## CELLULAR RESPONSES TO

### THE ANTI-CANCER DRUG, CISPLATIN

By

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### A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

## McMaster University

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# CELLULAR RESPONSES TO

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DOCTOR OF PHILOSOPHY (2001) (Biology) McMaster University Hamilton, Ontario

TITLE: Cellular Responses to the Anti-Cancer Drug, Cisplatin.

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NUMBER OF PAGES: xvii, 217

#### Abstract

*cis*-Diamminedichloroplatinum (cisplatin) and other platinum-based drugs, represent the best hope for cancer patients with ovarian tumours and many other types of solid tumours. Sadly, the clinical efficacy of cisplatin is often limited by the emergence of drug-resistant tumours. The research presented here is intended to expand understanding of the molecular characteristics that make cells resistant to cisplatin.

Cisplatin kills cells by damaging the genome and inducing cell suicide (apoptosis). Despite having been used for more than twenty years, the cellular responses responsible for this drug's cytotoxic effect remain fundamentally unknown.

When cisplatin reacts with DNA, a spectrum of addition products (adducts) are formed. Most of these adducts can be repaired by nucleotide excision repair (NER) and cells with defects in NER are extremely sensitive to the drug. In this thesis, we show that human and Chinese hamster ovary (CHO) cells with defects in NER are reduced in their capacity to repair cisplatin-damaged adenovirus (Ad) DNA.

Excision repair cross complementing gene-1 (ERCC1) is required for NER and many researchers have proposed that clinical cisplatin-resistance results from enhanced ERCC1 expression. To study the role of this gene in cisplatin resistance and DNA repair, we have constructed Ad viruses that express ERCC1. Cells infected with these viruses express very high levels of the protein. We report here that these very high expression levels actually impede DNA repair and make some human tumour cells cisplatin-sensitive.

The response to DNA damaging agents like cisplatin is dynamic. Upon exposure to cisplatin, many mechanisms that affect cell survival are induced. We show here that

some human tumour cells are much more sensitive to low doses of cisplatin (per unit of dose) than they are to higher doses. This hypersensitivity suggests that, at some critical dose of the drug, a mechanism that protects cells from death is triggered. Treating cells with other DNA damaging agents (including ultraviolet light (UV) and ionising radiation) before cisplatin exposure can induce this resistance. We have also induced resistance to low doses of cisplatin by infecting cells with UV-irradiated Ad suggesting that it is the DNA damage *per se* that triggers the mechanism of cisplatin resistance.

The Jun N-terminal kinase (JNK) is a family of mitogen-activated protein kinases (MAPKs) that responds to a broad range of cellular stresses. While the contribution of JNK activity to determining cell fate is complex, it is generally considered a proapoptotic factor. Several research groups have proposed that JNK activity is important in cisplatin-induced cell death.

By studying cisplatin-induced JNK activity in human xeroderma pigmentosum (XP) and Cockayne syndrome (CS) cells with well known DNA repair defects, we have shown that it is the persistence of cisplatin-DNA lesions that is associated with prolonged JNK activation. This is the first direct evidence that it is DNA lesions that promote JNK activity in cisplatin-treated cells. Further, we have used human cells with defective transcription-coupled repair (TCR) to show that damage in actively transcribing genes causes persistent JNK activity while persistent DNA damage in untranscribed regions does not. Our results support a role of JNK activation in cisplatin-induced cell death by showing that cells with defects in TCR are extremely sensitive to cisplatin while cells with defects in global genome repair are not.

#### Acknowledgements

I would like to thank my supervisor, Dr. Andrew Rainbow, for support and encouragement throughout my studies. I would also like to thank Dr. Gurmit Singh for helpful suggestions and for enduring many long meetings throughout his service on my committee. I greatly appreciate the willingness of Dr. JP Xu in joining my committee so late in my studies when I was in greatest need.

The staff of the Biology department has been very helpful to me through all of my graduate work. Their good humour and commitment is the glue (and the tape) that holds the building together.

I thank all those with whom I've shared many great experiences at McMaster and I would especially like to thank all those with whom I endured the long stretches between those 'great experiences'. I would also like to extend special appreciation to all those who have acknowledged me in their theses.

Thanks to Natalie and Susan for their affection and understanding through the darkest hours of my graduate studies.

Lastly, I'd like to thank my Mom and Dad and the rest of my family for their emotional support and for their unwavering confidence in me.

v

# **Table of Contents**

Abstract	page iii
Acknowledgements	v
Table of Contents	vi
List of Figures	xii
List of Tables	xiii
Preface	xiv
Abbreviations	xv
Chapter 1. Literature review	1
Cisplatin: history and chemistry	2
Cisplatin-based cancer treatment	6
Combination therapy	7
Dose and response	8
Hypersensitivity and induced resistance	12
How does cisplatin cause cell death?	13
Apoptosis and cisplatin	14
Cisplatin resistance	15

<b>Classical mechanisms of resistance</b>	15
Reduced intracellular accumulation	15
Intracellular nucleophile levels	16
DNA repair	17
Mismatch repair (MMR)	17
Nucleotide excision repair (NER)	18
NER in humans	19
Xeroderma pigmentosum (XP)	19
Cockayne syndrome (CS)	20
NER in Chinese hamster ovary cells	21
The human NER mechanism	21
The role of ERCC1 in cisplatin resistance	22
Measuring DNA repair	25
Host cell reactivation (HCR)	26
HCR of adenovirus	26
Regulatory molecules and cisplatin resistance	28
Mitogen activated protein kinases (MAPKs)	28
MAPKs and cisplatin resistance	29
p38 MAPK and cisplatin resistance	32
ERK activity and cisplatin resistance	32
JNK activity and cisplatin resistance	33
Current research	34

vii

Chapter 2. Materials and methods	37
Cell lines and fibroblast strains	38
Media and cell culture	39
Recombinant adenovirus constructs	41
Virus propagation and quantification	42
UV-irradiation of virus	43
UV-irradiation of cells	43
Cisplatin stock solutions	43
Cisplatin-treatment of virus	44
Cisplatin-treatment of cells	44
Clonogenic survival assay	44
Host cell reactivation of viral DNA synthesis	45
Host cell reactivation of viral $\beta$ -galactosidase expression	46
In vitro JNK assay	47
Chapter 3. Host cell reactivation of cisplatin-treated adenovirus	
is reduced in nucleotide excision repair deficient mammalian cells and several human tumour cells.	48
Preface	49
Abstract	50
Introduction	50
Materials and Methods	51
Results	52

Discussion

Acknowledgements					55
	References				55
Chap cispla	ter 4. Overexpression tin-treated DNA and	of human ERC can sensitise tur	CC1 can impede r mour cells to cisp	epair of latin	57
	Preface				58
	Abstract				59
	Introduction				60
	Materials and Method	ds			62
	Results				65
	Discussion				71

Chapter 5. Pre-exposure of human squamous carcinoma cells to low doses of γ-rays leads to an increased resistance to subsequent low-dose cisplatin treatment. 80

Preface	81
Abstract	82
Introduction	82
Materials and Methods	83
Results	84
Discussion	86
Acknowledgements	89
References	89

Chapter 6. Investigating mechanisms of induced cisplatin-resistance in mammalian cells. 92		
Preface	93	
Abstract	94	
Introduction	95	
Materials and Methods	98	
Results	102	
Discussion	114	

Chapter 7. Persistent DNA damage in active genes activates the Jun N-terminal kinase in human cells. 123

Abstract	124
Introduction	125
Materials and Methods	128
Results	130
Discussion	142

Chapter 8. Summary and Discussion	
Nucleotide Excision Repair (NER) and cisplatin resistance	159
Low dose hypersensitivity to cisplatin	161
Activation of the Jun N-terminal kinase	162

## **Chapter 9. References**

Appendix A. Studying the effects of several oncogenes on the cisplatin-resistance ofhuman fibroblasts.206

Appendix B. Nucleotide excision repair in genetically unstable CHO cells.

# List of Figures

	page
Figure 1-1. DNA damage caused by cisplatin.	5
Figure 1-2. Schematic representations of survival curves	11
Figure 1-3. Scheme showing the current model of the assembly of the human NER incision complex	24
Figure 1-4. The MAPK regulatory signal transduction pathways	31
Figure 4-1. Effect of ERCC1 expression on HCR of cisplatin-treated Ad in CHO cells.	73
Figure 4-2. Expression of exogenous ERCC1 is protective against cisplating in NER-deficient cells from complementation group 1.	n 76
Figure 6-1. The SCC-25 human tumour cell line is extremely sensitive to low doses of cisplatin.	104
Figure 6-2. The Chinese hamster ovary cell line, AA8, is extremely sensit to low doses of cisplatin.	ive 107
Figure 6-3. Infection with UV-irradiated adenovirus protects SCC-25 cells from subsequent low doses of cisplatin.	s 113
Figure 6-4. Cisplatin exposure causes no detectable increase in the HCR of UV-irradiated adenovirus.	116
Figure 6-5. Activation of JNK does not appear to be involved in induced cisplatin resistance.	119
Figure 7-1. The colony forming ability of cisplatin-treated XP-A fibroblast is greatly reduced compared to normal fibroblasts.	sts 132
Figure 7-2. Activation of JNK-1 is greater following cisplatin exposure in XP-A fibroblasts compared with normal fibroblasts.	135
Figure 7-3. Activation of JNK-1 in XP-A and normal fibroblasts.	138
Figure 7-4. Activation of JNK-1 is more persistent in XP-A fibroblasts compared with repair proficient fibroblasts.	141

Figure	7-5. Cells with defective transcription-coupled repair are sensitive to cisplatin while cells deficient in global genome repair are similar to normal cells in their cisplatin sensitivity.	144
Figure	7-6. Activation of JNK-1 in CS and normal fibroblasts.	147
Figure	7-7. Cisplatin-induced activation of JNK-1 in XP-C fibroblasts is similar to that in normal fibroblasts.	150
Figure	7-8. JNK-1 activity reaches a maximum 6 hours after cisplatin treatment in XP-C fibroblasts.	153
Figure	8-1. Model representing the role of JNK in determining the fate of cisplatin-treated mammalian cells.	165
Figure	A-1. The effects of oncogene expression on the cisplatin-sensitivity of primary human fibroblasts.	210
Figure	B-1. Host cell reactivation of $\beta$ -galactosidase synthesis in Chinese hamster ovary cell clones with delayed genomic instability.	215
Figure	B-2. Host cell reactivation of $\beta$ -galactosidase synthesis in Chinese hamster ovary cell clones with delayed genomic instability.	217

# List of Tables

	page
Table 2-1. Primary human fibroblasts used.	40
Table 2-2. CHO cells used.	41
Table 4-1. The effect of ERCC1 overexpression on HCR	71
Table 4-2. The effect of ERCC1 overexpression on the clonogenic surviv of HeLa cells following various 1 hour cisplatin treatments	al 70
Table 6-1. Pretreatment with UV or cisplatin protects AA8 and SCC-25 c from low doses of cisplatin	ells 109
Table 6-2. Infection with UV-irradiated Ad protects AA8 and SCC-25 cel from low doses of cisplatin	ls 110

### Preface

This thesis is comprised of a series of chapters in the form of journal articles. Two of these are currently published, and reproduced here (chapters 3 and 5). The remaining 3 manuscripts (chapters 4, 6, and 7) have been written to be submitted to Mutation Research, the International Journal of Radiation Biology and the Journal of Biological Chemistry, respectively. Where the work of others appears in this thesis, it is summarised in a preface to the chapter.

# Abbreviations

Ad	adenovirus
ADP	adenosine diphosphate
ATP	adenosine triphosphate
СНО	Chinese hamster ovary
cisplatin	cis-diamminedichloroplatinum(II)
CMV	cytomegalovirus
CS	Cockayne syndrome
CS-A	CS complementation group A
CSA	affected protein in CS-A
D <sub>37</sub>	dose required to cause a SF of 0.37 $(e^{-1})$
DIR	down-regulated by ionising radiation
DMIPS	digital microscope imaging platform system
DNA	deoxyribonucleic acid
DPC	DNA-protein crosslink
dsDNA	double stranded DNA
E1	early region 1 (of the Ad genome)
E3	early region 3 (of the Ad genome)
EDTA	ethylenediaminetetraacetic acid
ERCC	excision repair cross complementing
ERK	enzyme regulated kinase
FBS	foetal bovine serum
GGR	global genome repair
GSH	glutathione
Gy	gray
HCR	host cell reactivation
HEPES	N-2-hydoxyethylpiperazine-N'-2-
	ethanesulfonic acid
ICR	induced cisplatin resistance
IR	ionising radiation
IRR	induced radiation resistance
JNK	Jun N-terminal kinase

lacZ	β-galactosidase
LDH	low-dose hypersensitivity
LET	linear energy transfer
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	MAPK enzymatic kinase
MEKK	MEK kinase
a-MEM	alpha minimal essential medium
MMR	mismatch repair
MOI	multiplicity of infection
mRNA	messenger RNA
MT	metallothionein
NA-AAF	N-acetoxy-acetylaminofluorine
NER	nucleotide excision repair
ONGP	o-nitrophenyl β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PARP	poly-ADP ribosyl polymerase
PBS	phosphate-buffered saline
pfu	plaque forming units
pol	polymerase
PMSF	phenylmethylsulfonylfluoride
Pt-GG	cisplatin intrastrand crosslink (2 guanines)
Pt-AG	cisplatin intrastrand crosslink (between
	adenine and guanine)
Pt-G/G	cisplatin interstrand crosslink (2 guanines)
RNA	ribonucleic acid
RPA	replication protein A
RIR	region of induced resistance
SDS	sodium dodecyl sulphate
SEM	standard error about the mean
SF	surviving fraction
SSB	DNA single strand break
SV40	simian virus 40
TE	Tris-EDTA
TCR	transcription-coupled repair

xvi

ſFII	transcription factor for RNA pol II
<b>Fris</b>	Tris(hydroxymethyl)aminomethane
JDS	unscheduled DNA synthesis
J <b>V</b>	ultraviolet light
XP	xeroderma pigmentosum
XP-A	XP complementation group A
XPA	affected protein in XP-A

# Chapter 1

Literature Review

#### **Cisplatin:** history and chemistry

*cis*-Diamminedichloroplatinum(II) (cisplatin) is a very useful drug in the treatment of cancer (Loehrer and Einhorn, 1984). The discovery of cisplatin was a fortunate event that resulted from investigating bacterial growth rates in electric fields. It was observed that the "field" was toxic when the electrical potential was created across platinum electrodes but not when electrodes of other metals were used. Ironically, platinum was chosen for these experiments because of its "well known chemical inertness" (Rosenberg, 1965). The primary cytotoxic agent was later identified as a square-planar molecule with two chloride ions and two ammine groups bound to a tetravalent platinum atom in the, *cis* arrangement (Rosenberg, 1985). This observation, followed by years of careful study has led to the development of a cancer treatment that has saved the lives of many people. Cisplatin and one of its analogues, carboplatin (see figure 1), are used successfully in treating a broad range of tumour types that are refractory to other treatment modalities (Giaccone, 2000; Ferguson and Pearson, 1996; Loehrer and Einhorn, 1984).

Cisplatin reacts readily and irreversibly with DNA and this is thought to endow cisplatin with its cytotoxicity (Sherman and Lippard, 1987). The first step in the cisplatin-DNA reaction is the nucleophilic attack on the platinum atom by the electronegative N7 atom of guanine that is exposed in the major groove of the DNA

double helix. This displaces one chloride ion and the second chloride ion is displaced by a second nucleophilic attack by a nearby electronegative site, usually the N7 position on a neighbouring purinic base. A number of platinum-based analogues are currently being investigated for their therapeutic value (Tallen et al., 2000) but only cisplatin and carboplatin are in common use. Cisplatin and carboplatin differ only in their so-called 'leaving groups'; carboplatin has a cyclobutanedicarboxylate group instead of chloride ions as is shown in figure 1-1.

Because the reactivity of cisplatin depends on the loss of chloride ions to solution, the drug is quite stable in solutions with a high chloride-ion concentration. Since the chloride ion concentration is much greater in the extracellular fluid than in the cytosol, the drug is stable in the blood plasma and much more reactive within cells. This property is thought to limit the drug to reactions with targets within cells (Sherman and Lippard, 1987) but experimental proof of this is unclear (Jennerwein and Andrews, 1995).

This two-step addition produces a spectrum of damage that causes local distortions in the DNA (Rice et al., 1988; Malinge et al., 1994). 65% of addition products (adducts) are cross-links between adjacent guanine residues on the same strand (Pt-GG) and 25% of adducts link a guanine residue with an adjacent adenine residue (Pt-AG). The remaining adducts are mostly lesions that bind two guanines on the same strand with one intervening base (Pt-GNG). Cross-links between guanines on opposite strands (Pt-G/G) and DNA-protein cross-links are also produced with low frequency (Eastman, 1986). Carboplatin is less reactive but forms adducts that are chemically identical, except that carboplatin does not show preferential formation of the Pt-GG

Figure 1-1. Cisplatin, carboplatin, and transplatin and the adducts they produce. A. Cisplatin and carboplatin are commonly used in cancer treatment. Transplatin is the non-therapeutic geometric isomer of cisplatin.
B. The most common adducts formed when cisplatin reacts with DNA are shown. The Pt-GG and the Pt-AG adducts comprise about 90% of adducts while the other diagrams represent less frequent events.



Pt-G/G

Pt-DNA-protein

cross-link (Blommaert et al., 1995). No consensus exists as to which species of lesion accounts for cisplatin's cytotoxicity but induction and removal of the Pt-AG adduct has been shown to correlate best with survival in some mammalian cells (Fichtinger-Schepman et al., 1995). Cisplatin's geometric isomer, transplatin, is much less toxic than cisplatin and has been of no therapeutic use. This difference in toxicity is likely because the geometry of transplatin does not allow the formation of the Pt-GG or Pt-AG lesions and the platinum-DNA adducts that are formed are quickly removed (Ciccarelli et al., 1985).

Each species of adduct causes different amounts of DNA bending and unwinding and each type interferes with DNA replication and gene transcription in many prokaryotic and eukaryotic systems (Heiger-Bernays et al., 1990; Comess et al., 1992; Corda et al., 1991; Corda et al., 1992; Corda et al., 1993; Mello et al., 1995; Bulmer et al., 1996). Recent evidence suggests that cisplatin may not impede transcription directly but by preventing the initiation of transcription. The presence of cisplatin-damaged DNA inhibits transcription *in trans* from undamaged templates in human tumour cell extracts while the Pt-GG lesion does not detectably interfere with transcript elongation by human RNA polymerase II (RNA polII) (Cullinane et al., 1999). It is likely that the degree to which cisplatin adducts prevent DNA function depends on the organism and detecting the impedence is sensitive to the nature of the assay used.

#### Cisplatin-based cancer treatment: usefulness and limitations

While intrinsic cisplatin-resistance varies greatly between different cancers, most tumours are initially sensitive to the drug and acquire resistance through the course of treatment (McGuire and Ozols, 1998; Loehrer and Einhorn, 1984). Clearly, the cellular attributes that cause resistance are clinically important and have been the subjects of intense research. It is hoped that clinical protocols can be modified to maximise tumour killing while minimising treatment-related morbidity.

In addition to its antitumour activity, cisplatin is extremely toxic to healthy tissue and the morbidity associated with the drug limits its use. Prevalent side-effects associated with cisplatin treatment include: renal toxicity, peripheral neuropathy, ototoxicity, electrolyte imbalances (particularly of magnesium), bone marrow suppression, and infertility (Cooley et al., 1994). The dose-limiting toxicity is most often damage to the kidneys.

#### **Combination therapy**

Clinicians have experimented extensively in combining platinum-based chemotherapy with other cancer treatments. Therapeutic gains have been achieved by combining cisplatin or carboplatin with the mitosis inhibitor, paclitaxel, and the topoisomerase II inhibitor, etoposide (Choy et al., 1999; Giaccone, 2000). An obvious candidate therapy to combine with platinum-based chemotherapy is radiotherapy. Ionising radiation (IR) causes DNA strand breaks and oxidative base damage and is effective in the treatment of many tumour types for which cisplatin is also effective.

Cisplatin-IR combination therapy has been used effectively to treat many tumour types (Wheeler and Spencer, 1995).

Combination treatment with cisplatin and radiotherapy have proven to be more effective than radiotherapy alone in treating non-small cell lung cancer and some squamous cell carcinoma patients (Hazuka et al., 1994; Crissman et al., 1987). This effect may result from cisplatin-induced radiosensitisation caused by an inhibition in the repair of radiation-induced strand breaks (Dolling et al., 1998). Other workers have reported *in vitro* results that show low cisplatin or  $\gamma$ -ray doses can actually be protective against subsequent exposure to ionising radiation or cisplatin (Marples et al., 1997; Caney et al., 1999).

Unavoidably, combination treatment protocols have been established through a lengthy and difficult process of trial and error. The effectiveness of these therapies depends on tumour type, treatment schedule, and certainly many unknown factors. It is our hope that better fundamental understanding of tumour cell biology will allow the design of more effective treatment protocols that will limit treatment-related morbidity while improving the outcome for patients.

#### **Dose and response**

The *in vitro* technique that best correlates with clinical sensitivity of tumour cells to cancer treatment is thought to be the clonogenic assay (Malaise et al., 1987). This assay uses as its end-point the ability of treated cells to grow and multiply into a colony (usually  $\geq$  32 cells) that is visible to the eye. The number of surviving cells is expressed as a ratio of the number of colonies in treated population compared with untreated cells and this is referred to as the surviving fraction (SF). When cells are treated with a DNA damaging agent, the likelihood of cell survival is traditionally thought to depend on the number of DNA lesions produced and the toxicity of each lesion. That is, as the number of cytotoxic events increases, the probability that the cell will survive should decrease. Based on this rationale, we can use statistical functions to predict SF of cells from the absorbed dose.

The Poisson distribution can be used to predict survival where a single event is sufficient to kill the cell. Here 'a' is the dose given as an average number of events in each cell, and P(x) is the probability of x events in any given cell:

$$P(x) = \frac{(a^{x}e^{-a})}{x!}$$
  
∴ SF = P(0) =  $e^{-a}$ 

A scheme representing the surviving fraction in this situation is shown in panel A of figure 2. The function that relates dose and SF depends on the quality of the treatment and the characteristics of the cells. For example, if death can result from either a single lethal event <u>or</u> from the addition of two independent 'sub-lethal' events the SF is illustrated in panel B of figure 1-2. In this case the SF is described by the following equation:  $(a + 8a^2)$ 

$$SF = e^{-(\alpha a + \beta a^2)}$$

Figure 1-2. Schematic representations of survival curves. Panels A and B represent curves showing the predicted survival of cells using the 'single target, single hit' model and the 'single target, multiple hit' model, respectively. Panel C shows the type of dose-response expected from a population of cells with a sensitive sub-population (solid line) compared with a curve representing a homogeneous, resistant population (dotted line). Panel D represents a survival curve showing a hypersensitive region (HSR) and a region of induced resistance (RIR)(solid line) compared with the expected survivorship of a homogeneous resistant population of cells (dotted line).



Dose

This is the linear quadratic model wherein the coefficients,  $\alpha$  and  $\beta$ , indicate the relative importance of two different mechanisms of cell death. As well as being useful in predicting survival, these models have been important in establishing theory from empirical results (Tubiana et al., 1990).

#### Hypersensitivity and induced resistance

These models are quite accurate in describing the survival of cells that are treated with relatively high doses of DNA damaging agents like cisplatin or ionising radiation (IR). Recently, however it has become accepted that, at least for IR and cisplatin, these models are not good at describing the survival of some mammalian cells following low doses. Many cell types are much more sensitive (per unit of dose) to low doses than would be predicted from models based on higher doses (Joiner et al., 2001; Caney et al., 1999; Marples et al., 1997; Begg, 1986). This phenomenon is observable in survival curves of human tumour cells with a characteristic region of extreme sensitivity (low dose hypersensitivity (LDH)) that is commonly referred to as 'the blip' (Skov, 1994).

While scientific opinion is, by no means, unanimous (Zaider et al., 1996), these observations are increasingly being attributed to inducible molecular responses that either protect cells from DNA damage or increase tolerance of damaged DNA (Marples et al., 1997; Wouters and Skarsgard, 1997; Skov, 1999; Joiner et al., 2001). Alternatively, it has been proposed that this observed hypersensitivity is a consequence of mixed populations of cells (Lambin et al., 1993; Wouters et al., 1996). That is, there may be an extremely sensitive sub-population of cells that are eliminated from the surviving

population at the lowest doses. This sub-population may be sensitive because of their position in the cell cycle as mammalian cells have been shown to be most sensitive to DNA damage during mitosis (Chapman et al., 1999). There are several cogent statistical arguments against this 'sensitive subpopulation' hypothesis. First, in order to account for the observed LDH, the putative subpopulation would have to be much more sensitive than any published reports have shown (Wouters and Skarsgard, 1997). Secondly, the SF that would result from treating a mixed population of cells would be a simple sum of the subpopulations and no region of induced resistance (RIR) would result (compare panels C and D in figure 2)(Skov, 1999).

There is considerable interest in studying the clinical implications of LDH. It is hoped that treating patients with many small, temporally spaced fractions (hyperfractionation) of radiation or chemotherapy will improve therapeutic effects, decreasing the total dose required. While there is no published report of therapeutic gain with hyperfractionation, there are promising preliminary results in the radiotherapy of patients with prostate cancer and carcinomas of the head and neck (Joiner et al., 2001).

#### How does cisplatin cause cell death?

Cisplatin has been used successfully to treat cancer for over 20 years and yet the process by which it kills cells remains fundamentally unknown. For many years, the antitumour activity of the drug was thought to result from a specific species of lesion that could be mutagenic (Rosenberg, 1985). When this putative adduct could never be identified, the consensus shifted to the theory that cisplatin killed cells by directly interfering with DNA function (Sherman and Lippard, 1987). That is, since cisplatin adducts distort DNA sufficiently to impede DNA and RNA polymerases, cell death resulted from the inability to produce mRNA for the synthesis of essential proteins, or failure to complete the synthesis phase of the cell cycle. It later became clear that the process was much more complex with the discovery that cytotoxicity does not appear to correlate with inhibition of DNA replication (Sorenson and Eastman, 1988) or mRNA synthesis (Corda et al., 1991; Mello et al., 1995). The capacity of each cisplatin lesion to block RNA polymerase is different and does not appear to correlate with the ability to cause cell death (Fichtinger-Schepman et al., 1995). It is now generally accepted that cisplatin-treated cells die, not because of a passive inability to survive but predominantly by inducing an active, programmed, process of cell suicide (Eastman, 1999).

#### Apoptosis and cisplatin.

Apoptosis is the energy-dependent, tightly-mediated process by which cells kill themselves. This cell death is essential in the normal development of all multicellular organisms and in their response to many genotoxic agents. Apoptosis is necessary in preserving the health of organisms by removing damaged cells from tissues without compromising the health of the tissue. This active cell death is distinctive from 'nonapoptotic' necrotic cell death. Apoptosis is characterised by cell shrinkage, caspasemediated proteolysis in the cytoplasm, DNA degradation, and nuclear condensation (reviewed by Salvesan and Dixit, 1997 and Gonzalez et al., 2001). Some dysregulation of apoptosis appears to be obligatory in the development and progression of cancer; however, many cancer treatments depend on apoptosis for their therapeutic effect.

