MITOCHONDRIAL ALTERATIONS IN TUMOUR CELLS

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ABSTRACT

Drug resistance limits the clinical efficacy of many cancer treatment modalities. Mutations, as a result of genomic instability within tumours, are thought to generate subpopulations of tumour cells which are less responsive to cytotoxic agents. This thesis investigates the hypothesis that drug resistance is associated with alterations in the mitochondria of cancer cells and these mitochondrial alterations provide a means to restore drug sensitivity in drug-resistant cell populations.

A human ovarian carcinoma model was used to further characterize mitochondrial changes associated with cisplatin resistance (Chapter 1). It was observed that mitochondria in a cisplatin-resistant variant, C13*, accumulated and retained more of a lipophilic cation, rhodamine 123 (Rh123), compared to its parental line, 2008. This extended mitochondrial retention of lipophilic cations rendered C13* cells sensitive to another lipophilic cation, dequalinium chloride (Deca), compared to 2008 cells. Moreover, combinations of Deca and cisplatin induced synergistic cell kill in both cell types. These data suggest that disruption of mitochondrial function may sensitize tumour cells, including cisplatin-resistant tumour cells, to the cytotoxic effects of cisplatin.

An association between mitochondrial alterations and resistance to another type of cancer therapy was also examined (Chapter 2). Photofrin II-

mediated photodynamic therapy (PDT) is thought to destroy tumour cells, *in vitro*, by disrupting mitochondria. It was observed that a variant resistant to Photofrin II-mediated PDT, RIF-8A, contained mitochondria that differed structurally and functionally compared to its parental line, RIF-1. This was the first report to associate mitochondrial changes with PDT resistance. Similarities between RIF-8A mitochondria and C13* mitochondria at the ultrastructural level, suggested that RIF-8A may be resistant to cisplatin compared to RIF-1 cells. This hypothesis was supported by the observations that RIF-8A cells were cross-resistant to cisplatin (Chapter 3). These data indicate that mitochondrial characteristics may modulate the sensitivity of tumour cells to certain cytotoxic agents.

Since cisplatin's cytotoxicity is believed to be mediated through its interactions with nuclear DNA, the mechanisms through which mitochondria influence cisplatin sensitivity, are unclear. Several of the steps involved in the repair of cisplatin-DNA lesions require ATP hydrolysis. Therefore, an increase in mitochondrial activity may augment the production of ATP, which can then be used for DNA repair. A host cell reactivation (HCR) assay indicated that C13* cells did not have an enhanced capacity to repair cisplatin-damaged DNA compared to either 2008 or RH4 cells suggesting that changes in the mitochondrial membrane potential do not influence the repair of cisplatin-DNA lesions.

Independent observations have implicated the proto-oncogene, c-*fos*, as a potential regulator of mitochondrial activity and/or cisplatin sensitivity. This hypothesis was examined in three different model systems (Chapter 5). In both instances where there was an overexpression of the c-*fos* gene there was no corresponding increase in mitochondrial membrane potential. Similarly, in C13* cells which have an elevated mitochondrial membrane potential compared to 2008 cells, there was no significant difference in either c-*fos* mRNA or protein levels. Cells that overexpressed c-*fos* were resistant to cisplatin however, reducing c-*fos* expression did not sensitize cells to cisplatin. These data suggest that alterations in both mitochondria and c-*fos* expression may modulate cisplatin sensitivity but these two characteristics are not interdependent.

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LIST OF ABBREVIATIONS

Ad 5	adenovirus type 5
cisplatin	*cis*-diamminedichloroplatinum(II)
CRE	cyclic AMP response element
CS	Cockayne's syndrome
Deca	dequalinium chloride
FCCP	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
GSH	glutathione
GST	glutathione-*S* transferase
Hpd	hematoporphyrin derivative
IC_{50}	drug concentration that inhibits cell growth by 50%
IPTG	isopropyl β-D-thiogalactopyranoside
MDR	multi-drug resistance
MSV	murine sarcoma virus
MT	metallothionein
NAO	10*N*-nonyl acridine orange
NER	nucleotide excision repair
PDT	photodynamic therapy
Rh123	rhodamine 123
ROS	reactive oxygen species
SIE	*sis*-inducible element

LIST OF ABBREVIATIONS (cont'd)

SRE	serum response element
TRE	TPA responsive element
XP	Xeroderma pigmentosum
UV	ultraviolet

PREFACE

This thesis contains five original manuscripts, four of which have been published and one that has been submitted for publication. A letter granting permission for reproduction of these manuscripts in this thesis has been obtained from the respective publishers and accompanies each published manuscript (Chapters 1-4). Each manuscript is contained within a thesis chapter and the references cited in each manuscript are listed at the end of each chapter. References which are not provided within a manuscript are listed at the end of the thesis. Page numbers of each manuscript have been adjusted for continuity within the thesis.

LIST OF PUBLICATIONS: R.A. Moorehead

1. Moorehead, R.A. and Singh, G. Expression of c-*fos* and mitochondrial membrane potential in cisplatin resistance (submitted to Journal of Biologicaly Chemistry)

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INTRODUCTION

Drug resistance is a major limitation in improving cancer cure rates. Resistance can be categorized as either intrinsic or acquired, both of which appear to result from spontaneous mutations in the unstable tumour cell genome (1,2). In intrinsic resistance, a majority of the tumour cells are unresponsive to a particular chemotherapeutic agent without prior exposure to that agent. In contrast, tumours with acquired resistance initially respond to chemotherapy, however, a small number of tumour cells are resistant to the chemotherapeutic agent. Eventually, these tumour cells replicate, generating a tumour predominantly composed of the drug-resistant phenotype. Therefore, re-exposure of the tumour to the initial chemotherapeutic agent proves ineffective (1-3).

In addition to spontaneous mutations, chemotherapeutic agents themselves may contribute to the generation of drug-resistant tumours. Many chemotherapeutic agents, including platinum-based agents, alkylating agents and anthracyclines, can generate DNA mutations. DNA damaging agents interfere with DNA transcription, synthesis, or repair, therefore, in addition to being cytotoxic these agents promote further DNA mutation (1). Appearance of drug-resistant tumours is particularly common following treatment with the platinum-based chemotherapeutic agent, *cis*-diamminedichloroplatinum(II) (cisplatin) and thus, cisplatin resistance has been extensively investigated (4).

