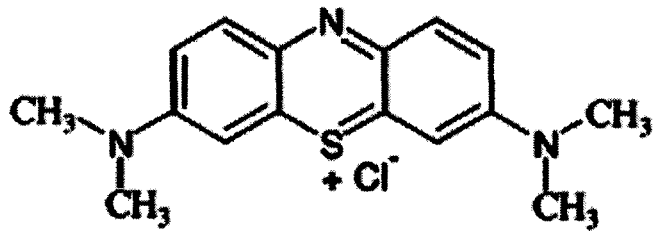

Figure 1.5 — Chemical structure of methylene blue. (Adapted from Floyd *et al.*, *Antiviral Res.* 2004; 61: 141-151.)

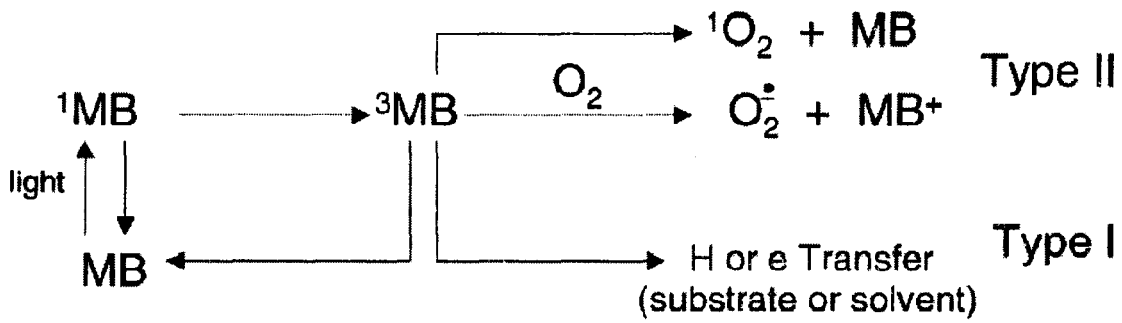

Figure 1.6 — Photochemical reactions of methylene blue (MB). Light excites ground state MB to an excited singlet state (^{1}MB). ^{1}MB has equal probability of returning to ground state MB or becoming an excited triplet state (^{3}MB). ^{3}MB can decay slowly back to a ground state or react via a type I or type II reaction. In the presence of oxygen (O_2), the most rapid reaction produces singlet oxygen ($^1O_2$) and ground state MB. However, superoxide anions ($O_2^{\bullet -}$) can also be formed from ^{3}MB and O_2, although the reaction proceeds at a much slower rate. In a type I reaction, ^{3}MB reacts with a substrate or solvent to mediate hydrogen (H) atom or electron transfer. (Adapted from Floyd *et al.*, *Antiviral Res.* 2004; 61: 141-151.)

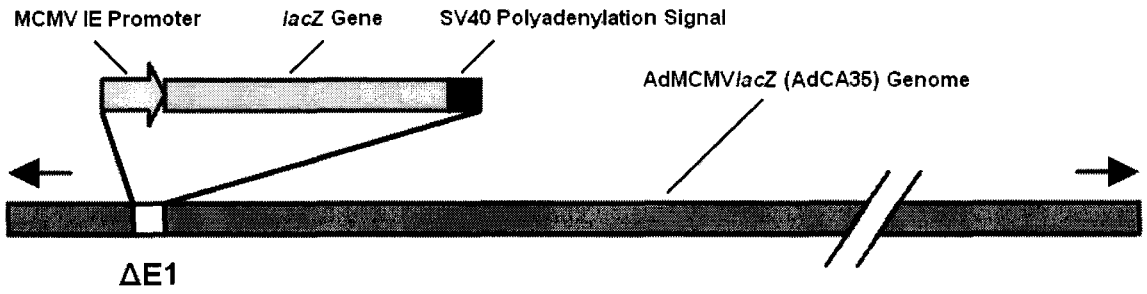

Figure 1.7 — Diagram of the recombinant AdMCMV*lacZ* (AdCA35) virus genome. The AdMCMV*lacZ* vector is a non-replicating virus that expresses β-galactosidase using the *Escherichia coli lacZ* gene under the control of the murine cytomegalovirus (MCMV) immediate early (IE) promoter, inserted into the deleted E1 region, which is responsible for adenoviral replication. Solid arrows represent the inverted terminal repeats of the adenoviral genome. (Adapted from Addison *et al.*, *J. Gen. Virol.* 1997; 78: 1653-1661.)

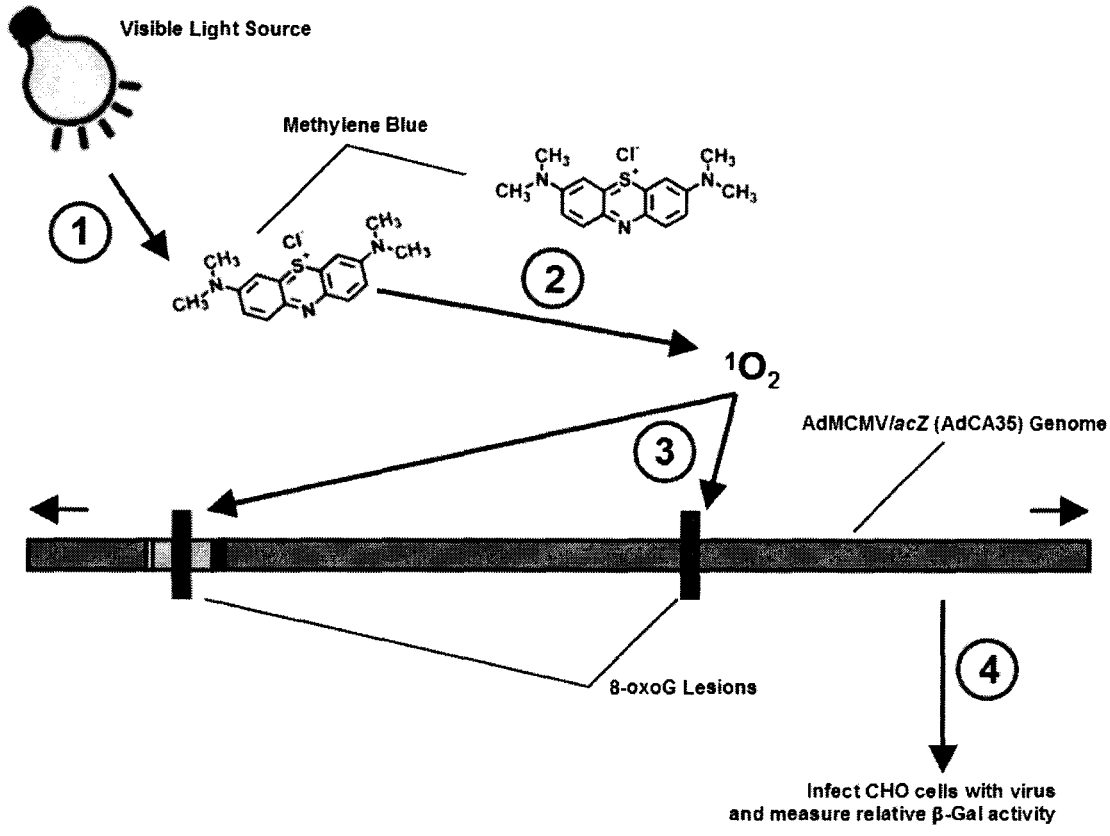

Figure 1.8 — Methylene blue (MB) plus visible light (VL) treatment of the AdMCMV*lacZ* (AdCA35) virus in a host cell reactivation (HCR) assay. **1** — A VL source is used to excite MB, which is present in the medium along with the virus particles. **2** — The excited MB produces singlet oxygen ($^1O_2$). **3** — The $^1O_2$ reacts with the viral DNA to produce 8-hydroxyguanine (8-oxoG) lesions. **4** — The treated virus is infected into Chinese hamster ovary (CHO) cell lines. Proper β-galactosidase (β-gal) expression in mammalian cells is expected to only occur from transcription of a lesion-free *lacZ* gene (found on the AdCA35 recombinant virus), therefore measuring the β-gal activity indicates how much repair has occurred to remove those lesions relative to other cell lines.

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CHAPTER 2

Host cell reactivation of a methylene blue plus visible light-treated reporter gene in base and nucleotide excision repair-deficient Chinese hamster ovary cell lines

1.0 Abstract

Methylene blue (MB), a type II photosensitizer, produces singlet oxygen upon excitation by visible light (VL) that reacts with DNA to produce predominantly 8-hydroxyguanine (8-oxoG) lesions. The base excision repair (BER) pathway, specifically the short-patch repair mechanism of BER, has been shown to repair 8-oxoG lesions in DNA. Previous reports have shown an involvement of some nucleotide excision repair (NER) proteins, including XPG and CSB, in BER. In the present study, a non-replicating recombinant adenovirus expressing the β-galactosidase (β-gal) reporter gene under the control of the murine cytomegalovirus immediate early promoter (AdMCMV*lacZ*) was employed to examine the role of NER proteins in the BER of [MB+VL]-induced DNA damage in select Chinese hamster ovary (CHO) cell lines. Host cell reactivation (HCR) of β-gal activity for [MB+VL]-treated AdMCMV*lacZ* was examined in a number of BER- and NER-deficient CHO cell lines. Results show a decreased HCR capacity of [MB+VL]-induced DNA damage in the *CSB*-deficient UV61 cell line, when compared to the parental AA8. This suggests an involvement of CSB in BER of [MB+VL]-induced damage. In contrast, the *XRCC1*-deficient EM9 showed an increased HCR capacity when compared to the parental AA8. This suggests a beneficial role for an *XRCC1* deficiency or for the specific gain-of-function gene mutation in the *XRCC1* gene for the repair of [MB+VL]-induced damage. Similarly, the *ERCC1*-deficient UV20 showed an increased HCR capacity when compared to the parental AA8. In contrast, another *ERCC1*-deficient cell line, 30PV, and the *ERCC1* knock-out cell line, CHO-7-27, showed no significant increase in β-gal activity when compared to the parental CHO-K1. It is

possible that the mutation in the *ERCC1* gene in UV20 results in a dominant gain-of-function for repair of [MB+VL]-induced DNA damage.

2.0 Introduction

Cellular systems must contend with a wide variety of DNA-damaging agents, and as such have developed a number of repair pathways to ensure the integrity of the genome throughout successive generations. One such pathway is known as nucleotide excision repair (NER) and involves the removal of bulky lesions from DNA and their release as part of an oligonucleotide fragment (as reviewed by Friedberg, 2001). Base damage can originate from numerous sources, including ultraviolet (UV) exposure. For example, UVC causes cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts in DNA (as reviewed by Hanawalt, 2002). Two distinct subpathways of NER have been identified: transcription-coupled repair (TCR) and global genome repair (GGR). TCR involves the removal of DNA lesions from the transcribed strand of transcriptionally active DNA, whereas GGR involves the removal of DNA lesions from the non-transcribed strand and the remaining non-transcribing regions of the genome (as reviewed by Friedberg, 2001). NER is highly conserved across species and has been associated with three autosomal recessive syndromes in humans: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). These syndromes have led to the isolation of a number of NER proteins from different complementation groups including seven XP complementation groups (XPA→XPG) and two CS complementation groups (CSA and CSB).

Another important repair mechanism that has been identified is the base excision repair (BER) pathway. BER involves the removal of single damaged bases in DNA and their excision as free bases, leaving apurinic or apyrimidinic (AP) sites in the DNA (as reviewed by Friedberg, 2001). BER is responsible for DNA lesions that cause minor helix distortions, and the removal of base damage caused by environmental agents (as reviewed by Slupphaug *et al.*, 2003). Damage repaired by BER arises from oxidation by reactive oxygen species, hydrolytic processes, ionizing radiation, alkylation from nitosamines and other sources (as reviewed by Slupphaug *et al.*, 2003). Like NER, two distinct subpathways for BER have also been identified: short- and long-patch repair. Short-patch repair results in a single-nucleotide repair patch, whereas long-patch repair results in a two to eight-nucleotide-long repair patch. Numerous previous studies have suggested a role for some NER proteins in the BER of certain lesions. For example, the XPG protein, involved in the 3' incision of lesion-containing oligonucleotide fragments in NER, has been shown to aid in recruitment of a DNA glycosylase to sites of oxidized pyrimidines in BER (as reviewed by Lindahl and Wood, 1999). Additionally, the CSB protein has been associated with the BER of 8-hydroxyguanine (8-oxoG) lesions (as reviewed by Pastoriza Gallego and Sarasin, 2003), the most common purine lesion.

Methylene blue (MB) is a type II photosensitizer, which, upon excitation with visible light (VL), produces singlet oxygen (as reviewed by Slameňová *et al.*, 2002) that results in 8-oxoG lesions in DNA (Floyd *et al.*, 1989) and minimal strand breaks (Epe *et al.*, 1993). UVA also produces oxidative damage such as 8-oxoG, but additionally creates other base modifications such as thymine glycol (as reviewed by Slupphaug *et al.*,

2003). MB plus VL therefore mimics the effects of UVA exposure, in that in produces 8-oxoG lesions in DNA, however it does not produce other base modifications in large quantities, allowing nearly exclusive research on 8-oxoG repair. Unrepaired 8-oxoG lesions are highly mutagenic, as they can mispair with adenine, resulting in G:C to T:A transversions (Epe *et al.*, 1993). Previous reports have shown that 8-oxoG lesions are almost exclusively repaired by the BER pathway, and that the short-patch repair mechanism is preferentially employed (as reviewed by Dianov *et al.*, 2001).

In the present study, the role of NER genes in the BER of [MB+VL]-induced damage was investigated in BER- or NER-deficient Chinese hamster ovary (CHO) cell lines using host cell reactivation (HCR) assays. The HCR assay employs a non-replicating recombinant adenovirus, AdMCMV*lacZ*, expressing the bacterial *lacZ* β-galactosidase (β-gal) reporter gene under the control of the murine cytomegalovirus (MCMV) immediate early promoter (Addison *et al.*, 1997). Assays involve infecting BER- or NER-deficient CHO cells with [MB+VL]-treated AdMCMV*lacZ* and observing β-gal activity. Increasing exposure to the photosensitizer is expected to produce an increasing number of 8-oxoG lesions in the reporter gene. Proper β-gal expression in mammalian cells is expected to only occur when lesions in the reporter gene are absent or repaired, such that HCR of β-gal activity is thought to be reflective of the cell line's repair capacity for [MB+VL]-induced damage. By examining HCR of the [MB+VL]-treated reporter gene in various mutant BER- and NER-deficient CHO cell lines and parental, wild-type CHO cell lines, the involvement of several NER genes in the repair of [MB+VL]-induced DNA damage was determined.

3.0 Materials & Methods

3.1 Cell Lines

Chinese hamster ovary (CHO) cell lines employed include: AA8 (parental); UV5 (RCG-2; XPD); UV20 (RCG-1; *ERCC1*); UV24 (RCG-3; XPB); UV41 (RCG-4; XPF); UV61 (RCG-6; CSB); UV135 (RCG-5; XPG); and EM9 (provided by Dr. Larry Thompson [Lawrence Livermore National Laboratory, Livermore, CA] with the help of Dr. Gordon Whitmore [Physics Division, Ontario Cancer Institute, Toronto, ON]). The *XRCC1*-deficient EM9, isolated based on its hypersensitivity to ethyl methanesulfonate, has a deficiency in excision repair caused by defective rejoining of anomalous DNA strand breaks (Thompson *et al.*, 1982). Additionally, another *ERCC1*-deficient cell line, 30PV, and an *ERCC1* knock-out cell line, CHO-7-27, were employed and compared to their parental strain, K1 (provided by Dr. Miria Stefanini [Instituo di Genetica Biochimica ed Evoluzionistica del C.N.R., Pavia, Italy]). All cell cultures were grown in α-minimum essential media (α-MEM: HyQ MEM Alpha Modification with L-glutamine, ribonucleosides and deoxyribonucleosides) supplemented with 10% fœtal bovine serum (FBS) and 1% antibiotic-antimycotic (100 μg/mL penicillin G sodium, 100 μg/mL streptomycin sulfate and 250 ng/mL amphotericin B in 0.85% saline) obtained from Gibco-BRL. Cultures were maintained in 37°C humidified air containing 5% CO_2.

3.2 Methylene Blue

Stock methylene blue solution was prepared by dissolving 0.02 g methylene blue trihydrate (C.I. 52015; methylthionine chloride, $C_{16}H_{18}ClN_3S \cdot 3H_2O$, FW 373.9) in 20.0 mL phosphate-buffered saline (PBS: 140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4 [pH 7.4]) that was warmed to 37°C. The resulting mixture was filter-sterilized in the dark through a 0.2 μm filter to create a 1000 μg/mL solution. Stock solution was then aliquoted into volumes of 1 mL or less and stored at -20°C in the dark.

3.3 Virus

The virus employed (provided by Dr. Frank Graham [Department of Biology, McMaster University, Hamilton, ON]) is a non-replicating adenovirus (Ad) that expresses the bacterial *lacZ* β-galactosidase reporter gene under the control of the murine cytomegalovirus (MCMV) immediate early (IE) promoter (Addison *et al.*, 1997). The reporter gene was inserted into the deleted E1 region of the genome, making the virus non-replicable. The virus is referred to as AdMCMV*lacZ*, or simply AdCA35. Stock solutions of virus were stored at -20°C.

3.4 Host Cell Reactivation Assay

Cells were counted on a haemocytometer and seeded for confluence (4×10^4 cells per well) in a 96-well microtitre plate in supplemented α-minimum essential media (α-MEM) and allowed to incubate in humidified air for 18-24 hours at 37°C and 5% CO_2.

A 3.6 mL volume of 40 μg/mL methylene blue (MB) solution was prepared in a small (35 × 10 mm) Petri dish using cold phosphate-buffered saline (PBS) and kept on ice under aluminium foil with minimal ambient lighting. A small, sterile stir-bar was added to the Petri dish to aid in mixing the contents of the solution. An appropriate volume of stock AdMCMV*lacZ* (10^{10} pfu/mL; pfu = plaque-forming units) was added to the Petri dish containing the MB solution to obtain the desired multiplicity of infection (MOI) for the experiment. The solution was kept on ice and under aluminium foil while the contents of the Petri dish were mixed by the stir-bar using a stir plate (VWR Dylastir Stirrer). A 400 μL aliquot of the solution was removed and added to a 2 mL volume of unsupplemented α-MEM and kept in the dark on ice. The solution was then exposed to visible light (VL) using a 1000 W bulb (General Electric, GE R1000) at a distance of 41 cm from the source for a specified time while continuously being stirred on a stir plate and kept on ice with the Petri dish lid and aluminium foil temporarily removed. Another 400 μL aliquot was removed from the Petri dish to a different 2 mL volume of unsupplemented α-MEM and kept in the dark on ice. Various other aliquots were subsequently removed after increasing time points, allowing for cumulative exposure to VL. A background level was obtained by preparing a 400 μL volume of 40 μg/mL MB in PBS and adding it to a 2 mL volume of unsupplemented α-MEM.