#### **Cisplatin resistance**

For obvious reasons, the mechanisms that underlie cisplatin resistance are of profound importance. In this work we present results of experiments designed to investigate these processes. Since the cytotoxic effect of cisplatin is thought to result from DNA damage, reducing the amount of DNA damage has classically been considered the important factor in determining the fate of cisplatin-treated cells. However, it has become clear that there is considerable range in the capacity of cells to tolerate DNA damage by regulating different aspects of cell physiology. Here, proposed mechanisms of cisplatin resistance will be loosely grouped into 'classical' and nonclassical or 'regulatory' processes.

#### **Classical mechanisms of cisplatin resistance**

#### Reduced intracellular accumulation

Since cisplatin reacts quickly with target molecules after entering the cytosol, the effective intracellular cisplatin concentration is thought to be affected predominantly by mediating the influx of the drug rather than drug export. Cisplatin enters the cell readily, either through transmembrane channels or by high capacity facilitated diffusion (Gately and Howell, 1993; Endo et al., 2000). Reduced accumulation of cisplatin has been reported in some resistant non-small cell lung carcinoma lines (Bungo et al., 1990) but

not others (Hospers et al, 1990a; Hospers et al., 1990b). Decreased platinum accumulation has also been shown in some, but not all cisplatin-resistant ovarian carcinoma lines (Andrews et al., 1989) and cisplatin sensitivity has been shown to correlate with drug accumulation in naïve bladder carcinoma cell lines (Koga et al., 2000)

While it is inarguable that decreasing intracellular cisplatin concentrations will protect cells from toxicity, there is no clear evidence that this is a predominant mechanism in clinical cisplatin resistance.

#### Intracellular nucleophile levels

Several cellular molecules bind to cisplatin at physiological concentrations thereby preventing interaction with molecular targets that are important to cytotoxicity. These include the sulphydryl compounds glutathione (GSH) and metallothionein (MT) (Dedon and Borch, 1987).

GSH levels have been shown to be elevated in cisplatin-resistant ovarian (Mistry et al., 1991; Godwin et al., 1992), kidney and some, but not all, bladder cancer cell lines (Ahn et al., 1994; Koga et al., 2000) and inhibition of GSH synthesis can sensitise some (Mistry et al., 1991) but not all cancer cell lines (Andrews et al., 1985).

MT is a small, cysteine-rich protein that can confer cisplatin resistance in some rodent cells and MT levels have been shown to correlate with resistance in many different human tumour cells (Kelley et al., 1988). In many ovarian cancer cells however there is no relationship between MT expression and cisplatin resistance (Schilder et al., 1990).

#### **DNA Repair**

The recognition and repair of platinated DNA is essential to the normal cellular response to cisplatin exposure. Two distinct DNA repair systems have been investigated extensively to determine their part in the cisplatin-induced stress response, mismatch repair (MMR) and nucleotide excision repair (NER).

#### Mismatch Repair (MMR)

MMR is a mechanism that recognises and corrects unpaired or mispaired nucleotides after DNA replication. Defects in MMR allow genomic instability and patients with inherited defects in MMR are predisposed to cancer, particularly hereditary non-polyposis colon cancer (Karran and Bignani, 1994; Fishel and Kolodner, 1995).

Interestingly, defects in this DNA repair system can confer resistance to some DNA damaging agents, including cisplatin (Karran and Bignani, 1994). Reduced MMR has been associated with resistance to cisplatin in ovarian, endometrial, and colon carcinoma lines (Drummond et al., 1996; Aebi et al., 1996; Fink et al., 1996) and cells with targeted disruption of MMR genes are more resistant to cisplatin than their normal counterparts (Fink et al., 1997).

The processes that relate MMR and cisplatin toxicity are not clear. The MMR complex recognises the most frequent cisplatin-DNA adduct within the context of a synthetic dsDNA oligomer (Yamada et al., 1997; Duckett et al., 1996). Since MMR cannot repair platinated DNA (Aebi et al., 1996), it is possible that adduct recognition begins a repair process that can not be completed. It has been proposed that some

intermediate complex in the aborted repair process initiates a pro-apoptotic signal. Thus, cells with a reduced capacity to recognise damage would have a greater cisplatin tolerance (Perez, 1998). The nature of this putative signal is not known.

#### Nucleotide Excision Repair (NER)

Many workers have proposed that enhanced repair of cisplatin-damaged DNA is important in both intrinsic and acquired cisplatin resistance (Masuda et al., 1990; Parker et al., 1991; Lai et al., 1988; Johnson et al., 1994; Ferry et al., 2000; Damia et al., 1998). While the repair of interstrand crosslinks is not well understood, intrastrand adducts are corrected predominantly by nucleotide excision repair (Sheibani et al., 1989; Hansson et al., 1990; Bulmer et al., 1996).

Nucleotide excision repair (NER) is a complex process that repairs many types of DNA damage that affect only one strand including that caused by ultraviolet light (UV) and cisplatin (reviewed by Sancar, 1994; and Hoeijmakers, 1993a and 1993b). A broad range of substrates can be recognised by NER by virtue of the DNA distortion caused rather than the specific chemistry of the lesion (Gunz et al., 1996).

In prokaryotes, NER is essential to the survival of cisplatin-treated bacteria (Keller et al., 2001). NER is accomplished by the co-ordinated action of six proteins. UvrA, UvrB, UvrC, UvrD recognise the lesion and excise an oligomer of 12-13 nucleotides in length (Lin and Sancar, 1990). DNA polymerase I then fills the gap with new DNA and DNA ligase completes the repair (Sancar, 1994).
In prokaryotic NER, DNA that is actively being transcribed is repaired much faster than the rest of the genome (Mellon and Hanawalt, 1989). This process is mediated by a gene product (transcription-repair coupling factor, TRCF) that recruits the repair machinery to interact with transcription complexes and displaces RNA polymerase that is stalled at sites of DNA damage (Selby and Sancar, 1995a and 1995b). This preferential repair is termed transcription-coupled repair (TCR).

## NER in humans

As is the prokaryotic system, the repair of actively transcribed DNA is somehow coupled to transcription and is much faster than in the rest of the genome (global genome repair, GGR). However, NER is much more complex in the human system requiring many more factors (Hoeijmakers, 1993b). Homozygous defects in any of these genes causes serious morbidity. However, the study of these patients and cultured explants of their tissues has been extremely useful in investigating NER. There are two main categories of human NER-deficiency syndromes, xeroderma pigmentosum (XP) and Cockayne syndrome (CS).

#### Xeroderma pigmentosum (XP)

XP is a rare, autosomal-recessive condition involving dry skin (xeroderma) abnormal pigmentation (pigmentosum), sun-sensitivity and a marked predisposition to skin cancer. Some severe cases are complicated by neuron degradation that causes growth retardation and neurological abnormalities (Friedberg et al., 1995). Eight complementation groups of XP have been identified by cell fusion studies (XP-A through XP-G and XP-V). Cells from XP patients in groups A, B, D, and G have general defects in both GGR and TCR. XP-C cells efficiently repair actively transcribed genes but are unable to repair DNA damage in inactive DNA (Venema et al., 1991; van Hoffen et al., 1995). XP individuals in group E have the mildest symptoms and the affected XP-E cells are proficient in TCR but complete repair of bulk DNA in these cells can be delayed (Hwang et al., 1999; Galloway et al., 1994).

The cause of the eighth XP complementation group (XP-variant (XP-V)) remained unknown until 1999 when Masutani et al. attributed the UV-sensitivity in XP-V to mutations in the gene that encodes the DNA polymerase  $\eta$  (pol  $\eta$ ). This polymerase is necessary for the error-free by-pass of damaged DNA during replication (Masutani et al., 1999).

# Cockayne's syndrome (CS)

CS is characterised by many developmental defects including physical and mental retardation, premature ageing, degenerative pigmentary retinopathy, neurological abnormalities, and characteristic facies with a narrow, prominent nose and sunken eyes. CS patients are sun-sensitive but have no predisposition to skin cancer (Vermeulen et al., 1997).

Like XP, CS is a heterogeneous disorder with two complementation groups (CS-A and CS-B). Initial studies with CS cells did not detect any defect in NER despite extreme sensitivity to UV light (Andrews et al., 1978). The first identified defect in CS cells was their failure to resume transcription following exposure to UV (Mayne and Lehmann, 1982). These observations led to the identification of defects that specifically preclude TCR (Venema et al., 1990; van Hoffen et al., 1993).

# NER in Chinese hamster ovary cells

In addition to human studies, researchers have successfully used Chinese hamster ovary (CHO) cells to identify NER genes. Hundreds of UV-sensitive cell lines were identified and sorted into groups by cell fusion analysis (Busch et al., 1989). About 11 complementation groups were identified and the affected genes have been named excision repair cross complementing (ERCC) genes. Most of the genes identified have functionally equivalent human homologues. For example, the CHO ERCC4 is equivalent to the human XP-F and the human nomenclature for the genes is now commonly used.

For one CHO complementation group, ERCC1, no individuals with homozygous defects in the human homologue have been identified. Targeted disruption of ERCC1 causes severe runting and multiple defects in mice so it is presumed to be a lethal condition in humans (Weeda et al., 1997). Because there is no human syndrome associated with ERCC1, the CHO nomenclature persists for this gene.

# The human NER mechanism

While the general scheme of NER is conserved from prokaryotes to humans, NER is more complicated in the human system (Hoeijmakers, 1993b). The reconstitution of human NER with purified proteins has allowed detailed analysis of the assembly and

activity of the NER complex (Aboussekhra et al., 1995). The current model of NER complex assembly is shown in figure 3. The damage is recognised and the DNA is partially opened by two heterodimers, XPA with replication protein A (XPA/RPA); and XPC together with the human homologue of the S. *cerevisiae* protein 23 (XPC/HHRad23B) as shown in panel B. The transcription factor II H (TFIIH) includes the helicases, XPB and XPD and is responsible for locally unwinding the double helix. The endonuclease, XPG, then enters the complex 3' to the lesion and the XPC/HHRad23B dimer is released. Finally the ERCC1/XPF heterodimer is recruited to the 5' end of the melted segment. The structure-specific endonuclease activity of XPG and ERCC1/XPF excise a fragment of about 30 nucleotides. This oligomer is replaced by a DNA polymerase ( $\delta$  or  $\varepsilon$ ) and the repair is completed by a DNA ligase (for review see Wood, 1997).

### *The role of ERCC1 in cisplatin resistance*

A number of studies have suggested that ERCC1 levels contribute to cisplatin resistance by enhancing NER. Increased ERCC1 mRNA levels in untreated ovarian carcinomas correlate with a negative clinical response to platinum-based chemotherapy (Dabholkar et al., 1994). Analysis of an *in vitro* system of cisplatin-resistant ovarian carcinoma suggests that ERCC1 levels may be important in regulating the rate of NER in the cisplatin response (Ferry et al., 2000). However, simply increasing ERCC1 mRNA levels may not increase the cellular concentration of the gene product as the ERCC1 protein is very unstable when it is not associated with its binding partner, XPF (Sjibers et Figure 1-3. Scheme showing the current model of the assembly of the human NER incision complex. A. A bulky adduct is introduced into the double stranded DNA molecule. B. The first identifiable complex formed at sites of DNA damage includes XPA, XPC, RPA, HHR23B, and TFIIH. C. The XPG excinuclease is then recruited to the complex and XPC/HHR23B is concomitantly lost. D. Finally, the XPF/ERCC1 excinuclease heterodimer is recruited. (Adapted from Wakasugi et al., 1998)



al., 1996). Despite this, ERCC1 levels have been proposed as the rate-limiting quantity in the NER of cisplatin adducts. This assertion is based on the kinetics of repair in the extracts of ovarian carcinoma cells (Ferry et al., 2000). While moderate increases in ERCC1 expression may improve NER and cause cisplatin resistance, overexpression of exogenous ERCC1 can sensitise mammalian cells and interfere with NER (Bulmer and Rainbow, this work; Bramson and Panasci, 1993).

#### **Measuring DNA repair**

There are many experimental techniques used to assess NER in mammalian cells. To detect repair of genomic DNA these methods either directly quantify DNA lesions or measure the amount of new DNA synthesis in the repair process (termed unscheduled DNA synthesis (UDS)). Alternatively, cellular repair can be assessed by measuring the capacity of a cell to reactivate DNA that had been damaged before being introduced into the host cell (host cell reactivation (HCR)). The most important difference between these two strategies has been the potential of HCR to distinguish between inducible and constitutive (non-inducible) mechanisms of repair. That is, it is not possible to cause DNA damage to cellular DNA without applying stress to the cell. HCR, in principle, can measure constitutive levels of DNA repair in untreated cells. Using these techniques, our lab and others have detected and examined DNA repair mechanisms that are inducible by cellular stresses (Colicos et al., 1991; McKay et al., 1997; Valerie and Singhal, 1995).

# Host Cell Reactivation (HCR)

Deficiency in the HCR of cisplatin- and UV-modified DNA has been identified in mutant cells with known NER defects (Sheibani et al., 1989; Protic-Sabljic and Kraemer, 1985). Unfortunately, the range of these assays is limited by the low levels of plasmid uptake in many normal cells compared with tumour cell lines or cell lines transformed with viral antigens (Canaani et al., 1986). Additionally, many of the transfection techniques used to introduce plasmid DNA into cells stimulate stress responses that are similar to the response to DNA damage, including cell cycle arrest (Renzing and Lane, 1995). To circumvent these difficulties, our lab, and others, have used adenovirus (Ad) to carry damaged DNA into cells for assessing HCR (Bulmer et al., 1996; Francis and Rainbow, 1999; Valerie and Singhal, 1995).

# HCR of Adenovirus

Adenovirus (Ad) is a non-enveloped virus with a dsDNA genome of about 35 kb (Fields, 1996). The manipulation of the Ad genome has proven to be a convenient method of introducing foreign DNA into cells and Ad is a promising vector for gene therapy (Hitt et al., 1995 and 1999). Ad enters the cell by receptor-mediated endocytosis in clathrin-coated pits (Chardonnet and Dales, 1970a and 1970b; Nemerow, 2000). For most, but not all, Ad serotypes, the receptor has been identified as the 46-kDa immunoglobin, coxsackie adenovirus receptor (CAR) that has no known cellular function (Bergelson et al., 1997). Once released from the vesicle, the Ad genome is actively transported to the nucleus where it passes through the nuclear pore by classical nuclear

import that depends on the Ad hexon protein (Saphire et al., 2000). Inside the nucleus, the Ad genome forms nucleosome-like structures (Tate and Philipson, 1979) that resemble the structure of genomic DNA.

Unlike many of the transfection procedures for introducing foreign DNA into cells, infection with the constructed Ad vectors does not appear to elicit the DNA damage induced stress response or interfere with normal DNA synthesis in the host cell (Blagosklonny and el-Deiry, 1996). This allows the assessment of constitutive levels of DNA repair. Recently, however, the act of Ad infection requires the activation of the phosphatidylinositol-3-OH kinase (PI3'K), promoting the phosphorylation of signalling intermediary Crk-associated substrate (p130<sup>CAS</sup>) (Li et al., 2000).

The vectors used in this study are of two types: E1-deleted and E3-deleted. The early-1 (E1) region of the Ad genome is required for the transactivation of the rest of the genome. E1-deleted viruses, therefore, are not able to replicate in most cell lines and must be propagated in cells transformed with Ad-E1 (Graham et al., 1977). In contrast, the early-3 (E3) region of the Ad genome encodes a protein that interferes with the *in vivo* immune response and so is not required for viral replication in cultured cells. In either type of recombinant Ad, the deleted portions of the genome can be replaced with cartridges carrying foreign DNA (Hitt et al., 1995).

Two different endpoints for HCR of damaged Ad are assessed in this study. In the case of the E3-deleted vectors we measure the amount of viral DNA synthesis, whereas the E1-deleted Ad carry the b-galactosidase gene and HCR is determined by measuring the expression level of this reporter gene.

### **Regulatory molecules and cisplatin resistance**

It is becoming increasingly clear that cellular cisplatin resistance may be determined by the inactivation or the enhanced expression of regulatory molecules. A great deal of research has studied the roles of the countless oncogenes in the cisplatin response. These molecules govern the complex processes of cell cycle progression and apoptosis. Our focus in investigating these molecules has been the mitogen-activated protein kinase (MAPK) family.

# Mitogen activated protein kinases

There are three main families of MAPKs; the c-Jun N-terminal Kinases (JNKs), the Extracellular signal Regulated Kinases (ERKs) and the p38 family (p38 MAPKs). These molecules are essential to the normal cellular response to a broad range of stimuli including growth factors, physical, chemical, and osmotic stresses (Ronai, 1999). These pathways have been shown to mediate cellular processes such as; differentiation, cell cycle progression, and cell death and are highly conserved in all eukaryotes (Schaeffer and Weber, 1999).

The general scheme of action is the same for all of these MAPKs and is described in figure 4. They act to phosphorylate serine/threonine residues on a broad range of effector molecules, typically promoting gene expression by activating transcription factors (Erickson et al., 1990; Songyang et al., 1996). The immediate upstream activators of the MAPKs are the MAPK kinases (MAPKKs) and are often referred to as MAPK Enzymatic Kinases (MEKs). MAPKKs are activated by phosphorylation by MAPKK kinases (MAPKKKs) often called MEK kinases (MEKKs). The activity of the MAPKKKs depends upon a number of events including, directly or indirectly the status of plasma membrane associated proteins, notably Ras.

These are not linear pathways through which a single input signal is translated into a single output signal. At each level of the signal transduction cascade, the actions of several molecules may converge on a single effector, or a single kinase may act upon a number of targets. Each kinase recognises specific residues in their target molecules and protein function depends on these specific phosphorylation events. Further, it is likely that the phosphorylation of some residues cause conformational changes of the target protein that could either preclude or allow subsequent phosphorylation. This makes the sequence as well as the combination of different phosphorylation events important in MAPK regulation. An additional level of complexity is added to MAPK regulation through interactions with so-called 'scaffold proteins' that act to aggregate the enzymes within the cell (Yasuda et al., 1999, Ito et al., 1999). This astounding complexity gives the cell the potential to respond appropriately to an infinite array of biological, chemical, and physical stimuli. It is this subtlety that has caused difficulty in fitting empirical observations of the MAPK regulatory system to simple, explanatory models.

#### Mitogen activated protein kinases and cisplatin resistance.

MAPKs could conceivably affect cisplatin resistance by regulating any of the aforementioned classical modes of cisplatin resistance or by changing the inclination of Figure 1-4. The MAPK regulatory signal transduction pathways. A. The general scheme of MAPK activation is shown. Each step in the mechanism is mediated by the activation of downstream target proteins by enzymatic phosphorylation of specific residues. B. A diagram showing some of the molecules that impinge upon the activity of the three main families of MAPK. Some of their downstream effectors are also shown.



A



the cell to apoptosis. In general, JNK and p38 activity tend to be associated with stressinduced apoptosis while ERK is often associated with growth factor induced survival and proliferation (Schaeffer and Weber, 1999). These trends, however, do not appear to be consistent with the identified roles of the MAPKs in the cisplatin-induced stress response.

## p38 MAPK activity and cisplatin resistance

p38 MAPK is not induced by cisplatin in the human ovarian carcinoma line, SK-OV-3 (Persons et al., 1999). However, p38 does appear to be induced by cisplatintreatment of NIH 3T3 mouse embryonic fibroblasts (Sanchez-Prieto et al., 2000). While p38 can protect human melanoma cells from UV-induced apoptosis (Ivanov and Ronai, 2000), the role of p38 in cisplatin-sensitivity is not clear.

### ERK activity and cisplatin resistance

The ERK family of MAPK is thought to be activated by cisplatin exposure (Persons et al., 1999; Wang et al., 2000). It has been reported that the activation of ERK is required for cisplatin-induced apoptosis in the HeLa human cervical carcinoma line (Wang et al., 2000). Also, the activation of the Ras protein, which is known to be an upstream effector of the ERKs, is associated with cell sensitivity in mouse cells and in human melanoma lines (Gao et al., 1995; Fokstuen et al., 1997). However, the inhibition of ERKs in ovarian tumour cell lines, SK-OV-3 and Caov-3, made them cisplatinsensitive (Cui et al., 2000; Persons et al., 1999; Hayakawa et al., 1999).

# JNK activity and cisplatin resistance

Activation of the JNK pathway has been shown to be important in cisplatin resistance in many different cell systems. Activation of this enzyme cascade affects gene regulation through a set of transcription factors including c-Jun, JunD, ATF-2, Elk-1, Sap-1, and p53 (Ip and Davis, 1998; Fuchs et al., 1998a; Minden and Karin, 1997). The phosphorylation of these proteins by the JNK family of MAP kinases generally enhances their ability to promote transcription of many genes through a number of different promoter elements (Davis, 1999). For example, AP-1 sites are present within the promoters of many genes that may be important in cisplatin resistance. These include those that encode mdr-1, c-myc, topoisomerase I, thymidylate synthase, metallothionein IIa, glutathione-S transferase, and ERCC1 (Scanlon et al., 1991b; Li et al., 1999). For these reasons, JNK function has been studied extensively with respect to cisplatin resistance.

Despite a lack of direct evidence, cisplatin is thought to activate JNK in human tumour cells through the DNA damage it causes (Persons et al., 1999, Potapova et al., 1997). There is no evidence suggesting a mechanism that detects cisplatin adducts and initiates a JNK signal.

The role of the JNK pathway in determining cell fate following cisplatin treatment is controversial. When the activity of c-Jun in human tumour cell lines is blocked by the expression of a dominant negative construct, the repair of cisplatin-DNA adducts is impaired and the cells were more sensitive (Gjerset et al., 1999; Hayakawa et al., 1999; Potapova et al., 1997). However, mouse cells with no functional jun are cisplatinresistant compared with their normal counterparts (Sanchez-Perez et al., 1999). Exogenous expression of dominant negative forms of the upstream activators of JNK (MEKK-1 and SEK-1) protect cells from cisplatin cytotoxicity (Sanchez-Perez, 1999; Zanke et al., 1996). The cessation of the JNK signal has been attributed to the activity of the MAP kinase phosphatase CL100 (CL100/MKP-1) in human 293T cells (Sanchez-Perez et al., 2000). It is possible that persistent JNK activity following cisplatin exposure in human tumour cells promotes caspase-mediated apoptosis (Sanchez-Perez and Perona, 1999) while JNK activity immediately after cisplatin exposure may be protective (Gjerset et al., 1999).

## **Current Research**

This thesis presents our investigations of the cellular responses to cisplatin. In chapter 3 we describe a convenient host cell reactivation (HCR) assay which measures the synthesis of viral DNA in cells infected with cisplatin-damaged adenovirus (Ad). We show that both human and Chinese hamster ovary (CHO) cells with known defects in nucleotide excision repair (NER) are reduced in their capacity to reactivate cisplatin-damaged DNA. Alterations in DNA repair capacity have been shown to be important in the resistance of some tumour cells to cisplatin (Chao, 1994; Zeng-Rong et al., 1995; Zhen et al., 1992; Yen et al., 1995; Lai et al., 1995). In this work we show that HCR of cisplatin-treated Ad is capable of identifying differences in repair among several human tumour cell lines.

Chapter 4 describes our use of a recombinant adenovirus vector to express very high levels of the human DNA repair gene, ERCC1, in a number of mammalian cell types. We present data here that shows very high levels of this DNA repair protein can cause sensitivity to cisplatin in the human cervical carcinoma cell line HeLa. ERCC1 overexpression also compromises the ability of many different mammalian cell types to reactivate cisplatin-damaged Ad.

Our initial studies of low-dose hypersensitivity (LDH) are presented in chapter 5 of this thesis. We report LDH to cisplatin and increased resistance to higher doses of cisplatin for the human squamous cell carcinoma, SCC-25. We also show the absence of LDH in the cisplatin-resistant SCC-25/CP cell line, which was isolated by repeated exposure of SCC-25 cells to increasing doses of cisplatin (Teicher et al., 1987). Also shown is that pretreatment of SCC-25 with acute low-dose  $\gamma$ -irradiation results in an increased resistance to a subsequent low-dose cisplatin challenge, whereas acute low-dose  $\gamma$ -irradiation given after the cisplatin challenge dose did not increase resistance.

We extended our investigation of low dose cisplatin hypersensitivity in chapter 6. We show that treatment of SCC-25 cells as well as AA8 CHO cells with UV, cisplatin, or IR is protective against cell death caused by subsequent low doses of cisplatin. To test the possibility that it is damaged DNA *per se* that induces cisplatin protection, we used adenovirus to carry damaged DNA into undamaged cells. The introduction of exogenous, damaged DNA reduces the hypersensitivity to low cisplatin doses in the SCC-25 tumour cell line. This provides strong support for the hypothesis that the

35

presence of damaged DNA within cells triggers a physiological response that protects against cell death induced by cisplatin.

In chapter 7 we investigate the activation of the mitogen-activated protein kinase (MAPK), Jun N-terminal kinase (JNK) in cells that have been exposed to cisplatin. We have used human cells with known defects in nucleotide excision repair (NER) to investigate the role of cisplatin-DNA lesions in the induction of JNK and cell death. We present evidence here that cisplatin induces a prolonged activation of JNK in cells that are deficient in the repair of transcriptionally active DNA. Further, this prolonged activation is associated with extreme cisplatin sensitivity.

In this thesis we have endeavoured to understand the relationship between cisplatin-induced DNA damage and the toxicity of the drug. It is our hope that the body of knowledge, of which this work is a part, will ultimately increase the usefulness of cisplatin in treating cancer. Chapter 2

# Materials and Methods

# Cell lines and Fibroblast strains:

Human fibroblast strains and Chinese hamster ovary (CHO) cell lines used in this study are described in tables 2-I and 2-II, respectively. CHO cells were kindly provided by Dr. L. Thompson of the Lawrence Livermore National Laboratory, California, USA; Dr. G. Whitmore, Physics Division, Ontario Cancer Institute, Toronto, Ontario; and Dr. D. Busch, Department of Environmental and Toxicological Pathology, Armed Forces Institute of Pathology, Washington, D.C., USA. Primary human fibroblasts were purchased from NIGMS Human Genetic Cell Repository, Camden, N.J., USA. Human tumour cell lines used in this study were obtained from a number of sources. HeLa, (cervical carcinoma) were obtained from the ATCC. HT29 (colon carcinoma), 2008 and C13 (ovarian carcinoma lines) were kindly provided by Dr. G. Singh, Hamilton Regional Cancer Centre, Hamilton, Ontario. SKOV-3 (ovarian carcinoma) were provided by Dr. S. Bacchetti, McMaster University and the squamous cell carcinoma line (SCC-25) were obtained from Dr. J. Lazo, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA. The SV-40 transformed fibroblast lines GM 637 and AG02804 were purchased from NIGMS, Camden, NJ, USA. The human embryonic kidney cell line, 293, expresses early region 1 (E1) portion of adenovirus from serotype 5 (Ad5) permitting the replication of adenovirus constructs that lack E1 expression (Graham et al., 1977). 293

38

cells were kindly provided by Dr. F. L. Graham (McMaster University, Hamilton, Ontario).