Cisplatin is considered one of the most effective antitumour agents for a large number of solid tumours but its effectiveness is limited by the frequent emergence of drug-resistant tumours (4-6). In ovarian cancer patients treated with cisplatin, occurrence of cisplatin-resistant tumours can approach 60% (6). The importance of even a slight alteration in sensitivity to cisplatin was demonstrated in ovarian cancer patients receiving cisplatin-based therapy. This study showed a significant correlation between cisplatin dose-intensity and survival in ovarian cancer patients (7). However, as with most chemotherapeutic agents, cisplatin's dose intensity is limited by organ toxicity including nephrotoxicity (which is now reduced by chloruresis/hydration therapy), myelosuppression, ototoxicity, neurotoxicity, and gastrointestinal toxicity (8-11). Thus, even slight alterations in a tumour's responsiveness to cisplatin can affect the outcome of the treatment. Therefore, sensitizing either the original tumour or cisplatin-resistant populations to cisplatin, should provide a more favorable outcome for the patient.

In attempt to understand the mechanism(s) through which tumour cells become resistant to cytotoxic agents, drug-resistant variants have been generated and characterized. These variants are generated by exposing tumour cells to a concentration of drug that results in approximately 1 % survival, followed by re-growth of surviving colonies. Following several selection cycles, variants possessing various magnitudes of relatively stable drug resistance can be obtained. Variants which have acquired resistance to a particular drug can serve two purposes (i) they can provide information on a drug's mechanism of

action. It is assumed that the mechanism through which the variant employs to become resistant will provide insight into the critical intracellular target(s) of that particular drug and (ii) these variants serve as models for investigating strategies for overcoming drug resistance. It is conceivable that identification of mechanisms involved in cisplatin resistance may provide strategies to restore tumour cell drug sensitivity.

cis-diamminedichloroplatinum(II) (Cisplatin)

Cisplatin was discovered serendipitously by Rosenberg *et al.* (12) while investigating the effect of electrical current on bacterial growth. It was observed that a product of the platinum electrodes (eventually identified as cisplatin), rather than electrical current, inhibited bacterial cell division. Subsequently, cisplatin has been shown to be effective against a wide range of tumours. The ability of cisplatin to interact with intracellular targets is augmented in the presence of low chloride concentrations where hydrolysis of chloride from the parent drug is favoured, generating aquated, more active forms of the drug (13-15).

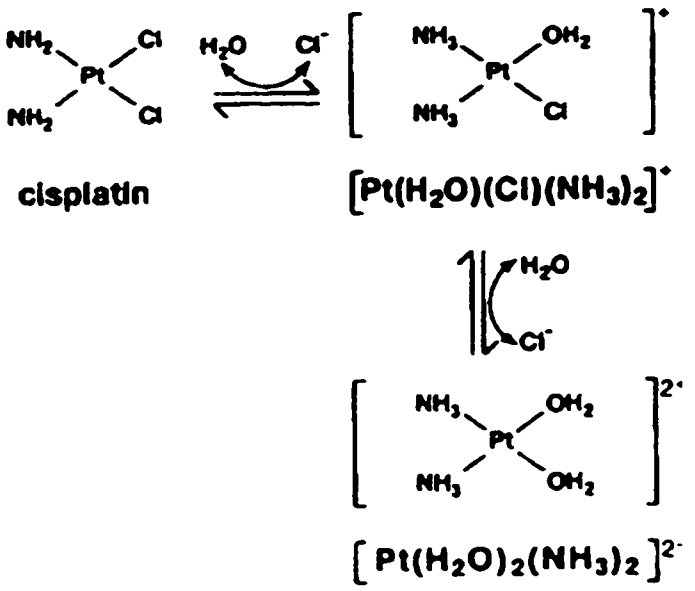

Figure 1. Cisplatin in its parental form and aquated species. This figure was adapted from (16).

In environments such as blood and plasma where chloride concentrations are in excess of 100 mM, the less active, parental form of the drug is relatively stable (4). Once inside a cell, low intracellular chloride concentrations (approximately 20 mM in epithelia cells) promotes the loss of chloride ions and the generation of active, aquated species of the drug (15,17). Active forms of cisplatin can interact with DNA (forming intrastrand adducts and interstrand cross-links), RNA, proteins and thiol containing compounds. Fractionation studies have demonstrated in kidney tissue that cisplatin is distributed in the following organelles from highest to lowest concentration (per mg protein); mitochondria > cytoplasm > nuclei > microsome (18,19) whereas in a human squamous carcinoma cell line, the distribution of cisplatin in organelles was nuclei > cytoplasm > mitochondria > lysosomes (20).

Cisplatin preferentially binds to the N7 position of purines to form a number of monofunctional and bifunctional DNA adducts (4). The relative abundance of cisplatin-DNA adducts are approximately 40-75% d(GpG)Pt, 10-20% d(GpNpG)Pt, 15-25% d(ApG)Pt while monoadducts, DNA-protein cross-links, and interstrand crosslinks comprise less than 5% of the total cisplatin-DNA adducts (21-24). Although only about 1% of total intracellular cisplatin eventually interacts with DNA it is believed that cisplatin-induced DNA lesions are primarily responsible for cytotoxicity (25). Cisplatin-DNA lesions are capable of interfering with synthesis, replication, unwinding, transcription and possibly repair of DNA (4,17).

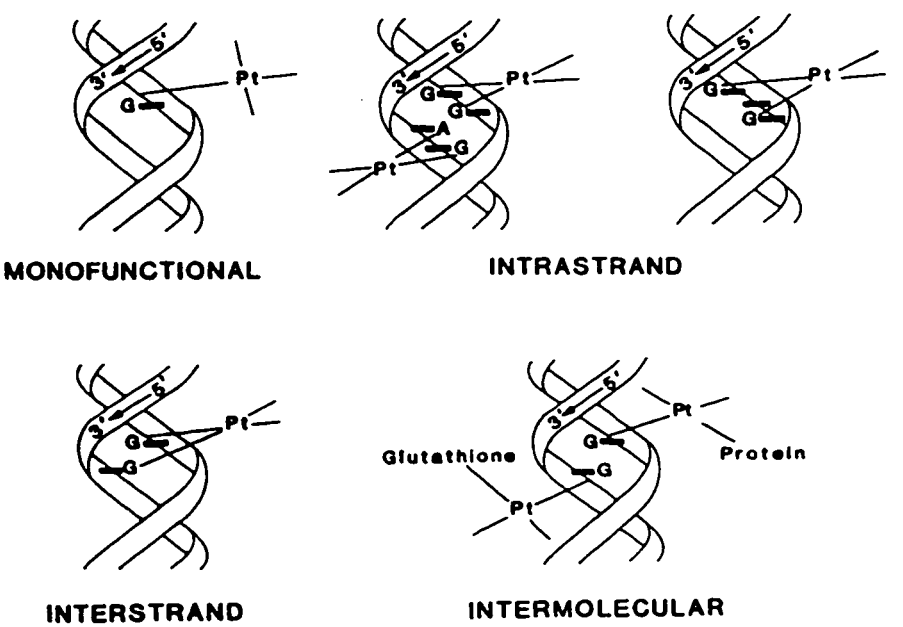

Figure 2. Different DNA lesions induced by cisplatin. This figure was adapted from (26).