Following the 18 to 24-hour incubation period, and immediately after completing the VL exposure to the virus solution, the media was aspirated from each well of the 96-well plate. Virus solutions were mixed using a vortex (Scientific Products S8220 Deluxe Mixer) and wells were given 40 μL of the treated virus/unsupplemented α-MEM solution

and subsequently incubated for 90 minutes in a humidified incubator at 37°C and 5% CO_2. These conditions permit the AdMCMV*lacZ* virus to infect the cells. Every well of a row received a 40 μL aliquot of solution that differed from the other rows in the length of exposure time to VL. Therefore, one row would receive aliquots containing untreated virus; one row would contain the background aliquot (no virus); and the other rows would receive aliquots containing virus treated with increasing periods of VL exposure. After incubation for 90 minutes, an additional 160 μL of warmed, supplemented α-MEM was added to each well (bringing the total volume to 200 μL per well) in order to stop the infection. The plate was further incubated in 37°C humidified air and 5% CO_2 until the desired time depending on the experimental conditions had elapsed (6, 12 or 24 hours) and the cells were to be harvested.

Quantitation of β-galactosidase (β-gal) activity was achieved by aspirating the media from each well, replacing it with 60 μL of chlorophenol red-β-D-galactopyranoside (CPRG) solution (0.01% Triton X-100, 1 mM $MgCl_2$, 100 mM phosphate buffer at pH 8.3), and reading the absorbance at 570 nm on a 96-well plate reader (EL 340 Microplate Bio Kinetics Reader, BIO-TEK Instruments). The β-gal enzyme catalyses the hydrolysis of CPRG into two component products: chlorophenol red and β-D-galactose. Chlorophenol red, as its name suggests, absorbs light at approximately 570 nm and produces a red solution, while its precursor molecule, CPRG, does not. Therefore an increase in light absorbance can be used to measure β-gal activity when CPRG is present. Absorbance values were obtained at several time intervals following the addition of the CPRG solution and a saturation curve for one control well from each cell line was

produced. Data from any point of the exponential portion of the saturation curve was used to produce the host cell reactivation curves discussed in the Results section. The average point for each measurement was obtained from quadruplicate wells and the mean absorbance background level was subtracted from each averaged point.

4.0 Results

4.1 Host Cell Reactivation of Visible Light-Treated Compared to Methylene Blue plus Visible Light-Treated AdMCMVlacZ

Host cell reactivation (HCR) of β-galactosidase (β-gal) activity from an AdMCMV*lacZ* recombinant virus treated with visible light (VL) alone compared to that treated with methylene blue (MB) plus VL was examined in several Chinese hamster ovary (CHO) cell lines. Results show a negligible effect of VL on β-gal activity relative to the effect of MB plus VL (Figure 2.1). An analysis of HCR experiments of VL-treated AdMCMV*lacZ* show a significant reduction in β-gal activity relative to untreated virus in K1 only but not AA8, UV41 or UV61 following exposure to VL for 30 seconds when β-gal activity was assessed 6 hours after infection (Table 2.1). After accumulating 60 seconds of VL exposure, only UV61 and K1 show a significant reduction, and this reduction is absent following 90 seconds of VL exposure. However, there were no significant differences observed amongst cell lines when compared to the parental AA8 cell line. These results suggest that although VL is capable of inducing some DNA damage, the AA8 normal and other cell lines appear to be affected equally as no significant differences were observed in relation to AA8. Additionally, the effects are

negligible in comparison to damage induced by MB plus VL, as the β-gal activity of the reporter gene is substantially reduced upon treatment with MB plus VL in comparison to treatment with VL alone. This suggests that only a small fraction of the damage induced by MB plus VL can be attributed to VL exposure, as otherwise equal repair proficiency would be observed in both treatment regimens.

4.2 Host Cell Reactivation of Methylene Blue plus Visible Light-Treated AdMCMVlacZ is Time-Dependent

Host cell reactivation (HCR) of β-galactosidase (β-gal) activity was examined in Chinese hamster ovary (CHO) cell lines at varying times following infection with methylene blue (MB) plus visible light (VL)-treated AdMCMV*lacZ*. Figure 2.2 shows a comparison of HCR curves obtained from the same experiment when viewed at 6 hours and 12 hours after infection. It can be seen that HCR of the [MB+VL]-treated reporter gene was significantly reduced in UV61 cells compared to parental AA8 cells when scored for β-gal activity at 6 hours after infection, but less so when scored at 12 hours after infection. HCR curves for UV61 cells were similar to those for AA8 cells when scored at 24 hours after infection (data not shown). This suggested a deficiency in the rate of repair of [MB+VL]-induced DNA damage in the reporter gene. Based on this result, subsequent HCR assays were carried out by scoring for β-gal activity at 6 hours after infection.

4.3 Host Cell Reactivation of the Methylene Blue plus Visible Light-Treated Reporter Gene in Several Mutant Chinese Hamster Ovary Cells

Host cell reactivation (HCR) of a methylene blue (MB) plus visible light (VL)-treated *lacZ* reporter gene was observed in selected base excision repair (BER)- and nucleotide excision repair (NER)-deficient Chinese hamster ovary (CHO) cell lines. Previous experiments involving HCR of β-gal activity from UVC-treated AdMCMV*lacZ* in NER-deficient CHO cell lines showed significant reductions in repair compared to that in NER-proficient parental cell lines (Table 2.2). These results indicate a deficiency in NER in these cell lines as UVC radiation induces cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts that are repaired by the NER pathway (as reviewed by Kim *et al.*, 2002).

Representative HCR curves from [MB+VL]-treated AdMCMV*lacZ* are shown in Figure 2.3. These results were obtained by scoring for β-gal activity 6 hours after infection using 40 μg/mL MB and a virus multiplicity of infection (MOI) of 20 plaque-forming units (pfu) per cell. It can be seen that Figure 2.3-A shows a significant reduction in β-gal activity in UV61 cells when compared to that in the normal AA8 cells. Conversely, the *ERCC1*-deficient UV20 and the *XRCC1*-deficient EM9 both show increased HCR capacity when compared to the normal AA8 (Figure 2.3-B). However, another *ERCC1*-deficient cell line, 30PV, as well as the *ERCC1* knock-out, CHO-7-27, does not show a reduction in HCR for β-gal activity when compared to that in the normal, CHO-K1 (Figure 2.3-D).

The length of exposure to VL required to reduce β-gal activity for the given MB concentration to 37% of the untreated control (D_{37}) was determined from the HCR curves and averages were compared to the normal AA8 (or K1 in the case of CHO-7-27 and 30PV). The D_{37} values were used as a measure of the HCR capacity of each cell line with respect to the type of damage introduced to the *lacZ* reporter gene. Therefore cell lines with a higher mean D_{37} value are able to repair DNA damage introduced by MB plus VL better than cell lines with a lower mean D_{37} value. The resulting D_{37} analyses are summarised in Table 2.3 and Figure 2.4. Two-sample independent *t*-tests were performed to compare absolute D_{37} values obtained from each HCR experiment and showed that UV61 (RCG-6; CSB) had a significantly lower HCR capacity compared to the parental AA8 ($P < 0.05$). Conversely, the *XRCC1*-deficient EM9 was shown to have a significantly higher HCR capacity when compared to AA8 ($P < 0.05$). Similarly, the *ERCC1*-deficient UV20 had a significantly higher HCR capacity than AA8 ($P < 0.05$). In contrast, the *ERCC1*-deficient 30PV and the *ERCC1* knock-out, CHO-7-27, showed no difference in HCR capacity when compared to the normal K1.

5.0 Discussion

Previous studies have implicated the involvement of certain nucleotide excision repair (NER) genes in base excision repair (BER). In this study, host cell reactivation (HCR) assays were employed that introduced a methylene blue (MB) plus visible light (VL)-treated recombinant adenovirus carrying a β-galactosidase (β-gal)-expressing reporter gene into selected Chinese hamster ovary (CHO) cell lines with NER or BER

gene deficiencies. Results showed a significant decrease in the repair capacity of [MB+VL]-induced DNA damage in the *CSB*-compromised UV61 cell line when compared to its parental, AA8 (Table 2.3). This suggests an involvement of the CSB protein in the repair of [MB+VL]-induced damage. MB plus VL has been shown to produce primarily 8-hydroxyguanine (8-oxoG) lesions in DNA (Floyd *et al.*, 1989), which are repaired mainly by the short-patch repair mechanism of BER (as reviewed by Dianov *et al.*, 2001). This information corresponds to existing data that implicates CSB in the repair of 8-oxoG lesions (as reviewed by Pastoriza Gallego and Sarasin, 2003).

Specifically, Sunesen *et al.* showed that a deficiency in *CSB* led to a decrease in DNA incision activity when deficient cell lines were confronted with 8-oxoG lesions (Sunesen *et al.*, 2002). This deficiency was not observed when other DNA lesions such as thymine glycols were present, and the deficiency could be rescued with transfection of a functional *CSB* gene (Sunesen *et al.*, 2002). Similarly, Tuo *et al.* also found a decrease in 8-oxoG incision activity in *CSB* mutant and null cell lines when compared to wild-type cells (Tuo *et al.*, 2001). Moreover, Tuo *et al.* showed an accumulation of 8-oxoG lesions in *CSB*-deficient cell lines following exposure to γ-irradiation (Tuo *et al.*, 2001). Osterod *et al.* also showed decreased repair of 8-oxoG in *CSB*-deficient mouse cells (Osterod *et al.*, 2002). Additionally, there may be some evidence for a role of CSB in a transcription-coupled repair (TCR) pathway of BER (Le Page *et al.*, 2000), similar to its role in NER (as reviewed by van Gool *et al.*, 1997). However, questions have recently arisen about the validity of such experiments, and several articles implicating a TCR role for CSB in

BER have been retracted (Le Page *et al.*, 2005; Check, 2005). Nevertheless, it is clear that there is some involvement of CSB in the removal of 8-oxoG lesions from DNA.

Any specific role for CSB in the repair of [MB+VL]-induced DNA damage has yet to be determined. However, despite several retracted papers on the subject, it remains possible that a TCR pathway exists for BER and that CSB is involved. Although it has been suggested that RNA polymerases would not stall on the non-bulky lesions repaired by BER, it has been proposed that a number of other proteins (such as BRCA1 or BRCA2) may associate with 8-oxoG lesions, thereby causing RNA polymerase II (RNAPII) to stall upon encountering the proteins during transcription (as reviewed by Pastoriza Gallego and Sarasin, 2003). The CSB protein may subsequently be recruited to the site to aid in the removal of RNAPII, as is the case for NER (as reviewed by van Gool *et al.*, 1997). Also, the CSB protein may have an entirely different role, as yet unseen, such as the dual role of XPG in incision during NER (as reviewed by Stary and Sarasin, 2002) and the recruitment of DNA glycosylases in BER (Clarkson, 2003).

Interestingly, the *XRCC1*-deficient cell line, EM9, showed an increased repair capacity for [MB+VL]-induced damage when compared to the parental AA8 (Table 2.3). This suggests that a deficiency in XRCC1, or the particular mutation in EM9 cells is beneficial to the removal of [MB+VL]-induced lesions. In normal cells, XRCC1 is involved in the short-patch BER pathway, forming a heterodimer with DNA ligase III (as reviewed by Boiteux and Le Page, 2001). Specifically, XRCC1 acts as a scaffold protein to bring DNA ligase III and DNA polymerase β (pol β) together during the final stages of short-patch repair (as reviewed by Lindahl and Wood, 1999). The increased β-gal

activity observed in EM9 cells may be due to a number of factors. It has been shown that XRCC1 is involved in the ligation step of short-patch BER, but is not involved in long-patch BER (Cappelli *et al.*, 1997). It is possible that in the absence of a functional short-patch repair system, [MB+VL]-induced damage is repaired by the long-patch repair mechanism, which is known to be able to repair 8-oxoG lesions. It has been proposed that an alternate role for XRCC1 is to promote single nucleotide incorporation by DNA pol β, rather than excessive nucleotide incorporation (as reviewed by Cappelli *et al.*, 1997). This is supported by evidence from Kubota *et al.* who showed that elevated repair patch size was observed in EM9 cells during short-patch repair (Kubota *et al.*, 1996). DNA pol β is involved in both short- and long-patch repair, and the elimination of a functional XRCC1 may remove some limitations on pol β, allowing it to perform excessive repair. It has also been shown that XRCC1 is not required enzymatically for BER, and although XRCC1 protein levels were greatly reduced in EM9 cells when compared to the parental, AA8, introduction of recombinant XRCC1 failed to significantly rescue the deficiency in ligation in short-patch BER (Cappelli *et al.*, 1997). Therefore, repair of [MB+VL]-induced damage may not specifically require the presence of XRCC1, and some other mechanism may help in the final stages of BER. Alternatively, DNA ligase III may function better in the absence of XRCC1 when confronted with [MB+VL]-induced DNA damage.

The *ERCC1*-deficient UV20 cell line also showed increased β-gal activity when compared to the parental AA8 (Table 2.3). Further investigation of the involvement of *ERCC1* showed no significant difference in the HCR of [MB+VL]-treated

AdMCMV*lacZ* in either the *ERCC1*-deficient 30PV or the *ERCC1* knock-out 7-27 cell line, when compared to the parental K1. The conflicting data makes it difficult to hypothesize an involvement of *ERCC1* in the BER of [MB+VL]-induced DNA damage. Previous experiments involving HCR of a UVC-treated reporter gene have similarly led to differences in relative β-gal activity amongst these cell lines (Table 2.2). It is interesting to note that there was no significant difference in β-gal activity of the [MB+VL]-treated reporter gene in the *XPF*-deficient UV41 cell line when compared to AA8, suggesting XPF and ERCC1 might not interact during BER as they do in NER. Hsia *et al.* showed a three-fold increase in 8-oxoG lesions in *ERCC1* knock-out mice when compared to control mice (Hsia *et al.*, 2003), suggesting a role for *ERCC1* in the removal of 8-oxoG lesions. It is possible that overexpression of *ERCC1* may also be detrimental to repair, as previous studies have indicated that overexpression of *ERCC1* cells is associated with such things as liver fibrogenesis, cancer and drug resistance (Fautrel *et al.*, 2005). It is also possible that a specific gain-of-function mutation has arisen in the *ERCC1* gene of UV20 cells. Further experimentation is required to determine whether *ERCC1* is involved in BER of [MB+VL]-induced DNA damage. For example, a functional copy of the *ERCC1* gene could be introduced into the deficient cell lines, and β-gal activity could be observed following infection of [MB+VL]-treated AdMCMV*lacZ*.

There were no significant differences in the HCR of β-gal activity from [MB+VL]-treated AdMCMV*lacZ* in UV5, UV24, UV41 or UV135 cells, suggesting a lack of involvement of XPD, XPB, XPF and XPG, respectively, in the repair of lesions

caused by such damage. In contrast, a majority of the cell lines employed in this study have all shown a significant reduction in the HCR of β-gal activity from AdMCMV*lacZ* treated with UVC radiation (Table 2.2). This indicated the ability of the HCR assay to detect the NER deficiency in these cell lines, and further suggests that the damage caused by MB plus VL is not repaired by NER. In general, β-gal activity was only slightly reduced as a result of VL exposure alone in all cell lines tested (Table 2.1). It is likely that VL exposure itself introduces DNA damage to the viral genome, however it is negligible when compared to the level of damage introduced through MB plus VL exposure and only results in an overall reduction in β-gal activity of 0 to 30% of the untreated control. Moreover, there were no significant differences in β-gal activity of a VL-treated reporter gene found amongst the cell lines examined.

Additionally, observed deficiencies in repair appear to be time-dependent, at least for the UV61 cell line (Figure 2.2). HCR of the [MB+VL]-treated reporter gene was significantly reduced in UV61 cells compared to parental AA8 cells when scored for β-gal activity at 6 hours after infection, but not when scored at 12 and 24 hours after infection. This result suggests that the observed deficiency in repair of the [MB+VL]-damaged reporter gene in UV61 cells is a deficiency in the rate of repair. Further experimentation is required to determine whether the increased HCR capacity of UV20 and EM9 cells compared to AA8 cells reported here results from an increased rate of repair in these cells.

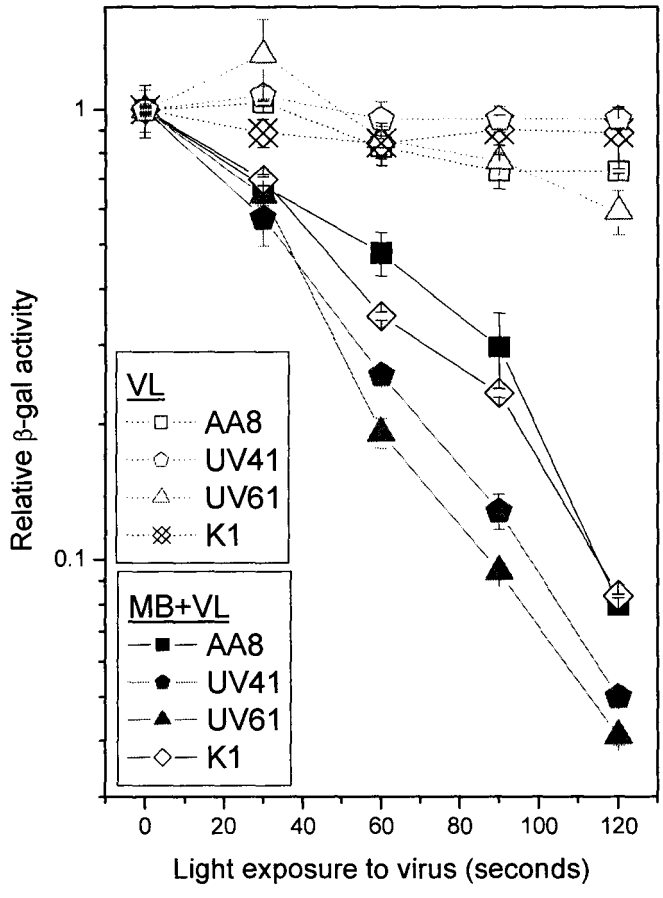

Figure 2.1 — Host cell reactivation of β-galactosidase (β-gal) activity for VL- and [MB+VL]-treated AdMCMV*lacZ* virus in selected Chinese hamster ovary cells. Cells were infected with untreated, VL- or [MB+VL]-treated AdMCMV*lacZ* (as indicated) and subsequently harvested 6 hours after infection. Conditions include 40 μg/mL MB and an MOI of 5 pfu/cell. The result shown is a representative experiment and includes AA8 (normal; ■ / □), UV61 (▲ / △), UV41 (⬟ / ⬠) and K1 (normal; ◇ / ※). Data points shown are the average value ± SE obtained through triplicate determinations from one experiment. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.).