# Media and cell culture

All cells were cultured in monolayers bathed in  $\alpha$ -minimum essential media ( $\alpha$  - MEM) supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic-antimycotic (GibcoBRL). Cultures were grown in a humidified chamber at 37°C and 5% CO<sub>2</sub>. Cells were passaged into new plates by treatment with 0.1% trypsin (GibcoBRL) in phosphate buffered saline (PBS: 140mM NaCl, 2.5mM Na<sub>2</sub>HPO<sub>4</sub> and 1.75mM KH<sub>2</sub>PO<sub>4</sub>) and adding aliquots of the cell suspension into fresh, supplemented  $\alpha$ -MEM in new plates.

NIGMS	Cell Strain	Repair	Source
Designation		Phenotype	
GM 37		Normal	NIGMS repository
GM 38		Normal	NIGMS repository
GM 9503		Normal	NIGMS repository
GM 969		Normal	NIGMS repository
GM 8399		Normal	NIGMS repository
GM 5509	XP12BE	XP-A	NIGMS repository
GM 13025	XPCS1BA	XP-B	NIGMS repository
GM 13026	XPCS2BA	XP-B	NIGMS repository
GM 677	XP2BE	XP-C	NIGMS repository
GM 10881	XP1BE	XP-C	NIGMS repository
GM 2096	XP1MI	XP-C	NIGMS repository
GM 510	XP1PW	XP-C	NIGMS repository
GM 3615	XP1BR	XP-D	NIGMS repository
GM 4313	XP2YO	XP-F	NIGMS repository
GM 3542	XP3YO	XP-F	NIGMS repository
GM 3021	XP2BI	XP-G	NIGMS repository
GM 1856	CS3BE	CS-A	NIGMS repository
GM 1428	CS7SE	CS-B	NIGMS repository
GM 739	CS1AN	CS-B	NIGMS repository

Table 2-I: Primary human fibroblasts used

1 able 2-II: CHO cells use
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Mutant Designation	ERCC Group
AA8	Parental
UV20	1
UV5	2
UV24	3
UV41	4
UV135	5
UV61	6
UV86	6

# Recombinant adenovirus constructs

Each adenovirus (Ad) construct has been modified in either the early region 1 (E1) or the early region 3 (E3). Deletion or inactivation of E3 does not affect the propagation of Ad in cell culture. Gene products encoded in E1 are necessary for replication so virions without E1 expression will only replicate in human cells that express the Ad E1 genes in *trans* (Graham et al., 1977). The E1 and the E3 vectors carrying ERCC1 were both produced in our lab (Colicos and Rainbow, 1991; Gu and Rainbow, unpublished results). The control viruses expressing the b-galactosidase gene from the deleted E1 and E3 regions were the Ad5-LacZ 5 and the Ad5HCMVsp1lacZ, respectively and they were kindly provided by Dr. F.L. Graham, McMaster University.

# Virus propagation and quantification

Viruses used were grown and titred in 293 cells as has been previously described (Graham and Prevec, 1991). 293 cells in 150mm tissue culture plates at 75-90% confluence were infected with about 4 plaque forming units (pfu) per cell suspended in 2mL of serum-free  $\alpha$ -MEM per plate. Virus was allowed 60-90 minutes to adsorb to the cells before the cells were refed with 20mL of supplemented medium. 3-5 days following infection the cells were harvested by scraping and pelleted by centrifugation. The pellet was resuspended in 100µL of 10% glycerol in PBS for each plate of infected cells collected. These viral suspensions were then subjected to three cycles of freezing and thawing to disrupt the cells. Insoluble debris was pelleted by centrifugation and the supernatant was transferred to cryovials and stored at  $-20^{\circ}$ C.

The titration of viral stocks was performed on monolayers of 293 cells. Typically, serial dilutions of between  $10^6$  and  $10^9$  were performed in serum-free  $\alpha$ -MEM and 0.3 mL of resulting suspension was used to infect each well of a 6-well plate. Following a 1-1.5 hour infection, cells were overlaid with 10 mL of 0.5% agarose in minimum essential medium F11 with 1% antibiotic/antimycotic and 0.1% yeast extract at 44°C. 7-10 days following infection, the overlay was removed by inverting the plates and the monolayer was stained with 0.5% methylene blue in 70% ethanol. Plaques were counted by eye and the viral titres are expressed in plaque forming units per mL of suspension (pfu/mL).

# UV-irradiation of virus

Virus was irradiated on ice with constant stirring in a 35 mm tissue culture plate. A germicidal lamp (General Electric model G8T5) was used to provide UV light (primarily at 254 nm) with fluence rates between 2 J/m<sup>2</sup>s and 10 J/m<sup>2</sup>s were used, as measured by a J-255 shortwave UV meter (UVP, San Gabriel, CA).

# UV-irradiation of cells

All cell irradiations were performed in monolayers bathed in small volumes of PBS (40mM, 200mM, 500mM, or 1mL per sample for 96-well, 24-well, 6-well, or 100mm plates, respectively). Cells were irradiated at room temperature with a model G8T5 (General Electric) germicidal bulb predominantly producing at 254 nm at fluence rates of 1 or 2  $J/m^2s$ .

# Cisplatin stock solutions

The cisplatin used in this work was either purchased as a powder (Sigma) or as a solution of 1mg/mL (Faulding). Initially, a fresh 1mM stock solution of cisplatin in PBS or low chloride PBS (4 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>) was prepared for each experiment. In the interests of cost, convenience and consistency it was preferable to make stock solutions in 0.2M NaCl that were sufficient for many experiments and were stable at 4°C for at least 6 months as assessed by cytotoxicity. Cisplatin stocks intended for clinical use (1mg/mL cisplatin, 9mg/mL NaCl, 1mg/mL

mannitol) are commercially available (Faulding) and this proved to be the most convenient and consistent source of cisplatin.

# Cisplatin treatment of virus

Because the reactivity of cisplatin is dependent on the chloride ion concentration, all Ad was treated with cisplatin in a suspension with 50mM concentration of chloride ion. This was achieved by using a ratio of low-chloride PBS (7 mM Cl<sup>-</sup>) and serum-free  $\alpha$ -MEM (140 mM Cl<sup>-</sup>). Cisplatin was added to the virus suspension to give the desired final drug concentration. The virus was incubated with the cisplatin in closed tubes for 24 hours at 37°C. Treatment was stopped by addition of serum-free  $\alpha$ -MEM sufficient to give a suitable concentration of virus for infection.

# Cisplatin treatment of cells

Appropriate dilutions of cisplatin were made in the same media as that in which the cells were being grown to avoid any effect of changes in serum concentration on the cells. Cisplatin treatments were stopped by aspirating away the cisplatin-containing media and washing the cells once with the appropriate media and then replaced with fresh cisplatin-free media.

# Clonogenic survival assay

Cells were seeded at low density into either 6-well tissue culture plates or 100mm tissue culture dishes so as to give 50-150 colonies per sample. The seeding efficiency

varies between different cell lines and this was taken into account when determining the number of cells added to each sample plate. Cells were counted manually with a haemocytometer and the cell suspension was mixed thoroughly before being added to the plates. Cells were allowed at least three hours before the media was disturbed in order to allow the cells to adhere to the substrate. Depending on the duplication rate of the cells, the plates were incubated for 7-14 days at which time the media was removed and colonies were stained with methylene blue or crystal violet at 0.5% in 70% ethanol and 10% methanol. Cell groups of  $\Box$ 32 cells were counted as surviving colonies and relative survival was calculated by comparison with mock treated samples.

### Host cell reactivation of viral DNA synthesis

Cells were seeded overnight into either 24-well or 96-well plates with a cell number appropriate to give a monolayer of ~75% confluence. Cells were then infected with the appropriate virus construct for 90 minutes at a multiplicity of infection of 40 plaque forming units (pfu) per cell in serum-free  $\alpha$ -MEM. Virus that had not adsorbed to the cells was removed by aspirating 2-4 hours after infection and cells were overlayed with whole,  $\alpha$ -MEM.

At an appropriate time after infection, cells were lysed with a pronase solution (4 mg/mL pronase, 40 mM Tris (pH 8.0), 40 mM EDTA, 2.4% SDS) for 1.5 hours. DNA samples extracted from 24-well plates were then extracted by a standard phenolchloroform technique (Sambrook et al., 1989) and were precipitated in cold absolute ethanol and 0.2 M NaCl. The DNA was pelleted by centrifugation at 13 000 g for 20

45

minutes and resuspended in TE buffer (10 mM Tris, 1 mM EDTA (pH 7.8)). These samples were then blotted onto GeneScreen Plus (DuPont) as per the manufacturer's instructions. The lysates collected from samples in 96-well plates were denatured by the addition of an equal volume of 1.0 N NaOH, 50 mM EDTA for 1 hour and then transferred directly onto the GeneScreen Plus. The quantity of viral DNA was determined by hybridisation of a <sup>32</sup>P-labelled probe for Ad2 DNA and imaging with phosphorimager technology.

# Host cell reactivation of viral $\beta$ -galactosidase expression

Transformed or immortalised cells were seeded at  $3.8 \times 10^4$  cell per well of a 96-well plate while  $1.9 \times 10^4$  fibroblasts were used per well. After incubation overnight, cells were infected with 10 pfu/cell of Ad5-lacZ that had been irradiated with UV. Virus that had not adsorbed to the cells was washed off and the cells were incubated with whole media for the appropriate length of time. Cells were lysed at  $37^\circ$ C for 20 minutes with NP-40 buffer (250 mM Tris, 1  $\mu$ M PMSF, and 0.5% NP-40 (pH 7.8)), followed by 10 minutes in 100mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5), 10 mM KCl, 1mM MgSO<sub>4</sub>, 50 mM 2-mercaptoethanol. OD<sub>405</sub> was determined at several times following addition of 0.1% on introphenol- $\beta$ -D-galactopyranoside (ONGP) in 100mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5) using a microtitre plate reader (Bio-Tek Instruments EL340 Bio Kinetics Reader). The reactivation of the UV-damaged reporter gene is evident in the extent of the colour reaction and is expressed herein as a relative value in comparison with unirradiated controls.

# In vitro JNK assay

The method used to determine kinase activity was similar to that used by Derijard et al. (1994). Cells were seeded (in supplemented  $\alpha$ -MEM) into 100mm plates  $(5 \times 10^{5})$  /plate). The following day, growth media was replaced with low-serum  $\alpha$ -MEM and cisplatin and UV treatments were started 24 hours later. At appropriate times after treatment, cells were collected in cold PBS and lysed in NP-40 lysis buffer (0.5% NP-40, 50mM Tris (pH 8), 150mM NaCl, 2mM EDTA, 100mM NaF, 1mM NaVO<sub>3</sub>, 10mM Na Pyrophosphate) with protease inhibitor cocktail (Boehringer Mannheim). Protein concentrations of cleared lysates were determined using the Micro BCA Protein assay (Pierce) and identical quantities of total cellular protein (30-150µg) were added to protein-G-sepharose beads (Pharmacia) and 1µg monoclonal antibody to human JNK-1 (Pharmingen). Immunoprecipitation was carried out overnight and immune complexes were washed twice with lysis buffer and twice more with kinase buffer (20 mM HEPES, 20 mM MgCl<sub>2</sub>, 10 mM β-glycerophosphate, 0.1 mM NaVO<sub>3</sub>, 2mg/mL pnitrophenylphosphate, 2mM DTT. The immune complexes were then incubated in 30  $\mu$ L of kinase buffer with 5 $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P] ATP and 2  $\mu$ g of GST-c-Jun(1-79) per sample for 20 minutes at 30°C. The substrate molecules were then separated by SDS-PAGE and kinase activity in the lysates was determined with phosphorimager technology.

# Chapter 3

Host Cell Reactivation of cisplatin-treated adenovirus is reduced in nucleotide excision repair deficient mammalian cells and several human tumour cells.

> J. Todd Bulmer, Kelly Davis, and Andrew J. Rainbow International Journal of Oncology (1996) 9: 1121-1127

# **Preface:**

Host cell reactivation (HCR) of damaged adenovirus has been used successfully by our lab and others to assess nucleotide excision repair of UV-irradiated DNA. Here, we extend this assay to the study of the reactivation of cisplatin-treated Ad.

At the time of publication, the role of NER in the repair of cisplatin-modified DNA had not been established and some NER genes were suspected to be unnecessary. By showing that each of the NER genes examined are required for HCR of cisplatindamaged Ad for repair of cisplatin

This chapter was originally published (International Journal of Oncology 9: 1121-1127) and is reproduced here. Some of the HCR experiments in human fibroblasts were performed by Kelly Davis. The rest of the experiments and the writing of the manuscript is my work.

# Host cell reactivation of cisplatin-treated adenovirus is reduced in nucleotide excision repair deficient mammalian cells and several human tumour cells

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Communicated by F.L. Graham, September 12, 1996

Abstract. Cisplatin is widely used for chemotherapy of a variety of human cancers. Cisplatin exerts its toxic effect by covalently binding to DNA, resulting in monofunctional adducts, intrastrand crosslinks, and interstrand crosslinks. Several recent reports suggest that the cellular capacity for DNA repair, especially nucleotide excision repair (NER), is an important determinant in the sensitivity of cells to cisplatin. We have used a sensitive host cell reactivation (HCR) technique to examine the repair capacity for cisplatindamaged DNA in several different mammalian cell types. HCR of cisplatin-damaged adenovirus (Ad) was reduced in all UV-sensitive NER deficient Chinese hamster ovary (CHO) cells examined (complementation groups 1 to 6) compared to NER proficient CHO cells. HCR of cisplatindamaged Ad was also reduced in fibroblasts from patients with xeroderma pigmentosum (XP) complementation groups A, B, C, D, F, and G compared to that in normal human fibroblasts. Differences in the HCR of cisplatin-treated Ad were also detected among human cancer cell lines, suggesting some tumour cells may be deficient in the NER of cisplatin-DNA adducts.

#### Introduction

Cisplatin is a chemotherapeutic agent used extensively in the treatment of many kinds of cancers (1) and is particularly effective in treating tumours of the gonads (2). The cytotoxic effect of cisplatin is widely believed to be a result of DNA damage (3,4). Cisplatin reacts with DNA through a two-step electrophilic addition to purine bases (5) which results in a spectrum of adducts that cause bending and local unwinding

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Key words: DNA repair, adenovirus, cisplatin, host cell reactivation, nucleotide excision repair

of the DNA (6,7) sufficient to stall both transcription and replication (8,9). While cisplatin is an extremely effective drug, the limit to its efficacy is the acquisition of drug resistance by tumours (10). The mechanisms by which tumour cells gain resistance are largely unknown although some studies have shown a correlation between cisplatin resistance and increased expression of some DNA repair genes (11,12). Cisplatin resistant cells have also been shown to have increased capacity to repair cisplatin-damaged DNA (13-17) and an increased level of p53 (18,19). Recently, p53 has been shown to be involved in DNA repair (20,21). While enhanced DNA repair capacity has yet to be proven as a primary mechanism of acquired drug resistance, there are a number of reports suggesting that the intrinsic resistance of tumour cells to killing by cisplatin varies with their constitutive capacity for DNA repair (13-17,22,23).

Nucleotide excision repair (NER) has been shown to be an important pathway in the repair of cisplatin-damaged DNA (23,24) and this process has been studied extensively through the use of repair deficient mammalian cells. Particularly useful have been a number of Chinese hamster ovary (CHO) cell lines isolated based on their sensitivity to UV-light (25). These CHO mutants have been valuable in the isolation of human DNA repair genes by complementation analysis as reviewed by Hoeijmakers (26). Defects in some of these genes have been discovered to cause the human UVsensitive syndrome xeroderma pigmentosum (XP), Cockayne syndrome (CS) or trichothiodystrophy (TTD). Many of these repair deficient mammalian cells show an increased sensitivity to cisplatin (27).

DNA repair capacity has been effectively assessed in the past using host cell reactivation (HCR) of DNA-damaged virus which can be quantitated in several ways such as viral antigen production (28-30), plaque forming ability (31,32), reactivation of a reporter gene which has been inserted into a recombinant virus (33,34), and amount of viral DNA synthesis (35).

We report here a convenient HCR assay which measures the synthesis of viral DNA in cells that have been infected with cisplatin-damaged adenovirus type 5 (Ad5). We show that both human and CHO cells with known defects in nucleotide excision repair (NBR) are reduced in their capacity to reactivate cisplatin-treated Ad5. Alterations in DNA repair capacity have been shown to be important in the resistance of some tumour cells to cisplatin (14,22,36-38). In this work we show that HCR of cisplatin-treated Ad is capable of identifying differences in repair among several human tumour cell lines.

#### Materials and methods

Cells and virus. Parental CHO-AA8 cells and UV-sensitive UV20, UV5, UV24, UV41, UV135, and UV61 mutant CHO cell lines (from complementation groups 1 to 6 respectively) were provided by Dr L. Thompson, Lawrence Livermore National Laboratory, Livermore, CA, and Dr G. Whitmore, Physics Division, Ontario Cancer Institute, Toronto, Ontario. The UV-sensitive mutant CHO cell line UV86, from complementation group 6, was obtained from Dr D. Busch, Department of Environmental and Toxicological Pathology, Armed Forces Institute of Pathology, Washington, DC, USA.

The normal human fibroblast strain GM969 and xeroderma pigmentosum cell strains XP12BB (GM5509B), XPCS2BA (GM13026), XP1MI (GM2096), XP2BE (GM677), XP1BR (GM3615), XP2YO (GM4313), XP2BI (GM3021A) (from complementation groups A, B, C, C, D, F, and G respectively) were obtained from the NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research, Camden, NJ, USA.

The human cervical carcinoma HeLa cell line was obtained from the American Type Culture Collection, Rockville, MD, USA. The human colon carcinoma cell line HT29 and the ovarian carcinoma cell line 2008 were obtained from Dr G. Singh, Hamilton Regional Cancer Centre, Hamilton, Ontario, Canada. The ovarian carcinoma cell line SKOV-3 was obtained from Dr S. Bacchetti, Department of Pathology, McMaster University, Hamilton, Ontario, Canada. The squamous cell carcinoma cell line SCC-25 was obtained from Dr J.S. Lazo, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA and human 293 cells were obtained from Dr F.L. Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada.

All cells were grown in monolayer in alpha-minimal essential medium ( $\alpha$ -MEM) (Gibco-BRL Canada) with 1% antibiotic-antimycotic supplemented with 10% newborn calf serum (NCS) for CHO cells or 10% fetal bovine serum (FBS) for cells of human origin.

The virus used was Ad5-LacZ 5, a recombinant virus containing the  $\beta$ -galactosidase gene inserted into the deleted non-essential E3 region (39). This construct was kindly provided by Dr F.L. Graham, Departments of Biology and Pathology, McMaster University, Ontario, Canada. Viral stocks were grown and titred by plaque assay (40) on monolayers of 293 cells (41) and suspended in PBS to a titre of about 10° plaque forming units (pfu)/ml.

Treatment of virus. Ad5(lacZ) was treated with cisplatin in a suspension in which the chloride ion concentration was adjusted to 50 mM using an appropriate ratio of low chloride PBS and  $\alpha$ -MEM containing 1% antibiotic-antimycotic solution. Cisplatin solution was then added to give the appropriate final drug concentration and the suspension was incubated at 37°C for 24 hours.

Table I. HCR of cisplatin-treated Ad5.

Cell line	Mean relative <sup>a</sup> D <sub>37</sub>	No. of experiments
CHO cells	nin a settingat oʻra incheseran da sayniyada a Ta'lari T	
AA8(parental)	1	8
UV20 (1°)	0.17±0.03°	8
UV5 (2)	0.22±0.05	5
UV24 (3)	0.30±0.05	7
UV41 (4)	0.21±0.06	4
UV135 (5)	0.25±0.08	4
UV86 (6)	0.24±0.05	3
Human fibroblasts		
GM969 (normal)	- 1	7
XP12BE (XP-A)	0.20±0.06	3
XPCS2BA (XP-B)	0.27±0.11	3
XP1MI (XP-C)	0.53	1
XP2BE (XP-C)	0.48	1
XPIBR (XP-D)	0.27±0.12	3
XP2YO (XP-F)	0.70	1
XP2BI (XP-G)	0.18±0.04	3
Human tumour		
HeLa	.1	6
2008	1.81±0.22	6
SKOV-3	2.37±0.75	4
HT29	1.97±0.48	3
SCC-25	0.89	1
GM969	2.93±0.80	2

<sup>a</sup>relative values shown with respect to the first cell line shown in each group. <sup>b</sup>NER deficient complementation group. <sup>c</sup>standard error of the mean.

Quantification of viral DNA synthesis. CHO cells and human tumour cells were seeded either in Corning 96-well plates at 3.8x10<sup>4</sup> cells/well or in Nunc 24-well plates at 2x10<sup>5</sup> cells/well, whereas human fibroblasts were seeded at 1.9x104 cells/well in 96-well plates or 1x10<sup>5</sup> cells/well in the 24-well. Cell monolayers that had reached confluence at about 24 h after seeding were infected with either untreated or cisplatintreated Ad5-LacZ 5 at a multiplicity of infection (MOI) of 40 pfu/cell in a volume of 40 µl/well for 96-well plates or 200 ml/well for 24-well plates. Following viral adsorption for 90 min at 37°C, the infected cells were overlayed with 160 µl for 96-well plates or 1 ml for 24-well plates, of warm a-MEM supplemented with 10% NCS or FBS. 2-4 h after infection virus which had not adsorbed to the cells was removed by aspirating and replaced with warm growth medium.

At an appropriate time after infection, infected cells were lysed and DNA was transferred to a nylon membrane. Cells which were seeded in 24-well plates were lysed by addition of 200  $\mu$ l of pronase solution (4 mg/ml pronase. 40 mM Tris



Figure 1. HCR of cisplatin-treated Ad in CHO cells. Survival of viral DNA synthesis for cisplatin-treated Ad in NER deficient CHO mutants from complementation groups 1-6 in comparison to that in the NER proficient parental cell line, AA8 (m). A, UV20 (a) and UV5 (a) are from groups 1 and 2 respectively. B, UV24 (a) and UV135 (c) represent groups 3 and 5 respectively. C, UV41 (v) and UV86 (c) represent groups 4 and 6 respectively. Each point is the mean of 3 to 6 determinations from a single experiment (±SE).

pH 8.0, 40 mM EDTA pH 8.0, 2.4% SDS) and incubated for 1.5-3 h. The cell lysates were then stored at -20°C until phenol:chloroform extractions were performed and DNA was precipitated with two volumes of cold absolute ethanol with 0.2 M NaCl. DNA was then pelleted by centrifugation at 13,000 rpm for 20-30 min at 4°C after which pellets were resuspended in 40 µl of TE buffer (10 mM Tris, 1 mM EDTA) and slot blotted onto Gene Screen Plus (Dupont Cat.#NEF-976) according to the manufacturer's specifications. Infected cells from 96-well plates were lysed by first aspirating the overlay and adding 40 µl of proteinase K solution per well. After 1.5-2 h at 37°C, DNA was denatured by addition of 80 µl/well of 1.0 N NaOH with 50 mM EDTA. After a further 2 h at 37°C, samples were slot blotted onto Gene Screen Plus (Dupont Cat.#NEF-976) according to the manufacturer's specifications.

Slot blots were probed with <sup>32</sup>P-labelled Ad2 DNA and the relative viral DNA content in each sample was determined from the radioactivity of each slot blot using a phosphorimaging system.

#### Results

Host cell reactivation of cisplatin-treated virus in excision repair deficient cells. The survival of viral DNA synthesis for cisplatin-treated Ad was examined following infection of both excision repair proficient and excision repair deficient mammalian cells. The amount of viral DNA synthesized by each cell line following infection with cisplatin-treated virus was expressed as a fraction of that synthesized in the same cell line following infection with non-treated virus. Fig. 1 shows typical results for the survival of Ad DNA synthesis

following cisplatin treatment for infection of the nucleotide excision repair (NER) proficient CHO-AA8 cells and the NER deficient CHO cell mutants representing complementation groups 1 to 6 (42,43). It can be seen that survival of cisplatintreated virus was significantly reduced in all the excision repair deficient CHO cell lines tested compared to that in CHO-AA8 cells.

Survival curves were fitted to a linear quadratic equation, In (relative amount of viral DNA) =  $-(\alpha D+\beta D^2+\chi)$  and the parameters  $\alpha$ ,  $\beta$  and  $\chi$  were determined by least squares analysis in order to obtain a D<sub>37</sub> value for each survival curve. For each experiment, the D<sub>37</sub> value obtained for each CHO cell mutant was expressed relative to the D<sub>37</sub> value obtained in the CHO-AA8 parental line. The relative HCR values so obtained were taken as a measure of the DNA repair capacity of the cell and pooled results for a number of experiments are shown in Table I. A significant reduction in HCR for cisplatin-treated Ad was detected following infection of all the NER deficient CHO cell lines tested representing complementation groups 1 to 6, compared to that of the NER proficient CHO-AA8 parental cells.

Typical results for the survival of Ad DNA synthesis for cisplatin-treated Ad in human fibroblasts are shown in Fig. 2. Survival of cisplatin-treated virus was reduced in all the XP NER deficient strains tested compared to that in the NER proficient normal human fibroblast strain. Relative HCR values for the various XP fibroblast strains obtained from a number of experiments are shown in Table I. A significant reduction in HCR for cisplatin-treated Ad was detected following infection of the XP fibroblast strains representing complementation groups A, B, C, D, F and G, compared to that in the NER proficient fibroblasts.



Figure 2. HCR of cisplatin-treated Ad in normal human and XP fibroblasts. Survival of viral DNA synthesis for cisplatin-treated Ad in NER deficient human fibroblasts from XP groups A, B, C, D, F, and G in comparison to that in the repair proficient normal GM969 cell line (a). A, XP12BE (a) and XP2BI (a) are XP-A and XP-G cell lines respectively. B, XPCS2BA (a) and XP1BR (c) are XP-B and XP-D cell lines respectively. (C) XP1MI (v) and XP2YO (c) are XP-C and XP-F cell lines respectively. Each point is the mean of 2 to 4 determinations from a single experiment (±SE).



Figure 3. HCR of cisplatin-treated Ad in several human tumour cell lines. Survival of viral DNA synthesis for cisplatin-treated Ad in different human tumour cell lines. Results for HeLa cells (m) are included in each plot for comparison. A, GM969, normal human fibroblasts (•) B, Tumour cells HT29 (•) and SCC-25 (a). C, Ovarian tumour cells 2008 (o) and SKOV-3 (v). Each point is the mean of 4 determinations from a single experiment (±SE).

Host cell reactivation of cisplatin-treated virus in human tumour cells. Fig. 3 shows typical results for the survival of cisplatin-treated Ad in various human tumour cell lines. For comparison purposes each experiment included the HeLa cell line. Relative HCR values for the different tumour cell lines are shown in Table I for a number of experiments. The results indicate that HCR of cisplatin-treated Ad varied by about two-fold for the 5 different tumour cell lines tested and was significantly reduced in HeLa, 2008 and SCC-25 cells compared to that in the normal human fibroblast strain indicating some deficiency in the repair of cisplatin-damaged DNA for these tumour cells.

#### Discussion

Cisplatin causes cell death by reacting with DNA, forming a spectrum of adducts (5). While it is unclear at this time which cisplatin-DNA adduct is the primary cause of cytotoxicity of the drug, comparison of cisplatin with its less toxic analogue, carboplatin, shows that cytotoxicity of the drug correlates best with the number of intrastrand adducts induced between purine bases (44). This is consistent with observations that NER deficient mutants show extreme sensitivity to cisplatin treatment (23,27) since NER is involved in the repair of intrastrand adducts (45).