Acquired Resistance to Cisplatin

Cisplatin resistance has been examined primarily *in vitro,* through the selection of variants resistant to cisplatin compared to their parental line. Selection procedures used to generate cisplatin-resistant variants *in vitro*, have been shown to induce multiple changes that contribute to cisplatin resistance including: altered drug transport, increased metallothionein or glutathione levels, and altered DNA adduct formation and/or repair (13,17,26-31). Changes in mitochondria and increased proto-oncogene expression have also been associated with cisplatin resistance and these characteristics will be discussed in detail in Chapter 1 and Chapter 5, respectively. However, the classical P-glycoprotein-mediated MDR does not appear to play a role in cisplatin resistance (32-34).

In vitro, cisplatin resistance is often associated with decreased intracellular cisplatin concentrations which may be a result of either decreased accumulation or increased efflux of the drug (20,25,30,32,35-44). Decreased cisplatin accumulation has also been reported in cisplatin-resistant tumours *in vivo* (45-48). Proposed mechanisms responsible for regulating cisplatin accumulation include, altered plasma membrane phospholipid composition (49), expression of plasma membrane protein pump(s) for cisplatin which are distinct from P-glycoprotein (50,51) and altered Na^+/K^+ ATPase activity (50,52,53). However, not all studies have observed an association between cisplatin resistance and an accumulation defect. In addition, the magnitude of the accumulation defects do not appear to reflect the magnitude of the cisplatin

resistance (37,42,45,46,54-66). Variations in cell type and selection procedure employed to generate the cisplatin-resistant variants probably contribute to the contrasting observations. Moreover, understanding the mechanism(s) involved in decreased cisplatin accumulation is difficult since the mechanism through which cisplatin enters a cell has not been completely elucidated; both passive diffusion and active transport have been reported (17,43,52,67,68).

Another reported mechanism involved in cisplatin resistance is an elevation in scavenging molecules such as glutathione (GSH) and metallothionein (MT). Glutathione can reach concentrations of 10 mM within cells thus making it the most abundant intracellular thiol-containing molecule. Formation of cisplatin-glutathione complexes occurs in a 1:2 ratio via interaction of cisplatin with sulfur groups in GSH (69). In addition to sequestering cisplatin, GSH has been shown to bind to monofunctional cisplatin-DNA adducts and prevent their progression to bifunctional adducts (70,71). This prevention of bifunctional adduct formation may be important since monofunctional adducts are more readily repaired than bifunctional adducts (26). Although elevated levels of GSH have been found in cisplatin-resistant cells (72-74), the lack of association in other cisplatin-resistant variants (38,41,42,59,75,76), the relatively slow formation of cisplatin-GSH complexes (77) and inconsistent results with GSH depleting agents (20,78-80) have raised questions as to the importance of elevated levels of GSH in cisplatin resistance.

Glutathione-S transferases (GSTs) are a family of enzymes capable of augmenting the rate of glutathione-cisplatin conjugation that have also been implicated in cisplatin resistance (81). However, correlations between elevated GST levels and cisplatin resistance suffer the same inconsistencies as the association between GSH levels and cisplatin resistance (20,35,59,73-75,81-93).

MTs are a family of inducible proteins involved in heavy metal regulation such as controlling free intracellular zinc, copper and cadmium concentrations (55,94,95). Like GSH, MT contains thiol groups that can interact with cisplatin (96,97). Each MT protein is capable of sequestering approximately 10 cisplatin molecules and thus represents a potential regulator of free intracellular cisplatin (98). While transfection of cells with MT has been shown to induce cisplatin resistance (99) and cells lacking MT are hypersensitive to cisplatin (94), an association between elevated MT expression and cisplatin resistance has been demonstrated in some studies (20,55,59,76,94,99-103) but not in others (42,48,74,88,104-108). Furthermore, tumour biopsies or cell lines from patients with ovarian cancer showed neither a correlation between MT levels and response to cisplatin nor did MT levels change following cisplatin-based therapy (105,109).

Since cisplatin-DNA lesions are thought to be responsible for cisplatin's cytotoxicity, enhanced repair of these lesions represents a potential mechanism through which cells can become resistant to cisplatin. It has been observed that

cells from human repair deficiency syndromes (i.e. Xeroderma pigmentosum and Cokayne's syndrome) and repair-deficient rodent cells, are hypersensitive to cisplatin (110-114). In addition, inhibitors of DNA repair such as aphidicolin and cytarabine (ara-C) can potentiate cisplatin toxicity in tumour cells (115-120). These results suggest that repair of cisplatin-damaged DNA is an important determinant of cisplatin sensitivity. In fact, enhanced repair of cisplatin-DNA lesions has been demonstrated in several cell culture systems for cisplatin intrastrand adducts (25,115,116,121-125) and cisplatin interstrand crosslinks (37,126,127). However, increased repair in cisplatin-resistant variants cannot always be shown (56,57,59,61-63,66,106,107,128-132). Variability of tumour type, cisplatin dose, and type of lesion investigated has contributed to the inconsistent findings. Moreover, preferential repair of DNA damage from actively transcribed genes has been described (21,133-136). Actively transcribed genes represent < 1% of the total genome and thus studies which examine total genomic repair are incapable of detecting repair differences in actively transcribed genes (134). The few studies that have examined repair of cisplatin lesions in actively transcribed genes suggest that enhanced repair of cisplatin lesions in certain genes is associated with cisplatin resistance yet inconsistencies between these studies exist (37,126,127). Therefore, it appears that enhanced repair of cisplatin lesions may occur in some cell types resistant to cisplatin but further studies are required to assess the relative importance of

gene-specific versus total genomic repair as well as the relative contribution of the various lesions to cisplatin's cytotoxicity.

Another form of enhanced DNA repair associated with cisplatin resistance is the ability to tolerate unrepaired DNA lesions (63,127,137,138). It has been observed that in some cisplatin resistant cells, DNA transcription and/or synthesis can occur even in the presence of cisplatin-DNA lesions through a process termed, replicative bypass. In these cells, it is generally accepted that repair occurs following DNA replication but prior to mitosis (139-141). A number of mechanisms through which tolerance of cisplatin-DNA damage have been proposed (17). It has been suggested that DNA lesions are repaired from actively transcribed genes necessary for cell survival while DNA lesions in non-transcribed regions in the genome are tolerated. Enhanced post-replication repair in cisplatin-resistant variants has also been hypothesized (17).