Table 2.1 — The β-galactosidase (β-gal) activity of a reporter gene treated with visible light (VL) obtained from host cell reactivation assays in select Chinese hamster ovary (CHO) cell lines. The mean β-gal activities ± SE are reported relative to the untreated control, 0 seconds VL, and relative to AA8. Results relative to the untreated control report any significant reductions in β-gal activity from the control (*i.e.* results compared to 1), whereas results relative to AA8 compare any significant differences from AA8 following 30 (Time = 30s), 60 (Time = 60s), or 90 seconds (Time = 90s) of VL exposure. Results that are significantly different by the two-sample independent *t*-test ($P < 0.05$) are also indicated (Sig). The virus was subjected to VL emitted from a 1000 W source (General Electric, GE R1000) at a distance of 41 cm and an MOI of 5, 10 or 20 pfu/cell. Results obtained 6 hours after infection and are derived from three experiments with triplicate determinations. (MOI = multiplicity of infection; pfu = plaque-forming units.)

		Relative to Untreated			***Relative to AA8***		
Time	**Cell Line**	**β-gal**	**P Value**	**Sig?**	**β-gal**	**P Value**	**Sig?**
30s	AA8	0.95 ± 0.05	0.3962	No	1		
	UV41	0.94 ± 0.07	0.8896	No	0.99 ± 0.06	0.8448	No
	UV61	0.84 ± 0.26	0.5688	No	0.86 ± 0.22	0.5654	No
	K1	0.78 ± 0.06	0.0234	*Yes*	0.83 ± 0.06	0.0515	No
60s	AA8	0.80 ± 0.08	0.0694	No	1		
	UV41	0.92 ± 0.03	0.0659	No	1.18 ± 0.16	0.3118	No
	UV61	0.65 ± 0.12	0.0432	*Yes*	0.81 ± 0.11	0.1699	No
	K1	0.73 ± 0.08	0.0275	*Yes*	0.94 ± 0.17	0.7582	No
90s	AA8	0.80 ± 0.10	0.1025	No	1		
	UV41	0.81 ± 0.09	0.1081	No	1.03 ± 0.14	0.8548	No
	UV61	0.61 ± 0.14	0.0539	No	0.76 ± 0.17	0.2247	No
	K1	0.69 ± 0.14	0.0916	No	0.88 ± 0.18	0.5450	No

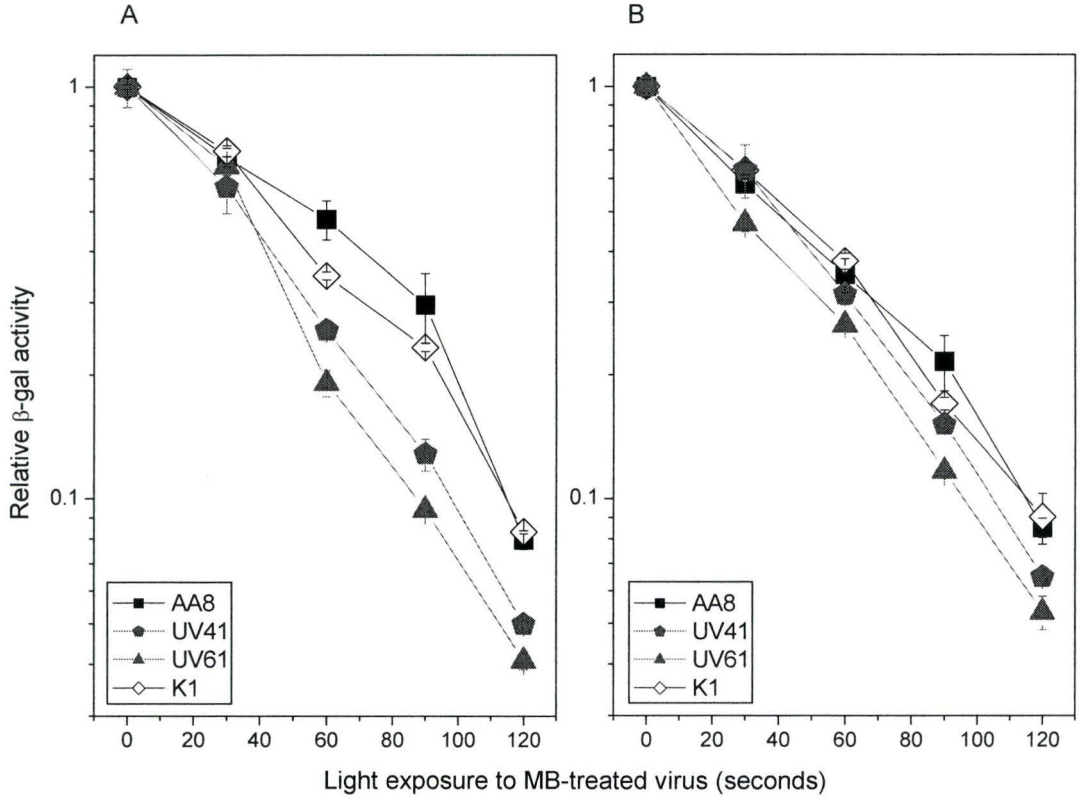

Figure 2.2 — Host cell reactivation of β-galactosidase (β-gal) activity for [MB+VL]-treated AdMCMV*lacZ* virus in selected Chinese hamster ovary cells observed at varying times following infection. Cells were infected with untreated or [MB+VL]-treated AdMCMV*lacZ* and subsequently harvested 6 hours (A) or 12 hours (B) after infection. Differences amongst the cell lines in β-gal activity are more clearly visible at the earlier time (6 hours), suggesting that at 12 hours, sufficient repair has occurred in gene deficient cell lines that no obvious differences are seen. Conditions include 40 μg/mL MB and an MOI of 5 pfu/cell. The results shown are from a representative experiment and include AA8 (normal; ■), UV41 (⬟), UV61 (▲) and K1 (normal; ◇). Data points shown are the average value ± SE obtained through triplicate determinations from one experiment. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.).

Table 2.2 — D_{37} values obtained from host cell reactivation assays of β-galactosidase activity for a UVC-treated AdMCMV*lacZ* virus in select Chinese hamster ovary cell lines. The average D_{37} ± SE and the average D_{37} ± SE relative to the parental AA8 are reported, in addition to the number of experiments (N) used to determine the values. Values that are relative to the K1 parental line (not AA8) are indicated (*). The virus was subjected to UVC radiation at a fluence rate 2 J/m^2/s and was introduced into the cells at an MOI of 1-2 pfu/cell. Results obtained 24 hours after infection. (MOI = multiplicity of infection; pfu = plaque-forming units.) (Private communication from J. Stavropoulos, McMaster University.)

Cell Line	N	Absolute D_{37} (J/m^2)	Relative D_{37}
AA8	9	131.29 ± 14.94	1
UV5	2	52.38 ± 9.08	0.32 ± 0.03
UV20	2	55.05 ± 6.50	0.34 ± 0.02
UV24	2	32.10 ± 3.85	0.27 ± 0.02
UV41	2	46.35 ± 2.70	0.36 ± 0.17
UV61	2	70.50 ± 20.48	0.66 ± 0.14
UV135	4	54.33 ± 2.98	0.40 ± 0.15
K1*	4	72.78 ± 3.12	1
30PV*	2	80.85 ± 2.15	1.12 ± 0.09
7-27*	2	30.50 ± 1.5	0.42 ± 0.02

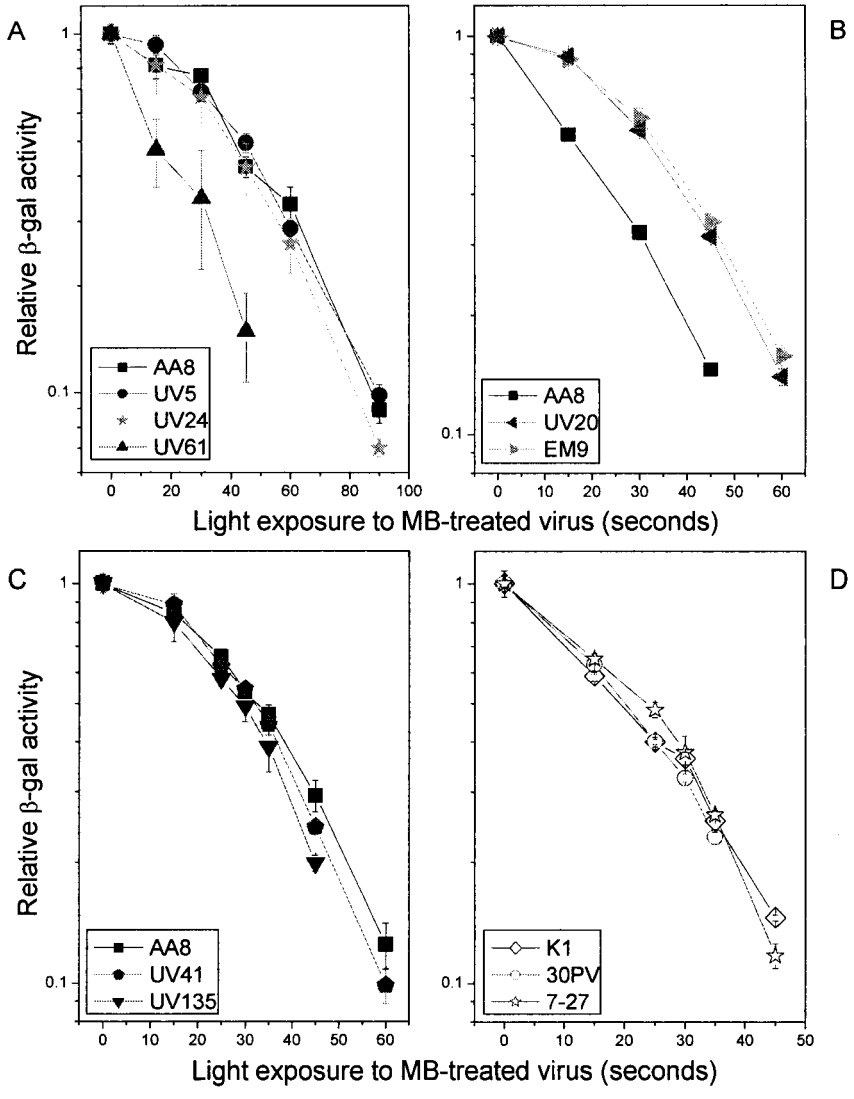

Figure 2.3 — Host cell reactivation of β-galactosidase (β-gal) activity for [MB+VL]-treated AdMCMV*lacZ* virus in selected Chinese hamster ovary cell lines. Cells were infected with untreated or [MB+VL]-treated AdMCMV*lacZ* and subsequently harvested 6 hours after infection. Conditions include 40 μg/mL MB and an MOI of 20 pfu/cell. Results shown are representative experiments and include AA8 (normal; ■), UV5 (●), UV20 (◀), UV24 (★), UV41 (⬟), UV61 (▲), UV135 (▼), EM9 (▶) as well as K1 (normal; ◇), 30PV (○) and 7-27 (☆). Data points shown are the average value ± SE obtained through triplicate or quadruplicate determinations from one experiment. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.).

Table 2.3 — D_{37} values obtained from host cell reactivation assays of β-galactosidase activity for [MB+VL]-treated AdMCMV*lacZ* virus in selected Chinese hamster ovary cells. The average D_{37} ± SE, and the average D_{37} ± SE relative to the parental AA8 are reported, in addition to the number of experiments (N) used to determine the values. Values that are relative to the K1 parental line (not AA8) are indicated (*). Absolute D_{37} values that are significantly different than the normal by the two-sample independent *t*-test ($P < 0.05$) are also indicated. The virus was subjected to 40 μg/mL MB at an MOI of 20 pfu/cell. Results obtained 6 hours after infection. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.)

Cell Line	N	Absolute D_{37} (seconds)	Relative D_{37}	P Value	Significant?
AA8	10	38.50 ± 2.45	1		
UV5	5	38.84 ± 3.62	0.94 ± 0.02	0.6215	No
UV20	4	46.35 ± 2.01	1.37 ± 0.07	0.0409	*Yes*
UV24	4	36.29 ± 5.65	0.89 ± 0.10	0.5864	No
UV41	4	37.99 ± 2.34	0.97 ± 0.03	0.7216	No
UV61	8	30.17 ± 2.29	0.80 ± 0.06	0.0454	*Yes*
UV135	3	37.56 ± 1.96	0.96 ± 0.03	0.5439	No
EM9	3	46.70 ± 2.95	1.47 ± 0.09	0.0250	*Yes*
K1*	5	31.86 ± 1.71	1		
30PV*	4	30.15 ± 2.43	0.94 ± 0.02	0.6141	No
7-27*	3	30.88 ± 1.72	0.96 ± 0.08	0.5746	No

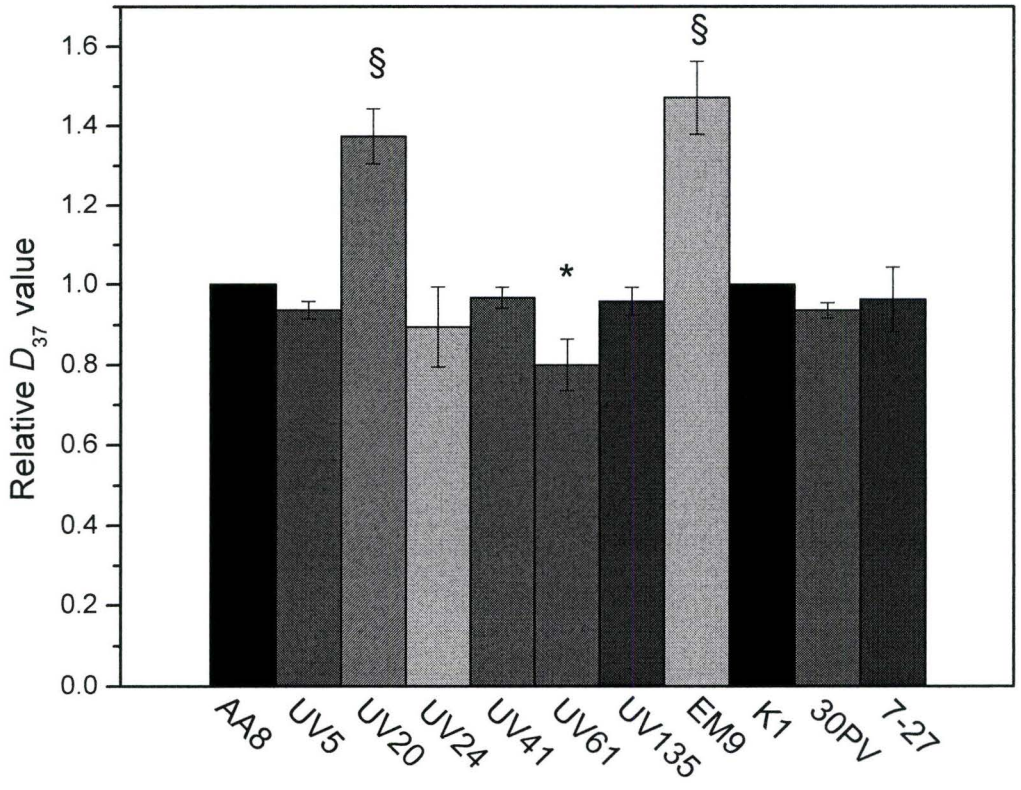

Figure 2.4 — Relative D_{37} values obtained from host cell reactivation assays of β-galactosidase activity for [MB+VL]-treated AdMCMV*lacZ* virus in selected Chinese hamster ovary cells. Results shown are the average ± SE of three to eight independent experiments, each with quadruplicate determinations. All values are relative to AA8, except K1, 30PV and 7-27, which are relative to K1. Absolute D_{37} values significantly lower (*) or higher (§) than the normal by the two-sample independent *t*-test ($P < 0.05$) are indicated. The virus was subjected to 40 μg/mL MB at an MOI of 20 pfu/cell. Results obtained 6 hours after infection. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.)

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CHAPTER 3

Inducible repair of DNA damage caused by methylene blue plus visible light in Chinese hamster ovary cells

1.0 Abstract

Methylene blue (MB), a type II photosensitizer, has been shown to produce large amounts of 8-hydroxyguanine (8-oxoG) lesions in DNA following excitation by visible light (VL). The removal of the highly mutagenic 8-oxoG lesion is performed almost exclusively by the base excision repair (BER) pathway, which removes single damaged bases and releases them as free bases. In contrast, the nucleotide excision repair (NER) pathway removes large, bulky lesions from DNA such as cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts formed by exposure to a component of ultraviolet (UV) radiation, UVC. Several NER proteins have been implicated in various aspects of BER, such as the NER protein, CSB, which has been associated with the removal of 8-oxoG lesions. AdHCMV*lacZ* (AdCA17) and AdMCMV*lacZ* (AdCA35) are non-replicating recombinant human adenoviruses that can efficiently infect a variety of mammalian cell types and express the β-galactosidase (β-gal) reporter gene under the control of the human or murine cytomegalovirus immediate early promoter, respectively. Previous published reports have indicated that pre-exposing cells to low levels of a DNA-damaging agent can induce the repair of certain types of DNA damage. In the present work, host cell reactivation (HCR) of β-gal activity for [MB+VL]-treated AdCA17 or AdCA35 was examined in several Chinese hamster ovary (CHO) cell lines that were either untreated or treated with low levels of UVC or MB plus VL. Pre-treatment of NER-deficient UV61 (RCG-6; CSB) and repair-proficient CHO-AA8 cells with UVC resulted in an enhanced HCR for [MB+VL]-treated AdCA17, although the results were

only significant for UV61 cells. Similarly, an enhanced HCR of [MB+VL]-treated AdCA35 was observed in AA8 cells, suggesting that the repair of the [MB+VL]-treated reporter gene is inducible by UVC. A significant enhancement in HCR of [MB+VL]-treated AdCA35 was not observed in AA8 cells following pre-treatment with MB, VL, or both, indicating that the detection of any induction in the repair of DNA damage from MB plus VL could not be made with the conditions of the HCR assay employed. However, the results do not preclude the possibility that a significant enhancement in HCR capacity could be observed using different conditions.