The HCR values for DNA synthesis of cisplatin-treated Ad in the UV-sensitive NER deficient CHO cell mutants from complementation groups 1-6 were reduced 3 to 5-fold compared with that in parental NER proficient CHO-AA8 cells (Table I). Similar HCR values have been reported for DNA synthesis of UV-irradiated Ad in CHO cell mutants from these complementation groups 1-6 (35). The relative amount of HCR for UV-irradiated Ad in these and other NER deficient cells correlates well with the UV-sensitivity of the cells themselves as measured by colony forming ability. suggesting that HCR of UV-irradiated Ad reflects the cellular DNA repair capacity important for clonogenic survival (35, 46,47). However, CHO cell mutants from complementation groups 1 and 4 are substantially more sensitive to cisplatin and other DNA interstrand cross-linking agents compared to CHO cell mutants from other complementation groups (23,26,48,49), yet HCR values for cisplatin-treated Ad were similar for the CHO cell mutants from complementation groups 1-6 tested. Similarly reduced HCR values for cisplatin-damaged plasmid have also been reported for the NER deficient CHO cell lines UV20, UV5 and UV41 from complementation groups 1, 2 and 4 respectively (23). Taken together these results might suggest that viral and plasmid reactivation assays are unable to detect differences in the cellular capacity for DNA interstrand cross-links. This is supported by the results of Poll et al (50) which show normal HCR levels for reactivation of cisplatin-treated SV40 DNA in Fanconi anemia (FA) cells, which are known to be deficient in cisplatin-induced interstrand DNA cross-links. Normal HCR levels in FA cells have also been reported for reactivation of Ad treated with psoralen plus near UV light (51). The results of Day et al (51) suggest that interstrand DNA cross-links induced in Ad DNA by psoralen plus near UV light are not repaired during reactivation of Ad in normal human cells.

Our results indicate a substantial decrease in the capacity of NER deficient CHO cell lines to repair cisplatin-damaged DNA. We also show a reduced capacity for human fibroblasts from XP patients from complementation groups A, B, C, D, F, and G to reactivate cisplatin-damaged Ad5. This is consistent with the finding that repair synthesis of a cisplatin-treated plasmid is reduced in cell extracts from XP groups A, C, D, and G (24) and that HCR of cisplatindamaged Ad5 is reduced in XP-A fibroblasts relative to that in a normal human cell line or several ovarian and testicular cell lines (29,51). XP-A and XP-F fibroblasts have also been shown to have a reduced colony forming ability following cisplatin exposure compared to normal fibroblasts (27) and XP-A fibroblasts are reduced in their ability to remove cisplatin adducts from the active gene, DHFR (53). Collectively, these results demonstrate the central importance of NER in the cellular response to cisplatin treatment and suggest an involvement of the following genes in the repair of cisplatin-damaged DNA: ERCC1, BRCC2/XPD, ERCC3/XPB, ERCC4/XPF, ERCC5/XPG, ERCC6/CSB, XPA and XPC.

Stable transfection of the UV20 CHO cell mutant from complementation group 1 with the ERCC1 gene results in an increased cellular resistance to cisplatin (54-56) giving further evidence for the involvement of the ERCC1 gene in the repair of cisplatin damaged DNA. The reduced HCR of cisplatin treated Ad for the UV24 CHO cell mutant from complementation group and the XP-B cells presented here indicates an involvement of the ERCC3 gene in the repair of cisplatin-damaged DNA. Previous studies have suggested ERCC3 is not a determinant of cisplatin resistance based on the finding that stable transfection of the CHO 27-1 mutant cell line from complementation group 3 with the ERCC3 gene does not increase cisplatin resistance (54). However, there is recent evidence that the CHO 27-1 cell is a double mutant with defects in the repair of both alkylation damage and UV-induced lesions (56). It is possible that both mutations in CHO 27-1 cells affect the repair of cisplatin-damaged DNA such that stable transfection with the ERCC3 gene alone does not lead to an increased cisplatin resistance of the cells.

The mechanism by which tumours acquire resistance to cisplatin has been the subject of intense research (57,58). The primary mechanisms proposed to account for this resistance are: decreased intracellular drug accumulation (59), cytosolic drug inactivation by increased expression of the thiols, glutathione and metallothionein (60), increased replicative by-pass of platinated DNA (61) and increased capacity to repair cisplatin-DNA adducts. While each of these mechanisms has been described in cisplatin resistant human tumour cell lines, cellular resistance has been shown to correlate best with increased DNA repair capacity in many tumour cell lines (62) although cell lines showing anomalous relationships between cell sensitivity and DNA repair have been described by some workers (63).

A number of assays have been employed to investigate the correlation between cisplatin resistance and DNA repair in human tumour cells. [<sup>3</sup>H]thymidine incorporation was found to be increased about two-fold in resistant cells that had an increased clonogenic survival of about 5-fold (63). While resistant sublines of the A2780 cells were shown to have decreased drug accumulation, their relative sensitivities correlated better with the rate of removal of platinum from genomic DNA (62). The human breast carcinoma cell line, MCF7-MLNr, selected for its resistance to alkylating agents, showed enhanced HCR of plasmid carrying the CAT reporter gene (37).

Many studies of the murine leukemia cell line L1210 and its cisplatin resistant derivatives indicated that the increased resistance was attributable to an enhancement of DNA repair (15,23,65). However, recent results have shown that the parental cell line, L1210, bears a spontaneous mutation which was shown by complementation analysis to be in the
NER gene XPG (66). While this result negates the assertion that the acquired resistance of L1210 sublines is a useful model for the resistance acquired by tumours in vivo, it confirms the results of others which have shown the necessity for proficient NBR in the intrinsic response of cells to cisplatin (22,23).

We show here a reduced HCR of cisplatin-damaged Ad in several human tumour cells suggesting a reduced capacity for NER of cisplatin-damaged DNA in these cells. Many other investigators have reported various types of DNA repair defects in human tumour cell lines (67-70), suggesting that various deficiencies in DNA repair may be an important aspect of their malignant phenotype.

#### Acknowledgements

Supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society. We wish to thank Dr Frank Graham for supplying the human 293 cells, and Drs. L. Thompson, D. Busch, G. Whitmore, G. Singh, S. Bacchetti and J.S. Lazo for providing the various cell lines used in this study.

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Chapter 4

Overexpression of human ERCC1 can impede repair of cisplatin-treated DNA and can sensitise tumour cells to cisplatin

J. Todd Bulmer and Andrew J. Rainbow

# Preface

This paper examines the role of ERCC1 in cisplatin sensitivity using adenovirus vectors to express high levels of exogenous ERCC1 in a number of different mammalian cell types.

All of the results and the writing in this manuscript are my own.

# Abstract

Cisplatin is a DNA damaging agent that is widely used in the treatment of cancer. The efficacy of cisplatin is limited by the development of resistant tumour cells. An increased capacity to repair cisplatin-damaged DNA has been reported in cells showing resistance to cisplatin. These findings have led workers to examine the roles of known DNA repair genes in cisplatin resistance.

ERCC1 is a subunit (with XPF) of the complex which makes an incision 5' to the lesion on the damaged strand and has been shown to be expressed at higher levels in tumour cells with acquired cisplatin resistance. We have constructed adenovirus (Ad) vectors expressing the human ERCC1 gene in order to study the hypothesis that ERCC1 activity is rate limiting in the nucleotide excision repair of cisplatin-DNA adducts and is thereby important in determining cisplatin resistance.

Recombinant Ad5 expressing a functional ERCC1 gene in the deleted E3 region (Ad5-ERCC1) was used to examine the effect of ERCC1 overexpression on the host cell reactivation (HCR) of Ad5 in other CHO and human tumour cell lines. HCR of Ad5-ERCC1 was greater than that of recombinant Ad5 with the control virus (Ad5-lacZ) in NER-deficient CHO cells from complementation group 1, but not in cells from groups 2, 4, and 5, demonstrating specific complementation by ERCC1. HCR in the repair proficient parental cell line was less in cells overexpressing ERCC1 indicating an inhibitory effect of ERCC1 on repair of cisplatin-damaged DNA. Reduced HCR was also observed in cells with defects in XP-F from complementation group 4. Further, we examined this effect in several human tumour cell lines and found that there was a significant reduction in HCR of cisplatin-damaged Ad5 in cells expressing exogenous ERCC1.

To study the role of ERCC1 in the resistance of mammalian cells to cisplatin we have used a non-lytic Ad vector expressing ERCC1 in the deleted E1 region (Ad-dE1-ERCC1). ERCC1 overexpression greatly increases the cisplatin resistance of the NER deficient cells from group 1, demonstrating complementation. However, the human tumour cell line, HeLa, is made more sensitive to cisplatin by the infection with Ad5-dE1-ERCC1. We conclude that high levels of ERCC1 expression can reduce the rates of NER of cisplatin damaged DNA and this can sensitise cells to cisplatin.

# Introduction

The development of drug resistant subpopulations within a tumour is the major obstacle to a positive clinical outcome with cisplatin treatment (Perez et al., 1993). There is, therefore, tremendous interest in this acquired drug resistance and a number of mechanisms may be involved. Decreased drug accumulation, enhanced bypass of cisplatin adducts during replication, increased expression of proteins that bind to and inactivate the drug, and improved DNA repair have each been shown to correlate with acquired cellular resistance to cisplatin (Gately and Howell, 1993; Mamenta et al., 1994; Chu, 1994; Eastman, 1991).

While it is unclear at this time which of these mechanisms of cisplatin resistance is most important to clinical outcome, the capacity for DNA repair has been shown to correlate best with drug resistance in many human tumour cell lines (Masuda et al., 1988; Zhen et al., 1992; Johnson et al., 1994). One mechanism which is essential to the repair of cisplatin-damaged DNA is nucleotide excision repair (NER) (Bulmer et al., 1996; Sheibani et al., 1989; Plooy et al., 1985).

ERCC1 is a subunit (with XPF) of the complex which associates with XPA and makes an incision 5' to sites of DNA damage which are recognised by NER (van Vuuren et al., 1993; Park and Sancar, 1994; Sijbers et al., 1996). The ERCC1 protein contains a nuclear localisation signal and a helix-turn-helix DNA binding domain (van Duin et al., 1986). Mammalian cells with defects in ERCC1 or XPF show cross-sensitivity to agents that form interstrand crosslinks and are more sensitive to cisplatin than other NER mutants (Collins, 1993; Damia et al., 1996) suggesting that the 5' excinuclease complex is involved in another repair pathway. This is consistent with evidence that the homologous complex in *Saccharomyces cerevisiae* (RAD1-RAD10) cleaves intermediate structures in the repair of damage events affecting both strands (Bardwell et al., 1994). Functional ERCC1 protein is necessary for repair of cisplatin-damaged DNA (Bulmer et al., 1996; Larminat and Bohr, 1994) and to the intrinsic resistance of cells to cisplatin (Bramson et al., 1993; Lee et al., 1993). Workers investigating the processes by which tumour cells acquire cisplatin resistance have proposed that increased ERCC1

expression may contribute to resistance. Two-fold increases in the levels of ERCC1 mRNA and protein have been found to correlate with poor clinical outcome in ovarian cancer patients (Dabholkar et al., 1992; Dabholkar et al., 1994; Yu et al., 1996). Similar (2-3 fold) increases in ERCC1 mRNA have also been identified in lymphocytes from patients with chronic lymphocytic leukemia resistant to DNA alkylation (Geleziunas et al., 1991). Additionally, some cisplatin-resistant ovarian cancer cells have been shown to have a 2-4 fold increase in ERCC-1 mRNA and protein levels following cisplatin treatment (Li et al., 2000; Li et al., 1998). While this modest increase in ERCC-1 expression correlates with resistance in these cell lines, some mammalian cells constructed to express much higher ERCC-1 levels were actually made more sensitive to cisplatin (Bramson and Panasci, 1993).

We have used recombinant adenovirus vectors that express the human ERCC1 gene to study the effect of ERCC1 overexpression on the repair of cisplatin-damaged DNA and on cisplatin resistance in CHO cells and human tumour cells.

## Materials and methods

## Cells and Virus

Parental CHO-AA8 cells and UV-sensitive UV20, UV5, UV41, UV135 mutant CHO cell lines (from complementation groups 1,2,4, and 5, respectively) were provided by Dr. Larry Thompson, Lawrence Livermore National Laboratory, Livermore, CA, with the help of Dr. Gordon Whitmore, Physics Division, Ontario Cancer Institute, Toronto, Ontario.

The human cervical carcinoma, HeLa cell line was obtained from the American Type Culture Collection, Rockville, MD, USA. Medical Research, Camden, N.J., USA. The human colon carcinoma cell line HT29, the ovarian carcinoma cell line 2008, and the cisplatin-resistant 2008-sub-line, C13 were obtained from Dr. G. Singh, Hamilton Regional Cancer Centre, Hamilton, Ontario, Canada. Human 293 cells were obtained from Dr. F. Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada.

All cells were grown in monolayer in alpha-minimal essential medium (α-MEM) (GIBCO-BRL Canada) with 1% antibiotic-antimycotic supplemented with 10% newborn calf serum (NCS) for CHO cells or 10% fetal bovine serum (FBS) for cells of human origin.

The recombinant viruses used in these experiments contain exogenous genes inserted into the deleted non-essential E3 region. The virus containing the  $\beta$ galactosidase gene (Ad5-lacZ) was kindly provided by Dr. F. L. Graham, Departments of Biology and Pathology, McMaster University, Ontario, Canada. A 1.1 kb ERCC1 expression cartridge was inserted under the endogenous, E3 promoter and carries the SV40 polyadenylation signal (Colicos and Rainbow, 1991). Viral stocks were grown as described previously (Hitt et al., 1995) on monolayers of 293 cells (Graham et al., 1977). Stocks were titred by plaque assay on human 293 cells and suspended in PBS to a titre of about 10<sup>9</sup> plaque forming units (pfu)/mL.

# Treatment of virus

Ad5-LacZ-5 or Ad5-ERCC1 were treated with cisplatin in a suspension in which the chloride ion concentration was adjusted to 50 mM using an appropriate ratio of low chloride PBS and  $\alpha$ -MEM containing 1% antibiotic-antimycotic solution. Cisplatin solution was then added to give the appropriate final drug concentration. This suspension was left at 37°C for 24 hours.

# Quantification of viral DNA synthesis

CHO cells and human tumour cells were seeded in Nunc 24-well plates at  $2 \times 10^5$  cells/well. Cell monolayers just reached confluence at about 24 hours after seeding and were infected with either untreated or cisplatin-treated Ad5-lacZ or Ad5-ERCC1 at a multiplicity of infection (MOI) of 40 pfu/cell in a volume of 200 µL/well. Following viral adsorption for 90 minutes at 37°C, the infected cells were overlayed with 1 mL of warm  $\alpha$ -MEM supplemented with 10% NCS or FBS. 2-4 Hours after infection virus which had not adsorbed to the cells was removed by aspirating and replaced with warm  $\alpha$ -MEM containing 10% serum and 1% antibiotic solution.

72 Hours after infection, infected cells were lysed and DNA was transferred to a nylon membrane. Cells which were seeded to 24-well plates were lysed by addition of 200  $\mu$ L of pronase solution (4 mg/mL pronase, 40 mM Tris pH 8.0, 40 mM EDTA pH 8.0, 2.4% SDS) and incubated for 1.5-3 hours. The cell lysates were then stored at -20°C until phenol:chloroform extractions were performed and DNA was precipitated with two volumes of cold absolute ethanol with 0.2 M NaCl. DNA was then pelleted by

centrifugation at 13 000 rpm for 20 - 30 minutes at 4°C after which pellets were resuspended in 40  $\mu$ L of TE buffer (10 mM Tris, 1 mM EDTA) and slot blotted onto Gene Screen Plus (Dupont Cat.#NEF-976) according to the manufacturer's specifications.

Viral DNA in each sample was probed with <sup>32</sup>P-labelled Ad2 DNA and the relative viral DNA content in each sample was determined from the radioactivity of each each slot blot using a phosphorimaging system.

# Colony forming assays:

Cells to be treated with cisplatin were seeded at  $5 \times 10^5$  per well of a 6-well plate and left over night. The cells were then infected (as above) with Ad5-dE1-ERCC1 at the desired MOI. 18 Hours after infection cells were typsinised and seeded at ~150 cells/well into new 6 well plates. 24 Hours after infection the cells were treated for 1 hour with supplemented media containing the desired concentration of cisplatin. The treatment solution was then removed and replaced with fresh, supplemented media and the plates were incubated for 5-7 days to allow colony formation. Plates were stained with methylene blue and colonies consisting of 32 or more cells were counted.

# Results

# HCR of Ad5-ERCC1 and Ad5-lacZ in CHO cell lines.

The effect of exogenous ERCC1 expression on DNA repair was examined in different mammalian cell types by comparing HCR of the Ad5 vector carrying the

ERCC1 expression cartridge to HCR of a control Ad5 vector carrying the repair inert lacZ gene 72 hours after infection. Functional expression of the ERCC1 gene product is evident in panel B of figure 4-1 where the HCR of cisplatin-treated Ad carrying the ERCC1 gene is substantially greater than that of the control Ad in NER deficient CHO cells from complementation group 1 (UV20). In panel A of figure 4-1, the NER proficient parental cell line, AA8, shows substantially lower HCR of Ad5-ERCC1 compared to Ad5-lacZ. Lower HCR of cisplatin-treated Ad5-ERCC1 by NER deficient CHO cells from complementation group 4 (UV41) is demonstrated in panel D while panel C of figure 4-1 shows similar HCR levels of both viruses in a CHO mutant cell line from group 2.

Survival curves were fitted to a linear quadratic equation, ln(Relative amount of viral DNA) = -  $(\alpha D + \beta D^2 + \chi)$  and the parameters  $\alpha$ ,  $\beta$ , and  $\chi$  were determined by least squares analysis in order to obtain a D<sub>37</sub> value for each survival curve. In each experiment the D<sub>37</sub> value for HCR of cisplatin-treated Ad5-ERCC1 was made relative to the D<sub>37</sub> for cisplatin-treated Ad5-LacZ for comparison between experiments. The mean values from all experiments are listed in table 4-I. There is a significant reduction in the HCR of cisplatin-treated Ad5-ERCC1 compared to Ad5-LacZ in NER proficient CHO cells (AA8) and in NER deficient CHO cells from complementation group 4 (UV41). There is no difference between the HCA of cisplatin-treated Ad5-ERCC1 and Ad5-LacZ in NER deficient CHO cell lines from complementation groups 2 and 5 (UV5 and UV135, respectively).

# HCR of cisplatin-treated Ad5-ERCC1 and Ad5-LacZ in human tumour cells.

We extended our investigation to human cells by comparing the HCR of cisplatintreated Ad5-ERCC1 to Ad5-LacZ in several tumour cell lines. Results were obtained as described above and are summarised in Table I. There is significantly lower HCR of the Ad vector expressing the ERCC1 gene than the lacZ vector in the ovarian, cervical, and colon carcinoma cell lines examined (2008, HeLa, and HT29, respectively). We also detected a decrease in HCR in C13, the cisplatin-resistant sub-line of 2008 but our results do not provide 95% confidence that this decrease is significant.

# Expression of exogenous ERCC1 affects cisplatin sensitivity.

To determine if the reduction in NER by ERCC1 overexpression affects the survival of cisplatin-treated cells, we used a non-replicating virus expressing ERCC1 in the deleted E1 region of Ad5 (Ad5-dE1-ERCC1). The functional expression of ERCC1 is demonstrated in figure 2. The extreme sensitivity of the NER deficient CHO cells is complemented by infecting cells 24 hours prior to infection with Ad5-dE1-ERCC1. Interestingly, the survival curves of the infected cells show resistant sub populations that increase with the ratio of virus to cell. The fraction of resistant cells in the population can be estimated by extrapolating the linear portions (doses > 1 $\mu$ M) of the survival curves back to the axis. For multiplicities of 0.5, 5, and 100 pfu/cell the resistant fractions were 3.6, 13, and 79%, respectively. The sensitivity of these subpopulations (in cytotoxicity per unit of dose) is similar for each infection condition suggesting that cisplatin resistance results from some critical level of ERCC-1 and any excess confers no further resistance.

No quantity of Ad5-dE1-ERCC-1 virus was sufficient to cause resistance in the UV20 cells to be similar to the repair proficient, parental cells, AA8. This may be a consequence of differences in these cells that is independent of ERCC-1 or may be a result of imprecise complementation of the human ERCC-1 gene in the CHO system. Expression of exogenous ERCC1 has no demonstrable effect on the survival of the repair proficient parental cell line, AA8.

The effect of ERCC1 overexpression was determined in the human cervical carcinoma cell line, HeLa. HeLa cells infected with Ad5-dE1-ERCC1 at 100 pfu/cell 24 hours before cisplatin treatment are more sensitive than cells similarly infected with the control virus expressing the  $\beta$ -galactosidase gene. The relative effect of ERCC1 is described in table I. ERCC1 overexpression reduces survival of cisplatin-treated HeLa cells to between 46 and 75% of controls at cisplatin doses of 1.25, 2, 4, 5, 7.5, and 10  $\mu$ M.

Table 4-I.		·	
CELL LINE	*RATIO OF HCR (Ad5-ERCC1/Ad5-lacZ) (±SE)	NUMBER OF EXPERIMENTS	
CHO cells			
AA8 (parental)	<sup>a</sup> 0.37±0.10	3	
UV20 (grp. 1)	<sup>b</sup> 3.20±1.49	4	
UV5 (grp. 2)	1.36±0.55	2	
UV41 (grp. 4)	<sup>a</sup> 0.49±0.04	5	
UV135 (grp. 5)	1.00±0.41	2	
Tumour cells			
2008	<sup>a</sup> 0.61±0.05	3	
HeLa	<sup>a</sup> 0.38±0.14	2	
HT29	<sup>a</sup> 0.35±0.08	2	
C13	0.82±0.21	3	

<sup>\*</sup> The D<sub>37</sub> value for the HCR of Ad5-ERCC1 have been made relative to those of Ad5-lacZ within each experiment and the mean of these values is shown. <sup>a</sup> Signifies significant decrease (p < 0.05) <sup>b</sup> Signifies significant increase (p < 0.05)

Tabl	e 4-	·II.
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Cisplatin Treatment to HeLa cells (µM)	Relative Survival (ERCC1/lacZ)	Number of Experiments	Standard Error	p-value
1.25	0.75	2	0.20	0.17
2.5	0.56	3	0.01	<0.001
° <b>4</b>	0.61	3	0.15	0.03
5	0.61	3	0.24	0.09
7.5	0.46	2	0.11	0.02
10	0.49	3	0.14	0.01

Table 4-II: The effect of ERCC1 overexpression on the clonogenic survival of HeLacells following various 1 hour cisplatin treatments.

# Discussion

ERCC1 is necessary in the normal response of mammalian cells to cisplatin because of its requirement for the repair of cisplatin-damaged DNA (Sheibani et al., 1989; Lee et al., 1994; Bulmer et al., 1996). This has made ERCC1 one protein considered a candidate as a rate-determining factor in the repair of cisplatin-damaged DNA. Since enhanced DNA repair has been described in some cell lines with acquired cisplatin resistance (Masuda et al., 1988; Dijt et al., 1988; Hospers et al., 1990; Zhen et al., 1992; Johnson et al., 1994), increased ERCC1 expression has been proposed as a mechanism of the acquired resistance which limits the clinical efficacy of the drug (Gosland et al., 1996). Efforts have been made to correlate ERCC1 expression with the clinical outcome for patients treated with cisplatin (Geleziunas et al., 1991; Dabholkar et al., 1992). Researchers have investigated this potential cause of clinical resistance in vitro by creating CHO cell lines overexpressing ERCC1 by stable transfection in the repair proficient CHO cells, AA8 (Bramson and Panasci, 1993). ERCC1 overexpression failed to improve clonogenic survival following treatment with cisplatin or melphalan beyond intrinsic levels and, in fact, caused increased sensitivity. However, in this study using a non-replicating Ad vector, we observe no effect of exogenous ERCC1 expression in AA8 cells (figure 4-1). It may be that ERCC1 expression in our system is of insufficient level or duration to cause sensitisation in CHO cells. ERCC-1 overexpression can make the human tumour cell line, HeLa, more sensitive to cisplatin

Figure 4-1: Effect of ERCC1 expression of HCR of cisplatin-treated Ad in CHO cells. Survival of viral DNA synthesis for cisplatin-treated Ad5-ERCC1 (●) is shown in comparison to Ad5-lacZ 5 (■) and the linear quadratic fit lines are shown (Ad5-ERCC1, dotted; Ad5-lacZ, solid). A) NER proficient parental cell line, AA8. B) Group 1 NER deficient CHO cells, UV20. C) Group 2 NER deficient CHO cells, UV5. D) Group 4 NER deficient cells, UV41.



(table 4-II). This may be because human cells are infected more efficiently than rodent cells and express higher levels of transgenes from non-replicating Ad vectors (Fields, 1996).

We have used HCR of a replicating Ad vector to study DNA repair in systems where ERCC1 is overexpressed. Our results show that HCR of cisplatin-treated Ad is reduced in CHO cells overexpressing ERCC1 and suggest that high levels of ERCC1 sensitise cells by reducing repair of cisplatin-damaged DNA.

Interestingly, ERCC1 overexpression causes a reduction of HCR in the NER deficient CHO cell line from complementation group 4 but not cells from complementation groups 2 and 5. This suggests that a high level of ERCC1 hinders some repair process which is still, at least partially, active in these NER deficient cells. Group 4 mutants have a defect in the XPF/ERCC4 gene product which associates tightly with ERCC1 in the complex which makes an incision 5' to the lesion on the damaged strand (van Vuuren et al., 1993). A recent report has demonstrated that the human XPF gene complements the NER defects in both CHO genes ERCC4 and ERCC11 (Sijbers et al., 1996). This intragenic complementation between groups 4 and 11 suggests that XPF is a multifunctional protein. This is consistent with previous reports that have shown involvement of the ERCC1-XPF complex in pathways which are involved in the repair of interstrand crosslinks as well as some ionising radiation induced DNA damage other than double strand breaks (Hoeijmakers, 1993; Murray et al., 1995).

Plasmid and viral HCR assays are unable to detect differences in cellular capacity for the repair of damage affecting both strands of the DNA (Poll et al., 1984; Day et al., Figure 4-2: Expression of exogenous ERCC1 is protective against cisplatin in NER- deficient cells from complementation group 1. The extreme sensitivity of NER-deficient cells from group 1 (UV20,  $\bullet$ ) is shown compared with the parental cell line (AA8,  $\bullet$ ) similarly infected with a control virus (Ad5-dE1-lacZ). The effect of exogenous ERCC1 expression on cisplatin sensitivity of UV20 cells was determined by infecting 24 hours before cisplatin treatment with Ad5-dE1-ERCC1 at 0.5 pfu/cell (diamonds, $\blacklozenge$ ), 5 pfu/cell (up-triangles, $\blacktriangle$ ), and 100 pfu/cell (down-triangles, $\heartsuit$ ). Hollow symbols represent the surviving fractions of the repair-proficient AA8 cells similarly infected with Ad5-dE1-ERCC1 at 0.5 pfu/cell (diamonds,  $\diamondsuit$ ), 5 pfu/cell (up-triangles,  $\triangle$ ), and 100 pfu/cell (down-triangles,  $\bigtriangledown$ ). Symbols represent the mean of 4 determinations (±SEM) within an experiment that is typical of 4 independent experiments.