Although cisplatin resistance has primarily been investigated *in vitro*, *in vivo* resistant tumours have also been examined. Similar inconsistencies are found *in vivo* as those found *in vitro*. While accumulation defects (46,48), elevated GSH levels (142,143), elevated GST levels (89,144), increased DNA repair (46) and elevated proto-oncogene expression (145) have been observed in models of cisplatin resistance *in vivo*, poor correlations between GSH levels (48,146), GST levels (143,147), MT levels (48,104,105), and DNA repair (142) have also been observed. In addition, some tumours resistant to cisplatin *in vivo* lost their resistance

in vitro unless they were grown as spheroids suggesting that tumour structure/architecture may also regulate drug sensitivity (47,148).

Investigations of cisplatin-resistant mechanisms are summarized in Table 1. This table illustrates the multifactorial nature of cisplatin resistance even within similar tumour types. It also demonstrates that depending on the tumour type and the protocol used to generate cisplatin-resistant variants, different mechanisms contribute to cisplatin resistance. The lack of a consistent mechanism through which cisplatin resistance is dependent upon has hindered the development of therapeutic interventions for cisplatin-resistant tumours.

Table 1. Cisplatin Resistance Mechanisms.

Resistance Mechanism	Cell Type	Reference
Decreased accumulation	human ovarian carcinoma	(25,32,36,37,40,42,45,48,54,63,87,131)
	human lung cancer	(44,60,88,106)
	human head and neck cancer	(20,91,149)
	various human cancer	(35)
	murine leukemia	(38,39,41,124)
	murine fibroblasts	(76)
No correlation between accumulation and sensitivity	human ovarian carcinoma	(37,42,45,54-57)
	human cervical carcinoma	(58)
	human lung cancer	(59,60)
	human bladder cancer	(61,62)
	human colon carcinoma	(63)
	human embryonal carcinoma	(64)
	human mammary epithelia	(65)
	murine immunocytoma	(46,66)
Increased GSH or GST levels	human ovarian carcinoma	(57,63,72,82,150)
	human melanoma	(83)
	human lung cancer	(59,84,88,90,106)
	human head and neck cancer	(20,91,149)
	human bladder cancer	(61,62)
	human embryonal carcinoma	(64)
	various human tumours	(35,73)
	murine leukemia	(39,74)
	rat lymphoma	(150)
	Chinese hamster ovary	(81,85)
No correlation between GSH or GST levels and sensitivity	human ovarian carcinoma	(36,42,48,55-57,82,87,109,147,150)
	human cervical carcinoma	(58)
	human lung cancer	(59,75,84,106)
	human prostate cancer	(85)
	human breast cancer	(86,92)
	human colon carcinoma	(63)
	various human tumours	(107)
	murine leukemia	(38,41)
	murine fibroblasts	(76,93,151)
Increased MT levels	human ovarian carcinoma	(55)
	human small cell lung cancer	(59)
	human testicular cancer	(100,101)
	human head and neck cancer	(20)
	human epithelia	(102)
	murine embryonic fibroblasts	(94)
	murine bladder tumour	(103)
	murine fibroblasts	(76,102)
	various human & murine cells	(99,101)
No correlation between MT levels and sensitivity	human ovarian carcinoma	(42,48,55,104,105)
	human lung cancer	(88,106)
	various human tumours	(107)
	murine fibroblasts	(105)
	murine leukemia	(74)
	porcine kidney epithelia	(108)

Resistance Mechanism	Cell Type	Reference
Increased DNA repair	human ovarian carcinoma human cervical carcinoma human mammary epithelia murine leukemia	(25,37,87,115,116,123,126,127) (58,122) (65) (124)
No correlation between DNA repair and sensitivity	human ovarian carcinoma human lung cancer human bladder cancer human colon carcinoma various human tumours murine immunocytoma	(56,57,63,129-131) (59,106,132) (61,62) (63) (107) (66)
Changes in mitochondria	human ovarian carcinoma	(152,153)
Overexpression of c-*fos*	human ovarian carcinoma human colon carcinoma	(154-156) (157)

Treatment of Cisplatin-Resistant Tumours

Little progress has been made in treating cisplatin-resistant tumours *in vivo*. Reduction of tumour glutathione levels using glutathione depleting agents appears limited since this treatment also resulted in increased nephrotoxicity (149,158). Currently, therapy aimed at reducing MT levels in tumours is not possible but induction of MT in normal tissues to reduce cisplatin's side effects has shown some promise (103,159). Inhibition of DNA repair by aphidicolin may prove ineffective since it remains unclear whether aphidicolin can inhibit repair in proliferating cells (74,115,117,125,149,160,161) and an *in vivo* trial did not show encouraging results (14). However, inhibition of DNA repair using cytarabine and hydroxyurea have provided better results (162,163).

Limited success in treating cisplatin-resistant tumours has lead ourselves and other investigators to examine alternate strategies. Several additional cisplatin resistance mechanisms have more recently been proposed including, altered signal transduction pathways, changes in mitochondria, and cytoskeletal modifications (reviewed in (17)). Changes in mitochondria associated with cisplatin resistance, particularly an increase in mitochondrial membrane potential, has been a focus of our laboratory. Mitochondria are the primary source of energy in most cells (164) and a class of agents, termed lipophilic cations, are capable of selectively disrupting mitochondria with elevated membrane potentials (165-168). Therefore, we propose that inhibition of

mitochondrial function may represent a strategy for treating cisplatin-resistant tumours.

Mitochondria: Structure and Function

Mitochondria contain an outer and an inner membrane which are separated by an intermembrane space. The inner mitochondrial membrane encompasses the matrix and contains the components of the electron transport chain. Five major complexes comprise the electron transport chain; NADH/ubiquinone oxidoreductase (complex I), succinate/ubiquinone oxidoreductase (complex II), ubiquinol/ferricytochrome *c* oxidoreductase (complex III), ferrocytochrome *c*/oxygen oxidoreductase (complex IV), and F_0F_1ATPase or ATP synthase (complex V) (169). Of the approximately 60 polypeptide components of the electron transport chain, 13 have been shown to be encoded by mitochondrial DNA (mtDNA) and synthesized within mitochondria (170). The remaining polypetides are encoded by nuclear DNA and must be imported into mitochondria (169). A certain amount of communication must exist between the nucleus and mitochondria to coordinate the synthesis of electron transport chain components but these signals remain unresolved (171).