2.0 Introduction

Several highly conserved repair mechanisms exist within the cell to effectively remove potentially harmful lesions that arise in DNA. The nucleotide excision repair (NER) pathway involves the removal of bulky lesions from the genome that can disrupt the normal structure of the DNA helix (as reviewed by Wood, 1996) and their release as part of an oligonucleotide fragment (as reviewed by Friedberg, 2001). Two distinct NER subpathways have been identified: transcription-coupled repair (TCR) and global genome repair (GGR). TCR involves the removal of DNA lesions from transcribed strands in active genes, whereas GGR involves the removal of lesions found throughout the genome, including those found in non-transcribed strands of active genes (as reviewed by Hanawalt, 2002). The NER pathway is responsible for repairing lesions such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts that are caused by a component of the ultraviolet (UV) light spectrum, UVC (200 – 280

nm) (as reviewed by Wood, 1996). Another repair mechanism is the base excision repair (BER) pathway, which is responsible for the removal of single damaged bases in DNA and their subsequent excision as free bases (as reviewed by Friedberg, 2001). BER removes minor helix-distorting lesions from DNA, as well as base damage caused by many environmental agents (as reviewed by Slupphaug *et al.*, 2003). Base damage repaired by BER can arise from such sources as reactive oxygen species, ionizing radiation and alkylating agents (as reviewed by Slupphaug *et al.*, 2003).

Methylene blue (MB) is a photosensitizer that has been shown to produce large amounts of 8-hydroxyguanine (8-oxoG) lesions in DNA following excitation by the visible light (VL) spectrum (Floyd *et al.*, 1989). The 8-oxoG lesion is the most common form of damaged purine (as reviewed by Slupphaug *et al.*, 2001) and can mispair with adenine, leading to G:C to T:A transversions (as reviewed by Epe *et al.*, 1993). The BER pathway has been shown to repair 8-oxoG lesions almost exclusively (as reviewed by Dianov *et al.*, 2001). However, many previously published reports have suggested a role for some NER proteins in the BER of certain lesions. The CSB protein, for instance, typically associated with the TCR pathway of NER, has been associated with the BER of 8-oxoG lesions (as reviewed by Pastoriza Gallego and Sarasin, 2003).

Several published reports have indicated the ability of cells to induce repair of certain DNA lesions by pre-exposing cells with low levels of a DNA-damaging agent. For example, Le *et al.* demonstrated that the repair of thymine glycols was enhanced in human lung carcinoma cells following exposure to a low dose (0.25 Gray) of ionizing radiation 4 hours prior to a higher dose (2 Gray) of radiation (Le *et al.*, 1998). In

addition, Germanier *et al.* have shown that TCR is inducible in Chinese hamster ovary (CHO) cells following irradiation with low levels of UVC (2 J/m^2) 3 hours prior to irradiation with higher levels of UVC (20 J/m^2) by measuring the repair of CPDs in a specific gene (Germanier *et al.*, 2000). Similarly, host cell reactivation (HCR) assays were able to view an enhanced ability to repair UVC-induced damage, such as CPDs and (6-4)-photoproducts, in normal human fibroblasts (Francis and Rainbow, 1999) and a parental Chinese hamster ovary (CHO) cell line (Liu and Rainbow, 2004) following pre-treatment of cells with UVC at a fluence of 15 or 9 J/m^2, respectively. Published reports have also indicated that an inducible effect is not always observed. For example, an enhanced HCR of a UVC-damaged reporter gene was not observed in TCR-deficient CHO-UV61 cells, which are deficient in the *CSB* gene, following exposure to UVC (Liu and Rainbow, 2004). Moreover, the timing of exposure to low levels of DNA-damaging agents prior to subsequent introduction of higher levels of the DNA-damaging agent has previously been reported as being a contributing factor in the repair of certain lesions. Pitsikas *et al.* showed that a delayed enhanced repair capacity of UVC-induced damage was observed in *CSB*-deficient human cells following exposure to 6 J/m^2 UVC 40 to 60 hours prior to the introduction of the additional UVC damage (Pitsikas *et al.*, 2005).

Due to the possible role of some NER proteins in the BER pathway, particularly involving the removal of 8-oxoG lesions, it would be beneficial to investigate whether an enhanced repair of [MB+VL]-induced damage is possible by stimulation of either BER or NER. In this study, the ability to induce BER of [MB+VL]-induced DNA damage by

pre-exposing cells to MB plus VL or UVC was investigated in select NER-deficient CHO cell lines using HCR assays.

3.0 Materials & Methods

3.1 Cell Lines

Chinese hamster ovary (CHO) cell lines employed include: AA8 (parental); UV5 (RCG-2; XPD); UV20 (RCG-1; *ERCC1*); and UV61 (RCG-6; CSB) (provided by Dr. Larry Thompson [Lawrence Livermore National Laboratory, Livermore, CA] with the help of Dr. Gordon Whitmore [Physics Division, Ontario Cancer Institute, Toronto, ON]). All cell cultures were grown in α-minimum essential media (α-MEM: HyQ MEM Alpha Modification with L-glutamine, ribonucleosides and deoxyribonucleosides) supplemented with 10% fœtal bovine serum (FBS) and 1% antibiotic-antimycotic (100 μg/mL penicillin G sodium, 100 μg/mL streptomycin sulfate and 250 ng/mL amphotericin B in 0.85% saline) obtained from Gibco-BRL. Cultures were maintained in 37°C humidified air containing 5% CO_2.

3.2 Methylene Blue

Stock methylene blue solution was prepared by dissolving 0.02 g methylene blue trihydrate (C.I. 52015; methylthionine chloride, $C_{16}H_{18}ClN_3S \bullet 3H_2O$, FW 373.9) in 20.0 mL phosphate-buffered saline (PBS: 140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4 [pH 7.4]) that was warmed to 37°C. The resulting mixture was filter-

sterilized in the dark through a 0.2 μm filter to create a 1000 μg/mL solution. Stock solution was then aliquoted into volumes of 1 mL or less and stored at -20°C in the dark.

3.3 Virus

The viruses employed (provided by Dr. Frank Graham [Department of Biology, McMaster University, Hamilton, ON]) are non-replicating adenoviruses (Ad) expressing the bacterial *lacZ* β-galactosidase reporter gene under the control of the murine or human cytomegalovirus (MCMV or HCMV, respectively) immediate early (IE) promoter (Addison *et al.*, 1997). The reporter gene was inserted into the deleted E1 region of the genome, making the viruses non-replicable. The viruses are referred to as AdMCMV*lacZ*, or simply AdCA35 for the virus under the control of the MCMV IE promoter, and AdHCMV*lacZ* or AdCA17 for the virus under the control of the HCMV IE promoter. Stock solutions of virus were stored at -20°C.

3.4 Pre-treatment of Cells with UVC

Cells were counted on a haemocytometer and seeded for less than confluence (3×10^4 cells per well) or confluence (4×10^4 cells per well) in a 96-well microtitre plate in supplemented α-minimum essential media (α-MEM) and allowed to incubate in humidified air for 20-24 hours at 37°C and 5% CO_2. Following the overnight incubation, the overlaying media was aspirated from each well of the microtitre plate and replaced with 40 μL of warmed phosphate-buffered saline (PBS). Lids were positioned such that wells not receiving UVC treatment remained covered, as UVC does not penetrate plastic.

Where applicable, cells were treated to UVC using a General Electric germicidal lamp (model G8T5) emitting light at predominantly 254 nm, at a fluence rate of 1 $J/m^2/s$ for 9 or 15 seconds, yielding a fluence of 9 or 15 J/m^2 (as specified). Following UVC or mock treatment, 160 μL of supplemented α-MEM was added to each well and cells were once again incubated until infection or immediately infected with virus treated with methylene blue (MB) plus visible light (VL), at a multiplicity of infection (MOI) of 20 plaque-forming units (pfu) per cell, as described below.

3.5 Pre-treatment of Cells with Methylene Blue plus Visible Light

Cells were counted on a haemocytometer and seeded for less than confluence (2×10^4 cells per well) in a 96-well microtitre plate in supplemented α-minimum essential media (α-MEM) and allowed to incubate in humidified air for 6 hours at 37°C and 5% CO_2. Following the incubation period, the overlaying media was removed and replaced with 200 μL of supplemented α-MEM containing a known concentration of methylene blue (MB) or phosphate-buffered saline (PBS) alone in α-MEM and returned to the 37°C and 5% CO_2 incubator for 20-24 hours.

Following the overnight incubation, the MB solution was aspirated from each well of the microtitre plate and wells were washed with 100 μL each of PBS. The PBS wash was also aspirated and a further volume of 60 μL PBS was added to each well for exposure to visible light (VL). Lids were removed and microtitre plates were exposed to VL using a 1000 W bulb (General Electric, GE R1000) at a distance of 83 cm for a specified duration. Lids were replaced and cells were immediately infected with

[MB+VL]-treated virus, at a multiplicity of infection (MOI) of 40 plaque-forming units (pfu) per cell, as described below.

3.6 Host Cell Reactivation

A 3.6 mL volume of 40 μg/mL methylene blue (MB) solution was prepared in a small (35 × 10 mm) Petri dish using cold phosphate-buffered saline (PBS) and kept on ice under aluminium foil with minimal ambient lighting. A small, sterile stir-bar was added to the Petri dish to aid in mixing the contents of the solution. An appropriate volume of stock AdMCMV*lacZ* or AdHCMV*lacZ* (10^{10} pfu/mL; pfu = plaque forming units) was added to the Petri dish containing the MB solution to obtain the desired multiplicity of infection (MOI) for the experiment. The solution was kept on ice and under aluminium foil while the contents of the Petri dish were mixed by the stir-bar using a stir plate (VWR Dylastir Stirrer). A 400 μL aliquot of the solution was removed and added to a 2 mL volume of unsupplemented α-minimum essential media (α-MEM) and kept in the dark on ice. The solution was then exposed to visible light (VL) using a 1000 W bulb (General Electric, GE R1000) at a distance of 41 cm from the source for a specified time while continuously being stirred on a stir plate and kept on ice with the Petri dish lid and aluminium foil temporarily removed. Another 400 μL aliquot was removed from the Petri dish to a different 2 mL volume of unsupplemented α-MEM and kept in the dark on ice. Various other aliquots were subsequently removed after increasing time points, allowing for cumulative exposure to VL. A background level was

obtained by preparing a 400 μL volume of 40 μg/mL MB in PBS and adding it to a 2 mL volume of unsupplemented α-MEM.

Following the desired pre-treatment conditions (with MB plus VL or UVC), and immediately after completing the VL exposure to the virus solution, the media was aspirated from each well of the 96-well plate. Virus solutions were mixed using a vortex (Scientific Products S8220 Deluxe Mixer) and wells were given 40 μL of the treated virus/unsupplemented α-MEM solution and subsequently incubated for 90 minutes in a humidified incubator at 37°C and 5% CO_2. These conditions permit the AdMCMV*lacZ* or AdHCMV*lacZ* virus to infect the cells. Every well of a row received a 40 μL aliquot of solution that differed from the other rows in the length of exposure time to VL. Therefore, one row would receive aliquots containing untreated virus; one row would contain the background aliquot (no virus); and the other rows would receive aliquots containing virus treated with increasing periods of VL exposure. After incubation for 90 minutes, an additional 160 μL of warmed, supplemented α-MEM was added to each well (bringing the total volume to 200 μL per well) in order to stop the infection. The plate was further incubated in 37°C humidified air and 5% CO_2 until the desired time depending on the experimental conditions had elapsed (6 or 40 hours) and the cells were to be harvested. In the instance that cells receive pre-treatment immediately prior to infection, virus solutions were prepared immediately in advance of pre-treatment to cells, and stirred using a vortex prior to infection. Cells were subsequently infected with virus immediately following pre-treatment conditions.

Quantitation of β-galactosidase (β-gal) activity was achieved by aspirating the media from each well, replacing it with 60 μL of chlorophenol red-β-D-galactopyranoside (CPRG) solution (0.01% Triton X-100, 1 mM $MgCl_2$, 100 mM phosphate buffer at pH 8.3), and reading the absorbance at 570 nm on a 96-well plate reader (EL 340 Microplate Bio Kinetics Reader, BIO-TEK Instruments). The β-gal enzyme catalyses the hydrolysis of CPRG into two component products: chlorophenol red and β-D-galactose. Chlorophenol red, as its name suggests, absorbs light at approximately 570 nm and produces a red solution, while its precursor molecule, CPRG, does not. Therefore an increase in light absorbance can be used to measure β-gal activity when CPRG is present. Absorbance values were obtained at several time intervals following the addition of the CPRG solution and a saturation curve for one control well from each cell line was produced. Data from any point of the exponential portion of the saturation curve was used to produce the host cell reactivation curves discussed in the Results section. The average point for each measurement was obtained from triplicate wells and the mean absorbance background level was subtracted from each averaged point.

4.0 Results

4.1 Pre-treatment of Cells with UVC

Host cell reactivation (HCR) of a methylene blue (MB) plus visible light (VL)-treated *lacZ* reporter gene was examined in several Chinese hamster ovary (CHO) cell lines that were treated with low levels of UVC radiation. A representative result of CHO cells exposed to 15 J/m^2 UVC prior to infection with [MB+VL]-treated AdHCMV*lacZ*

compared to untreated controls is shown in Figure 3.1. These results were obtained by scoring for β-galactosidase (β-gal) activity at 40 hours after infection. It can be seen in Figure 3.1 that pre-exposing cells to UVC resulted in a higher HCR curve for both the *CSB*-deficient UV61 cells and the parental AA8 cell line. The length of VL exposure to the virus required to reduce β-gal activity for the given MB concentration to 37% of the untreated control (D_{37}) was determined from the HCR curves and used as a measure of the HCR capacity of each pre-treated or untreated cell line with respect to the type of damage introduced into the *lacZ* reporter gene. Therefore a higher mean D_{37} value is indicative of a greater repair capacity for [MB+VL]-induced DNA damage than a lower mean D_{37} value. The resulting D_{37} analyses are summarized in Table 3.1. Two-sample independent *t*-tests were performed and show that the UV61 (RCG-6; CSB) cell line had a significantly higher HCR capacity in UVC-treated cells compared to untreated cells ($P < 0.05$). Although the enhanced HCR capacity observed in UVC-treated AA8 cells compared to untreated cells was not significant, an increased HCR capacity of β-gal activity of the [MB+VL]-treated reporter gene was observed in each repeat of the experiment. It is probable that this increase in HCR capacity following pre-treatment with UVC in AA8 cells would become significant after a successive experiment. A comparison of D_{37} values from untreated UV61 cells to those of untreated AA8 cells showed a mean relative D_{37} value of 0.81 ± 0.17, which is consistent with earlier findings (Chapter 2), although the results obtained from these experiments are not significant ($P = 0.3454$).

The assay was repeated with the AdMCMV*lacZ* virus at a lower UVC fluence of 9 J/m^2 and the time between infection and scoring for β-gal activity was reduced to 6 hours. Figure 3.2 shows a representative result of CHO cells treated with UVC 6 hours (Figure 3.2-A) and immediately (Figure 3.2-B) prior to infection with the [MB+VL]-treated virus. Absolute and relative D_{37} values are compared in Table 3.2. These results show that pre-treatment of UV5 (RCG-2; XPD) and the parental AA8 cell lines but not UV20 (RCG-1; *ERCC1*) cells with UVC 6 hours prior to infection with a [MB+VL]-damaged reporter gene results in a higher HCR capacity than untreated cells. A higher HCR capacity is also obtainable in UV5 cells when infection occurs immediately following pre-treatment with UVC radiation. The D_{37} values obtained from untreated cells are consistent with those from earlier HCR assays (Chapter 2), as UV20 cells show a higher D_{37} value relative to AA8, whereas no difference is observed between UV5 and AA8. Successive experiments are required to determine significance of the increased HCR capacity in AA8 and UV5 cells, as well as to determine the response of UV61 cells to [MB+VL]-treated AdMCMV*lacZ* following UVC treatment.

4.2 Pre-treatment of Cells with Methylene Blue plus Visible Light

Host cell reactivation (HCR) of β-galactosidase (β-gal) activity from a AdMCMV*lacZ* recombinant virus treated with methylene blue (MB) plus visible light (VL) was examined in a parental Chinese hamster ovary (CHO) cell line, AA8, that was either left untreated or treated with MB plus VL prior to infection. Cells were treated with MB and various light exposures and immediately infected with untreated or

[MB+VL]-treated AdMCMV*lacZ*. Typical results for HCR of the [MB+VL]-treated reporter gene in AA8 cells that were either left untreated or incubated with 10 μg/mL MB and subsequently exposed to various durations of VL are shown in Figure 3.3. It can be seen that the greatest enhancement in the HCR of β-gal activity of the damaged reporter gene for [MB+VL]-pre-treated cells compared to untreated cells was observed when conditions were 10 μg/mL MB and 14 minutes of VL exposure (Figure 3.1-B). Conditions other than 10 μg/mL MB and 14 minutes VL were applied, including different durations of light exposure (7 or 28 minutes VL; Figures 3.1-A and 3.1-C, respectively) and higher concentrations of MB (20 or 30 μg/mL MB) (data not shown). The length of exposure of the virus to VL required to reduce β-gal activity to 50% of the untreated control (D_{50}) was determined from the HCR curves for each condition and were used as a measure of the HCR capacity of the AA8 cells to repair the [MB+VL]-treated reporter gene. D_{50} values obtained from three independent experiments are shown in Table 3.3. It can be seen that compared to that for untreated cells, the average D_{50} values were greater for cells pre-treated with VL alone, MB alone and also MB plus VL. However, the enhanced HCR values due to pre-treatment with MB and VL were not significant.