1975). Our results suggest that there remains some repair function in NER deficient CHO cells from complementation group 4 which is involved in the repair of damage to a single strand.

The HCR of cisplatin-treated Ad5-ERCC1 was significantly lower than Ad5-lacZ in the cervical, ovarian, and colon carcinoma cell lines (HeLa, 2008, and HT29 cells respectively) examined. This inhibition of repair is consistent with the proposal that ERCC1 is a determinant of cisplatin resistance. Increased ERCC1 mRNA and protein levels have been detected in malignant tissues from patients not responding to treatment with cisplatin (Geleziunas et al., 1991; Dabholkar et al., 1992) and increased DNA repair has been reported in cell lines with acquired resistance to cisplatin (Masuda et al., 1988; Dijt et al., 1988; Hospers et al., 1990; Zhen et al., 1992; Johnson et al., 1994). This enhancement of DNA repair may be the result of a concerted, multifactor up-regulation of DNA repair activity of which ERCC1 is only a part. Indeed, increased expression of ERCC1 alone may give a cisplatin resistant phenotype when the increase is small, while extremely high levels sensitise cells.

The formation of the ERCC1-XPF complex is thought to stabilize both monomers as mutations in either gene which disrupt their interaction greatly reduce the cellular concentrations of both ERCC1 and XPF (Biggerstaff et al., 1993; Sijbers et al., 1996). A 20-fold increase in ERCC1 mRNA levels over wild type resulted in only a marginal increase in protein levels in NER deficient CHO cells from group 1 (Murray et al., 1995). Both of these results suggest that high levels of ERCC1 transcription should have no phenotypic effect since ERCC1 concentration and activity should be limited by the

endogenous levels of XPF. Clearly, cell phenotype is affected by extremely high levels of ERCC1 expression.

It has been proposed that high levels of ERCC1 cause cisplatin sensitivity by causing a rate of 5' incision that exceeds the capacity of the rest of the repair machinery. This could sensitise cells by causing incisions at sites that remain unrepaired (Bramson and Panasci, 1993). While our results do not address this possibility, this hypothesis seems unlikely in light of more recent results that show that the incision activity of ERCC1/XPF is an obligate follower of XPG incision. That is, the ERCC1/XPF incision site, 5' to the DNA lesion, is not a substrate for incision until the 3' incision has been made by the excinuclease, XPG. In fact, XPG is incision-competent in the presence or absence of ERCC1/XPF (Mu et al., 1996).

It is possible that ERCC1 must dissociate from the repair complex in order to allow completion of repair. At high levels, ERCC1 may prevent this dissociation and thereby block progression of the repair pathway (Bramson and Panasci, 1993). An analogous situation has been described in an *in vitro* E. *coli* system. High levels of uvrA prevents the dissociation of the uvrA<sub>2</sub> subunit and blocks incision by the uvrC endonuclease (Bertrand-Burggraf et al., 1991).

ERCC1 interacts with a number of different proteins in the process of NER (Park and Sancar, 1994). The introduction of exogenous ERCC1 may reduce repair by disturbing wild-type ratios of these proteins. High levels of ERCC1 may reduce DNA repair rates through the competition for damage sites between the ERCC1-XPF 5' incision complex and the ERCC1 monomer that does not carry endonuclease activity. ERCC1 bears a helix-turn-helix domain which is a putative DNA-binding motif which could allow the monomer to bind to sites of damage and block access to the lesion (van Duin et al., 1986). ERCC1 promotes UV-damage-specific binding by XPA through direct protein-protein interaction (Nagai et al., 1995; Li et al., 1995). At very high ERCC1 levels, this function may inhibit repair of cisplatin-damaged DNA by decreasing the specificity of XPA for damaged DNA resulting in the binding of undamaged genomic sequences which are similar in secondary structure to sites of UV lesions. This could effectively sequester XPA from sites of cisplatin adducts causing reduced repair.

None of these models address the specificity of the observed inhibition. The ERCC1 levels in cells infected with our vector enhance the repair of UV-damaged viral DNA (Colicos et al., 1991) and CHO cells overexpressing ERCC1 do not show increased UV-sensitivity (Bramson and Panasci, 1993). Additionally, the HeLa, human tumour cell line overexpressing a mutant form of ERCC-1 are more sensitive to the cross-linking agent mitomycin C, whereas high levels of wild type ERCC-1 did not affect mitomycin C sensitivity (Belt et al., 1991). Until now, overexpression of a DNA repair gene had not been shown to inhibit repair in mammalian cells. Indeed, overexpression of XPA may improve clonogenic survival of human cells following UV exposure (Cleaver et al., 1995). The specificity of these observations underscores the unexplored subtlety of DNA repair mechanisms in mammalian systems.

# Chapter 5

Pre-exposure of human squamous carcinoma cells to low doses of  $\gamma$ -rays leads to an increased resistance to subsequent low-dose cisplatin treatment.

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International Journal of Radiation Biology (1999) 75(8): 963-972

# Preface

This paper is the first report of cisplatin hypersensitivity and induced cisplatin resistance in a human tumour cell line. This hypersensitivity appears to be analogous to the hypersensitivity and induced resistance in human tumour cells irradiated with low LET ionising radiation.

The 7 experiments summarised in panel B of figure 1 were performed by me and I contributed to some of the statistical analysis and to the writing of the manuscript.

INT. J. RADIAT. BIOL 1999, VOL. 75, NO. 8, 963-972



82

Pre-exposure of human squamous carcinoma cells to low-doses of  $\gamma$ -rays leads to an increased resistance to subsequent low-dose cisplatin treatment

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(Received 9 December 1998; accepted 23 April 1999)

#### Abstract.

Purpose: To investigate low-dose hypersensitivity to cisplatin and increased resistance at higher doses of cisplatin for the human squamous carcinoma cell line SCC-25 and its cisplatin-resistant derivative SCC-25/CP, and to examine the effects of pre- and post-treatment of SCC-25 cells with low-doses of  $\gamma$ -rays on their resistance to cisplatin.

Materials and methods: SCC-25 and SCC-25/CP cells were treated with various cisplatin concentrations (0.1 to 20  $\mu$ M for 1 h) and assayed for survival using a conventional colony assay. For SCC-25, various doses of  $\gamma$ -rays (5 cGy to 2.5 Gy) were given either 10 or 60 min before the cisplatin challenge dose as well as either 10 or 60 min after the cisplatin challenge dose.

Results: Low-dose (0.5, 0.75 and 1  $\mu$ M for 1 h) hypersensitivity to cisplatin and increased resistance at higher doses was detected for the SCC-25 cell line, but not for its cisplatin-resistant derivative, SCC-25/CP. Pretreatment of SCC-25 cells with an acute low-dose of 5, 25 cGy or 1 Gy  $\gamma$ -rays given 60 min before a low-dose cisplatin challenge (0.1 and 1  $\mu$ M for 1 h) resulted in a significant increase in resistance (p=0.2, 0.01 and <0.001 respectively). For pretreatment of SCC-25 cells with similar lowdoses of  $\gamma$ -rays 10 min before the challenge cisplatin dose, the increased resistance was reduced or absent and was only significantly increased for pretreatment with 25 cGy and a challenge cisplatin dose of 0.1  $\mu$ M for 1 h (p=0.02). Similar acute low-doses of  $\gamma$ -rays given either 10 or 60 min after the challenge cisplatin dose did not increase resistance.

Conclusions: The human squamous carcinoma cell line SCC-25 showed a low-dose hypersensitivity to cisplatin followed by increased resistance at higher doses. Treatment of SCC-25 cells with low-doses of  $\gamma$ -rays can induce a protective effect to a subsequent low-dose cisplatin challenge.

#### 1. Introduction

Radiotherapy is an effective treatment for squamous cell carcinomas of the upper respiratory and digestive tract (Sweeney *et al.* 1994, Harari and Kinsella 1995, Ang 1998) and cisplatin is a highly effective chemotherapeutic agent in the treatment of

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squamous cell carcinomas of the head and neck and of the cervix (Al-Sarraf 1984). To improve further the treatment of certain tumours, there has been considerable interest in the combined modalities of radiotherapy and chemotherapy using cisplatin (Dewit 1987, Wheeler and Spencer 1995).

The European Organization for Research and Treatment of Cancer (EORTC) reported results of a randomized trial that showed that local cancer control and survival of patients with inoperable nonsmall cell lung cancer can be improved by combining radiotherapy with daily low-dose cisplatin, compared with radiotherapy alone (Schaake-Koning *et al.* 1994). Some additional clinical studies have found cisplatin to be a radiosensitizer (Crissman *et al.* 1987, Dewit 1987, Hazuka *et al.* 1994) whereas other studies have found no sensitization by cisplatin to radiation (Stewart *et al.* 1994, Blanke *et al.* 1995), or a possible increase in patient survival with a marked increase in toxicity (Hazuka *et al.* 1994).

Some laboratories have reported that cisplatin treatment can produce radiosensitization in a variety of mammalian cell lines (Begg et al. 1986, Dewit 1987, Raaphorst et al. 1996) and in some animal tumour models (Dewit 1987). A marked interaction occurs at relatively low radiation doses, in the 1-4 Gy range, which are used in the clinic (Korbelik and Skov 1989, Skov and MacPhail 1991, Walter et al. 1993). One mechanism of interaction currently believed to play a role in radiosensitization caused by cisplatin is an inhibition in the repair of radiationinduced DNA damage. It has been shown that cisplatin can inhibit the repair of radiation-induced damage when administered before or after radiation treatment (Carde and Laval 1981, Wilkins et al. 1993, Raaphorst et al. 1996, Dolling et al. 1998). The degree of repair inhibition has been reported to be dependent upon the sequence and timing of the combined treatments. In contrast, more recent reports indicate that low-dose cisplatin treatment  $(1.0 \,\mu g/m)$  for 30 min) 24 h before irradiation results in increased repair of DNA strand breaks and a reduction in

International Journal of Radiation Biology ISSN 0955-3002 print/ISSN 1362-3095 online © 1999 Taylor & Francis Ltd

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C. Caney et al.

radiation-generated micronuclei indicating an adaptive response (Dolling et al. 1998). However, the mechanism by which cisplatin inhibits, or enhances, cellular repair of radiation damage is not known (Dewit 1987, Wilkins et al. 1993). Other reports indicate that certain tumour cell variants isolated following fractionated exposures to low-dose Xirradiation acquire increased resistance to cisplatin (Hill et al. 1990, Dempke et al. 1992, Eicholtz-Wirth et al. 1993, Eicholtz-Wirth and Hietel 1994).

Several investigators have reported cell survival curves for radioresistant cell lines that exhibit a region of initial low-dose hyper-radiosensitivity (HRS) followed by a region of induced radioresistance (IRR) over the dose range 0.3-0.7 Gy, beyond which the response is typical of that seen in most survival curves (Skarsgard et al. 1991, Marples and Joiner 1993, Lambin et al. 1993, 1994, Singh et al. 1994). These results are consistent with the existence of a biological protective mechanism that reduces the cytotoxic effects of radiation and is triggered by some critical dose of radiation. Pretreatment of cells with a low-dose of X-rays before a challenge dose can induce IRR and abolish the HRS region (Marples and Joiner 1995). Other agents reported as having induced a similar protective effect against subsequent radiation exposure include mild hyperthermia (Cai and Jiang 1995), tritiated and <sup>14</sup>C-labelled thymidine (Skov and Marples 1995), hydrogen peroxide (Wolff 1992, Shadley 1994, Marples and Joiner 1995) and low-dose cisplatin (Marples et al. 1997).

Both excision repair and repair of single-strand breaks in DNA have been implicated in the mechanism of induced protection against subsequent radiation damage (Popoff et al. 1987, Skov and Marples 1995). This response could have important implications for clinical treatment involving combinations of cisplatin and radiation. In particular, the observed dependence of an inducible protective response on radiation dose (Skov and Marples 1995, Marples and Skov 1996), cisplatin concentration and the interval between the two treatments (Beck-Bornholdt et al. 1989, Skov and Marples 1995, Marples and Skov 1996) may turn out to be factors which can influence the patient response to combined treatments.

Cell survival curves showing low-dose hypersensitivity and increased resistance at higher doses have also been observed following cisplatin treatment of mouse fibrosarcoma (Rif-1) cells (Begg et al. 1986, Moorehead et al. 1994) and Chinese hamster ovary (CHO) cells (Marples et al. 1997). The present paper reports a low-dose hypersensitivity to cisplatin and increased resistance at higher doses of cisplatin for the human squamous cell carcinoma SCC-25. Reported too, is the absence of low-dose hypersensitivity in the cisplatin-resistant SCC-25/CP cell line, which was isolated by repeated exposure of SCC-25 cells to increasing concentrations of cisplatin (Teicher *et al.* 1987). Also shown is that pretreatment of SCC-25 cells with acute low-dose  $\gamma$ -irradiation (5 cGy to 1 Gy) results in an increased resistance to a subsequent low-dose cisplatin challenge (0.1 and 1  $\mu$ M cisplatin for 1 h), whereas acute low-dose  $\gamma$ -irradiation given after the cisplatin challenge dose did not increase resistance.

#### 2. Materials and methods

#### 2.1. Cell lines and culture conditions

Human squamous carcinoma cells of the tongue (SCC-25) were obtained through Dr John Lazo, Department of Pharmacology, University of Pittsburgh School of Medicine. The cisplatin-resistant cell line (SCC-25/CP) was developed by Teicher et al. of the Dana-Farber Cancer Institute, Division of Cancer Pharmacology, Boston, MA, by repeated exposure of SCC-25 cells to increasing concentrations of cisplatin (Teicher et al. 1987). The SCC-25 and SCC-25/CP cell lines were grown as monolayers in Dulbecco's modified eagle medium (DMEM-high glucose), supplemented with 10% foetal bovine serum,  $0.4 \,\mu g/ml$  final concentration hydrocortisone (dissolved in 10% ethanol) and 1% antibioticantimycotic solution (penicillin G sodium 10000 units/ml, streptomycin sulphate  $10\,000\,\mu$ g/ml, amphotericin B as Fungizone<sup>®</sup> 25 µg/ml in 0.85% saline) all from GibcoBrl Laboratories (Burlington, Ontario, Canada).

#### 2.2. Colony survival assay

Cell survival was assessed by a colony-forming assay. Cells were grown to semi-confluency, suspended by application of 2 ml of trypsin-EDTA (trypsin 0.5%, EDTA.4 Na 5.3 mm from GibcoBrl) and pelleted by centrifugation for 5 min at 1000 rpm and  $20^{\circ}$ C (IEC Centra-8R centrifuge, USA). Cell pellets were resuspended in growth medium, counted using a haemocytometer and serially diluted for seeding. Cells were seeded at 1800 SCC-25 cells per well and 1000 SCC-25/CP cells per well in Corning six-well plates or 100-mm Petri dishes (Fisher Scientific, Mississauga, Ontario, Canada), and had plating efficiencies of 20 and 35% respectively.

Cells were incubated overnight before treatment to ensure their adherence to the plate. Visual inspection under the light microscope revealed that <2%of the cells has completed one cell division before

treatment. After treatment, cells were incubated at 37°C in a humidified chamber containing 5% CO<sub>2</sub> and 95% humidity. Cells were permitted to grow for 5-7 days depending on colony growth in control samples. The doubling time for both the SCC-25 and SCC-25/CP cells was  $\sim$  28 h. When control samples contained colonies of at least 30 cells, colonies were fixed, stained with methylene blue and counted. Growth medium was removed from each well, samples were rinsed gently with PBS (140 mm NaCl, 2.7 mm KCl, 8.1 mm Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mm KH<sub>2</sub>PO<sub>4</sub>, pH 7.2; Fisher Scientific) and flooded with 0.5% methylene blue stain (Sigma Chemical Co., St Louis, MO, USA) dissolved in 70% methanol, 30% distilled water. Using a light microscope, a colony of >30 cells was counted as a survivor.

#### 2.3. Cisplatin treatment

Cisplatin was purchased from Sigma and was prepared as a  $333 \,\mu\text{M}$  stock solution in sterile PBS before each experiment. The stock solution was sterilized by filtration through a 0.2 micron Acrodisc (Gelman Sciences, Montreal, Quebec, Canada). Appropriate concentrations of drug were serially diluted from the stock solution using fresh growth medium. Each sample was incubated with the appropriate cisplatin concentration for 1 h while controls were incubated with fresh growth medium for the same duration. At the end of cisplatin treatment each sample was rinsed once with 3 ml fresh growth medium and then replenished with 5 ml fresh growth medium for the remainder of the experiment.

#### 2.4. Radiation treatment

Samples were irradiated at room temperature by  $^{60}$ Co  $\gamma$ -rays (Theratron 80). The dose-rate at the sample was 59.8 cGy/min and radiation doses ranging from 5 cGy to 2.5 Gy were used. The source to sample distance (SSD) was 80 cm and field size was  $30 \times 30$  cm. The lowest dose of 5 cGy was about a 5 s exposure and a 0.02 s correction for shutter error was necessary. Controls were mock irradiated. At the end of radiation treatment the samples received fresh growth medium.

#### 2.5. Combined cisplatin and radiation treatment

Radiation and drug treatments were carried out according to four different schedules. In the first two, cells were exposed to radiation before cisplatin treatment. An interval of 60 or 10 min was allowed between treatments. In the remaining two schedules, cells were treated with cisplatin followed by radiation either 10 or 60 min later.

#### 2.6. Statistical methods

Each datum point plotted in figures 2-6 is the mean of three independent experiments. The uncertainties are standard errors of the mean. A twosample Student's *t*-test was used to test for a difference between two means (Schefler 1988). The significance of the difference in surviving fraction between pretreated and non-pretreated cells was also determined using the  $\chi^2$ -test and the  $\chi^2$ -goodness of fit was used to quantify how well the survival curve for pretreated cells matches the survival curve for non-pretreated cells (Bevington 1969).

#### 3. Results

#### 3.1 Cell survival following cisplatin treatment

Figure 1 shows the clonogenic survival of SCC-25 and SCC-25/CP cells following treatment with cisplatin concentrations of  $1-20 \,\mu$ M. The cisplatin survival curve for the SCC-25 cell line was characterized by a low-dose hypersensitive region for doses up to  $1 \,\mu$ M cisplatin, followed by increased resistance for cisplatin concentrations  $> 1 \,\mu$ M. Whereas the SCC-25/CP cells were substantially more resistant to cisplatin and the survival curve for the SCC-25/CP cells did not show a region of low-dose hypersensitivity over the range of cisplatin doses examined (figure 1).

#### 3.2. Combined cisplatin and radiation treatment

Priming doses of  $\gamma$ -rays were given to cells at either 60 or 10 min before the challenge cisplatin doses. The effect of prior radiation exposure on cisplatin survival is shown in figure 2 for 5 cGy of y radiation given 60 min before the cisplatin challenge dose. The combined treatment curve has been corrected for cell death due to radiation. In general, cells pretreated with low-doses of radiation were more resistant to cisplatin concentrations of 0.1 and 1 µM compared with cells that were not pretreated. Figure 3 shows the changes in surviving fraction for 0.1, 1 and 10  $\mu$ M cisplatin as a function of the radiation pretreatment dose. It can be seen that pretreatment of cells with 5, 25 cGy or 1 Gyy-rays resulted in an increase in cell survival following cisplatin concentrations of 0.1 and 1 µM compared with non-pretreated cells. The significance of the difference in surviving fraction between pretreated and non-pretreated cells was determined using the  $\chi^2$ -test. Table 1 shows the results of the statistics



Figure 1. (A) Survival of SCC-25 (●) and SCC-25/CP (▼) cells treated for 1 h with concentrations of cisplatin ranging from 1 to 20 µm. Each datum point represents the mean±SE of 12 and three independent experiments for SCC-25 and SCC-25/CP respectively. (B) Survival of SCC-25 cells (●) over the low-dose region for cells treated with cisplatin for 1 h with concentrations of cisplatin ranging from 0.5 to 4 µm. Each datum point represents the mean±SE of seven independent experiments.





performed. The results from *t*-tests, P(t), between corresponding data points on the pretreated and non-pretreated survival curves showed individual data points for surviving fractions on the curves for pretreated cells were not significantly different (at a 95% confidence level) from corresponding points on the curve for non-pretreated cells. For example, the *t*-test determined that cells treated with 0.1 and 1  $\mu$ M, preceded by 5 cGy radiation, were only significantly more resistant than non-pretreated cells at an 87 and



Figure 3. Cisplatin survival as a function of radiation pretreatment dose given 60 min before cisplatin. Shows survival for cisplatin doses of 0.1 μM (Φ), 1 μM (Ο) and 10 μM (Ψ). Each datum point represents the mean ± SE of three independent experiments.

80% confidence level respectively. The individual  $\chi^2$ ,  $\chi_i^2$ , are relatively large for 5 cGy pretreatment and subsequent cisplatin doses of 0.1 and 1  $\mu$ M, when each cisplatin concentration is considered separately. However, the cumulative  $\chi^2$ , P( $\Sigma \chi_i^2$ ), summed over these first two cisplatin concentrations, is 0.02, indicating significance at a 98% confidence level. Thus, the survival for the 5 cGy pretreated cells for cisplatin concentrations of 0.1 and 1  $\mu$ M combined is significantly different from the survival in non-pretreated

	1997 - 1997 1997 - 1997				$P(\Sigma \chi_i^2)$
Pretreatment dose		0.1 µм	1 µм	10 µм	Σ0.1, 1 μм
5cGy	P(t)	0.13	0.20	0.90	
· · · · · · · · · · · · · · · · · · ·	$\chi_i^2$	3.52	2.33	0.02	0.02
25 cGy	$\mathbf{P}(\mathbf{t})$	0.18	0.10	0.44	
	$\chi^2_1$	2.65	4.40	0.74	0.01
l Gy	$\mathbf{P}(t)$	0.07	0.06	0.98	
	$\chi^2$	6.23	6.74	0.001	< 0.001
2.5 Gy	P(t)	0.19	0.69	0.79	
	x	2.45	0.19	0.08	0.10

Table 1. Effects of prior radiation exposure on cell survival following subsequent cisplatin treatment: statistical test results for data in figure 3.

On the left, the *i*-test p, P(t), comparing each daton point on the pretreatment curve with the corresponding data point on the cisplatin-only curve, are listed according to pretreatment dose and cisplatin concentration. The individual  $\chi^2$ ,  $\chi^2$ , is also listed according to pretreatment dose and cisplatin concentration. On the right, p for the cumulative  $\chi^2$ , summed over the cisplatin concentrations indicated, are listed.

cells at these cisplatin concentrations. Similarly, pretreatment of cells with 25 cGy and 1 Gy radiation increased cisplatin survival at the 99 and >99% confidence levels respectively, for the combined cisplatin concentrations of 0.1 and 1  $\mu$ M.

Pretreatment of cells with low-dose irradiation 10 min before a cisplatin challenge dose also resulted in an increased survival for low-dose cisplatin treatment. However, the increase in survival detected for a 10-min interval between radiation pretreatment and cisplatin was less marked than that detected for a 60-min interval (figure 4). The increase in survival due to pretreatment with 25 cGy radiation 10 min before 0.1  $\mu$ M cisplatin was significant with a p=0.02 as determined by a *t*-test. However, no other radiation-cisplatin concentrations produced a significant increase in survival.

Cisplatin survival was also examined when radiation was given at 10 and 60 min following cisplatin treatment. Figures 5 and 6 show that treating cells with radiation 10 and 60 min respectively, after cisplatin treatment, did not result in a significant increase in cell survival for cisplatin doses of 0.1, 1 or  $10 \,\mu$ m over the radiation dose range of 5 cGy to 2.5 Gy.

#### 4. Discussion

Accurate determination of cell survival following low-dose radiation and the detection of a low-dose hypersensitivity response has generally required methods with better precision than the conventional clonogenic survival assay. The improved methods to









detect low-dose cell survival use either a Dynamic Microscopic Image Processing Scanner (DMIPS) (Palcic and Jaggi 1986, Spadinger and Palcic 1992, 1993) or an improved cell plating protocol (Durand 1986, Watts et al. 1986, Skarsgard et al. 1991, Wouters and Skarsgard 1996). However, cisplatin survival curves characterized by a low-dose hypersensitive

### C. Caney et al.

Figure 6. Cisplatin survival as a function of radiation treatment dose given 60 min after cisplatin. Shows survival for cisplatin doses of 0.1 μM (●), 1 μM (O) and 10 μM (▼). Each datum point represents the mean±SE of three independent experiments.

region have also been observed in rodent cell lines using a conventional clonogenic survival assay (Moorehead et al. 1994, Begg et al. 1986). In the present work, a conventional clonogenic survival assay has been used and a low-dose hypersensitivity to cisplatin and increased resistance at higher cisplatin doses for the SCC-25 human squamous cell carcinoma line SCC-25 is reported. In contrast, the cisplatin survival curve for the SCC-25/CP cells was not characterized by hypersensitivity in the low-dose region. The cisplatin-resistant SCC-25/CP cell line was derived by repeated exposure of SCC-25 cells to increasing concentrations of cisplatin (Teicher et al. 1987). A similar result has been reported recently for CHO cells, where continuous exposure of cells to low-doses of cisplatin (0.01 µM) -induced resistance to subsequent cisplatin treatment and removed the low-dose hypersensitive region of the survival curve (Marples et al. 1997). The mechanism of the acquired cisplatin resistance in SCC-25/CP cells is multifactorial, involving plasma membrane changes, increased cytosol binding and decreased DNA cross-linking. The magnitude of the acquired resistance in SCC-25/CP cells (figure 1A) is substantially larger than that of the induced resistance confined to the hypersensitivity region in SCC-25 cells (figure 2). It appears, therefore, unlikely that the acquired cisplatin resistance of the SCC-25/CP cell line results entirely from the same process by which prior low-dose radiation exposure leads to the removal of low-dose

hypersensitivity from the cisplatin survival curve of the SCC-25 cell line.

It has been reported that small radiation doses can protect cells against subsequent radiation damage in a variety of cell types including plant (Hendry 1986), human tumour (Joiner 1994), hamster (Ikushima 1987) and human lymphocyte (Shadley 1994) systems. For some human tumour cells, the effect depends on the doses and the timing of both the pretreatment and the challenge radiation (Marples and Joiner 1995, Marples and Skov 1996). Agents other than radiation, including cisplatin, also appear to be protective against subsequent radiation damage (Wolf 1992, Shadley 1994, Cai and Jiang 1995, Marples and Joiner 1995, Skov and Marples 1995, Marples et al. 1997, Dolling et al. 1998). It has been proposed that the protective response elicited by pretreatment with certain agents is an induction of IRR over the otherwise HRS response, and that the inducing agents have a common mechanism for inducing the IRR response. There is evidence to suggest that the types of DNA damage caused by the pretreatment and by the challenge treatment are important in the induction of this protective response (Popoff et al. 1987, Skov and Marples 1995).

A more recent report indicates that exposure of human cells to 'priming' treatment of low-dose ionizing radiation 4h before a higher dose leads to the enhanced removal of thymine glycols after higher doses, providing evidence for an inducible base excision repair response in human cells (Le et al. 1998). There is also evidence that the induced response may involve increased synthesis of new proteins involved in DNA repair (Marples and Joiner 1995, Marples et al. 1997). Other evidence for an induced repair response in mammalian cells comes from studies that show an enhanced reactivation of DNA damaged viruses and reporter genes following pretreatment of cells with radiation (Jeeves and Rainbow 1979, 1983a, b, Francis and Rainbow 1995, 1999, McKay et al. 1997). It is not clear to what extent the induced response is specific to the type of damage that caused the induction. It may be that several mechanisms are induced, and DNA damage of one kind can induce protection against DNA damage of a different kind.