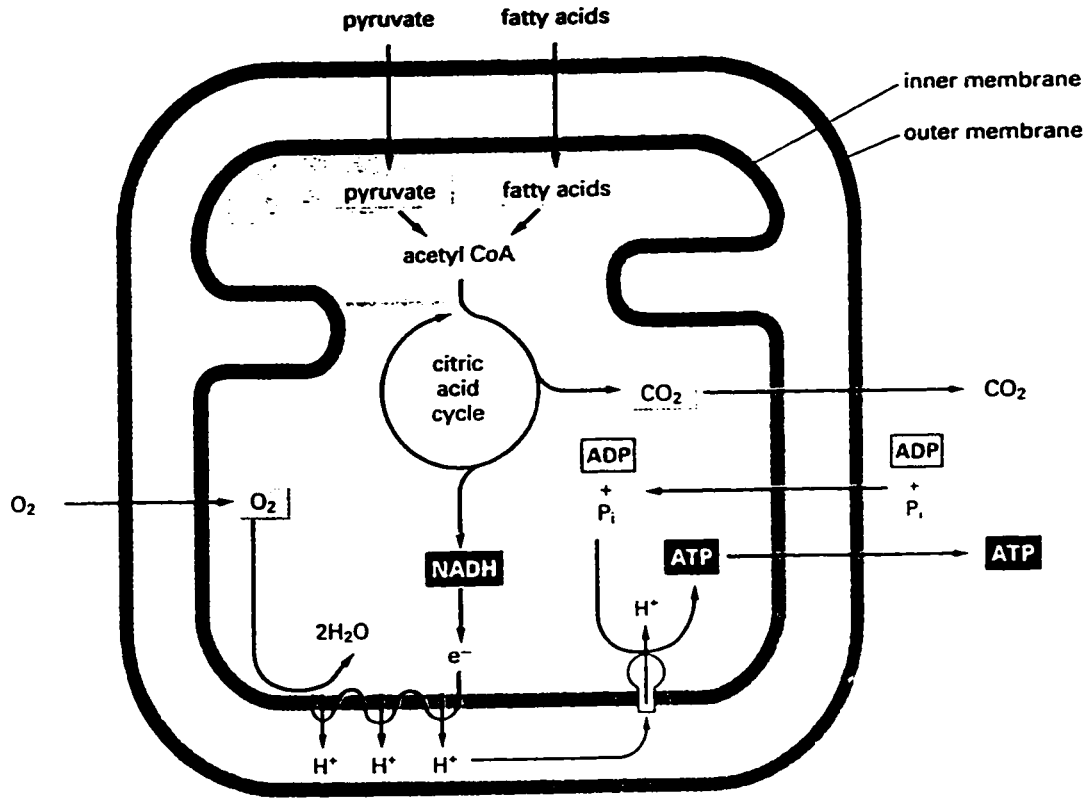

Figure 3. Schematic representation of a mitochondrion. This figure was adapted from (172).

Electrons are donated to the electron transport chain primarily from NADH and $FADH_2$, generated by the citric acid cycle (also known as tricarboxylic acid cycle or Krebs cycle) in the mitochondrial matrix. The citric acid cycle generates NADH, $FADH_2$, and CO_2 from acetyl CoA. NADH and $FADH_2$ transiently hold electrons and quickly pass these electrons to the electron transport chain enzymes. These electrons are then passed from one component to the next, releasing energy which is used to pump hydrogen ions across the inner mitochondrial membrane at three of the complexes; I, III, and IV (173-175).

Since the inner mitochondrial membrane is relatively impermeable to hydrogen ions, a hydrogen ion (H^+) concentration gradient is generated across the inner mitochondrial membrane with a low H^+ concentration in the mitochondrial matrix compared to the intermembrane space. This concentration gradient produces two components; a membrane potential, since hydrogen ions are positively charged and a pH gradient, since hydrogen ions also determine acidity (165). Together, the membrane potential and pH gradient are called the electrochemical gradient.

The electrochemical gradient, is utilized to drive the synthesis of ATP. As hydrogen ions pass through the F_0F_1ATPase, down their concentration gradient, ATP is synthesized from ADP and P_i (176-178). The electrochemical gradient is also essential to other cellular processes in addition to ATP synthesis. Transport of metabolites and ions as well as protein import and synthesis are dependent on an electrochemical gradient (179-184).

Changes in Mitochondrial Structure and Membrane Potential

The magnitude of the mitochondrial membrane potential differs depending on the cell type. The mitochondrial membrane potential in tissues, from highest to lowest, is as follows; cardiac muscle cells > skeletal muscle cells > smooth muscle cells > macrophages > hepatocytes > fibroblasts > resting neuronal cells > glial cells > keratinocytes > bladder epithelial cells and resting T and B lymphocytes (166,185,186). In addition to the magnitude of the mitochondrial

membrane potential being cell type-dependent, the relative contributions of a membrane potential and a pH gradient to the overall electrochemical gradient are cell type-specific. Significance of the cell-specific characteristics of mitochondria are poorly understood (165).

The mitochondrial structure and membrane potential also appears to vary depending on the state of an individual cell. Hackenbrock *et al.* (187,188) have observed that elevated rates of oxidative phosphorylation are associated with an increased electron density of the mitochondrial matrix and inner mitochondrial membrane. These electron-dense mitochondria are representative of mitochondria in a condensed form while less active, less electron-dense mitochondria are considered to be in an orthodox form. Ultrastructural changes in the mitochondria can occur within seconds following a stimulus of mitochondrial respiration and are reversible (188).

Several investigators have shown that the mitochondrial membrane potential is altered in exponentially growing cells compared to cells in a stationary growth phase (189), during different phases of the cell cycle (190-195), in cells induced to differentiate (166,189,195,196) and during cell migration (197). It has been proposed that the mitochondrial membrane potential is increased to augment ATP synthesis which is required to meet the energy demands of the cell (197).

Mitochondria and Cell Transformation

Changes in mitochondria including coupling of oxidative phosphorylation with glycolysis, altered calcium homeostasis, and the production of reactive oxygen species, have been associated with cell transformation (164,198-203). Interestingly, all of these characteristics can be associated with the actions of the electron transport chain. The coupling of oxidative phosphorylation and glycolysis has been investigated primarily in a rat hepatoma cell line. In this cell line, it has been observed that a high glycolytic rate is associated with a change in subcellular location of the hexokinase enzyme (204,205). Hexokinase is the initial enzyme in the glycolytic pathway where it converts glucose to glucose-6-phosphate, a process dependent on ATP hydrolysis. Normally this enzyme is located in the cytoplasm but in the rat hepatoma cell line, hexokinase is associated with the outer mitochondrial membrane. Altered localization of hexokinase is dependent on a N-terminal hydrophobic tail (206). This association between hexokinase and mitochondria provides hexokinase with preferential access to mitochondrial produced ATP and thus enhances the glycolytic capacity of a cell (204-206).