5.0 Discussion

In this study, host cell reactivation (HCR) assays were employed that introduced a methylene blue (MB) plus visible light (VL)-treated recombinant adenovirus carrying a β-galactosidase (β-gal)-expressing reporter gene into Chinese hamster ovary (CHO) cells

that were either left untreated or exposed to low levels of UVC radiation or MB plus VL. Results of UVC-treated CHO cells showed an enhanced HCR of β-gal activity from a [MB+VL]-treated AdHCMV*lacZ* virus in both UV61 (RCG-6; CSB) cells and the parental AA8 cell line when compared to untreated cells, although the results were only significant for UV61 (Figure 3.1 and Table 3.1). This suggests the ability to induce repair of [MB+VL]-induced DNA damage with low levels of UVC radiation in the *CSB*-deficient UV61 cell line, and likely the repair-proficient normal AA8. Although the enhanced HCR capacity observed in AA8 cells that were pre-exposed to UVC were not significant, an increased HCR was observed in each of the experiments, as measured by the D_{37} values. Interestingly, UVC damage has been shown to produce primarily bulky lesions in DNA, such as cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts (as reviewed by Kim *et al.*, 2002), which are repaired by the nucleotide excision repair (NER) pathway, not the base excision repair (BER) pathway that is responsible for removing lesions formed by MB plus VL (as reviewed by Dianov *et al.*, 2001). Low levels of UVC radiation have previously been shown to induce the NER pathway in AA8 cells, as measured by the increased ability to repair subsequent DNA damage formed by UVC (Liu and Rainbow, 2004). Curiously, UV61 cells did not demonstrate an enhanced repair capability following UVC exposure, suggesting that inducible repair of UVC damage requires the transcription-coupled repair (TCR) pathway of NER (Liu and Rainbow, 2004). In this study, the enhanced HCR of the [MB+VL]-treated reporter gene in the *CSB*-deficient UV61 cells suggests that inducible repair of [MB+VL]-induced damage does not involve CSB. However, a reduced HCR capacity

was observed in UV61 cells following the introduction of a [MB+VL]-treated reporter gene (Chapter 2), and D_{37} values obtained from HCR curves in this study support the idea of an involvement of CSB in the repair of DNA damage induced by MB plus VL. It is possible that after stimulation of repair mechanisms by an initial exposure to UVC radiation, other factors than the CSB protein contribute to an enhanced HCR capacity of the cells. For example, although the CSB protein may be involved in the repair of [MB+VL]-induced DNA damage, it may not be required. This is supported by data from Chapter 2 that indicated a reduction in the rate of repair only in UV61 cells compared to the parental AA8, as β-gal activity was similar for both the *CSB*-deficient UV61 and the repair-proficient AA8 cell lines following extended periods of incubation between infection and scoring. Previous reports involving HCR of UVC-induced damage in CSB-deficient cells have indicated that certain repair mechanisms require a delay between pre-treatment and introduction of a damaged reporter gene to obtain an inducible effect (Pitsikas *et al.*, 2005). This may be a result of other CSB-independent factors, such as the involvement of UV-damaged DNA binding (UV-DDB) proteins in the enhancement of repair (as reviewed by Pitsikas *et al.*, 2005).

Results of HCR assays involving the promoter-specific AdMCMV*lacZ* virus further suggest that the repair of [MB+VL]-induced DNA damage is inducible in CHO cells by low levels of UVC radiation (Table 3.2). However, an HCR enhancement was only observed in the parental AA8 cells exposed to UVC radiation 6 hours prior to infection and not in cells infected immediately following UVC exposure. These results suggest that a lag period is required to observe enhanced repair of [MB+VL]-induced

DNA damage after treatment with UVC. This is consistent with the HCR results reported in Table 3.1, as after 40 hours of incubation following infection, AA8 cells received sufficient time for any beneficial effects a lag period might afford to occur, including the upregulation of repair proteins.

MB plus VL has been shown to create large amounts of 8-hydroxyguanine (8-oxoG) lesions in DNA (Floyd *et al.*, 1989), which are repaired by the BER pathway (as reviewed by Dianov *et al.*, 2001). Therefore, it was of interest to observe whether the introduction of a small amount of [MB+VL]-induced damage in the cells could stimulate the BER pathway and result in an enhanced HCR capacity of the reporter gene. Although an enhanced HCR capacity was observed in some experiments (Figure 3.3), repeated experimentation showed no significant increase in the repair of [MB+VL]-induced DNA damage following pre-treatment of cells with MB, VL, or both (Table 3.3). The D_{50} values (taken because the D_{37} values were not always obtainable) relative to untreated cells show a large increase in β-gal activity in pre-treated cells compared to untreated cells. However, the standard error in all cases is quite large, and the mean absolute D_{50} value for untreated cells is larger than the D_{50} value of other conditions. This indicates the variability between experiments, where a large enhancement is observed in cells treated with MB, VL, or MB plus VL compared to untreated cells in some experiments, but is absent in other experiments. These results indicate that the detection of a significant induction in the repair of [MB+VL]-induced DNA damage by pre-treatment of cells with MB, VL, or both could not be made under the conditions of the HCR assay employed. This does not rule out the possibility that enhanced HCR of β-gal activity

from a [MB+VL]-treated reporter gene following pre-treatment of cells with MB plus VL may be detectable using different conditions of the HCR assay.

Interestingly, there were large enhancements observed in HCR capacity of VL-treated cells compared to untreated cells in some experiments, suggesting that it may be possible to induce repair of DNA damage caused by MB plus VL by merely exposing cells to light. However, the large variability in β-gal activity observed amongst repeated experiments precludes the possibility of a definitive statement at present regarding the ability of VL, MB, or MB plus VL to induce repair of DNA damage.

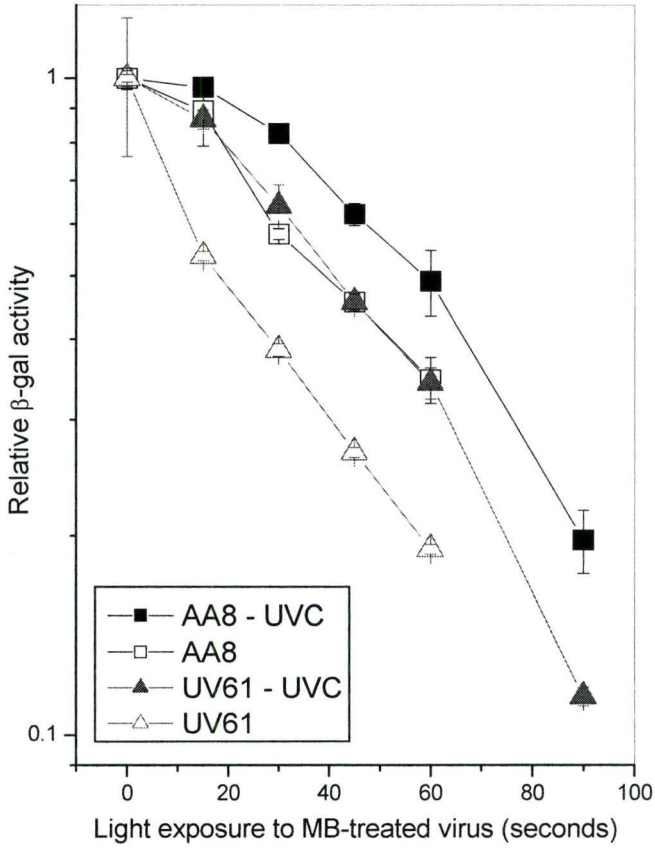

Figure 3.1 — Host cell reactivation of β-galactosidase (β-gal) activity for [MB+VL]-treated AdHCMV*lacZ* virus in select Chinese hamster ovary cell lines pre-treated with UVC radiation. Cells were exposed to 15 J/m^2 UVC radiation (solid shapes) immediately prior to infection with AdHCMV*lacZ* treated with 40 μg/mL MB and varying exposures to VL at an MOI of 20 pfu/cell and compared to untreated cells (open shapes). Cells were harvested 40 hours after infection and scored for β-gal activity. Results shown include AA8 (normal; ■ / □) and UV61 (▲ / △). Data points shown are the average value ± SE obtained through triplicate determinations from one experiment. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.)

Table 3.1 — D_{37} values obtained from host cell reactivation assays where UV61 and AA8 Chinese hamster ovary cell lines were exposed to 15 J/m^2 UVC radiation prior to infection with a [MB+VL]-treated AdHCMV*lacZ* virus. The average D_{37} ± SE for both untreated and UVC-treated cells, and the average D_{37} ± SE value for UVC-treated cells relative to untreated cells are reported. Results that were significantly different from the untreated cells (*i.e.* results compared to 1) by the two-sample independent *t*-test are also indicated ($P < 0.05$). Cells were exposed to UVC at a fluence rate of 1 J/m^2/s for 15 seconds immediately prior to infection with AdHCMV*lacZ* treated with 40 μg/mL MB and varying exposures to visible light at an MOI of 20 pfu/cell. Cells were harvested 40 hours after infection and scored for β-galactosidase activity. Results are pooled from three experiments with triplicate determinations. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.)

Cell Line	Absolute D_{37} (seconds)		Relative D_{37}	P Value	Significant?
	Untreated	UVC			
AA8	54.05 ± 4.43	71.80 ± 12.15	1.31 ± 0.13	0.0690	No
UV61	42.72 ± 5.99	68.87 ± 9.51	1.65 ± 0.22	0.0445	*Yes*

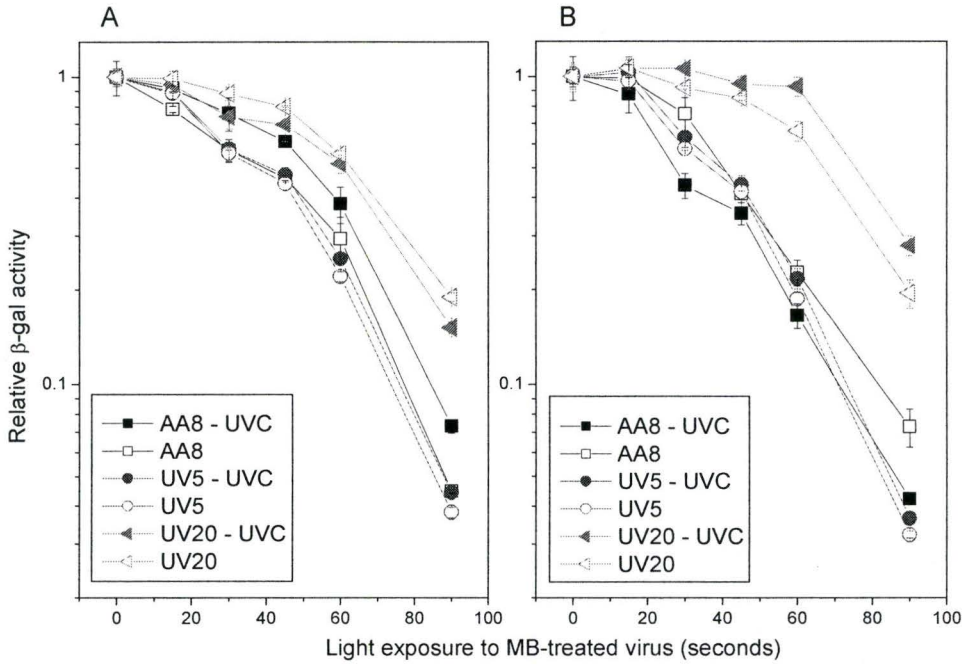

Figure 3.2 — Host cell reactivation of β-galactosidase (β-gal) activity for [MB+VL]-treated AdMCMV*lacZ* virus in select Chinese hamster ovary cell lines pre-treated with UVC radiation. Cells were exposed to 9 J/m^2 UVC radiation (solid shapes) 6 hours (A) or immediately (B) prior to infection with AdMCMV*lacZ* treated with 40 μg/mL MB and varying exposures to VL at an MOI of 20 pfu/cell and compared to untreated cells (open shapes). Cells were harvested 6 hours after infection and scored for β-gal activity. Results shown include AA8 (normal; ■ / □), UV5 (● / ○) and UV20 (◀ / ◁). Data points shown are the average value ± SE obtained through triplicate determinations from one experiment. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.)

Table 3.2 — D_{37} values obtained from host cell reactivation assays where select Chinese hamster ovary cell lines were exposed to 9 J/m^2 UVC radiation prior to infection with a [MB+VL]-treated AdMCMV*lacZ* virus. The average D_{37} ± SE for both untreated and UVC-treated cells, and the average D_{37} ± SE value for UVC-treated cells relative to untreated cells are reported (R-UVC). In addition, the average D_{37} ± SE value relative to AA8 are reported (R-AA8). Cells were exposed to UVC at a fluence rate of 1 J/m^2/s for 9 seconds either 6 hours (Time = 6h) or immediately (Time = 0h) prior to infection with AdMCMV*lacZ* treated with 40 μg/mL MB and varying exposures to visible light at an MOI of 20 pfu/cell. Cells were harvested 6 hours after infection and scored for β-galactosidase activity. Results are pooled from two experiments with triplicate determinations. (MB = methylene blue; VL = visible light; MOI = multiplicity of infection; pfu = plaque-forming units.)

Time	Cell Line	Absolute D_{37} (seconds)		Relative D_{37}	
		Untreated	UVC	R-UVC	R-AA8
0h	AA8	44.52 ± 3.17	45.37 ± 3.46	1.03 ± 0.15	1
	UV5	46.56 ± 0.65	51.66 ± 3.06	1.11 ± 0.08	1.05 ± 0.06
	UV20	73.49 ± 0.81	78.12 ± 4.79	1.06 ± 0.05	1.66 ± 0.10
6h	AA8	46.92 ± 5.57	54.72 ± 5.86	1.17 ± 0.01	1
	UV5	47.47 ± 1.53	52.88 ± 1.85	1.12 ± 0.08	1.02 ± 0.09
	UV20	71.99 ± 0.68	70.72 ± 2.55	0.98 ± 0.03	1.56 ± 0.20

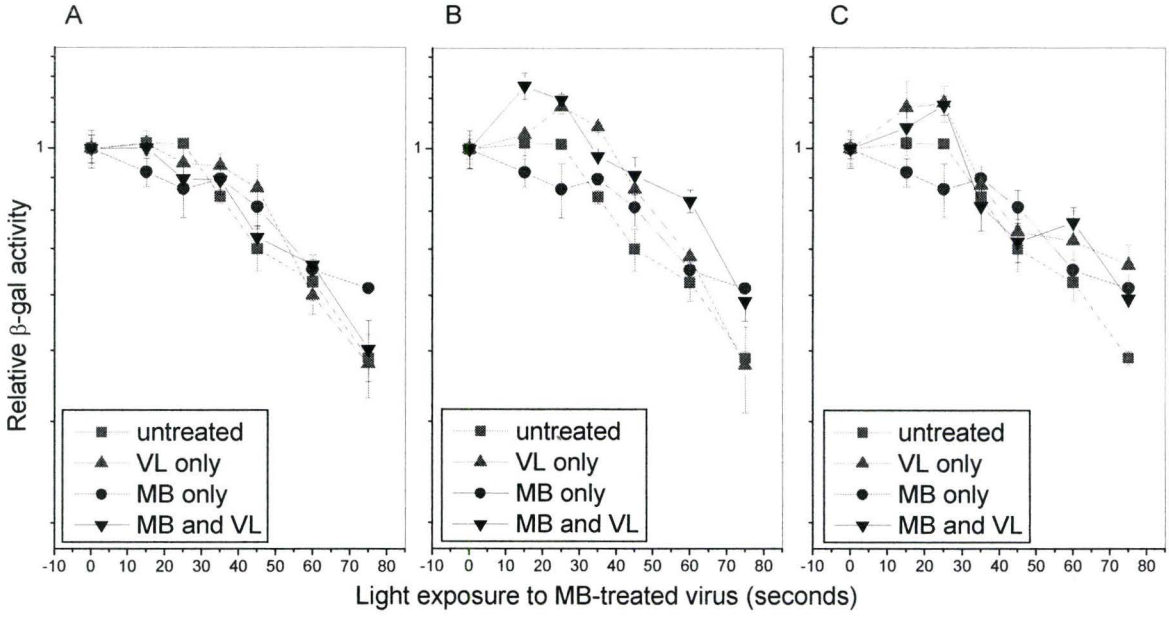

Figure 3.3 — Host cell reactivation of β-galactosidase (β-gal) activity for [MB+VL]-treated AdMCMV*lacZ* virus in CHO-AA8 cells pre-treated with MB (●) or VL (▲) or both (▼) and compared to untreated cells (■). Cells were incubated with α-MEM containing a MB concentration of 0 μg/mL or 10 μg/mL overnight (20-24 hours). Media was aspirated and replaced with PBS and cells were exposed to 7 (A), 14 (B), or 28 minutes (C) VL or left as a control immediately prior to infection with AdMCMV*lacZ* treated with 40 μg/mL MB and varying exposures to VL at an MOI of 40 pfu/cell. Cells were harvested 6 hours after infection and scored for β-gal activity. Data points shown are the average value ± SE obtained through triplicate determinations from one experiment. Cells incubated in 10 μg/mL MB or receiving VL exposure are indicated. (MB = methylene blue; VL = visible light; CHO = Chinese hamster ovary; α-MEM = α-minimum essential media; PBS = phosphate-buffered saline; MOI = multiplicity of infection; pfu = plaque-forming units.)

Table 3.3 — D_{50} values obtained from host cell reactivation assays where CHO-AA8 cells were pre-exposed to methylene blue (MB), visible light (VL) or both prior to infection with a [MB+VL]-treated AdMCMV*lacZ* virus. The average D_{50} ± SE, and the average D_{50} ± SE relative to untreated cells are reported. Results were not found to be significantly different than the untreated cells by the two-sample independent *t*-test ($P > 0.05$), as indicated. Cells were incubated with α-MEM containing a MB concentration of 0 μg/mL or 10 μg/mL overnight (20-24 hours). Media was aspirated and replaced with PBS and cells were exposed to 14 minutes VL or left as a control immediately prior to infection with AdMCMV*lacZ* treated with 40 μg/mL MB and varying exposures to visible light at an MOI of 40 pfu/cell. Cells were harvested 6 hours after infection and scored for β-galactosidase activity. Cells incubated in 10 μg/mL MB or receiving VL exposure are indicated. Results are pooled from four experiments with triplicate determinations. (CHO = Chinese hamster ovary; α-MEM = α-minimum essential media; PBS = phosphate-buffered saline; MOI = multiplicity of infection; pfu = plaque-forming units.)