In this study, radiation pretreatment 60 min before cisplatin treatment resulted in an increase in survival for low-doses of cisplatin. There was a significant increase in survival when 5 cGy pretreatment was given before 0.1 and 1  $\mu$ M cisplatin (p=0.02 for the  $\chi^2$ -test) as shown in figures 2 and 3 and table 1. An increase was also seen for the combined 0.1 and 1  $\mu$ M cisplatin survival for pretreatment radiation doses of 25 cGy (p=0.01) and 1 Gy (p<0.001) (figure 3).

These results are consistent with a radiation-induced response which is protective against subsequent cisplatin damage in SCC-25 cells. However, it is possible that the observed increase in cell survival in the hypersensitive low-dose region, which results from prior cell exposure to  $\gamma$ -rays, occurs because the radiation and cisplatin target the same subpopulation of cells. Pretreatment may eliminate the sensitive subpopulation so that the remaining cell population is more resistant to subsequent cisplatin treatment. However, the priming low-doses of radiation that remove the low-dose hypersensitivity to cisplatin typically do not cause sufficient cell kill to account for the observed increase in survival following a challenge dose. It is also possible that the observed increase in cell survival in the hypersensitive lowdose region results from some minor cell cycle redistribution during the 10- or 60-min interval between prior low-dose y-ray treatment and cisplatin challenge dose. However, it is unlikely that the differences in cisplatin sensitivity typically seen between different stages of the cell cycle together with the minor cell cycle redistribution possible in the short interval would be sufficient to account for the relatively large increase in cell survival observed. For this reason it appears more likely that the increased cisplatin resistance due to prior low-dose radiation exposure reported here represents a radiation-induced protective mechanism.

Since no radiation doses <5 cGy were explored here, it can only be assumed that the cisplatin protection induced by radiation is triggered by a threshold dose of radiation which is of the order of 5 cGy or less. No significant survival increase was detected for radiation priming doses >1 Gy. It can be seen from figure 3 that the induced survival increases for increasing doses of radiation pretreatment up to 1 Gy for an interval of 60 min between treatments. This observation suggests that the extent of radiation-induced cisplatin protection depends on the magnitude of the radiation pretreatment dose. This is consistent with the observations of radiation resistance caused by other agents (Wolff 1992, Shadley 1994, Marples and Joiner 1995, Marples and Skov 1996, Wouters and Skarsgard 1996). The priming y-ray doses (5 cGy to 1 Gy) found to increase resistance to subsequent low-dose cisplatin treatment in this report are similar to the priming doses of X-rays (5, 20 cGy and 1 Gy) reported to increase resistance to subsequent low-dose radiation treatment (Marples and Skov 1996, Marples et al. 1997). There is some evidence that the induced radiation resistance involves nucleotide excision repair (NER) (Skov et al. 1994), DNA single-strand break repair (Dolling et al. 1998) and/or base excision repair (BER) (Le et al.

1998). Since excision repair is an important determinant in the response of cells to cisplatin (Andersson et al. 1996, Bulmer et al. 1996) it is possible that the induced response leading to increased resistance to low-dose cisplatin reported here involves the excision repair pathway. However, it is also possible that the radiation-induced resistance to cisplatin in SCC-25 cells results from an induced recombination mechanism (Lehhnert and Chow 1997), enhanced tolerance of DNA damage (Hill et al. 1990, Dempke et al. 1992) or some other mechanism that increases cell survival such as an inhibition of apoptosis (Yount et al. 1998).

There are several reports showing that tumour cell variants isolated following fractionated exposures to X-irradiation acquire increased resistance to cisplatin (Hill et al. 1990, Dempke et al. 1992, Eicholtz-Wirth et al. 1993, Eicholtz-Wirth and Hietel 1994). In the case of a human teratoma cell line and a human ovarian tumour cell line, both enhanced repair and increased tolerance of DNA damage were implicated in the acquired resistance (Hill et al. 1990, Dempke et al. 1992). In these reports the acquired resistance of the isolated cells resulted in an increased survival following both low and high cisplatin doses, rather than an increased survival limited to the low-dose hypersensitivity region of the cisplatin survival curve reported here for SCC-25 cells. Furthermore, the fractionated X-ray exposure was given to cells over several weeks and the cells were allowed to re-populate and resume logarithmic growth between fractions, whereas in the experiments reported here, SCC-25 cells were given low-dose y-ray exposures either 10 or 60 min before the cisplatin challenge dose. It is not known whether the acquired cisplatin resistance of the tumour cells reported previously (Hill et al. 1990, Dempke et al. 1992, Eicholtz-Wirth et al. 1993, Eicholtz-Wirth and Hietel 1994) and the increased cisplatin resistance in the low-dose hypersensitivity region for SCC-25 cells reporter here result from a similar mechanism.

Some combinations of low-dose radiation and cisplatin treatment of SCC-25 cells resulted in an increased colony survival compared with that for untreated cells. For example, a surviving fraction >1 was observed for cells pretreated with radiation doses of 5, 25 cGy, 1 and 2.5 Gy either 60 or 10 min before treatment with 0.1  $\mu$ M cisplatin (figures 3–6). Surviving fractions that are >1 following combination treatment have been reported previously for CHO cells given a 1 h treatment of 1  $\mu$ M cisplatin 6 h before an X-ray dose of 10 cGy (Marples *et al.* 1997) and for Chinese hamster V79 cells treated with 20 cGy neutron radiation 4 h before an X-ray dose of 1 Gy (Marples and Skov 1996). This result was unexpected and it is not clear why this occurs. It is

possible that protective mechanisms induced by lowdose pretreatment together with repair mechanisms induced by the subsequent low-dose treatment result in a decrease in the number of cells that lose viability throughout the course of the assay due to spontaneously occurring cell damage compared with corresponding controls.

Low-dose irradiation 10 min before the cisplatin challenge dose also resulted in a cisplatin survival increase over the same pretreatment dose range, but only for  $0.1 \,\mu\text{M}$  cisplatin (p = 0.02) (figure 4). The cisplatin-protective effect induced by radiation is noticeably lower for the 10-min interval between radiation and cisplatin than for the corresponding 60-min schedule. This is consistent with an inducible protective effect that persists for at least 1 h. This is in agreement with observations by others. For example, pretreatment with an X-ray dose of 20 cGy can induce IRR and eliminate the HRS region if given 6h before a challenge dose but not if the challenge dose is given concurrently or at 24h after the priming dose (Skov et al. 1994, Marples and Joiner 1995, Marples and Skov 1996). Others have reported that clinically relevant concentrations of cisplatin prevented induced radiation resistance when present during radiation exposure, whereas pretreatment with 1 µm cisplatin 6 h before radiation exposure abolished the hypersensitive region seen at very low doses, suggesting that cisplatin can cause induced radiation resistance (Marples et al. 1997).

Radiation treatment given 10 or 60 min after cisplatin (figures 5 and 6 respectively) did not increase cisplatin survival. Clearly, the order in which the treatments are administered is a factor in the induction of a cisplatin survival increase by radiation pretreatment.

In the present work no significant effect of prior irradiation with 2.5 Gy on the survival of SCC-25 cells treated with 0.1, 1 or  $10 \,\mu$ m cisplatin was found. This suggests that there would be no change in tumour response to cisplatin treatment resulting from prior irradiation with clinically relevant doses of ~2 Gy, but that the effects of prior radiation treatment on cisplatin sensitivity could affect effect the response of cells receiving lower doses as would be found in the radiation field margins. It is thus possible that the protective effect of low-dose  $\gamma$ -ray exposure on a subsequent low-dose cisplatin challenge dose reported here could contribute to the clinical outcome of combined radiotherapy and chemotherapy using cisplatin.

#### Acknowledgements

This work was supported by an operating grant to A. J. R from the National Cancer Institute of Canada

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# 972

# Chapter 6

Investigating mechanisms of induced cisplatin-resistance in

mammalian cells

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# Preface

This manuscript was prepared for submisssion to the International Journal of Radiation Biology.

This set of experiments proceeds to examine the hypotheses that have been presented to explain induced radioresistance in the context of induce cisplatin-resistance and hypersensitivity.

Jim Stavropoulos contributed to this work by performing the series of experiments to identify low-dose hypersensitivity in the CHO cell line, AA8. The rest of the experiments and the writing of this manuscript are my work.

### Abstract

**Purpose:** To investigate the roles of DNA damage and the activation of the stress activated, Jun N-terminal kinase-1 (JNK-1) in the hypersensitivity of mammalian cells to the chemotherapeutic drug, cisplatin.

**Materials and Methods:** We investigated the hypothesis that DNA damaging agents activate a cellular mechanism that protects against low-doses of cisplatin. In human squamous cell carcinoma, SCC-25, and AA8 Chinese hamster ovary (CHO) cells, the effect of priming doses of UV and cisplatin on the cytotoxicity of low cisplatin doses was determined by colony forming assay. To examine the possibility that damaged DNA stimulates cisplatin resistance, we compared the cytotoxicity of low cisplatin doses in cells infected with a non-replicating adenovirus (Ad) that had been irradiated with UV (primarily at 254nm) to cells that had been infected with unirradiated Ad. JNK-1 activity was assayed by *in vitro* kinase assay following cisplatin treatment.

**Results:** Treatment of SCC-25 and AA8 cells with UV, cisplatin and IR is protective against cell death following subsequent cisplatin doses of 1 and 0.75  $\mu$ M, respectively. Infection of SCC-25 cells with UV-irradiated Ad caused increased cell survival following exposure to low doses of cisplatin compared with cells infected with unirradiated Ad. Neither low doses of cisplatin nor infection with UV-irradiated Ad caused an increase in

the activity of the stress responsive kinase, JNK-1. We have been unable to detect any cisplatin-induced increase nucleotide excision repair (NER).

**Conclusions:** Cisplatin, UV-light, and  $\gamma$ -rays can protect mammalian cells from subsequent treatment with low doses of cisplatin. The presence of UV-damaged DNA in the nucleus of mammalian cells prevents cisplatin hypersensitivity. Activation of JNK-1 does not appear to be involved in induced resistance to low doses of cisplatin and we have detected no cisplatin-induced increase in HCR.

## Introduction

The response of mammalian cells to genotoxic agents is dynamic and complex. These stresses affect cell metabolism and alter gene expression causing: cell cycle arrest; apoptosis; and induction of DNA repair. These responses determine the sensitivity of cells to genotoxic agents like ionising radiation (IR), ultraviolet light (UV), and cisplatin.

Cisplatin is a very useful drug in the treatment of many different types of cancer, particularly ovarian carcinomas and solid tumours of the head and neck. Unfortunately, malignant cells are often intrinsically resistant or they acquire resistance to the drug during treatment and this limits the efficacy of the drug (Hogberg et al., 2001; Loehrer and Einhorn, 1984). For this reason, the cellular responses to cisplatin are the subject of intense investigation.

It has been shown that some mammalian cells are much more sensitive to low doses of ionising radiation (reviewed by Joiner et al., 2001) and cisplatin (Marples et al., 1997, Caney et al., 1999; Begg, 1986) than they are to higher doses. This hypersensitivity can be relieved by treating cells with genotoxic stress prior to the challenge dose of *cis*Pt or IR (Caney et al., 1999; Marples and Joiner, 1995; Skov et al., 1995). These observations suggest that the increased cell survival per unit of dose at higher doses results from an inducible protective mechanism that can increase cell survival following exposures that exceed some threshold dose. This protective machanism could involve; protection from DNA damage, enhanced DNA repair, or down-regulation of apoptosis. Despite a number of important advances recently (Short et al., 2001; Robson et al., 2001; Marples and Joiner, 2000; Le et al., 1998), the mechanisms of induced resistance to cisplatin or IR have not been well characterised.

Cisplatin induces a number of cellular survival mechanisms and the signal that is thought to trigger these events is the presence of cisplatin-induced genomic damage (Perez et al., 1998). Cisplatin predominantly crosslinks neighbouring purines on the same DNA strand and, less frequently, forms crosslinks between strands and forms DNAprotein crosslinks. The predominant intrastrand adducts are substrate for nucleotide excision repair (NER) (Bulmer et al., 1996; Sheibani et al., 1989). Many other types of bulky lesions are substrate for NER, including UV-induced thymine dimers (for review see Wood et al., 1997).

A broad range of DNA damaging agents transiently induce resistance to low doses of low LET radiation (Skov et al., 1995; Marples and Joiner, 1995; Marples et al., 1994). Further, we have shown that treatment with IR can be protective against subsequent low doses of cisplatin (Caney et. al, 1999). Taken together, these data suggest that inducible protection is responsive to many different types of stimuli and may be protective against

96

many types of genotoxic stress. It is possible that these genotoxic stresses induce a generally protected state (for example, by suppressing apoptosis) or it may be that these stresses activate a number of independent cellular events that are protective against different specific challenges. In either case, the immediate stimulus which causes the protection remains unidentified.

DNA can be introduced into a broad range of mammalian cell types using adenovirus (Ad) as a vector (Hitt et al., 1995). This is a common method of gene delivery *in vitro* and may prove to be a clinically important modality in gene therapy of human genetic disease and cancer (Hitt et al., 1999). Ad enters the cell by receptormediated endocytosis and is rapidly transported into the nucleus where Ad DNA assumes a histone-like conformation (Tate and Philipson, 1979; and as reviewed by Nemerow, 2000). The recognition and repair of bulky lesions in Ad DNA has proven to be an excellent model system for studying cellular DNA processes and lesions in Ad DNA are thought to be processed by the same processes as genomic DNA (Bulmer et al., 1996; Francis and Rainbow, 1999). Here, we have used Ad to study the possibility that a signal initiated by DNA damage *per se* can induce cisplatin protection.

Induced protection against radiation induced cell death is likely achieved by either a reduced tendency to apoptosis or an enhancement of DNA repair (Joiner et al., 2001). One signaling pathway that is often associated with apoptosis is the Jun N-terminal kinase (JNK) pathway (Minden and Karin, 1997). JNK is up-regulated by many stimuli including UV, IR, cisplatin, and osmotic stress (Ip and Davis, 1998) and in some cases at levels that cause no detectable cell death. To our knowledge, the role of JNK and the

97

immediate early stress response has not been studied in the context of hypersensitivity and induced resistance.

While suppression of apoptosis may be important, a number of recent reports present cogent evidence that the important factor in induced resistance is enhanced DNA repair. Le et al., showed that repair of thymine glycols was enhanced by IR doses less than about 0.25 Gy (1998) and a factor that is down-regulated by very low IR fluences has been identified as a suppressor of single strand break repair (Robson et al., 1999; Robson et al., 2000). It is not clear how these repair activities contribute to the cisplatin response, if at all.

We have used a number of techniques to study the causes and mechanisms that affect the observed hypersensitivity of mammalian cells to cisplatin.

# **Materials and Methods**

#### Cells and Virus:

Human squamous cell carcinoma line, SCC-25, and the Chinese hamster ovary cell line, AA8, were grown in  $\alpha$ -MEM supplemented with fetal calf serum (10%), penicillin (100  $\mu$ g/mL), streptomycin (100  $\mu$ g/mL), and amphotericin B (250 ng/mL) (GibcoBRL).

The adenovirus used in these experiments is Ad5-LacZ 5 which contains the  $\beta$ -galactosidase reporter gene inserted in the place of the E1 region of the Ad5 genome. Virus was grown and titred in 293 cells (Graham et al., 1977).

# Irradiation:

Plates containing cells to be irradiated had the media replaced with 1 mL of warm PBS (in 100mm dish). The plates were then irradiated with a germicidal bulb emitting primarily at 254nm at an intensity of 1 J/m<sup>2</sup>s (GE model G8T5). Virus was irradiated at an intensity of 5 J/m<sup>2</sup>s in PBS on ice while being stirred.

# Colony forming assays:

In assessing the colony forming ability of cells infected with Ad, 5 X  $10^5$  cells were plated into each well of a 6-well plate overnight in supplemented  $\alpha$ -MEM at which point they were infected with damaged or undamaged Ad at a multiplicity of infection of 10 plaque forming units (pfu)/mL. 3 hours after infection, cells were trypsinized and seeded at low density into 6-well plates such that ~100 colonies resulted in each well. After allowing the cells to become adherent for 3 hours, the media was removed and replaced with whole media containing cisplatin. After 1 hour, the treatment media was removed and replaced with fresh, warm, supplemented  $\alpha$ -MEM. Plates were incubated in humidified chamber at 37°C for 7 days. The media was then removed and plates were stained with 0.5% methylene blue solution in 70% ethanol. Colonies with more than 32 cells were counted and scored with the relative survival being determined by comparing colony number in experimental samples to untreated controls.

In determining the effect of treatment with genotoxic agents on the sensitivity of cells to a subsequent cisplatin treatment,  $5 \times 10^5$  cells were plated into each well of a 6-well plate and allowed to adhere to the substrate overnight. Cells were then exposed to

the proscribed pre-treatment of UV light or cisplatin. Plates were then incubated for 3 hours; at which time they were trypsinized and seeded at low density into 6-well plates such that ~100 colonies resulted in each well and allowed to adhere to the substrate for 3 hours. 6 hours after the pretreatment cells were exposed to the challenge treatment of cisplatin, as above.

## Host cell reactivation assay:

DNA repair capacity was determined by a HCR assay as has been described previously (McKay and Rainbow, 1996). Briefly, cells were seeded into 96-well plates at 3.8 X 10<sup>4</sup> cells/well and incubated overnight. Cells were then infected with 10 pfu/cell of Ad5-lacZ that had been irradiated with UV, as described above. Virus that had not adsorbed to the cells was washed off and the cells were incubated with whole media for 24 hours. Cells were lysed at 37°C for 20 minutes with NP-40 buffer (250 mM Tris, 1  $\mu$ M PMSF, and 0.5% NP-40 (pH 7.8)), followed by 10 minutes in 100mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5), 10 mM KCl, 1mM MgSO<sub>4</sub>, 50 mM 2-mercaptoethanol. OD<sub>405</sub> was determined at several times following addition of 0.1% o-nitrophenol- $\beta$ -D-galactopyranoside (ONGP) in 100mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5) using a microtitre plate reader (Bio-Tek Instruments EL340 Bio Kinetics Reader). The reactivation of the UV-damaged reporter gene is evident in the extent of the colour reaction and is expressed herein as a relative value in comparison with unirradiated controls.

# JNK-1 assay:

Cells were seeded at 5 X  $10^{5}/100$  mm plate, infected or irradiated as described. At the appropriate time, cells were washed with 1 mL of ice cold PBS per plate and scraped into 1.5mL microfuge tubes and centrifuged at 3 000 g for 30 seconds to pellet the cells. The PBS was removed and 0.5 mL of NP-40 lysis buffer (0.5% Igepal (Boehringer Mannheim; 150 mM NaCl; 50 mM Tris (pH 8); 2mM EDTA; 100mM NaF; 10mM  $Na_4P_2O_7$ ) with protease inhibitors (Boehringer Mannheim cat.#1 836 170) was added to each sample and kept on ice for 30 minutes; being shaken every 5 minutes. Insoluble material was removed by centrifugation at 13 000g for 10 minutes at 4°C and the supernatant was transferred to new tubes. The total protein concentration of the lysates was determined by MicroBCA assay (BioRad cat. #500-0006). Within each experiment the amount of cellular protein used was normalized to a quantity between 0.1 and 0.4 mg and the volume of each sample was augmented to 0.6 mL with cold lysis buffer. 30 µL of 80% protein G sepharose beads (Pharmacia cat.#17-0618-01) and 1 µg of purified monoclonal antibody to human Jun N-terminal kinase-1 (α-hJNK-1, Pharmingen cat.#15701A) was added to each sample and the tubes were rotated overnight at 4°C. Beads were then washed twice with ice-cold lysis buffer and twice more with ice-cold kinase buffer (0.1XHEPES, 20mM MgCl<sub>2</sub>, 10mM β-glycerophosphate, 2mg/mL pnitrophenyl phosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>) and resuspended in 30 µL of kinase buffer containing 1 µg of GST-Jun and 1 mCi of  $^{32}$ P,  $\gamma$ -labeled ATP and the kinase reaction was allowed for 20 minutes at 30°C. Samples were then heated to 95°C for 3 minutes and then separated on a 12% denaturing, polyacrylamide gel. Gels were dried and

phosphorylation of the GST-Jun substrate was visualized by autoradiogram and quantitated using phosphorimager technology (Molecular Dynamics).

# **Results:**

Low concentrations of cisplatin are more cytotoxic (per unit of dose) than higher concentrations.

Conventional colony forming assays examining the survival of the human tumour cell line, SCC-25 (figure 6-1), and the Chinese hamster ovary (CHO) cell line, AA8 (figure 6-2) show that very low cisplatin concentrations are more effective (per unit of dose) in killing cells than are greater concentrations. In the AA8 cells this hypersensitivity is seen following 1 hour treatments at concentrations of up to 0.75  $\mu$ M whereas SCC-25 cells are hypersensitive to treatments up to and including 1  $\mu$ M. Hypersensitivity of CHO cells to cisplatin has been reported previously using a microscopic cell location assay (Marples et al., 1997). It is interesting to note that our results indicate that increasing the concentration of cisplatin can actually increase the surviving fraction (0.5-0.75  $\mu$ M compared with 1.0-2.0  $\mu$ M). This is very similar to results obtained with this cell line using automated techniques (Zhou and Skov, personal communication). **Figure 6-1. The SCC-25 human tumour cell line is extremely sensitive to low doses of cisplatin.** The clonogenic survival of SCC-25 cells following a one hour exposure to the shown concentrations of cisplatin is shown. The inset figure shows detail in the low dose region. The symbols represent the mean of 7 independent experiments of 4 replicates (±SEM).



Agents which damage DNA are protective against subsequent treatment with low doses of cisplatin.

We have reported previously that exposure to low doses of <sup>60</sup>Co  $\gamma$ -rays induces a response that protects SCC-25 cells to subsequent low doses of cisplatin (Caney et al., 1999). Here we present results suggesting that other DNA damaging agents can likewise increase the surviving fraction of cisplatin-treated SCC-25 and AA8 cells. We irradiated AA8 cells with 0.5 or 5 J/m<sup>2</sup> 6 hours before a 1 hour exposure to a 0.75  $\mu$ M cisplatin solution. This treatment was chosen as the challenge dose since this dose causes the minimum surviving fraction within the region of hypersensitivity (figure 6-2). At this dose, induced resistance would cause the greatest difference in surviving fraction. In this CHO cell line, a fluence of 0.5 J/m<sup>2</sup> causes no detectable decrease in cell survival while 5 J/m<sup>2</sup> causes a reduction in surviving fraction of about 10%. Both UV fluences were protective against cell death caused by a cisplatin exposure delivered 6 hours later and the pooled results for both pretreatments shows a significant increase in cisplatin resistance (p < 0.05, table 6-I).

We examined the effect of cisplatin pretreatment on a cisplatin challenge dose applied 6 hours later. The challenge dose of 1.5  $\mu$ M was chosen to exceed the putative threshold dose of induced resistance (~1  $\mu$ M) while having only a modest effect on survival in SCC-25 cells. A 1  $\mu$ M challenge dose was employed as the challenge dose to maximise the difference in outcome between pretreated and naïve cells. Our results suggest that priming doses of cisplatin increase resistance to subsequent low dose cisplatin challenge (table 6-1).

105

**Figure 6-2.** The Chinese hamster ovary cell line, AA8, is extremely sensitive to low doses of cisplatin. The clonogenic survival of AA8 cells following a one hour exposure to the shown concentrations of cisplatin is shown. The inset figure shows detail in the low dose region. The symbols represent the mean of 7 independent experiments of 4 replicates (±SEM).



# Infection with UV-irradiated Ad is protective against subsequent treatment with low doses of cisplatin.

We studied the importance of DNA damage in induced cisplatin resistance by delivering UV-irradiated DNA into the nuclei of naïve cells before a cisplatin challenge. Cells were infected with UV-irradiated or un-irradiated adenovirus (Ad). The dsDNA genome of the non-replicating Ad virus is efficiently targetted for nuclear import in infected cells (reviewed by Nemerow, 2001). Cells infected with UV-irradiated virus would thereby have UV-induced DNA damage introduced to the nucleus of unirradiated cells. We found that this exogenous, UV-damaged DNA induced protection of the SCC-25 cells to subsequent, low doses of cisplatin. Figure 6-3 shows the difference in survival between cells infected with Ad that had been irradiated with a fluence of 200 J/m<sup>2</sup> compared with cells that had been identically infected with unirradiated virus. Results of these preinfection experiments are summarised in table 6-II. By  $\chi^2$  analysis, preinfection with UV-irradiated Ad significantly improves survival of SCC-25 cells exposed to 0.5 or 1.0  $\mu$ M cisplatin 6 hours later (p < 0.05). Using the DMIPS apparatus (access and guidance kindly provided by Dr. K.A. Skov, British Columbia Cancer Research Centre) we also assessed the effects of UV-irradiated Ad infection in the CHO system. As shown in table 6-II, AA8 cells were protected from a 1 hour, 0.75  $\mu$ M challenge dose (p < 0.05).