Mitochondria are also capable of accumulating calcium and thus regulate intracellular calcium concentrations. Calcium uptake is dependent on the mitochondrial membrane potential and the intramitochondrial concentration of inorganic phosphate (207-209). Since the electrochemical gradient is used to power the exchange of ATP for ADP and P_i, the loss of the membrane potential

could limit intramitochondrial inorganic phosphate levels and thus the capacity to sequester calcium (210). Regulation of cytosolic calcium by mitochondria, under normal conditions, is probably minor since the affinity for calcium and its uptake rate are significantly greater in other organelles such as the endoplasmic and sarcoplasmic reticulum (209). Under stressful conditions, where cytoplasmic calcium levels are increased due to an influx of calcium across the plasma membrane, mitochondria have the capacity to sequester large amounts of calcium, in bound and precipitated forms, with little effect on their function (211,212). Since intracellular calcium levels are important determinants of calcium-mediated signal transduction and possibly activation of endonucleases involved in programmed cell death, the magnitude of the mitochondrial membrane potential may influence these processes (200). In addition, three mitochondrial dehydrogenases, pyruvate dehydrogenase, NAD-dependent isocitric dehydrogenase and oxoglutarate dehydrogenase are regulated by matrix calcium content (207,209,213,214). These three dehydrogenases are important in the TCA cycle and may affect the production of substrates (NADH or $FADH_2$) available to the electron transport chain. Activation of these dehydrogenases by calcium may augment rates of electron transport and possibly ATP production (215,216).

Oxidative phosphorylation consumes most of the body's oxygen and is the primary generator of reactive oxygen species (ROS) (217). In normal cells, mitochondria contain their own complement of superoxide dismutase and

glutathione that are capable of scavenging most of the ROS produced (218,219). In tumour cells, elevated activity of the electron transport chain may overwhelm these defense mechanisms, resulting in cellular damage such as lipid peroxidation as well as damage to nuclear DNA and mtDNA. Since genes for the electron transport chain are encoded by both the nuclear and mitochondrial genome, damage to either of these pools of genetic information may result in impaired electron transport. ROS production can be augmented by impaired electron transport through destabilization of reduced intermediates during electron transport. Destabilization may permit attack by oxygen or electrophiles further generating reactive oxygen intermediates. ROS's have been implicated in intracellular signaling and calcium release from mitochondria (211).

A number of studies have shown that tumour cells accumulate and retain more lipophilic cations than normal cells thus suggesting that tumour cells have an elevated mitochondrial membrane potential relative to normal cells. Since cells elevate their mitochondrial membrane potential during periods of activity such as during cell migration and proliferation (189,197) it is not surprising that tumour cells maintain an elevated mitochondrial membrane potential since they are often characterized by increased rates of proliferation and migration (165). A study of over 200 cell lines/types indicated that tumour cells, including tumours derived from kidney, ovary, pancreas, lung, adrenal cortex, skin, breast, prostate, cervix, vulva, colon, liver, testis, esophagus, trachea, and tongue had elevated mitochondrial membrane potentials/Rh123 accumulation compared to

cells from normal tissue (166,167). The magnitude of difference has been estimated to be at least 60 mV (220,221). This increase in mitochondrial membrane potential is not observed in cells from leukemias, lymphomas, neuroblastomas or osteosarcomas (166,185).

Mitochondria and Drug Resistance

In addition to their involvement in cell transformation, mitochondria may also regulate drug sensitivity. Mitochondrial alterations have recently been associated with cisplatin resistance. Variants which acquired cisplatin resistance, possessed an approximate 2-fold elevation in their mitochondrial membrane potential compared to their parental line (152). Selection of revertants of the cisplatin-resistant variant that had lower mitochondrial membrane potentials restored cisplatin sensitivity (153). These revertants were similar to the cisplatin-resistant variant with respect to glutathione levels, metallothionein levels and platinum accumulation suggesting that it was the reduction in mitochondrial membrane potential that restored cisplatin sensitivity. Furthermore, it has been shown that elimination of mtDNA from cisplatin-resistant cells, and thus functional mitochondria, restores cisplatin sensitivity (222).

Mitochondrial alterations associated with cisplatin resistance, in some cell variants, suggests that (i) cisplatin sensitivity may partially depend on the mitochondrial characteristics of a cell and/or (ii) cisplatin is capable of effecting

mitochondria. These proposals are consistent with the inhibitory effects cisplatin has on renal function (10,223). It was observed that cisplatin induced mitochondrial swelling in the kidney proximal tubules in mice. The mitochondrial swelling was associated with a decrease in Rh123 accumulation, oxygen consumption and cyctochrome *c* oxidase protein level suggesting that the mitochondrial function was affected by cisplatin (224). Nephrotoxicity was thus implicated to result from disruption of mitochondrial function.

Cisplatin has also been shown to bind mtDNA and the number of cisplatin lesions may be greater in mtDNA than in nuclear DNA (225-227). Mitochondrial DNA is more susceptible to DNA-damaging agents than nuclear DNA because mtDNA (i) lacks protective histone and non-histone proteins (228), (ii) has only one origin of replication (201), and (iii) is attached to the inner mitochondrial membrane putting it in close proximity to the components that generate ROS and metabolize certain compounds (229-231). Moreover, cisplatin-induced lesions in mtDNA may not be repaired (227). Mitochondria contain several repair enzymes, uracil DNA glycosylase, AP endonuclease and mtDNA polymerase γ that are encoded in the nucleus and transported into mitochondria (232,233) but it has been reported that mitochondria cannot repair cisplatin intrastrand adducts or UV pyrimidine dimers (234). Therefore, it is possible that cisplatin lesions on nuclear DNA and/or mtDNA effects transcription of, or induces mutations in, the genes which encode the electron transport chain components. Altered electron transport chain function may, in turn, induce changes in calcium homeostasis,

production of ROS, coupling of oxidative phosphorylation and glycolysis or ATP production as previously described. Damage to mtDNA and the effects on mitochondrial function have been examined in a recent review published in *Pharmacology and Therapeutics* (170) from our laboratory.

This thesis is concerned with the further characterization of mitochondrial alterations associated with resistance to certain cancer treatments (Chapters 1, 2, and 3). An observed increase in accumulation and retention of lipophilic cations in cisplatin-resistant tumour cells prompted us to examine the efficacy of lipophilic cations either alone or in combination with cisplatin (Chapter 1). In an attempt to describe a mechanism through which mitochondrial alterations would render cells resistant to cisplatin, DNA repair was examined in cells varying in their mitochondrial characteristics (Chapter 4). In addition, a potential regulator of mitochondrial membrane potential, namely c-*fos* was examined (Chapter 5). Data suggested that both c-*fos* overexpression and elevations in mitochondrial membrane potential were associated with cisplatin resistance but the two characteristics were not interdependent. Therefore, cisplatin-resistant cells containing an elevated mitochondrial membrane potential are not more efficient at repairing cisplatin intrastrand adducts and the elevation in mitochondrial membrane potential is not associated with and overexpression of c-*fos*. However, these cells can be effectively treated with agents that disrupt mitochondrial function such as lipophilic cations.