Pre-treatment Conditions	Absolute D_{50} (seconds)	Relative D_{50}	P Value	Significant?
Untreated	43.07 ± 13.48	1		
VL Only	40.70 ± 11.98	1.71 ± 0.96	0.4866	No
MB Only	41.55 ± 15.75	1.51 ± 0.71	0.5022	No
MB plus VL	40.99 ± 13.92	1.66 ± 0.93	0.5058	No

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CHAPTER 4

Survival of base and nucleotide excision repair-deficient Chinese hamster ovary cell lines following treatment with methylene blue or methylene blue plus visible light

1.0 Abstract

Methylene blue (MB) is a thiazine dye that is becoming increasingly more useful as a diagnostic and therapeutic drug in such areas as methemoglobinemia, malaria and cancer due in part to its effectiveness as a photosensitizer. MB, upon excitation with visible light (VL), produces 8-hydroxyguanine (8-oxoG) lesions in DNA almost exclusively by means of singlet oxygen generation. The base excision repair (BER) pathway, responsible for removing single damaged bases from DNA, has previously been implicated in the repair of 8-oxoG lesions. Another repair mechanism known as nucleotide excision repair (NER) removes bulky lesions from DNA, and several NER proteins have been implicated in some aspects of BER. Although the effect of MB plus VL has been studied in some detail in DNA systems, investigations into its effects on whole cells have been limited. In the present study, clonogenic survival of Chinese hamster ovary (CHO) cells following treatment with MB alone or MB plus VL was examined in two parental repair-proficient cell lines (AA8 and K1), NER-deficient mutants from rodent complementation groups (RCG) 1 through 6, and the BER-deficient cell line EM9. The concentration of MB, or the duration of VL exposure that is required to reduce clonogenic survival to 37% of the untreated control (D_{37}) was used to measure the survival of CHO cells following treatment with MB alone or MB plus VL, respectively. The uptake of MB per cell was similar in all CHO cell lines tested. The sensitivities of the NER-deficient CHO cell lines from RCGs 2, 4 and 5 and the BER-deficient EM9 cell line to MB alone were within the range obtained for the two repair-

proficient CHO normal cell lines, AA8 and K1. In contrast, the UV20 cell line from RCG-1 was more sensitive to MB alone compared to the normal cell lines. Additionally, both UV24 (RCG-3; XPB) and UV61 (RCG-6, CSB) showed a decreased sensitivity to MB alone when compared to the normal AA8 cell line. The results of Chapter 2 indicated a reduced repair of [MB+VL]-damaged DNA only in UV61 cells and an enhanced repair in UV20 and EM9 cells compared to that in the repair-proficient normal cells. In contrast, the sensitivity of cells to MB plus VL was greater in the UV24, UV135 (RCG-5; XPG) and the *XRCC1*-deficient EM9 cell lines compared to that of the range obtained for the two repair-proficient normal cell lines, AA8 and K1. This suggests that [MB+VL]-induced DNA damage to cells and its repair do not play a major role in the survival of cells following treatment with MB plus VL. It appears likely that damage to cellular components other than DNA, such as protein, lipid and biological membranes, play a more important role in cell killing by MB plus VL.

2.0 Introduction

Methylene blue (MB) is a type II photosensitizer that produces singlet oxygen upon excitation by the visible light (VL) spectrum (as reviewed by Slameňová *et al.*, 2002). It has been shown to produce 8-hydroxyguanine (8-oxoG) lesions in DNA (Floyd *et al.*, 1989) with minimal strand breaks (Epe *et al.*, 1993). The base excision repair (BER) mechanism has been shown to repair 8-oxoG lesions almost exclusively (as reviewed by Dianov *et al.*, 2001). BER involves the removal of single damaged bases in DNA and results in their excision as free bases, as its name suggests (as reviewed by

Friedberg, 2001). In contrast, another highly conserved repair mechanism, nucleotide excision repair (NER), involves the removal of bulky lesions from DNA and their removal as part of an oligonucleotide fragment (as reviewed by Friedberg, 2001). A number of NER proteins have previously been reported to be involved in the BER pathway, such as XPG (as reviewed by Lindahl and Wood, 1999) and CSB (as reviewed by Pastoriza Gallego and Sarasin, 2003).

Past studies involving MB plus VL often focus on plasmids (Seah and Burgoyne, 2001), naked DNA (Floyd *et al.*, 1989) or viruses (Floyd *et al.*, 2004), and published reports concerning the effects of MB plus VL to whole cells have been limited. However, the potential use of MB as a human therapeutic agent, particularly with respect to malaria (Vennerstrom *et al.*, 1995), methemoglobinemia (Chui *et al.*, 2005) or cancer identification (Salhab *et al.*, 2005), has garnered increased attention to the effects of MB on living cells. An exploration of the effects of MB plus VL on whole cells is useful, as MB can also be used in photodynamic therapy. For example, Zeina *et al.* demonstrated that 100 μg/mL MB, upon excitation by VL from a 250-W source for 10 minutes, was effective at killing prokaryotic bacteria such as *Staphylococcus aureus*, but that eukaryotic species such as *Candida albicans* were much less susceptible to killing, even after 60 minutes of VL exposure (Zeina *et al.*, 2001).

Previous colony-forming assays involving photoactivated-MB have shown a large reduction in cultured human HeLa tumour cell colony formation following incubation with 5 μmol/L MB and exposure to a VL-emitting laser for 30 to 40 seconds (Schmidt *et al.*, 1991). Even in the absence of VL exposure, MB was shown to have a toxic effect on

the cells, reducing the relative survival to 77% of the untreated control (Schmidt *et al.*, 1991). The [MB+VL]-treated cells showed an even greater reduction to 16% of the untreated control (Schmidt *et al.*, 1991). Waki *et al.* demonstrated how cultured rodent cells incubated with 2 μM MB for 24 hours could be selectively killed with targeted white light, most likely from the creation of singlet oxygen and its subsequent interaction with various biological molecules like DNA, membrane lipids and other proteins (Waki *et al.*, 2005). The involvement of a reactive oxygen (ROS) species such as singlet oxygen in cell death is corroborated by evidence from Kirszberg *et al.* who showed that erythroleukemic cells incubated with 2 μg/mL MB that is subsequently activated resulted in higher cell death and a greater amount of ROS than normal peripheral blood mononuclear cells (Kirszberg *et al.*, 2005). The amount of ROS was measured by incubating cells with a non-fluorescent probe, dihydrorhodamine 123, which can become oxidized into the fluorescing rhodamine 123 upon interaction with ROS, and determining fluorescence intensity (Kirszberg *et al.*, 2005). Although the effectiveness of MB plus VL at killing certain cell types has been explored in published reports, the mechanism of cell death by MB plus VL has not been established in any detail. In order to examine the role of DNA damage and repair in the sensitivity of cells to MB plus VL, the clonogenic survival of selected BER- and NER-deficient Chinese hamster ovary (CHO) cell lines was investigated following treatment with MB or MB plus VL.

3.0 Materials & Methods

3.1 Cell Lines

Chinese hamster ovary (CHO) cell lines employed include: AA8 (parental); UV5 (RCG-2; XPD); UV20 (RCG-1; *ERCC1*); UV24 (RCG-3; XPB); UV41 (RCG-4; XPF); UV61 (RCG-6; CSB); UV135 (RCG-5; XPG); and EM9 (provided by Dr. Larry Thompson [Lawrence Livermore National Laboratory, Livermore, CA] with the help of Dr. Gordon Whitmore [Physics Division, Ontario Cancer Institute, Toronto, ON]). The *XRCC1*-deficient EM9, isolated based on its hypersensitivity to ethyl methanesulfonate, has a deficiency in excision repair caused by defective rejoining of anomalous DNA strand breaks (Thompson *et al.*, 1982). Additionally, another parental line was used, CHO-K1. All cell cultures were grown in α-minimum essential media (α-MEM: HyQ MEM Alpha Modification with L-glutamine, ribonucleosides and deoxyribonucleosides) supplemented with 10% fœtal bovine serum (FBS) and 1% antibiotic-antimycotic (100 μg/mL penicillin G sodium, 100 μg/mL streptomycin sulfate and 250 ng/mL amphotericin B in 0.85% saline) obtained from Gibco-BRL. Cultures were maintained in 37°C humidified air containing 5% CO_2.

3.2 Methylene Blue

Stock methylene blue solution was prepared by dissolving 0.02 g methylene blue trihydrate (C.I. 52015; methylthionine chloride, $C_{16}H_{18}ClN_3S \cdot 3H_2O$, FW 373.9) in 20.0 mL phosphate-buffered saline (PBS: 140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4 [pH 7.4]) that was warmed to 37°C. The resulting mixture was filter-

sterilized in the dark through a 0.2 μm filter to create a 1000 μg/mL solution. Stock solution was then aliquoted into volumes of 1 mL or less and stored at -20°C in the dark.

3.3 Clonogenic Survival Assay

Cells were counted on a haemocytometer and the desired number (300 cells per well) was seeded in a 6-well plate in 2 mL of supplemented α-minimum essential media (α-MEM). Following a 6-hour incubation period in 37°C humidified air and 5% CO_2, which allowed the cells to adhere to the surface of the plate, the media was aspirated from each well and replaced with 1 mL of supplemented α-MEM containing the desired concentration (0 – 80 μg/mL) of methylene blue (MB). MB concentrations were prepared with a set volume of supplemented α-MEM and variable volumes of phosphate-buffered saline (PBS) and MB to obtain the desired 1 mL volume. Plates were incubated again, this time for an overnight period (20 - 24 hours) and kept under aluminium foil to minimise the effects of ambient lighting.

Following the 20-hour incubation period, the media was aspirated from each well and the cells were washed with 1 mL of warmed PBS. The PBS was then aspirated from the wells and replaced with another 2 mL of warmed PBS. Plates requiring exposure to visible light were then placed under a 1000 W bulb (General Electric, GE R1000) at a distance of 83 cm from the source for a specified period of time. After illumination (or after adding PBS in the case of non-illuminated plates), the overlaying PBS was aspirated from each well and replaced with 2 mL of supplemented α-MEM. Plates were placed

under aluminium foil in humidified air at 37°C and 5% CO_2 for an incubation period of 5 days.

Following the incubation period, the plates were removed from incubation and the media was aspirated from each well. Approximately 1 mL of crystal violet solution (63% absolute ethanol, 27% H_2O, 10% methanol, 5 g/L crystal violet) was added to each well to stain colonies over a period of one hour. Plates were then submerged in water to remove any excess crystal violet solution. Colonies were subsequently counted using a VWR hand tally counter. Colonies containing a minimum of 32 cells were considered to have survived treatment.

3.4 Uptake of Methylene Blue by Chinese Hamster Ovary Cells

Cells were counted on a haemocytometer and seeded for confluence (7.5×10^5 cells per well) in a 12-well plate in 1.6 – 3.2 mL of supplemented α-minimum essential media (α-MEM) and allowed to incubate in humidified air for 6 hours at 37°C and 5% CO_2. Subsequent to the 6-hour incubation period that permitted the cells to adhere to the surface of the well, the media was aspirated from each well and replaced with 1 mL of supplemented α-MEM containing the desired concentration of methylene blue (MB). MB concentrations were prepared with a set volume of supplemented α-MEM and variable volumes of phosphate-buffered saline (PBS) and MB to obtain the desired 1 mL volume. Plates were incubated again, this time for an overnight period (20 - 24 hours) and kept under aluminium foil to minimise ambient lighting.

Following the 20-hour incubation period, the media was aspirated from each well and the cells were washed with 1 mL of warmed PBS. The PBS was also aspirated from the wells and replaced with either 500 µL of 2× trypsin-EDTA (0.5% trypsin, 5.3 mM EDTA • 4Na), for wells whose cells were to be counted, or 375 µL lysis buffer (0.01% Triton X-100, 1 mM $MgCl_2$, 100 mM phosphate buffer at pH 8.3), for wells whose absorbance was to be determined.

For cells that were trypsinised, 1.5 mL of supplemented α-MEM was added and the solution was mixed several times with a micropipette. The contents of the well were removed to a 15-mL Falcon tube and the number of cells within the solution was determined using a Z1 Coulter Particle Counter (Beckman Coulter) counting particles in the range of 9.672 µm – 21.66 µm. The number of cells present in a well following incubation with each concentration of MB at the time of lysis allowed for absorbance values to be corrected for a single cell.

Cells being lysed were incubated in humidified air at 37°C and 5% CO_2 for one hour while lysis occurred. Following lysis, 200 µL of each well's solution was removed to a new well from a 96-well microtitre plate. Absorbance at 662 nm (the optimal wavelength for MB) was determined using a microplate reader (SAFIRE, Tecan). It has previously been predicted that such a wavelength would be the most effective for permitting [MB+VL]-mediated processes (as reviewed by Floyd *et al.*, 2004). Absorbance per cell was plotted against MB concentration for each tested cell line.

4.0 Results

4.1 Effects of Unabsorbed Methylene Blue plus Visible Light on Chinese Hamster Ovary Cells

Preliminary experiments on the clonogenic survival of selected Chinese hamster ovary cell lines treated with methylene blue (MB) and immediately exposed to visible light (VL) (as opposed to allowing cells to absorb MB overnight) showed a reduced surviving fraction in the normal AA8 cells compared to the repair-deficient UV5 and UV61 cells. In these experiments, cells were seeded in a 6-well plate and permitted a 6-hour incubation period at 37°C and 5% CO_2, during which time the cells adhered to the surface of the wells. Following the 6-hour incubation period, MB was added to the wells and plates were exposed to VL, with the MB present in the extracellular media. Representative results of these preliminary experiments that included MB in the media during illumination are shown in Figure 4.1. The increased susceptibility to [MB+VL]-induced damage in the normal when compared to the repair-deficient cell lines was similar for both fixed MB concentration and variable VL exposure (Figure 4.1-A) and *vice versa* (Figure 4.1-B). The increased clonogenic survival of the nucleotide excision repair-deficient UV5 and UV61 compared to the repair-proficient AA8 was unexpected, as the repair-proficient AA8 cell line was hypothesised to have equal or better survival capability. These preliminary results suggested that clonogenic survival might be affected by damage to the exterior of the cell, such as membrane damage. The procedure was therefore modified to allow absorption of MB by the cells, thereby increasing the potential to observe DNA damage and reducing the potential damage to the outside of the

cells. This decision subsequently required an investigation into whether MB was absorbed differently amongst the tested cell lines.

4.2 Uptake of Methylene Blue by Chinese Hamster Ovary Cells

The uptake of methylene blue (MB) per cell of select base excision repair (BER)- and nucleotide excision repair (NER)-deficient Chinese hamster ovary (CHO) cell lines was investigated. An experiment was designed whereby the amount of MB absorbed by a particular cell line over a 20-24 hour period could be determined relative to other cell lines based on the absorbance per cell observed at the optimal wavelength for light absorption by MB. Representative results are shown in Figure 4.2. The expected increasing absorbance with increasing MB concentration is demonstrated in Figure 4.2-A. A linear relationship between relative absorption per cell and concentration was assumed over the 0 to 40 μg/mL MB range as shown in Figure 4.2-B and the slopes were obtained from the line-of-best-fit for each cell line. This assumption is substantiated by the high correlation coefficients, or *r*-values, obtained from each linear fit of data. An *r*-value of +1 would indicate a perfect, positive correlation between absorption per cell and MB concentration. The *r*-values obtained from each graph were all very close to +1, such as an *r*-value of +0.98764 for UV41, obtained from one experiment and shown in Figure 4.2-B. The resulting slopes were compared to the normal AA8 and showed no significant difference among the cell lines with multiple experiments, as summarised in Table 4.1. This suggests that there was no significant difference in absorption of MB at low

concentrations amongst the CHO cell lines observed following a 20-24 hour incubation period.

4.3 Effects of Methylene Blue Alone on Chinese Hamster Ovary Cells

The effects of methylene blue (MB) alone on clonogenic survival of base excision repair (BER)- and nucleotide excision repair (NER)-deficient Chinese hamster ovary (CHO) cells were investigated. The concentration of MB required to reduce survival to 37% of the untreated control (D_{37}) was used as a measure of cell sensitivity to MB alone. Representative survival curves are shown in Figure 4.3 and analyses of D_{37} values obtained from multiple experiments are summarised in Table 4.2 and Figure 4.4. There were no significant differences in the absolute D_{37} values obtained for each of the cell lines. The P values shown in Table 4.2 relate to the relative D_{37} values of each cell line compared to that for the AA8 cell line obtained in individual experiments. It can be seen that although both UV24 and UV61 showed an increased resistance to MB alone when compared to the parental AA8, the NER-deficient CHO cells from rodent complementation groups (RCGs) 2 through 5 and the BER-deficient EM9 cells all showed a sensitivity to MB alone which was within the range of the two repair-proficient normal lines, AA8 and K1. In contrast, the UV20 (RCG-1; *ERCC1*) cell line showed increased sensitivity compared to the normal cells.

4.4 Effects of Absorbed Methylene Blue plus Visible Light on Chinese Hamster Ovary Cells

The effects of methylene blue (MB) plus visible light (VL) on clonogenic survival of base excision repair (BER)- and nucleotide excision repair (NER)-deficient Chinese hamster ovary (CHO) cells were also investigated. A suitable MB concentration was determined from MB absorption assays and similar clonogenic survival assays involving MB alone and no VL. A concentration of 10 μg/mL MB was selected, due to the similarity in uptake at that concentration amongst all the CHO cell lines tested, as well as the low toxicity observed due to MB alone at lower concentrations. The duration of VL exposure required to reduce survival of cells to 37% of the unexposed control that received no VL (D_{37}) was used as a measure of sensitivity to MB plus VL. Representative results are shown in Figure 4.5 and analyses of D_{37} values obtained from multiple experiments are summarised in Table 4.3 and Figure 4.6. It can be seen that the sensitivity of cells to MB plus VL was similar in the NER-deficient cells from rodent complementation groups (RCGs) 1, 2, 4 and 6 compared to that of the two repair-proficient normal cell lines, AA8 and K1. In contrast, the sensitivity was greater in UV24 (RCG-3; XPB), UV135 (RCG-5; XPG) and the *XRCC1*-deficient EM9 cell lines compared to the normal cell lines.