Та	ble	6-I

Treatment ( $\mu$ M)( $\pm$ SEM)S. F.Exps.AA80.75mock UV0.91 $\pm$ 0.02-30.750.5 J/m²0.98 $\pm$ 0.031.07 $\pm$ 0.0630.755 J/m²0.99 $\pm$ 0.041.09 $\pm$ 0.0630.75pooled1.08 $\pm$ 0.046SCC-251.0Mock0.88 $\pm$ 0.04-3	p-value	# of	Relative	S. F.	Pretreatment	Cisplatin	Cells
( $\mu$ M) AA8 0.75 mock UV 0.91 ± 0.02 - 3 0.75 0.5 J/m <sup>2</sup> 0.98 ± 0.03 1.07 ± 0.06 3 0.75 5 J/m <sup>2</sup> 0.99 ± 0.04 1.09 ± 0.06 3 0.75 pooled 1.08 ± 0.04 6 SCC-25 1.0 Mock 0.88 ± 0.04 - 3		Exps.	S. F.	(±SEM)		Treatment	
AA8 $0.75$ mock UV $0.91 \pm 0.02$ -3 $0.75$ $0.5 \text{ J/m}^2$ $0.98 \pm 0.03$ $1.07 \pm 0.06$ 3 $0.75$ $5 \text{ J/m}^2$ $0.99 \pm 0.04$ $1.09 \pm 0.06$ 3 $0.75$ pooled $1.08 \pm 0.04$ 6SCC-25 $1.0$ Mock $0.88 \pm 0.04$ -3						(μM)	
Mile $0.75$ $1.001 \pm 0.02$ $0.91 \pm 0.02$ $5$ $0.75$ $0.5 \text{ J/m}^2$ $0.98 \pm 0.03$ $1.07 \pm 0.06$ $3$ $0.75$ $5 \text{ J/m}^2$ $0.99 \pm 0.04$ $1.09 \pm 0.06$ $3$ $0.75$ pooled $1.08 \pm 0.04$ $6$ SCC-25 $1.0$ Mock $0.88 \pm 0.04$ $ 3$		3	<b></b>	$0.91 \pm 0.02$	mock UV	0.75	<b>AA</b> 8
$0.75$ $5 \text{ J/m}^2$ $0.99 \pm 0.04$ $1.09 \pm 0.06$ $3$ $0.75$ pooled $1.08 \pm 0.04$ $6$ SCC-25 $1.0$ Mock $0.88 \pm 0.04$ $ 3$	0.16	3	1.07 ± 0.06	$0.98 \pm 0.03$	$0.5 \text{ J/m}^2$	0.75	7 11 10
0.75 pooled $1.08 \pm 0.04$ 6 SCC-25 1.0 Mock $0.88 \pm 0.04$ - 3	0.13	3	$1.09 \pm 0.06$	$0.99 \pm 0.04$	5 J/m <sup>2</sup>	0.75	
SCC-25 1.0 Mock 0.88 ± 0.04 - 3	0.05	6	$1.08 \pm 0.04$		pooled	0.75	
SCC-25 1.0 Mock 0.88 ± 0.04 - 3							
		3	85	$0.88\pm0.04$	Mock	1.0	SCC-25
1.0 1.5 $\mu$ M 1.02 $\pm$ 0.01 1.17 $\pm$ 0.06 3	0.06	3	$1.17\pm0.06$	$1.02\pm0.01$	1.5 μM	1.0	
(cisplatin)					(cisplatin)		

Table 6-II	
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Cell	Cisplatin	UV-	Surviving	Relative	Number	p value
Line	Exposure	fluence to	Fraction	Surviving	of Exp.	
	(µM)	virus	(±SEM)	Fraction		
		(J/m <sup>2</sup> )				
SCC-25	0.5	0	$0.89\pm0.02$	1	6	
	0.5	200	$0.98 \pm 0.02$	$1.10\pm0.05$	6	0.05
	0.5	1000	$0.93\pm0.04$	$1.04\pm0.07$	6	0.29
SCC-25	1.0	0	$0.85\pm0.04$	1	6	
	1.0	200	$0.92 \pm 0.01$	$1.09\pm0.07$	6	0.12
	1.0	1000	$0.91 \pm 0.03$	$1.08 \pm 0.08$	6	0.19
AA8	0.75	0	$0.94\pm0.01$	1	5	
	0.75	200	$1.00\pm0.01$	$1.06 \pm 0.02$	5	0.02

# Low doses of cisplatin do not enhance DNA repair of UV irradiated DNA

We have used the host cell reactivation (HCR) of UV-irradiated Ad to assay the effect of cisplatin exposure on nucleotide excision repair. The Ad used in these experiments contains a  $\beta$ -galactosidase reporter cartridge in the deleted E1 region of the

genome. When the viral DNA is damaged before being introduced into the cells, the expression of the reporter gene depends on the repair mechanisms of the host cell. In these experiments, the HCR of UV-irradiated Ad was used to assess NER. UV was used as the genotoxic agent because it is impractical to treat the virus with difficulties of removing all of the cisplatin from the virus preparation before infection. We wanted to exclude the possibility that residual cisplatin in the virus preparation was, itself, affecting DNA repair. We have not detected any increase in the HCR of UV-irradiated Ad resulting from cisplatin pre-treatment in either the AA8 or the SCC-25 cell line. Figure 6-4 shows the relative reporter gene expression in AA8 and SCC-25 cells that had been exposed to 1 hour treatments of 1 mM or 4 mM 6 hours before infection with the damaged reporter. Similar results were obtained when the pretreatment was administered immediately before infection.

# JNK-1 is not activated by low doses of cisPt

We used and *in vitro* kinase assay to determine if JNK-1 is part of a mechanism of induced cisplatin resistance. We could detect no change in JNK-1 activity at 3, 6, or 24 hours after any cisplatin doses that might be relevant to low dose hypersensitivity ( $\leq 8\mu$ M). We also detected no change in JNK activity in cells infected with UV-irradiated Ad (data not shown). Very high cisplatin doses (sufficient to decrease survival to < 10-6) did induce a modest increase in JNK activity 24 hours after exposure (figure 6). Our preliminary results suggest a similar lack of low dose cisplatin response from other MAPK molecules (ERK1/2 and p38) in these cells (data not shown). While we cannot

Figure 6-3. Infection with UV-irradiated adenovirus protects SCC-25 from subsequent low doses of cisplatin. Tumour cells were infected with Ad that had been irradiated with 200 J/m<sup>2</sup> ( $\blacksquare$ ) or mockirradiated Ad ( $\bullet$ ). 6 hours after infection, cells were exposed to a 1 hour cisplatin challenge at the given doses. Symbols represent the mean of 6 independent experiments of 4 replicates (±SEM).



exclude the involvement of JNK activity and the immediate early MAPK stress response in induced cisplatin resistance (ICR), it does not appear that these molecules contribute to ICR.

# **Discussion:**

DNA damage is thought to kills cells by inducing apoptosis and DNA damage is also known to induce protective responses. It is the balance, or perhaps the sum, of these two signals that determines the fate of cells carrying DNA damage. In systems where hypersensitivity is observed, the frequency of DNA damage sufficient to induce cell death does not induce protection. For each different genotoxic agent, the nature of these two apparently contradictory signals will be somewhat, if not entirely, different. It is the relative strengths of these signals that determines the threshold of induced resistance. For natural agents like oxidative stress, UV and ionising radiation, it is likely that doses that trigger protection will not cause significant cytotoxicity. That is, there would be little advantage to inducing a protective response only after a significant fraction of cells are dead. For a synthetic agent, like cisplatin, there is no such expectation. It is likely that this difference accounts for our ability to detect cisplatin hypersensitivity using conventional colony forming techniques while automated techniques have been required to detect hypersensitivity to IR. Neither a signal that could induce a protective response nor a definitive death signal have been identified in cisplatin-treated cells. Our work here is intended to investigate these signals and their consequences.

Figure 6-4. Cisplatin exposure causes no detectable increase in the HCR of UV-irradiated adenovirus. The expression of  $\beta$ -galactosidase in SCC-25 (panel A) and AA8 (panel B) cells infected with UV-irradiated Ad is shown relative to cells infected with unirradiated virus. Cells were treated for 1 hour with media containing 0 ( $\bigcirc$ ), 1 $\mu$ M ( $\Box$ ), or 4 $\mu$ M ( $\Delta$ ) cisplatin 6 hours before infection. Each symbol represents the mean of 3 independent experiments of 4 replicates. Error bars have been omitted for clarity. Each mean value is within the confidence intervals of the comparable points.



# Is induced cisplatin-resistance analogous to induced radioresistance?

There is some indirect evidence that ICR and IRR may be attributable to the same, or at least analogous, mechanisms. Most noteworthy are observations that cisplatin protects against radiation hypersensitivity (Marples et al., 1997) and exposure to radiation appear to be protective against low doses of cisplatin (Caney et al., 1999). While we certainly can not conclude that ICR and IRR share any analogous mechanisms, the comparison is useful in this discussion.

# What is the signal for induced protection?

Exposure to IR, UV and cisplatin can each induce protection against cisplatin toxicity. By using Ad to deliver the damaged DNA, we provide support for the hypothesis that it is DNA damage *per se* is the triggering event in this protection. The spectra of DNA damage caused by IR, UV, and cisplatin are each very different. More than 90% of the lesions in cisplatin or UV modified DNA affect only one strand and are repaired by NER. IR, on the other hand, causes mostly strand breaks and base modifications. One structural intermediate that is common among these treatments is the preseince of single strand breaks (SSBs). SSBs have previously been proposed as the trigger for induced radioresistance (Skov et al., 1995; Marples and Joiner, 1995). Our results are consistent with this possibility assuming that the transient presence of strand breaks within the NER complex cause the same signal as strand breaks in the rest of the genome. Figure 6-5. Activation of JNK-1 does not appear to be involved in induced cisplatin-resistance. SCC-25 cells were exposed to the cisplatin concentrations shown for 1 hour. 24 hours after treatment, the activity of JNK-1 in lysates was determined by *in vitro* kinase assay. The image shows the JNK-1 substrate, GST-Jun, and the activity of the kinase is evident in the intensity of the P32 signal in each band. The signal was quantified using phosphorimager technology and the relative increase in JNK activity is shown below the autoradiograph.



# Relative JNK-1 Induction

# What is the protective mechanism?

It has been proposed that the inducible processes that prevent cell death from low doses of cisplatin and IR result from either an enhancement of DNA repair or a reduction in apoptotic tendency (Joiner et al., 2001).

Apoptosis is regulated by a subtle and elaborate mechanism that responds to a broad range of physical, chemical, and biological signals. Small changes induced in this mechanism could conceivably impart a general anti-apoptotic protection against a broad range of cellular stresses. This would be consistent with observations that many different types of cellular stresses can induce resistance to low doses of agents that produce very different DNA lesions.

The JNKs comprise one family of MAPK molecules that is part of the complex intermediate-early response to UV, IR and cisplatin (for review, see Ip and Davis, 1998, Watters, 1999). JNK regulates gene expression through phosphorylation of a number of transcription factors and can affect survival more directly by affecting the stability of the apoptotic factor p53 (Fuchs et al., 1998a and 1998b). JNK is generally thought to be a 'pro-apoptotic' factor that is activated within seconds of very low UV fluences (our unpublished results). Here we have detected only a moderate change in JNK activity 24 hours after exposure to very high cisplatin concentrations. If alterations in MAPK activity contribute to induced resistance it appears unlikely that these changes will be detectable with conventional *in vitro* kinase assays.

There is increasing evidence that DNA repair is the mechanism of induced radioresistance. Robson et al. recently identified a protein (DIR1) that is transiently

120

repressed by low doses of IR (1999) and showed that that antisense interference of DIR1 increased the rate of single strand break repair and protected cells from low radiation doses (Robson et al., 2000).

Recently it has been reported that chemical inhibition of poly-(ADP-ribose) polymerase (PARP) precluded induced radioresistance in the V79 CHO line (Marples and Joiner, 2000). PARP is associated with the breaking and rejoining of DNA strands (Soldatenkov and Smulson, 2000). To our knowledge, PARP has not been associated with any repair function of cisplatin-modified DNA. If PARP is the effector of induced radioresistance it seems unlikely that this mechanism is the same that accounts for induced cisplatin resistance.

There is no inducible radioresistance in CHO cells with defects in the NER gene ERCC1 (Skov et al., 1994). When irradiated in hypoxic conditions, these same cells are more sensitive then their parental line and exogenous expression of ERCC1 corrects this sensitivity (Murray et al., 1996). Taken together, these data suggest that, at least for CHO cells, induced radioresistance depends on some inducible role of ERCC1 in the repair of DNA damage that is not caused through oxygen radical intermediates. DNAprotein crosslinks and UV-like damage have been proposed to be substrate for this alternative repair mechanism (Murray et al., 1996).

Cisplatin-damaged DNA is repaired predominantly by nucleotide excision repair (NER) (Sheibani et al., 1997; Wood, 1997). However, a few cisplatin-induced adducts do not appear to be substrates for NER. Interstrand cross-links, and DNA-protein crosslinks together account for less than 2% of DNA-bound platinum in cisplatin-treated cells (Eastman, 1986) and may be repaired by some type of cross-link repair pathway. ERCC1 is part of a heterodimer (with XPF) that acts as a structure-specific endonuclease in NER (Wood, 1997). Cells with defects in XPF or ERCC1 are more sensitive to cisplatin than other NER mutants suggesting repair functions beyond a role in NER (Collins, 1993). ERCC1/XPF is a putative resolvase and this activity may be important in recombinational repair of cisplatin-DNA interstrand cross-links (Kuraoka et al., 2000). This evidence is consistent with a role of ERCC1 in the inducible repair of DNA-protein cross-links or some other DNA damage that is repaired neither by NER nor strand break repair mechanisms.

For many cancer patients, platinum-based chemotherapy represents their best chance for survival (Hogberg et al., 2001). Despite years of research, the events that govern cisplatin's cytotoxicity are fundamentally unknown. Our attempts to understand the mechanisms of low-dose cisplatin sensitivity have been motivated by two end goals. First, it may be possible to achieve therapeutic gain by redesigning treatment protocols to take advantage of tumour hypersensitivity. Secondly, the molecules and processes of induced cisplatin resistance may also be involved in the intrinsic or acquired resistance that so often limits clinical success. With greater understanding, it may be possible to develop novel treatments by manipulating these factors. Chapter 7

Persistent DNA damage in active genes activates the Jun N-terminal kinase

in human cells

J. Todd Bulmer and Andrew J. Rainbow

# Abstract

The Jun N-terminal kinases (JNKs) are activated by many biological, physical, and chemical stimuli, including the chemotherapeutic agent, cisplatin. Cisplatin causes DNA damage that kills cells predominantly by inducing apoptosis. This active cell death is not well understood but is thought to depend on the activities of the MAP kinases, including JNK. The importance of JNK in determining cell fate following cisplatin treatment remains controversial. JNK activity has been shown, in different systems, to be associated with both cisplatin resistance and sensitivity. The mechanism by which cisplatin activates JNK is unknown but it is thought to be induced by DNA damage. The primary pathway that repairs cisplatin-DNA adducts is nucleotide excision repair (NER). NER is a complex process that recognises and removes many types of bulky DNA lesions from the genome and uses the undamaged strand as template to replace the excised fragment. We have used well-characterised NER-deficient human fibroblasts to study the activation of JNK by cisplatin.

Cisplatin exposure causes a dose-dependent increase in JNK activity in NERdeficient xeroderma pigmentosum (XP) group A (XP-A) fibroblasts that exceeds 10-fold 24 hours after a 1 hour 400µM exposure. This is much greater than the modest, 2-fold increase we measure in similarly treated, repair-proficient fibroblasts. In fact, cisplatin induces a transient increase in JNK-1 activity of about 10-fold in normal and DNA repair-deficient fibroblasts. In normal fibroblasts, though, this activation declines to about 2-fold 24 hours after treatment while in XP-A fibroblasts, this 10-fold activation persists.

Cells from patients with XP in complementation group C (XP-C) are competent in the repair of actively transcribing DNA strands but are not able to recognise lesions in inactive DNA. Cockayne's syndrome (CS) is a disease that results from a cellular inability to repair active DNA strands while having a normal capacity for repairing inactive DNA. SV40-transformed CS fibroblasts were extremely sensitive to cisplatin, compared with repair proficient cells but surprisingly the XP-C fibroblasts were not. As in the XP-A strains examined, the activation of JNK-1 in primary CS fibroblasts is prolonged compared with normal fibroblasts. XP-C fibroblasts, however, showed only a transient JNK activation similar to the normal response.

Taken together these data provide strong evidence that persistent damage within the transcribed strand of active genes causes persistent JNK activation. Further, it is this damage that correlates with cisplatin-induced cell death.

# Introduction

Cisplatin is a very useful drug in treating many types of cancer. Sadly, intrinsic or acquired drug resistance of the malignancy often limits clinical success using cisplatin-based chemotherapy (Loehrer and Einhorn, 1984). Despite extensive research, the processes that govern cisplatin resistance remain largely unknown. The cytotoxicity of cisplatin is thought to result from the local DNA distortion caused when cisplatin binds to genomic DNA (Rosenberg, 1985, Jordan and Carmo-Fonseca, 2000). This damage induces apoptosis through signalling pathways that are not well understood (Dempke et al., 2000). In order to devise novel means of overcoming clinical cisplatin resistance, it will be essential to understand these pathways.

The mitogen-activated protein kinase (MAPK) family of signalling molecules (that includes p38, ERK and JNK) has been shown to be important in determining cell fate following cisplatin treatment (Cui et al., 2000, Persons et al., 1999, Wang et al., 2000, Dempke et al., 2000). Activation of the JNK enzyme cascade affects gene regulation through a set of transcription factors that includes c-Jun, JunD, ATF-2, Elk-1, and Sap-1 (Ip and Davis, 1998). The phosphorylation of these proteins by JNK family members generally enhances their ability to promote transcription of their target genes including many that could directly affect cisplatin resistance like: metallothionine IIa, topoisomerase I, mdr-1, thymidylate synthase, and glutathione-S transferase (Scanlon et al., 1991). JNK also has multiple roles in regulating the activity of the important proapoptotic factor, p53 (Schreiber et al., 1999, Fuchs et al., 1998a, Fuchs et al., 1998b). It is clear that the JNK pathway contributes in many different ways to determining the fate of cisplatin-treated cells. In this light, it is not surprising that the activity of the JNK pathway has been shown, in different systems, to correlate with both resistance and sensitivity. Mouse cells with no functional c-Jun are resistant to cisplatin (Sanchez-Perez and Perona, 1999), while the expression of a dominant negative Jun impedes the repair of cisplatin-damaged DNA and sensitises human tumour cells (Gjerset et al., 1999, Hayakawa et al., 1999, Potapova et al., 1997). When JNK activity is blocked in RIF-1
cells by the expression of a dominant negative form of its activator, SEK, the resulting cells were more resistant to cisplatin than the parental line (Zanke et al., 1996). Other workers have produced strong evidence that persistent JNK activity is associated with the cisplatin-induced apoptosis in human 293 cells (Sanchez-Perez et al., 2000). It is clear that the contribution of JNK activity to determining cell fate following cisplatin treatment is not simply to promote *or* prevent cell death. It is likely that the strength and duration of JNK activity within the context of other cell signals determine its effect.

The persistence of JNK activity appears to be important in determining if it will act to promote survival or cell death (Sanchez-Perez et al., 1998). The cessation of the JNK signal has been attributed to the activity of the MAPK phosphatase CL100 (CL100/MKP-1) (Sanchez-Perez et al., 2000). Despite a lack of direct evidence, the activation and maintenance of JNK signalling is thought to depend on the presence of cisplatin-induced DNA lesions (Potapova et al., 1997, Nehme et al., 1999, Persons et al., 1999).

Nucleotide excision repair (NER) is the enzymatic pathway that recognises and repairs most of the DNA damage caused by cisplatin (Zamble et al., 1996). Patients with xeroderma pigmentosum (XP) or Cockayne's symdrome (CS) harbour homozygous, germ-line mutations in genes that are essential to NER (reviewed in de Boer and Hoeijmakers, 2000). These genes and their products have been well characterised and NER has been recreated *in vitro* from purified proteins (Aboussekhra et al., 1995). CS cells have a specific defect that blocks the preferential repair of the transcribed strand of active genes (transcription-coupled repair (TCR)) and this defect correlates, in some

cases, with the reduced activation of JNK following UV-irradiation suggesting that competent TCR can contribute to JNK activation (Dhar et al., 1996). Others have presented data that suggests DNA mismatch repair is necessary for recognition of cisplatin-DNA adducts and induction of JNK as well as the tyrosine kinase c-Abl (Nehme et al., 1999). and the loss of mismatch repair is a characteristic of many cisplatin resistant tumours (Aebi et al., 1996). We have used human cells with known defects in NER to investigate the role of cisplatin-DNA lesions in the induction of JNK and cell death. In this report we present evidence that cisplatin treatment induces a prolonged activation of JNK-1 in cells that are deficient in the repair of transcriptionally active DNA. Further, this prolonged activation is associated with extreme cisplatin sensitivity. This new information expands our understanding of the clinical cytotoxicity of this drug that may allow the development of new platinum-based treatment strategies.

### **Materials and Methods**

*Cells:* Primary and SV40-transformed fibroblasts used in these experiments were obtained from National Institute of General Medical Sciences Repository (Camden, NJ). All cells were grown as monolayers in supplemented  $\alpha$ -MEM (FBS (10%), penicillin (100mg/mL), streptomycin (100mg/mL), and amphotericin B (250ng/mL) in a humidified growth chamber (5% CO<sub>2</sub>).

*Cisplatin treatment:* Cells were treated for 1 hour with appropriate concentrations of cisplatin. For JNK assays, cisplatin was dissolved in low-serum  $\alpha$ -MEM (FBS (0.1%), penicillin (100mg/mL), streptomycin (100mg/mL), and amphotericin B (250ng/mL)) and

for colony forming assays cisplatin was dissolved in supplemented  $\alpha$ -MEM. Treatment was stopped by removing the cisplatin solution, washing the monolayer and refeeding the cells with fresh low-serum  $\alpha$ -MEM. For colony forming experiments, all cisplatin treatments and washes were performed in supplemented  $\alpha$ -MEM.

Colony forming assays: Cells were seeded at low density (sufficient ~100 colonies per 100mm dish) in supplemented  $\alpha$ -MEM. 4 hours after seeding, cells were treated with either cisplatin or UV. Primary and SV40-transformed fibroblasts were incubated for 10 to 14 days. Plates were stained and colonies with >30 cells were counted as survivors and relative survival was calculated by comparison with mock-treated controls.

In vitro *JNK assay:* The method used to determine kinase activity was similar to that used by Derijard et al. (1994). Briefly, cells were seeded (in supplemented  $\alpha$ -MEM) into 100mm plates (5 x 10<sup>5</sup>/plate). The following day, growth media was replaced with low-serum  $\alpha$ -MEM and cisplatin and UV treatments were started 24 hours later. At appropriate times after treatment, cells were collected in cold PBS and lysed in NP-40 lysis buffer (NP-40 (0.5%), Tris (50mM, pH 8), NaCl (150mM), EDTA (2mM), NaF (100mM), NaVO<sub>3</sub> (1mM), Na Pyrophosphate (10mM)) with protease inhibitor cocktail (Boehringer Mannheim). Protein concentrations of cleared lysates were determined using the Micro BCA Protein assay (Pierce) and identicle quantities of total cellular protein (30-150µg) were added to protein-G-sepharose beads (Pharmacia) and 1µg monoclonal antibody to human JNK-1 (Pharmingen). Immunoprecipitation was carried out overnight and immune complexes were washed twice with lysis buffer and twice more with kinase buffer (HEPES (20 mM), MgCl<sub>2</sub> (20 mM),  $\beta$ -glycerophosphate (10 mM), NaVO<sub>3</sub> (0.1 mM), p-nitrophenylphosphate (2mg/mL), DTT (2mM)). The immune complexes were then incubated in 30 µL of kinase buffer with 5µCi of [ $\gamma$ -<sup>32</sup>P] ATP and 2 µg of GST-c-Jun(1-79) per sample for 20 minutes at 30°C. The substrate molecules were then separated by SDS-PAGE and kinase activity in the lysates was determined with phosphorimager technology.

## Results

The activation of JNK-1 following cisplatin exposure is greater in NER-deficient, XP-A fibroblasts – Fibroblasts from patients with group-A xeroderma pigmentosum are deficient in an early step in NER and are consequently sensitive to DNA-damaging agents like cisplatin (Fig. 7-1). Cisplatin activates JNK-1 in many cell types (Persons et al., 1999, Sanchez-Perez et al., 1998, Kharbanda et al., 1996) and has been shown in some situations to correlate with cell death (Sanchez-Perez et al., 1998, Hayakawa et al., 1999, Cui et al., 2000, Potapova et al., 1997). We studied the role of cisplatin-DNA adducts in the activation of JNK-1 by comparing normal fibroblasts with fibroblasts with no detectable NER. Extracts were collected from XP-A fibroblasts (XP12BE) and repair proficient fibroblasts (GM9503) 24 hours after exposure to different concentrations of cisplatin. JNK-1 was immunoprecipitated from these extracts and kinase activity was assessed. Relative JNK-1 activity increased to much greater levels in XP-A cells compared with normal cells. The increase in JNK-1 activity is dose-dependant in XP-A fibroblasts and increased 10-fold in cells exposed to 400µM of cisplatin compared with Figure 7-1: The colony forming ability of cisplatin-treated XP-A fibroblasts is greatly reduced compared to normal fibroblasts. The relative survival of an XP-A cell strain (XP12BE, ●) is shown relative to normal fibroblasts GM9503 (■) and GM 38A (▲). Symbols represent the average of three triplicate experiments (±SEM).



untreated cells. There was only a modest increase of less than 2–fold in similarly treated normal fibroblasts (Fig. 7-2). In order to ensure that these observations are not specific to the two cell strains examined, we went on to study a number of normal and repair-deficient fibroblast strains. Each of the three primary fibroblast strains from XP-A individuals (XP12BE, XP1WI, and XP4LO) demonstrated elevated activation of JNK-1 compared with four different repair proficient strains (Fig. 7-3). While the fold-increase in JNK-1 activity 24 hours varied among the XP-A strains, each of the strains showed significantly greater activation following the 400µM exposure (6 to 13-fold) than each of the repair proficient strains (1.5 to 2-fold). We conclude that JNK-1 activation following cisplatin-treatment is greater in cells that are unable to repair cisplatin-damaged DNA. This strongly suggests that the presence of cisplatin-DNA lesions is an important signal in the induction of JNK-1 activation by cisplatin.

*Cisplatin-DNA adducts cause persistent JNK-1 activity.* We continued our investigation by studying the time-course of JNK-1 activation following cisplatin exposure in primary fibroblasts. We exposed fibroblasts to a 400µM cisplatin solution for 1 hour and assessed JNK-1 activity in lysates collected at several subsequent times. JNK-1 activity increases similarly in normal and XP-A fibroblasts for about 5 hours after the start of treatment (Fig. 7-3). After about 5 hours, however, JNK-1 activity in repair-proficient fibroblasts begins to decrease while JNK-1 activity in the XP-A strain remains ~10-fold higher for at least 30 hours after treatment. These experiments were also performed with other XP-A Figure 7-2: Activation of JNK-1 is greater following cisplatin-exposure in XP-A fibroblasts compared with normal fibroblasts. A) Relative activation of JNK-1 24 hours following a 1 hour treatment at the doses shown in an XP-A (XP12BE, ●) and normal fibroblasts (GM9503, ■). Values were derived using phosphorimager analysis of gels shown in B (GM9503) and C (XP12BE).



and normal strains (XP4LO and GM969, respectively) and similar results were found (data not shown). These data are consistent with those in Fig. 7-2 where only a modest increase in JNK-1 activity was detected in normal cells 24 hours after treatment. We conclude that it is the presence of cisplatin-DNA adducts that is the signal for JNK-1 activation and the persistence of these adducts causes a persistent JNK signal.

Cisplatin-induced DNA-damage in transcriptionally active DNA causes cell death. Nucleotide excision repair (NER) deficient cells that have a specific defect in the preferential repair of transcriptionally active DNA can be derived from patients with Cockayne's Syndrome (CS) (Venema et al., 1990). In vitro studies have shown that there are two complementation groups (CS-A and CS-B) (Tanaka et al., 1981). In contrast, within the heterogeneous disorder, xeroderma pigmentosum (XP), there is a complementation group (XP-C) that is competent in the preferential repair of active genes while being unable to repair lesions throughout inactive regions of the genome (Kantor et al., 1990). We have taken advantage of these well-characterised cellular phenotypes to study the importance these two NER sub-pathways in the cellular response to cisplatin. We treated SV40-transformed fibroblasts from XP-C (XP4PA) and CS-B (CS1AN) individuals and compared the clonogenic survival to that in repair proficient fibroblasts (Fig. 7-5). CS-B cells were extremely sensitive to cisplatin suggesting persistent lesions within transcribed DNA cause cell death. These cells have been reported to be sensitive to UV-induced DNA-lesions (Francis et al., 2000). Interestingly, the SV40-transformed XP-C fibroblast line showed no marked sensitivity to cisplatin compared with the normal

Figure 7-3: Activation of JNK-1 in XP-A and normal fibroblasts: Relative activation of JNK-1 24 hours following a 1 hour treatment at the doses shown in XP-A cells, GM5509(O), GM1630(I), GM544(A) and in repair proficient cells,  $GM9503(\Box)$ ,  $GM969(\bigcirc)$ , GM8399 ( $\diamondsuit$ ), and  $GM38A(\bigtriangleup)$ . Symbols represent the means of 2 to 7 experiments (±SEM).



lines, AG02804, or AR5 (data not shown). These cells have been shown to be sensitive to UV (Francis et al., 1997) and the XPC gene product is required for normal repair of cisplatin-damaged DNA (Bulmer et al., 1996).