Chapter 1 Mitochondrial alterations associated with cisplatin resistance can be targeted with lipophilic cations.

Exploitation of the mitochondrial membrane potential was initially used to localize mitochondria within living cells. Johnson *et al.* (235) showed that the lipophilic cation, Rh123, could accumulate in, and stain mitochondria in living cells. Subsequent studies demonstrated that lipophilic cations, such as Rh123, possess two properties that make them ideal for examining mitochondria; they are lipophilic which enables passive movement across the hydrophobic membranes of mitochondria and they are positively charged and thus accumulate in mitochondria in response to the negative charge inside mitochondria (220,221,236,237). The most commonly used lipophilic cation, Rh123, possesses one additional property, it is fluorescent, thereby permitting the detection of mitochondria with confocal microscopy, fluorescent microscopy or flow cytometry. Rh123 also had the distinction of being the least toxic of the lipophilic cations tested (165).

Another lipophilic cation commonly used to measure mitochondrial membrane potential is [^{3}H]TPP$^+$. This compound has also been used to show that tumour cell mitochondria accumulate more lipophilic cations than normal cells and retain lipophilic cations for extended periods of time compared to normal cells (220,238). Both of these characteristics have been suggested to reflect an increase in mitochondrial membrane potential in tumour cells

compared to normal cells. Accumulation of lipophilic cations in response to the mitochondrial membrane potential can be explained mathematically by the Nernst equation (239-241). This equation can be used to measure the magnitude of the mitochondrial membrane potential or predict the amount of lipophilic cation accumulation for a given membrane potential.

Measurement of a mitochondrial membrane potential within living cells is complicated by the presence of a plasma membrane potential which is generated by the plasma membrane Na^+/K^+-ATPase. It has been demonstrated that the plasma membrane potential can be dissipated by inhibiting the Na^+/K^+-ATPase or by incubating cells in media containing 137 mM KCl (220). Similarly, the mitochondrial membrane potential can be dissipated by azide plus oligomycin, valinomycin or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (165,220,237,242,243). Thus, Rh123 accumulation in response to either the plasma or mitochondrial membrane potential can be measured.

Similar properties that permit mitochondrial accumulation of Rh123, at low doses, contribute to Rh123's cytotoxic effects at high doses. At high doses, accumulation of the positively charged Rh123 reduces the inside negative charge within mitochondria and thus dissipates the mitochondrial membrane potential (165,244). Since the mitochondrial membrane potential is one of the components that drives ATP synthesis, Rh123 accumulation can inhibit ATP synthesized via oxidative phosphorylation (190,244,245). It has been shown that following Rh123 administration, glycolysis increased within cells prior to cell

death, presumably in an attempt to compensate for reduced capacity of mitochondria to synthesize ATP (246). In addition to its ability to inhibit oxidative phosphorylation, Rh123 has also been observed to effect mitochondrial protein synthesis (184), protein transport into mitochondria (247,248), protein kinase C activity (249), and calmodulin activity (250). Therefore, Rh123 may directly effect mitochondria or mitochondria may serve as a drug reservoir to accumulate and slowly release Rh123. Once released, Rh123 can interact with other intracellular targets.

Rh123's use as an antitumour agent was based on its selective accumulation and retention in tumour cells compared to normal cells (166). Lampidis *et al.* (167) observed that human MCF-7 breast cancer cells and human CRL pancreatic carcinoma cells were more sensitive to Rh123 than normal African green monkey kidney epithelia (CV-1) cells or normal marsupial kidney epithelial (PtK-1) cells. Animal epithelial cells were used since no "normal" human epithelial cell line was available at the time (168). Furthermore, this study suggested that Rh123 inhibited tumour cell growth by inhibiting oxidative phosphorylation. In MCF-7 cells, it was observed that the combination of Rh123 and 2-deoxyglucose, an inhibitor of glycolysis, resulted in 100 % tumour cell kill. This observation is consistent with the idea the Rh123 inhibits mitochondrial ATP production and combinations of Rh123 with an inhibitor of glycolytic ATP production, reduces the ATP levels below that which are necessary for cell survival. Subsequent studies have confirmed that a number of

tumour cells have greater accumulation and retention of lipophilic cations compared to normal cells *in vitro* (220,238,251) and that this enhanced accumulation and retention resulted in increased sensitivity to lipophilic cations (238,252). Rh123's antitumour activity has also been demonstrated *in vivo* (253-255). In mice bearing either Ehrlich ascites tumour cells or MB49 bladder carcinoma cells, treatment with Rh123 prolonged survival by 260% and 180%, respectively.

However, high Rh123 concentrations are required to kill tumour cells, and thus another lipophilic cation, dequalinium chloride (Deca), has been examined as an antitumour agent. Like Rh123, Deca accumulates in mitochondria (168) where it can inhibit oxidative phosphorylation (165,244). Deca is unique from Rh123 in that it has been used as an over-the-counter antimicrobial agent in topical ointments, throat lozenges and mouthwashes, for the past 30 years (168). Furthermore, Deca is a divalent cation and thus more potent than the monovalent, Rh123 (168). In addition to its effects on mitochondria, Deca has also been observed to intercalate into DNA (256), and inhibit the activities of calmodulin and protein kinase C (250,257-259).

Deca was shown to be 125-fold more toxic to the human breast carcinoma cell line, MCF-7, compared to normal epithelial (CV-1) cells as determined at an IC_{50} by a colony forming assay following a 3 hour treatment (168). It is interesting to note that continuous exposure of cells to Deca reduced its selectivity for MCF-7 cells to approximately 3-fold. These results suggest that

Deca's selectivity for tumour cells may be a consequence of increased retention of the lipophilic cation. After a 3 hour Deca exposure, cells are grown in drug-free media and thus if Deca is not retained by the cells most of it will diffuse out of cells into the surrounding media. In contrast, with continuous Deca exposure, retention is not as important since diffusion of Deca out of the cell is less probable since the media still contains high concentrations of Deca.

Growth, migration and invasion of melanoma cells, *in vitro,* have also been observed to be inhibited by Deca and inhibition of these cellular processes were association with changes in mitochondrial ultrastructure (260). *In vivo* Deca has been shown to possess antitumour activity against MB49, a mouse bladder carcinoma and CX-1, a human colon carcinoma cell line (168). In this study, Deca prolonged mouse survival by 190 % which was greater than several other therapeutic agents examined which included cisplatin, methotrexate, and 5-fluorouracil.