5.0 Discussion

Preliminary experiments in this study observed the effects of methylene blue (MB) plus visible light (VL) on clonogenic survival of a repair-proficient parental and

two repair-deficient mutant Chinese hamster ovary (CHO) cell lines, where MB was present in the illuminated media (Figure 4.1). Results suggested that clonogenic survival was affected by extracellular [MB+VL]-mediated damage and assays were subsequently modified to allow overnight absorption of MB by cells in order to increase the likelihood of observing the effects of damage to DNA. Such a scenario is supported by previous reports that have shown that MB migrates from the cytoplasm to the nucleus after exposure to light in rat epithelial cells (Rück *et al.*, 1992). The presence of MB in the nucleus would permit DNA damage from [MB+VL]-derived singlet oxygen.

Previous published reports have shown a toxic effect of MB alone in living cells. Schmidt *et al.* found that a concentration of 10 μmol/L MB had a toxic effect on the human HeLa tumour cell line during colony-forming assays (Schmidt *et al.*, 1991). Additionally, MB was shown to produce skin and fat necrosis of the breast following its injection for diagnostic purposes in a patient with breast cancer (Salhab *et al.*, 2005). The reports of MB toxicity are consistent with the results of the current work, where clonogenic survival assays were employed to examine the effects of increasing MB concentrations with minimal ambient lighting on various base excision repair (BER)- and nucleotide excision repair (NER)-deficient CHO cell lines. Results showed a substantial toxic effect at high concentrations of MB on all cell lines tested (Figure 4.3). However, only the UV20 cell line showed a significant increase in senstitivity to MB alone compared to the two repair-proficient normal cell lines, AA8 and K1 (Table 4.2). Both UV24 and UV61 had a higher surviving fraction of colonies following incubation with MB compared to AA8, although the sensitivity was within the range of the two repair-

proficient normal cell lines. The damage introduced to cells during incubation with MB alone should not result in singlet oxygen-mediated DNA damage specifically, as VL has not activated MB. Therefore it is likely that cell survival is affected by damage created elsewhere in the cell. For instance, as MB has been shown to accumulate in the cytoplasm prior to illumination (Rück *et al.*, 1992), damage may arise to cell organelles and ultimately lead to cell death. An analysis of relative uptake of MB per cell suggested no differences in absorption amongst the various CHO cell lines (Table 4.1).

To determine the effects of MB plus VL on clonogenic survival of selected BER- and NER-deficient CHO cell lines, a MB concentration of 10 μg/mL was employed and the duration of light exposure was varied. This concentration is consistent with a number of other published reports that used the same MB concentration (Slameňová *et al.*, 2002). The variable effects of MB alone on clonogenic survival were effectively eliminated as illuminated colonies were compared to non-illuminated colonies that were also incubated with 10 μg/mL MB. Results showed differences in clonogenic survival from MB plus VL amongst the various cell lines. However, with the exception of UV24, UV135 and EM9, an analysis of D_{37} values shows that survival of the other NER-deficient cell lines was within the range presented by the two repair-proficient parental lines (Table 4.3). These results suggest that UV24, UV135 and EM9 cells are more sensitive to [MB+VL]-induced damage than other repair-proficient or NER-deficient cell lines.

It is possible that the observed deficiencies in clonogenic survival following treatment with MB plus VL are due to deficiencies in DNA repair; suggesting XPB, XPG and XRCC1 are involved in the repair of [MB+VL]-induced DNA damage. The

increased sensitivity to MB plus VL of the BER-deficient EM9 cells relative to all the other cell lines tested is consistent with the deficiency in short-patch BER of EM9 cells. Short-patch BER has been shown to repair the most prominent DNA lesion caused by MB plus VL, 8-hydroxyguanine (8-oxoG) (as reviewed by Dianov *et al.*, 2001), and the XRCC1 protein that is deficient in EM9 cells is involved in the final stages of short-patch BER (as reviewed by Boiteux and Le Page, 2001). In contrast, the host cell reactivation (HCR) assays (Chapter 2) indicated an increased repair of [MB+VL]-induced DNA damage in EM9 cells compared to that in normal cells.

In addition, the increased sensitivity of the UV24 and UV135 cells to MB plus VL did not correlate with the normal capacity for HCR of [MB+VL]-damaged DNA in these cells. Likewise, the reduced HCR capacity of the UV61 cells did not correlate with the normal sensitivity of UV61 cells themselves to MB plus VL and the increased HCR capacity of UV20 cells did not correlate with the normal sensitivity of UV20 cells to MB plus VL. Taken together, these results suggest that the variability in clonogenic survival amongst the cell lines following MB plus VL does not result from the differences in NER among the cell lines tested and further suggest that [MB+VL]-induced DNA damage to cells and its repair by NER do not play a significant role in the survival of CHO cells following treatment with MB plus VL. It appears more likely that the MB plus VL treatment results in cell death due to peripheral damage to the cells. For example, singlet oxygen, which is produced by MB, has been implicated in protein, lipid and biological membrane damage (as reviewed by Tuite and Kelly, 1993). In addition, singlet oxygen has been shown to activate the nuclear factor-κB (NF-κB) transcription factor (as

reviewed by Matroule and Piette, 2000). Although NF-κB activation traditionally serves to prevent apoptosis and programmed cell death, it has sometimes been implicated as becoming a transcriptional activator of proapoptotic genes (as reviewed by Luo *et al.*, 2005), as is the case with murine clonal osteoblasts (Kitajima *et al.*, 1996). MB itself has previously been shown to activate the NF-κB transcription factor in human lymphocytes and monocytes (Piret *et al.*, 1995), and it may have an effect on clonogenic survival of the CHO cell lines tested. Due to the manner in which the mutant cell lines were derived from the parental lines (as reviewed by Friedberg *et al.*, 1995), mutations in the genome were likely created in sections of the DNA other than the respective BER or NER genes. Such modifications may play a role in clonogenic survival of [MB+VL]-induced damage, such as altering genes associated with signaling pathways such as NF-κB. Additionally, previous studies involving other photosensitizers have suggested various consequences of whole-cell treatment. For example, the singlet oxygen-producing rose bengal photosensitizer, which has previously been shown to produce similar damage to that produced by MB (Epe *et al.*, 1988), has demonstrated the ability to form protein peroxides within human monocyte-like cells upon excitation by VL (Wright *et al.*, 2003). Protein peroxides can have a negative effect on cell survival, leading to dimerization, aggregation, fragmentation, unfolding, and conformational changes of cellular proteins (as reviewed by Wright *et al.*, 2003). Similarly, another thiazine dye that is structurally related to MB, toluidine blue, has been shown to produce fractured DNA in human leukemia cells that led to apoptosis (Tremblay *et al.*, 2002).

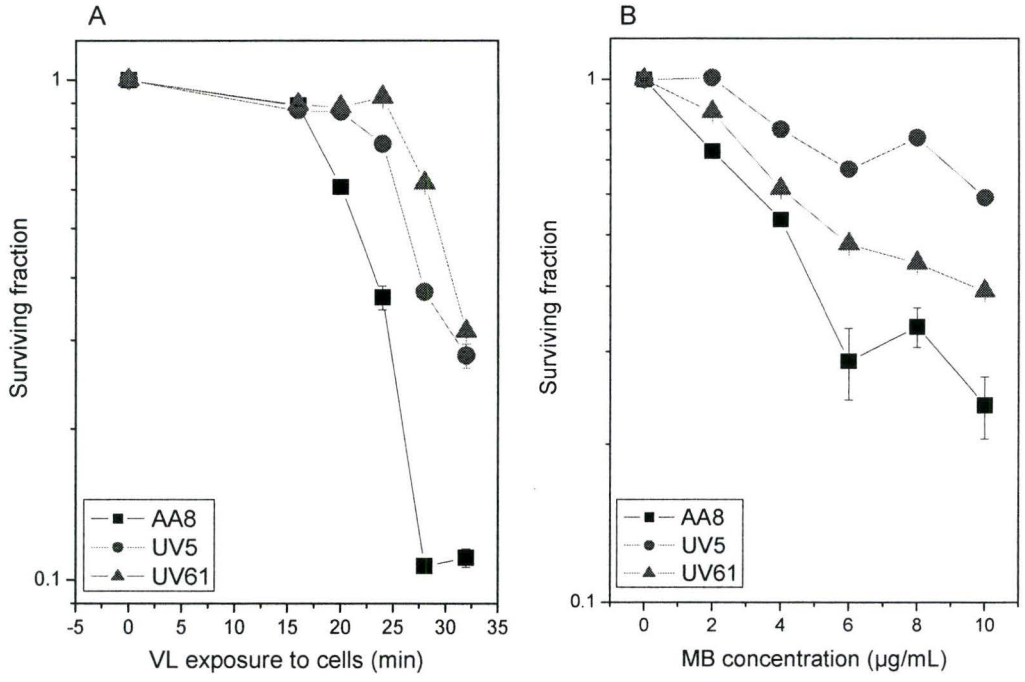

Figure 4.1 — Clonogenic survival of selected Chinese hamster ovary cell lines following treatment with methylene blue (MB) plus visible light (VL) where MB was present during illumination. Cells were seeded in a 6-well plate and given either 10 µg/mL MB and variable durations of VL exposure (A) or 28 minutes of VL and increasing concentrations of MB (B). MB was present in the wells during illumination with VL. Colonies were counted six days after treatment. Results shown are representative experiments and include AA8 (normal; ■), UV5 (●) and UV61 (▲). Data points shown in each graph are the average value ± SE obtained through triplicate determinations from one experiment.

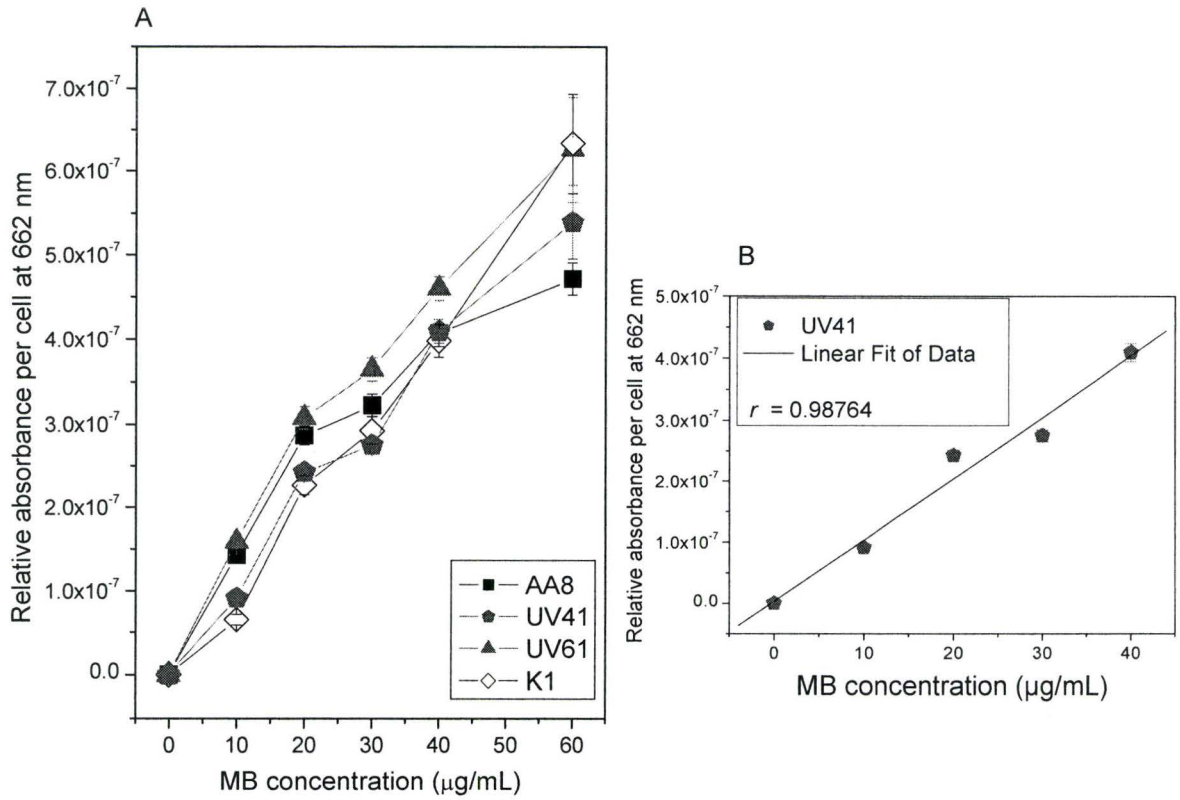

Figure 4.2 — Relative uptake of methylene blue (MB) per cell for selected Chinese hamster ovary cell lines. (A) Cells were incubated in humidified air at 37°C and 5% CO_2 with varying concentrations of MB overnight (20-24 hours). Media was removed and cells were lysed. The absorbance of the resulting solution was obtained from a 96-well mictrotitre plate reader and the value was corrected for the number of cells present (determined from Coulter Counter). The results shown are from a representative experiment and include AA8 (normal; ■), UV41 (⬟), UV61 (▲) and K1 (normal; ◇). Data points shown are the average value ± SE obtained through triplicate determinations from one experiment. (B) Relative absorbance per cell was assumed to be linear over the range of concentrations from 0 to 40 μg/mL MB. A representative graph displaying the line-of-best-fit for UV41 is shown using the data from the graph in A.

Table 4.1 — Slope values obtained from relative uptake per cell of increasing methylene blue (MB) concentrations in selected Chinese hamster ovary cell lines where uptake over the range of 0 to 40 μg/mL was assumed to be linear. The average slope ± SE, and the average slope ± SE relative to the parental AA8 are reported, in addition to the number of experiments (N) used to determine the values. Results where $N \geq 2$ were analysed by the two-sample independent *t*-test and relative uptake in each tested cell line was shown not to be significantly different than the normal. The cells were incubated with increasing MB concentrations over a 20-24 hour period and subsequently lysed and absorbance of lysate was observed at 662 nm (the optimal wavelength for MB) and calculated relative to the number of cells counted following the same treatment.

Cell Line	N	Absolute Slope ($\mu g/mL\ MB^{-1}$)	Relative Slope	P Value	Significant?
AA8	4	$2.00\times10^{8} \pm 3.49\times10^{9}$	1		
UV5	2	$2.50\times10^{8} \pm 3.17\times10^{9}$	1.01 ± 0.08	0.8808	No
UV20	1	2.41×10^{8}	1.03		
UV24	1	2.02×10^{8}	0.86		
UV41	3	$1.92\times10^{8} \pm 5.48\times10^{9}$	1.05 ± 0.10	0.6714	No
UV61	3	$2.01\times10^{8} \pm 4.54\times10^{9}$	1.08 ± 0.03	0.0628	No
UV135	1	2.59×10^{8}	1.10		
EM9	1	2.45×10^{8}	0.95		
K1	2	$1.44\times10^{8} \pm 4.20\times10^{9}$	0.96 ± 0.07	0.6160	No

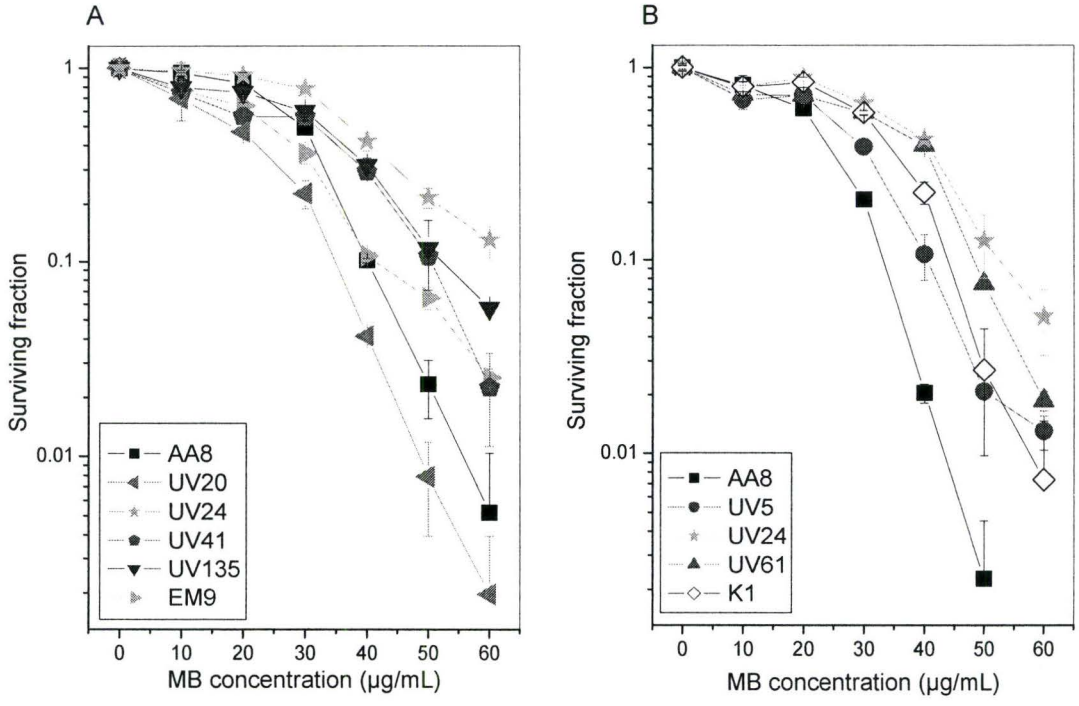

Figure 4.3 — Clonogenic survival of selected Chinese hamster ovary cell lines following overnight incubation with increasing concentrations of methylene blue (MB). Cells were seeded in a 6-well plate at 300 cells/well and incubated in humidified air at 37°C and 5% CO_2 with varying concentrations of MB overnight (20-24 hours). MB was aspirated the following day and colonies were subsequently counted five days later. Results shown are representative experiments and include AA8 (normal; ■), UV5 (●), UV20 (◀), UV24 (★), UV41 (⬟), UV61 (▲), UV135 (▼), EM9 (▶) and K1 (normal; ◇). Data points shown in each graph are the average value ± SE obtained through duplicate determinations from one experiment.

Table 4.2 — D_{37} values obtained from colony survival assays of exposure to methylene blue (MB) alone in selected Chinese hamster ovary cell lines. The average D_{37} ± SE, and the average D_{37} ± SE relative to the parental AA8 are reported, in addition to the number of experiments (N) used to determine the values. Results that are significantly different than the normal by the two-sample independent *t*-test ($P < 0.05$) are also indicated. Cells were seeded in a 6-well plate at 300 cells/well and incubated in humidified air at 37°C and 5% CO_2 with varying concentrations of MB overnight (20-24 hours). MB was aspirated the following day and colonies were subsequently counted five days later.