Cisplatin-DNA adducts within active genes maintain JNK-1 activation. Having determined that cisplatin-induced DNA damage is important in the maintenance of JNK activation and that the persistence of damage within active genes appears to be related to cell death, we were interested in determining if the location of the DNA damage is important in maintaining JNK activation. We assessed JNK-1 activity following cisplatin treatment in primary fibroblasts from patients with CS and XP-C. JNK-1 activity is elevated in CS-A (CS3BE) and CS-B (CS1AN) fibroblasts 24 hours after cisplatin treatment compared with similarly treated repair proficient fibroblasts (Fig. 7-6) suggesting that persistent cisplatin lesions within the transcribed strand of active genes causes the maintenance of high levels of JNK-1 activity. However the fold-induction of JNK-1 is less in these CS strains than in the XP-A strains examined. This observation may be attributable to residual NER in CS cells. That is, CS cells repair damage in the genome without preference for active regions and this slow repair of active sequences may be sufficient to diminish the signal to maintain JNK-1 activity 24 hours after exposure.

Cells from XP individuals from complementation group C (XP-C) can efficiently repair strands of DNA actively being transcribed but are unable to recognise lesions in transcriptionally inactive DNA. We used these cells to further examine the role of Figure 7-4: Activation of JNK-1 is more persistent in XP-A fibroblasts compared with repair proficient fibroblasts. The relative activity of JNK-1 following exposure to  $400\mu$ M cisplatin for 1 hour is shown in XP-A (XP12BE,  $\textcircled{\bullet}$ ) and normal fibroblasts (GM9503,  $\textcircled{\bullet}$ ).



persistent cisplatin-DNA adducts in the activation of JNK-1. 24 hours after being exposed to cisplatin, fold-increase in JNK-1 activity in the two XP-C strains (XP1BE and XP1PW) was similar to the increase seen in normal fibroblasts (Fig. 7-7). While these data suggest that the removal of cisplatin-DNA lesions in the bulk of the genome does not affect the activation of JNK-1, it is also possible that the defect in XP-C precludes JNK-1 signalling. That is, the inability to recognise lesions in the bulk of the genome may inhibit the mechanism that triggers JNK-1 activity. To assure that cisplatin does induce JNK-1 in XP-C fibroblasts we exposed XP1PW fibroblasts to 400µM and measured JNK-1 activation at subsequent times (Fig. 7-8). Kinase activity reached a maximum of about 10-fold around 6 hours after exposure and gradually decreased to about 3-fold 24 hours after exposure. The time period of this decrease corresponds to the removal of cisplatin-DNA lesions (Bingham et al., 1996, Zamble et al., 1996) and further supports our contention that it is the persistence of adducts in the transcribed strand of active genes that maintains JNK activity.

#### Discussion

The JNK-1 molecule responds to many genotoxic stresses and many of its upstream activators have been well-characterised (Schaeffer and Weber, 1999). What remain unknown are the events that translate these physical or chemical stresses into biological signals. Cisplatin, like ultraviolet light (UV), causes a spectrum of DNA lesions that block transcription and DNA replication and most of this DNA damage is repaired by nucleotide excision repair (NER). The activation of the JNK pathway by UV Figure 7-5: Cells with defective transcription-coupled repair are sensitive to cisplatin while cells deficient in global genome repair are similar to normal cells in their cisplatin sensitivity. The relative clonogenic survival of cells is shown for SV40-transformed CS-B (CS1AN, ●), XP-C (XP4PA, ■) and normal fibroblasts (AG02804, ▲). Symbols represent the mean result of 2-4 experiments of 3 replicates (±SEM). Some error bars lie within symbols.



light is, at least primarily, the result of cytosolic events involving molecules at the plasma membrane and appears to be independent of DNA damage (Devary et al., 1993). In cisplatin-treated cells, however, increased JNK activity is thought to result from DNA damage (Persons et al., 1999, Potapova et al., 1997). Until now, the importance of cisplatin-DNA adducts in activating JNK has been inferred from comparison with the less cytotoxic stereoisomer, transplatin. Transplatin does not detectably activate JNK and this is thought to be because transplatin-DNA adducts are quickly repaired (Ciccarelli et al., 1985, Persons et al., 1999, Potapova et al., 1997). However, if cisplatin-induced JNK activation is mediated by interactions with other biomolecules like lipid or protein, then transplatin could also be expected to interact differently with these other targets, failing to activate JNK. We have tested this possibility by comparing JNK activation in primary fibroblasts with differing capacities to repair DNA. Xeroderma pigmentosum group A (XP-A) fibroblasts are sensitive to cisplatin and have a complete inability to perform NER. In these NER deficient cells, cisplatin adducts persist in the genome much longer than in repair proficient cells.

JNK activity in the fiobroblasts tested reaches a maximum 4-6 hours after cisplatin treatment. This coincides with the completion of the two-step cisplatin-DNA reaction (Sherman and Lippard, 1987). This is much slower than UV-induced activation that reaches a maximum only a few minutes after exposure in all cells tested (Derijard et al., 1994 and our unpublished results). There is a greater increase in JNK activity 24 hours after cisplatin-exposure in XP-A compared with normal fibroblasts. This difference is evident because there is a decrease in JNK activity that begins 6 hours after



treatment and this time period is that over which most cisplatin adducts are repaired in normal cells (Bingham et al., 1996, Dijt et al., 1988). Taken together, these data strongly support the notion that the DNA adducts *per se* promote JNK function.

XP is a heterogeneous disorder that encompasses eight known complementation groups that have defects in some aspect of NER. Cells from complementation group C (XP-C) retain the ability to efficiently repair the transcribed strand of active genes (transcription-coupled repair, TCR) but are unable to recognise and repair DNA damage to the rest of the genome (global genome repair, GGR) (Venema et al., 1991). Cisplatin exposure causes only a transient activation of JNK in XP-C fibroblasts that is similar to the activation seen in repair proficient cells. Since the cisplatin lesions in untranscribed regions of the genome remain unrepaired in XP-C fibroblasts, we conclude that these lesions are not important in the maintenance of the JNK signal. These data are consistent with a number of results that have shown the importance of intragenic DNA damage in the activation of cell signals (Yamaizumi and Sugano, 1994). XP-A and CS fibroblasts have been shown to have a lower threshold for UV-induced accumulation of several genes compared with TCR-proficient XP-C or normal fibroblasts (McKay et al., 1998, Blattner et al., 1998). Interestingly, a number of these gene products (for example p53 and metallothionein IIa) are affected by JNK activity directly through post-translational modification, gene promotion, or both.

p53 is a commonly altered gene in human malignancy (Hollstein et al., 1991) and the protein affects cell physiology through a barrage of functions. p53 mediates apoptosis, cell cycle progression, and DNA repair and each of these are thought to be Figure 7-7: Cisplatin-induced activation of JNK-1 in XP-C fibroblasts is similar to that in normal fibroblasts. JNK-1 activity in lysates collected 24 hours after exposing XP-C fibroblasts (XP1PW,  $\blacktriangle$ , and XP1BE,  $\blacklozenge$ ) to given doses of cisplatin. Symbols represent the means of 2 experiments (±SEM). For comparison, the mean results from all XP-A ( $\textcircled{\bullet}$ ) and normal strains ( $\blacksquare$ ) are included.



important in cisplatin resistance (Ko and Prives, 1996, McKay et al., 1999, Dempke et al., 2000). Activity of the MAPK family is essential to regulating p53 in the stress response. A number of amino acid residues within the p53 gene are targets for phosphorylation by MAPKs and both p38 and JNK have been shown to be important regulators of p53. In mouse cells, JNK (through c-Jun) represses the p53 gene (Schreiber et al., 1999); can stabilise and activate p53 in response to stress (Fuchs et al., 1998a, Buschmann et al., 2001); and targets p53 for ubiquitin-mediated degradation in nonstressed cells (Fuchs et al., 1998b). Buschmann and co-workers (2001) recently showed that cisplatin failed to activate JNK in normal human fibroblasts 1 hr after a 100µM exposure. This is consistent with our results showing that JNK activation in human fibroblasts only reaches a maximum 4-6 hours after treatment. It remains to be shown that cisplatin-induced JNK activation causes the specific phosphorylation (Thr-81) that is essential to the stabilisation and activation of p53 following UV-irradiation and other cell stress.

Kinase activity of p38 has been shown to promote cisplatin- and UV-induced activation of p53 and apoptosis (Sanchez-Prieto et al., 2000, Bulavin et al., 1999). Other workers, however, have shown that p38 is not activated by cisplatin in human tumour cells (Persons et al., 1999). Since tertiary conformation appears to affect the ability of p53 to accept phosphorylation (Bulavin et al., 1999), it is likely that the order and duration of the different MAPK signals that converge at p53 determine its role in response to genotoxic stress. This complexity may explain how p53 activation, despite being a predominantly pro-apoptotic factor, has been shown in some systems to be Figure 7-8: JNK-1 activity reaches a maximum 6 hours after cisplatin treatment in XP-C fibroblasts. JNK-1 activity is shown in cisplatintreated cells relative to untreated cells. XP-C fibroblasts (XP1PW) were exposed to a 400 $\mu$ M cisplatin solution for 1 hour and lysates were collected at the times shown and assayed for JNK-1 activity. Symbols represent the mean of 3 experiments (±SEM).



associated with cisplatin resistance (Brown et al., 1993, Yazlovitskaya et al., 2001). Clearly, p53 is an important effector of JNK and the elucidation of this interaction will be essential in understanding the role of JNK in clinical cisplatin resistance.

Expression of metallothionein confers resistance to cisplatin by binding to the drug, reducing the effective intracellular concentration (Kelley et al., 1988). Expression of the human metallothionein IIa gene (hMTIIa) is controlled, in part by JNK activity, through the AP-1 sequence encoded in its promoter (Scanlon et al., 1991). Some cisplatin-resistant cancer cells have been shown recently to have greater expression of a gene that is driven by the MTIIa promoter compared with a cisplatin-sensitive counterpart (Vandier et al., 2000). It may be that MTIIa expression as part of a early response is important in the protective actions of JNK within the early hours after cisplatin exposure.

One group has reported a defect in the UV-induced activation of the JNK pathway in CS-B suggesting that the CSB gene product is required for the detection of DNA lesions (Dhar et al., 1996). We find no evidence of subnormal JNK induction following cisplatin or UV-irradiation in CS-B fibroblasts (data not shown). It may be that some defect in the CS cells is accountable for lower levels of induced kinase activity compared with the XP-A cells studied. However, we think this difference is more likely caused by the non-preferential NER in CS cells that would be much slower than the intact TCR in XP-C or normal fibroblasts

The nature of the signal that is initiated at the transcription-blocking lesions is not well understood but is thought to involve the stalled RNA polymerase II (polII) (Ljungman et al., 1999, Blattner et al., 1998). Clearly, though, the initiation of these cell signals does not require the ubiquitylation or the degradation of polII since these actions are defective in CS cells while the ability to activate the JNK cascade remains intact (McKay et al., 2001, Bregman et al., 1996).

Cisplatin exposure mediates the kinase activity of JNK through at least two separate biochemical signals (Sanchez-Perez et al., 2000). The first is the signal to activate the JNK and this signal appears to be dependent on the known upstream activators, SEK1 and MEKK1 (Sanchez-Perez and Perona, 1999). The second signal is mediated by the MAPK phosphatase, CL100/MKP-1, that has been shown to decrease cisplatin-induced act JNK activity (Sanchez-Perez et al., 2000). Our results indicate that it is this phosphatase-mediated down-regulation of JNK is forestalled by the presence of cisplatin-DNA adducts.

The role of JNK in determining clinical success with cisplatin has not been defined. The *in vitro* technique for assessing cell survival that is most predictive of clinical sensitivity, at least for ionising radiation, is the colony forming assay (Malaise et al., 1987). We have employed this assay here to investigate the effect of different DNA repair defects on the survival of cisplatin-treated cells. The extreme sensitivity of SV40-transformed CSB fibroblasts compared with XP-C and normal fibroblasts suggests that, in addition to maintaining JNK activity, these lesions cause cell death. This correlation is consistent with the conclusions of others who have reported that persistent JNK activation promotes apoptosis in human cells (Sanchez-Perez et al., 1997). It may be transient JNK activation, on the other hand, that is important in the induction of a number

of protective mechanisms like cell cycle arrest, thiol expression or DNA repair. These multiple roles for JNK, within a complex response to cisplatin, could explain some seemingly contradictory observations. For example, the expression of a dominant interfering cJun construct in human tumour cells has been shown to; enhance p53-mediated apoptosis, impede NER, and cause cisplatin sensitivity (Potapova et al., 1997, Gjerset et al., 1999, and Hayakawa et al., 1999). NIH 3T3 cells with targeted deletion of cJun, however, are resistant to cisplatin (Sanchez-Perez and Perona, 1999). This deletion precludes some downstream signalling, presumably without affecting the immediate early (IE) functions of JNK suggesting that the IE actions of JNK are protective. This model is consistent with a recent report that the phosphatase activity of CL100/MKP-1 protects human 293T cells from apoptosis by dephosphorylation of JNK (Sanchez-Perez et al., 2000).

Cisplatin is a synthetic compound that produces a unique spectrum of DNA lesions. For this reason, the cellular response to the drug is surely an incidental combination of mechanisms that have been evolutionarily designed for other purposes. This is evident in the slow repair and relative cytotoxicity of cisplatin-DNA adducts (Zamble et al., 1996, Dijt et al., 1988) compared with UV-induced DNA lesions. Within this context, it is not surprising that many different cell mechanisms have apparently contradictory roles in cisplatin resistance.

We demonstrate here the importance of lesions within transcriptionally active genomic DNA in the prolonged activation of the MAPK, JNK-1. This prolonged activation is associated with reduced survival in fibroblasts that are compromised in their ability to perform TCR. While the mechanisms are not well understood, it is clear that JNK is an important part of the elaborate mechanism that governs the cellular 'decision' to live or die in response to cisplatin exposure. Understanding these mechanisms will allow the clinical use of cisplatin to be modified to maximise efficacy while minimising the morbidity of platinum-based chemotherapy. Chapter 8

# Summary and Discussion

## Nucleotide Excision Repair (NER) and cisplatin resistance

We have reported here that intact NER is essential for normal repair of cisplatindamaged DNA. Previously, it had been suggested that some components of NER (particularly the ERCC3/XPB helicase) were not required for the repair of cisplatin induced adducts (Lee et al., 1993). We show here that very high levels of the NER gene ERCC1 can inhibit repair of cisplatin-damaged DNA. Many workers, however, have proposed that levels of the ERCC1 gene product are rate-limiting in NER and have suggested that increased ERCC1 contributes to clinical drug resistance (Ferry et al., 2001). This apparent contradiction demonstrates that the 2-10-fold increases in ERCC1 expression in cisplatin resistant cells cause a very different biological outcome than the very high levels employed in this study. This is consistent with results from other workers in our lab showing that the effect of ERCC1 overexpression on HCR is affected by the multiplicity of virus used to infect (Gu and Rainbow). Clearly, NER is a subtle mechanism and the stoichiometry of the components is crucial to efficient function. Beyond this, the repair-inhibition caused by ERCC1 overexpression in not understood.

We note, though, that ERCC1 overexpression reduces host cell reactivation (HCR) in Chinese hamster ovary (CHO) cells with defects in the ERCC4/XPF gene but not in other repair deficient mutants. ERCC4 forms a heterodimer with ERCC1 that makes the incision 5' to the lesion in NER. Our observation suggests that the repair function that is inhibited remains intact in CHO cells from complementation group 4. Human XPF has long been known to complement the defect in ERCC4 mutant CHO cells. More recently, though, expression of human XPF has additionally been shown to compensate for the mutation in repair deficient cells from complementation group 11 (Sijbers et al., 1996). This intragenic complementation suggests that XP-F has at least two separable roles in DNA repair. ERCC1 overexpression reduces HCR of cisplatintreated adenovirus (Ad) but not UV-irradiated Ad. Taken together, these data suggest that the repair inhibition we have described affects a function of ERCC4 that is specifically important in the repair of cisplatin-DNA adducts and does not appear to require functional NER.

Understanding the relationship between ERCC1 and DNA repair will be essential to identifying a causative role for ERCC1 in clinical cisplatin resistance. While this relationship has been studied in cell-free systems, it has been impracticable in intact cells. The ERCC1-expressing Ad vectors used in this study have been designed to express at very high levels. This has limited our ability to use Ad to examine the effect of a modest elevation of ERCC1 levels in living cells. The construction of Ad vectors containing ERCC1 expression cassettes that are driven by low-level or inducible promoters would allow a more informative study by allowing precise control of expression.

## Low dose hypersensitivity to cisplatin

We have shown here that some mammalian cells are extremely sensitive to low doses of cisplatin. If the conditions of this hypersensitivity can be created or maintained in malignant tissue during cancer treatment, there is potential for considerable therapeutic gain. This is the premise for experimental treatment protocols currently being tested for maximising the efficacy of radiotherapy through hyperfractionation (Joiner et al., 2001). Additional clinical implications of our work stem from our observations that DNA damaging agents can preclude hypersensitivity to subsequent cisplatin exposure. This general cross-resistance may account for the failure of some treatment protocols that combine cisplatin and ionising radiation.

Besides the clinical implications of our study, we have used a conventional colony forming assay to demonstrate that the presence of damaged DNA in the nucleus of cells triggers protection from exposure to low concentrations of cisplatin. By employing a similar experimental approach along with more sensitive techniques like flow cytometry and computerised microscopy it could readily be determined if UV-damaged DNA can confer protection to low doses of ionising radiation. This result would suggest the extent to which radioresistance and cisplatin-resistance are induced through similar mechanisms.

A gene product (DIR) has recently been identified that is down regulated by very low fluences of ionising radiation and is a suppresser of single strand break repair (Robson et al., 2000). Disruption of DIR expression with anti-sense sequences has been shown to promote resistance to ionising radiation. The role of DIR in induced cisplatin resistance would further elucidate the commonalties of induced resistance to both of these agents.

Further, virus constructs with different biological activities could be employed to characterise the mechanisms of DNA damage recognition. As we have shown elsewhere in this work, damage to active genes can cause different results than damage within non-coding DNA. By using a transcriptionally 'dead' Ad construct to deliver damaged DNA we could determine the importance of the transcriptional machinery in the signalling that induces a protected cellular condition.

### Activation of the Jun N-terminal kinase

We show here that the activation of the Jun N-terminal kinase is prolonged in human cells with defects in the removal of cisplatin-DNA adducts from transcriptionally active DNA (transcription-coupled repair (TCR)) but not in cells with a specific defect in the repair of non-coding DNA (global genome repair (GGR)). This persistent JNK activation has been shown to be associated with cisplatin-induced, caspase-mediated apoptosis in human cells (Sanchez-Perez et al., 1998). Consistent with these observations, we find that cells with TCR defects are extremely sensitive to cisplatin while GGR deficient cells are not. Taken together, our results support a model as illustrated in figure 1. The implications of these results are important. We suggest that prolonged activation of the JNK pathway may be required for cisplatin-induced cell death. Many avenues of this research need to be explored. Most obviously, the kinetics of cisplatin-induced JNK activity should be compared between sensitive tumour cell lines
and their cisplatin-resistant counterparts. This would be an informative test for a hypothetical role for JNK in clinical cisplatin resistance.

Another important extension of this research would be to investigate the persistence of JNK activity following UV-irradiation of these human cell lines with well-characterised DNA repair defects. While a great deal of research has focused on UV-induced activation of the JNK pathway, the persistence of this activation remains largely unexplored. These experiments would provide important evidence that could lend clarity to the general processes that govern cell death induced by DNA damage.

**Figure 8-1. Model representing the role of JNK in determining the fate of cisplatin-treated mammalian cells.** Persistent cisplatin-induced DNA damage in active genes is associated with prolonged activation of JNK and reduced survival. The signal that maintains JNK activity is initiated by DNA damage and may be mediated by the JNK phosphatase MKP-1. While prolonged JNK activation is associated with apoptotic cell death, transient activation may contribute to DNA repair and cell survival.



Chapter 9

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## Appendix A

Studying the effects of several oncogenes

on the cisplatin resistance of human fibroblasts

J. Todd Bulmer, Bruce C. McKay, and Andrew J. Rainbow

Adenovirus is a promising vector for therapeutic gene therapy. Many of the genes proposed as having possible therapeutic value in cancer treatment encode proteins that are involved in cell cycle regulation. We have obtained a number of adenovirus vectors that have been engineered to express proteins that affect cell cycle regulation. These include: the anti-oncogene, p53; the cell cycle inhibitor, p21; and the retinoblastoma protein (pRb)-controlled transcription factors E2F-1, E2F-4, and DP-1. These Ad constructs were kindly provided to us by: Dr. J. R. Nevins at Duke University, Chapel Hill, N.C., USA; Dr. F.L. Graham, McMaster University; and Dr. T.C. Thompson, Baylor College of Medicine, Houston, TX, USA.

The p53-pRb pathway for cell cycle control has been described in detail (for review, see Muller H. and Hellin K., (2000) *Biochim Biophys Acta* **1470**(1):1-21). Very briefly, pRb binds to transcription factors that promote cell cycle progression, sequestering from their genomic sites. pRb is phosphorylated in a cyclin-dependent manner, and this releases the transcription factors, thereby activating them and promoting cell cycle progression. p53 can prevent this cell cycle progression by transactivating the cyclin-cdk inhibitor, p21<sup>WAF1</sup>.

We have also obtained Ad expressing viral oncogenes. The large T antigens from Simian virus (SV40LT) and polyomavirus (PyLT) bind to and inactivate pRb and

207

SV40LT also binds to and inactivates p53. These viruses were kindly provided by Dr. F. L. Graham.

We infected a primary human fibroblast line (GM 38A) with these vectors and treated them with cisplatin 24 hours later. Preliminary results are shown in Figure A-1.

Expression of p21 causes resistance to high doses of cisplatin and the transcription factor E2F-1 sensitised cells. This is consistent with the current thinking that a recoverable arrest in the cell cycle can be protective against DNA damage. Expression of SV40LT also sensitised cells, consistent with its role in cell cycle promotion. Interestingly, our preliminary data show that p53 or E2F-4 overexpression do not appear to sensitise these fibroblasts. Early results with the PyLT and DP-1 show little effect on cisplatin sensitivity.

As the use of Ad in cancer treatment increases, possible interaction between transgene expression and conventional therapies will become more prevalent. *In vitro* studies will be important preparation for this kind of clinical investigation.

Figure A-1: The effects of oncogene expression on the cisplatinsensitivity of primary human fibroblasts. The surviving fraction of GM 38A primary fibroblasts infected with non-replicating Ad expressing different oncogenes is shown here relative to the surviving fraction of cells similarly infected with the control virus, Ad5sp1LacZ. A) p21 overexpression significantly increased the survival of cisplatin treated cells ( $\blacktriangle$ ) while E2F-1 reduced the surviving fraction ( $\blacksquare$ ). B) p21 infected cell survival ( $\bigstar$ ) is shown compared with cells in which SV40LT is overexpressed (O). C) The mean relative survival of p53 (O) and E2F-4 ( $\bigstar$ ) are shown. Each symbol represents 2-3 independent experiments of 3 replicates.



Appendix B

Nucleotide excision repair in

genetically unstable CHO cells

J. Todd Bulmer and Andrew J. Rainbow

One of the DNA lesions induced by ionising radiation (IR) is the double strand break (dsb). These dsbs can lead to chromosomal rearrangement during the mitotic division after exposure. Interestingly, some dsbs can promote chromosomal rearrangements in the descendants of cells many generations after exposure (for review see Morgan et al., (1998) *Mut. Res.* 404(1-2): 125-8). The mechanisms that cause this delayed genomic instability are not known. One possible explanation for these observations is that exposure to dsbs causes some impairment of DNA repair that allows genetic instability.

We were kindly given a set of Chinese hamster ovary cells that had been irradiated along with their un-irradiated counterparts by Dr.C. Limoli, Department of Radiation Oncology, University of California, San Francisco. Some of the irradiated clones we received were known to display delayed chromosomal instability while others did not. We used our viral host cell reactivation (HCR) assay in an attempt to identify DNA repair differences that correlated with the chromosomal instability phenotype. Additionally, we examined the effect of UV-irradiation before Ad infection on HCR with the possibility in mind that there was a difference in the inducible repair capacity of these cells.

For optimal expression we used an Ad construct expressing b-gal from the deleted E1 region and driven by the mouse cytomegalovirus (MCMV) promoter. Our preliminary results with these clones showed no clear correlation between genomic instability and the constitutive or the inducible HCR of UV-irradiated Ad (compare panels A and B in figure B-1). We found differences in HCR in clones that differed only in their passage number so we attempted to control for this by comparing cells of the same passage number. This became impractical as the cells varied greatly in their replicative rates with some clones doubling in less than 24 hours while some of the clones had doubling times of 4 or 5 days. This extreme variation in growth rate also made experiments difficult to perform and appeared to affect the infectivity of the virus and caused great variation in the reporter gene expression between cell lines.

While we certainly cannot conclude that DNA repair in general, or NER in particular, is not involved in delayed chromosomal instability, it is likely that it will be very difficult to detect these differences with conventional HCR techniques. Figure B-1: Host cell reactivation of  $\beta$ -galactosidase synthesis in Chinese hamster ovary cell clones with delayed genomic instability. CHO cells with delayed chromosomal instability (A, clone 138) and without delayed chromosomal instability (B, clone 114) were infected (10 pfu/cell) with a non-replicating Ad construct expressing  $\beta$ -galactosidase ( $\beta$ -gal) driven by the mouse cytomegalovirus (MCMV) major late promoter (CA35-1). The relative  $\beta$ -gal expression is shown for cells pretreated with 0 ( $\blacktriangle$ ), 10 J/m<sup>2</sup> ( $\textcircled{\bullet}$ ), or 30 J/m<sup>2</sup> ( $\textcircled{\bullet}$ ) of UV 1 hour before infection. The mean of 4 replicates in a typical experiment is shown (±SEM)



Figure B-2: Host cell reactivation of  $\beta$ -galactosidase synthesis in Chinese hamster ovary cell clones with delayed genomic instability. CHO cells that had not been irradiated (A, clone Choc4) and one that had been irradiated but displayed no chromosomal instability (B, clone 102) were infected (10 pfu/cell) with a non-replicating Ad construct expressing  $\beta$ -galactosidase ( $\beta$ -gal) driven by the mouse cytomegalovirus (MCMV) major late promoter (CA35-1). The relative  $\beta$ -gal expression is shown for cells pretreated with 0 ( $\blacktriangle$ ), 10 J/m<sup>2</sup> ( $\textcircled{\bullet}$ ), or 30 J/m<sup>2</sup> ( $\blacksquare$ ) of UV 1 hour before infection. The mean of 4 replicates in a typical experiment is shown (±SEM)