Cell Line	N	Absolute D_{37} (μg/mL MB)	Relative D_{37}	P Value	Significant?
AA8	6	31.78 ± 3.78	1		
UV5	3	36.89 ± 3.30	1.07 ± 0.08	0.4525	No
UV20	3	22.16 ± 5.84	0.76 ± 0.07	0.0282	*Yes*
UV24	3	37.17 ± 4.35	1.54 ± 0.11	0.0087	*Yes*
UV41	3	37.93 ± 2.39	1.06 ± 0.05	0.3026	No
UV61	3	48.64 ± 4.19	1.41 ± 0.12	0.0244	*Yes*
UV135	3	33.35 ± 8.65	1.14 ± 0.10	0.3442	No
EM9	3	30.04 ± 8.42	1.03 ± 0.12	0.8474	No
K1	3	39.33 ± 5.19	1.51 ± 0.21	0.0714	No

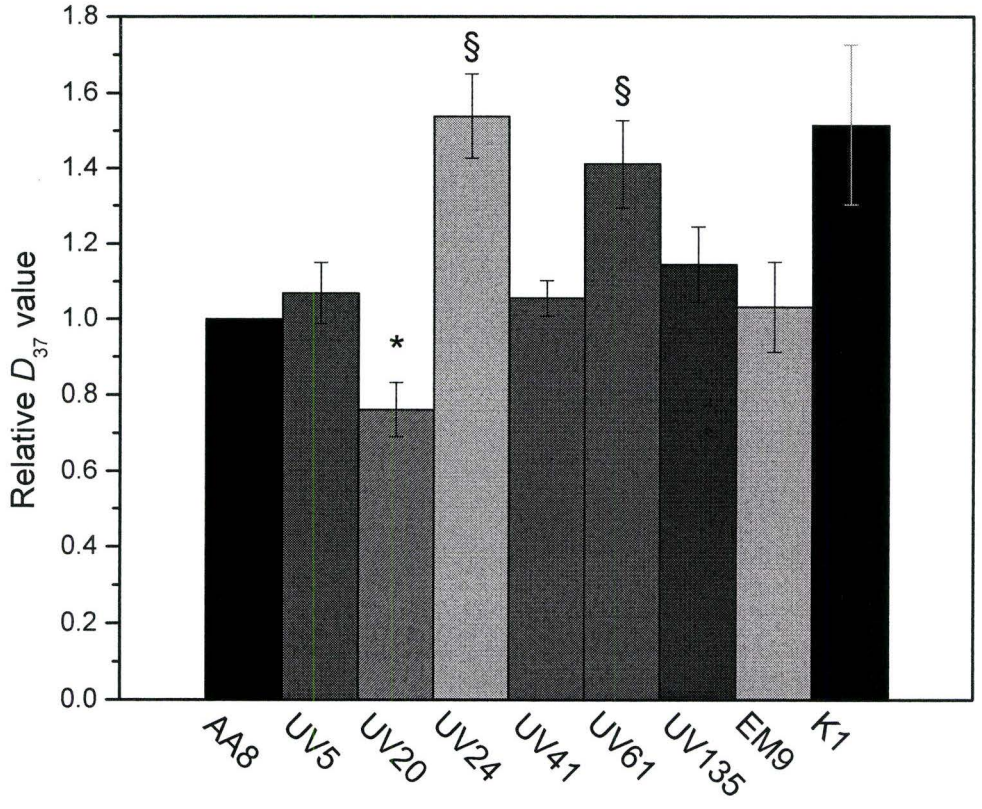

Figure 4.4 — Relative D_{37} values obtained from clonogenic survival assays of selected Chinese hamster ovary (CHO) cells treated with methylene blue (MB) alone. Results shown are the average ± SE of three experiments (or six experiments for AA8) relative to the normal, AA8. Values significantly lower (*) or higher (§) than the normal by the two-sample independent *t*-test ($P < 0.05$) are indicated. CHO cells were seeded at 300 cells/well in a 6-well plate and incubated overnight (20-24 hours) at 37°C and 5% CO_2 with various concentrations of MB. Unabsorbed MB was subsequently aspirated from the media and colonies were stained and counted five days later.

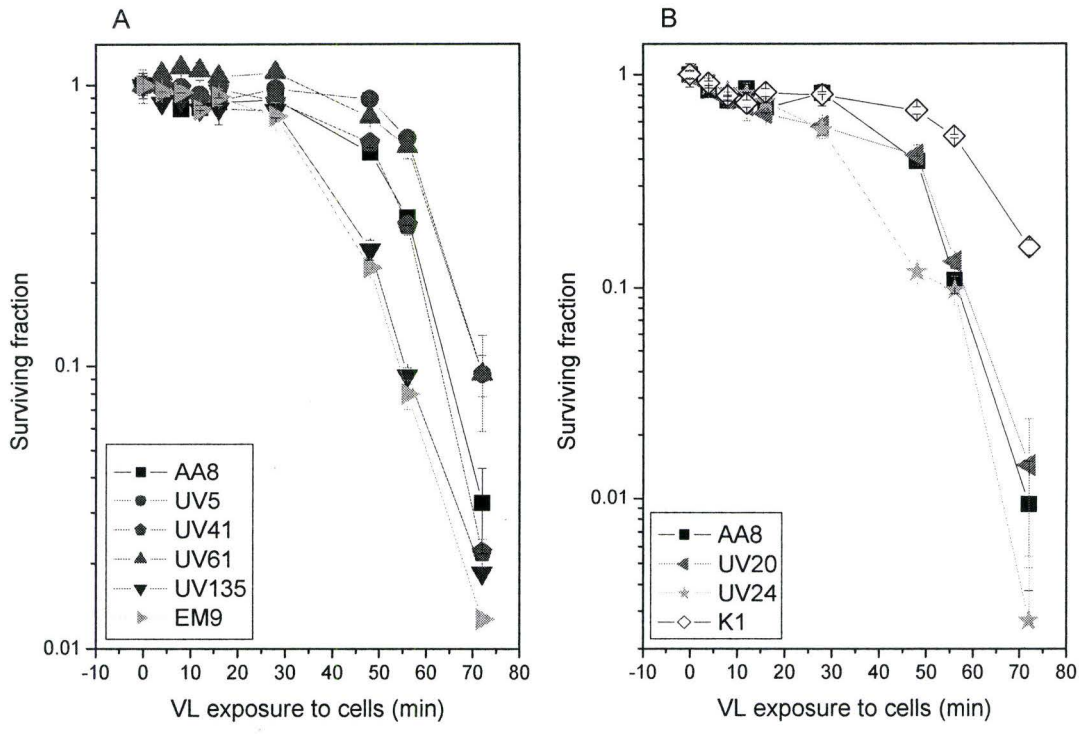

Figure 4.5 — Clonogenic survival of selected Chinese hamster ovary cell lines following overnight incubation with 10 μg/mL methylene blue (MB) and subsequent treatment with increasing lengths of visible light (VL) exposure. Cells were seeded in a 6-well plate at 300 cells/well and incubated in humidified air at 37°C and 5% CO_2 with a MB concentration of 10 μg/mL overnight (20-24 hours). MB was aspirated the following day and cells were subsequently exposed to VL for varying periods of time. Colonies were counted five days later. Results shown are representative experiments and include AA8 (normal; ■), UV5 (●), UV20 (◀), UV24 (★), UV41 (⬟), UV61 (▲), UV135 (▼), EM9 (▶) and K1 (normal; ◇). Data points shown in each graph are the average value ± SE obtained through duplicate determinations from one experiment

Table 4.3 — D_{37} values obtained from colony survival assays of exposure to methylene blue (MB) plus visible light (VL) in selected Chinese hamster ovary cell lines. The average $D_{37} \pm$ SE, and the average $D_{37} \pm$ SE relative to the parental AA8 are reported, in addition to the number of experiments (N) used to determine the values. Results that are significantly different than the normal by the two-sample independent *t*-test ($P < 0.05$) are also indicated. Cells were seeded in a 6-well plate at 300 cells/well and incubated in humidified air at 37°C and 5% CO_2 with MB at a concentration of 10 μg/mL overnight (20-24 hours). MB was aspirated the following day and cells were exposed to increasing periods of exposure to VL. Colonies were subsequently counted five days later.

Cell Line	N	Absolute D_{37} (minutes)	Relative D_{37}	P Value	Significant?
AA8	5	52.80 ± 2.26	1		
UV5	2	48.81 ± 11.87	0.91 ± 0.20	0.6884	No
UV20	2	50.38 ± 1.47	0.94 ± 0.08	0.4852	No
UV24	2	33.08 ± 0.09	0.62 ± 0.07	0.0310	*Yes*
UV41	3	56.04 ± 3.04	1.03 ± 0.02	0.2486	No
UV61	3	57.89 ± 1.29	1.04 ± 0.06	0.5170	No
UV135	3	37.56 ± 2.44	0.72 ± 0.02	0.0003	*Yes*
EM9	3	30.23 ± 6.34	0.55 ± 0.10	0.0113	*Yes*
K1	3	66.62 ± 10.14	1.33 ± 0.16	0.1066	No

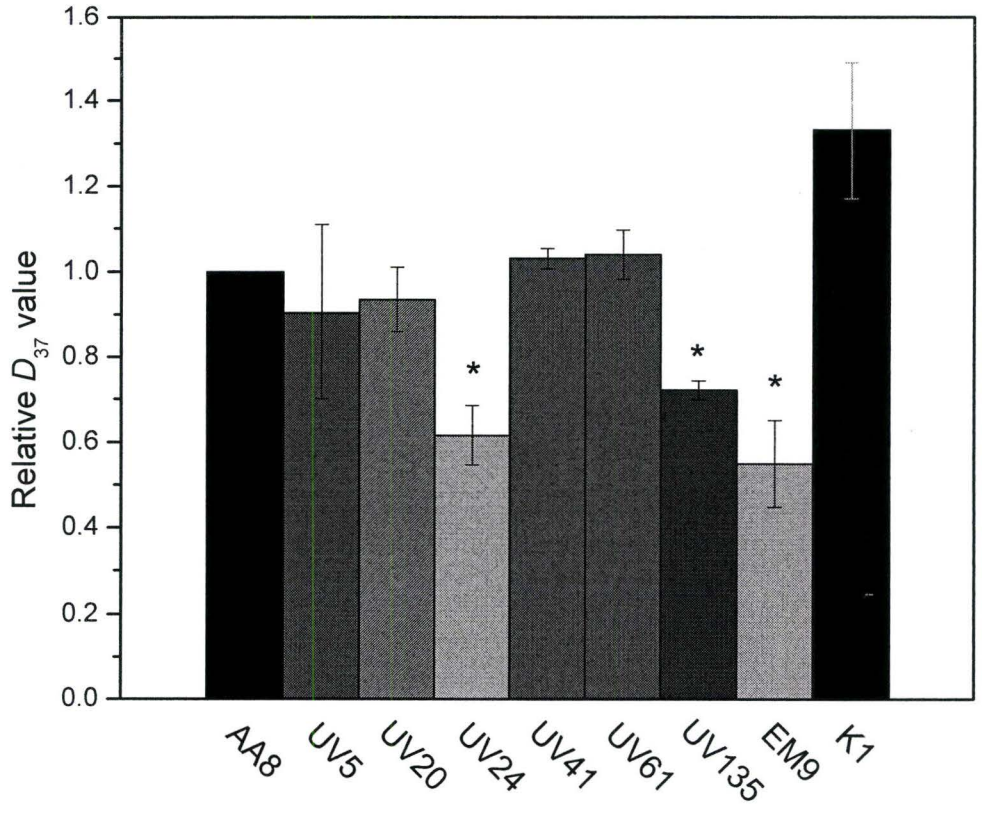

Figure 4.6 — Relative D_{37} values obtained from clonogenic survival assays of selected Chinese hamster ovary (CHO) cells treated with methylene blue (MB) plus visible light (VL). Results shown are the average ± SE of two to three experiments (or four experiments for AA8) relative to the normal, AA8. Values significantly lower (*) than the normal by the two-sample independent *t*-test ($P < 0.05$) are indicated. CHO cells were seeded at 300 cells/well in a 6-well plate and incubated overnight (20-24 hours) at 37°C and 5% CO_2 with 10 μg/mL MB. Unabsorbed MB was subsequently aspirated from the media and plates were exposed to increasing durations of VL exposure. Colonies were stained and counted five days later.

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CHAPTER 5

Summary and Future Initiatives

1.0 Summary

Host cell reactivation (HCR) of a β-galactosidase (β-gal) reporter gene treated with methylene blue (MB) plus visible light (VL) in select Chinese hamster ovary (CHO) cell lines showed differences between the parental cell lines and some cell lines deficient in nucleotide excision repair (NER) or base excision repair (BER). Specifically, the *XRCC1*-deficient EM9 cells showed a higher HCR of β-gal activity from a [MB+VL]-treated reporter gene relative to the parental AA8, suggesting a beneficial role for an XRCC1 deficiency or a specific gain-of-function gene mutation in the *XRCC1* gene for the repair of [MB+VL]-induced DNA damage. Similarly, an increased HCR capacity was observed in the *ERCC1*-deficient UV20 cell line when compared to AA8 cells, but interestingly both the *ERCC1*-deficient 30PV cells, and the *ERCC1* knock-out cell line, CHO-7-27, showed no significant increase in the HCR of β-gal activity compared to the parental K1. The *CSB*-deficient UV61 cells showed a significant reduction in HCR capacity compared to AA8 cells, suggesting a role for CSB in the repair of [MB+VL]-induced DNA damage.

The clonogenic survival assays showed an increased sensitivity to MB plus VL in the *XRCC1*-deficient EM9 cells as well as the *XPB*-deficient UV24 and *XPG*-deficient UV135 cell lines. The increased sensitivity to MB plus VL in the BER-deficient EM9 cells is consistent with the role of XRCC1 in short-patch BER, which has previously been shown to repair 8-hydroxyguanine (8-oxoG) lesions. However, these results are inconsistent with the increased HCR capacity of [MB+VL]-treated DNA observed in

EM9 cells. It may be that in the short-term, the increased HCR capacity is due to an initial increase in the rate of repair, whereas in the long-term, the deficiency in XRCC1 negatively affects survival. This may be due to a possible increase in the long-patch BER of 8-oxoG lesions, which has previously been shown to repair 8-oxoG lesions, although at a reduced rate than short-patch repair. However, the conflicting data suggests that the observed deficiencies in clonogenic survival amongst the various cell lines are likely an effect of [MB+VL]-mediated damage in other areas of the cell than DNA.

The lack of reproducibility in the HCR of β-gal activity from [MB+VL]-treated AdMCMV*lacZ* in cells pre-exposed to MB plus VL might be a result of the damage introduced to the cells by MB plus VL itself. For example, the absolute sensitivity of each cell line following treatment with MB plus VL was shown to vary somewhat between successive experiments, although the sensitivity relative to other cell lines remained more consistent. Therefore pre-treatment of cells with MB plus VL may reduce survival or inhibit growth of cells to different degrees between experiments. The resulting changes in cell density may affect the HCR capacity of the [MB+VL]-treated reporter gene in the pre-treated cells compared to untreated cells.

Pre-treatment of CHO-AA8 cells with UVC showed an enhanced HCR capacity for the [MB+VL]-treated reporter gene in all experiments. However, further experimentation is required to determine whether these results are significant. If so, this suggests that BER of [MB+VL]-treated DNA is inducible. An enhanced HCR of β-gal activity of the [MB+VL]-treated reporter gene was also observed in UV61 cells pre-treated with UVC compared to untreated cells, and results of UVC pre-treatment

experiments involving AdMCMV*lacZ* also seem to suggest that BER is inducible by exposure to low levels of UVC radiation.

2.0 Future Initiatives

Several aspects of these experiments could be further explored. In the host cell reactivation (HCR) assays, the conflicting results obtained between the *ERCC1*-deficient UV20 and the other *ERCC1*-deficient cell line, 30PV, as well as the *ERCC1* knock-out, CHO-7-27 should be further investigated. It would be of interest to re-introduce a functional copy of the *ERCC1* gene by means of another recombinant adenovirus, thereby restoring the proper gene product, and subsequently viewing the effects on HCR of DNA damage caused by methylene blue (MB) plus visible light (VL). Similarly, it would be of interest to restore the *CSB* function to UV61 cells and view whether such an act is able to rescue the deficiency in HCR capacity observed in UV61 cells compared to the parental AA8. The increased β-galactosidase (β-gal) activity observed in EM9 compared to the normal AA8 cells also warrants further investigation. For example, it may be possible to knock-down the activity of *XRCC1* in the normal AA8 to view whether it is the absence of XRCC1 that produces a beneficial HCR effect in EM9 cells. Further experimentation is required for both UV20 and EM9 cells to examine β-gal activity of [MB+VL]-treated AdMCMV*lacZ* at later times following infection (*i.e.* at 12 or 24 hours after infection) to determine whether the increased HCR capacity is a result of an increased rate of DNA repair.

Further experimentation is required to determine conclusively whether the base excision repair (BER) of a [MB+VL]-treated reporter gene is inducible by MB, VL, or both. Although it seems possible to induce the repair of DNA damage caused by MB plus VL by pre-treatment of cells with small amounts of [MB+VL]-induced damage, ideal conditions have not yet been identified that would permit such an observation. Additionally, the possibility of inducing repair by VL alone is worth investigating. Pre-treatment of cells with UVC radiation showed an increased HCR capacity of [MB+VL]-induced DNA damage, suggesting such repair is inducible. However, more experimentation is required to confirm or deny significance of the enhanced HCR capacity observed in pre-treated cells compared to untreated cells. An investigation into the difference in repair obtained with lag phases of various lengths between pre-treatment and infection should also be explored, as well as the effect of cell density on β-gal activity.

With respect to the clonogenic survival assays, experiments should be conducted to quantify the amount of 8-hydroxyguanine (8-oxoG) lesions being formed in the nuclear DNA of cells from various cell lines. Differences may be observed between cell lines showing an increased susceptibility to [MB+VL]-induced damage and less susceptible cell lines. An investigation into whether reduced survival is affected by the presence of various reactive oxygen species (ROS) quenchers would also be of interest. For example, the addition of a singlet oxygen quencher may help in understanding the differences in survival between cell lines. Additionally, it may be beneficial to conduct clonogenic survival assays with other oxidizing agents and photosensitizers in an effort to

produce more DNA damage and less damage to the surrounding cell, in order to observe the repair of oxidative damage.