Although effective on their own, lipophilic cations may represent a class of agents that can sensitize tumour cells to existing chemotherapeutic agents. Few studies have examined the efficacy of "antimitochondrial" agents such as lipophilic cations, in combination with existing chemotherapeutic agents. To my knowledge only one study, in addition to the one we published in *The International Journal of Oncology* (261), has investigated lipophilic cations in combination with an existing chemotherapeutic agent. Both studies examined the interaction between lipophilic cations and cisplatin (251,261). It was

reasoned that an effective combination of chemotherapeutic agents should disrupt two different essential intracellular functions. Therefore, we examined the interaction between cisplatin which is believed to kill cells through damaging DNA and lipophilic cations which apparently acts primarily through the disruption of mitochondrial function.

Using a Taper liver tumour (TLT-H) cell line it was observed that Deca was more potent than Rh123 and resulted in greater tumour cell kill than cisplatin (261). Furthermore, both Rh123 and Deca selectively killed TLT-H cells compared to normal human fibroblasts. Examination of the interaction between cisplatin and lipophilic cations showed that both Rh123 and Deca induced synergistic tumour cell kill when combined with cisplatin. These data suggest, at least *in vitro*, that combinations of lipophilic cations and cisplatin are superior to either agent alone.

Combinations of lipophilic cations and cisplatin have also proved effective *in vivo*. Christman *et al.* (251) demonstrated that the addition of Deca to the cisplatin treatment of nude mice, injected intraperitonealy with the human ovarian carcinoma cell line OVCAR 2774P, increased survival beyond either agent alone. Since cisplatin is one of the primary chemotherapeutic agents used in the treatment of ovarian cancer it is interesting to note that median survival for mice treated with cisplatin alone was approximately 50 days while the median survival for the combination of cisplatin and Deca was approximately 200 days. Since cisplatin's efficacy is limited by organ toxicity (8-11) combining

it with lipophilic cations might reduce the concentration of cisplatin required without compromising tumour cell kill.

In light of these encouraging results, surprisingly little has been done to extend this work. The following manuscript further characterizes the mitochondria alterations associated with cisplatin resistance in a human ovarian carcinoma model system and investigates whether an increase in mitochondrial membrane potential can be exploited to selectively kill cisplatin-resistant tumour cells. Furthermore, the lipophilic cation, Deca was used to examine whether inhibition of mitochondrial function could sensitize tumour cells and/or cisplatin-resistant tumour cells, to cisplatin.

All the data in this manuscript was obtained from experiments which were conducted by R.A. Moorehead.

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May 21, 1997

Dr. Kenneth D. Tew
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1. Sensitisation of Cisplatin-Resistant Cells Using Dequalinium Chloride. Roger, A. Moorehead and Gurmit Singh. *Cellular Pharmacology* 2:311-317, 1995.

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Cellular Pharmacology (1995), 2, 311–317

Sensitisation of cisplatin-resistant cells using dequalinium chloride

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Summary

The cisplatin-resistant variant (C13*) required 8.5 ± 1.4-fold more time to efflux 50% of the Rh123 from their mitochondria compared with the parental human ovarian carcinoma cell line (2008). The mitochondria of the RH4 cells (Rh123-resistant variants of the C13* cells) accumulate Rh123 to a similar extent as the 2008 cells but they require 2.6 ±0.9-fold more time to remove 50% of the Rh123 relative to the 2008 cells. This decreased efflux of lipophilic cations appears to reflect the 7.1 ± 1.7-fold and 4.4 ± 1.3-fold increase in sensitivity to a 3 h exposure to dequalinium chloride (Deca) in the C13* and RH4 cells respectively, relative to the 2008 cells. The mitochondrial Rh123 accumulation in the 2008 cells increased significantly (maximum of 2.5 ± 0.5-fold) following a 1 h cisplatin exposure whereas neither the C13* nor RH4 cells had significant increases in their mitochondrial Rh123 accumulation following cisplatin treatment. In an attempt to exploit the mitochondrial alterations of the C13* cells, cisplatin treatment was combined with the lipophilic cation Deca. At low cisplatin concentrations the interaction of cisplatin and Deca was synergistic at the 50% effect level in both the 2008 and C13* cells. Furthermore, using a Deca concentration of 0.625 μM the IC_{50} for cisplatin was reduced approximately 93-fold in the C13* cells whereas this concentration of Deca only reduced the IC_{50} approximately three-fold in the 2008 cells. Thus the cisplatin-resistant variant is sensitised to cisplatin by the addition of the lipophilic cation, Deca, during drug exposure.

Keywords: *cisplatin; mitochondrial membrane potential; lipophilic cations*

Introduction

Various mechanisms have been reported to contribute to cisplatin (*cis*-diamminedichloroplatinum(II)) resistance including altered drug transport, increased glutathione or metallothionein levels, altered DNA adduct formation and enhanced DNA repair (reviewed in refs 1–5). In the human ovarian carcinoma cell line 2008 and its cisplatin-resistant variant C13* some of these differences exist and could contribute to cisplatin resistance.[6–8] In addition to the classical mechanisms of cisplatin resistance, the C13* cells have elevated mitochondrial and plasma membrane potentials compared with 2008 cells.[6] To further investigate the contribution of the altered membrane potentials to cisplatin resistance, Zinkewich-Péotti and Andrews[7] selected C13* cells for resistance to rhodamine 123 (Rh123) and generated the variant RH4. Rh123 is a lipophilic cation that accumulates in response to and dissipates the plasma and mitochondrial membrane potentials.[9,10] The RH4 cells were similar to the 2008 cells in both their membrane potentials and their cisplatin sensitivity. Interestingly, while the RH4 cells are more sensitive to cisplatin than the C13* cells, the P-glycoprotein, glutathione, metallothionein content and platinum accumulation in the RH4 and C13* cells is similar.[7] Furthermore, the ability of RH4 cells to repair cisplatin intrastrand adducts is similar to the C13* cells.[11] Thus, the apparent difference between the C13* and RH4 cells is the altered membrane potentials.

The mitochondrial membrane potential and the pH gradient comprise the mitochondrial electrochemical gradient which is the driving force for ATP production in the mitochondria.[12] The mitochondria synthesise ATP from ADP, P_i, and energy released from hydrogen ions moving down their electrochemical gradient through the F_1F_O ATPase. Therefore, the mitochondrial membrane potential is a critical component in the synthesis of ATP. Certain agents, such as the lipophilic cations Rh123 and Deca, can accumulate in the mitochondria in response to the mitochondrial membrane potential and dissipate this gradient.[9,10] Rh123 has been shown to inhibit mitochondrial ATP production and initially stimulate glycolysis before eventually killing the cell.[13] As lipophilic cations target a critical site within the cell, other than the DNA, they have been used *in vitro*[14] and *in vivo*[15] to enhance cisplatin's efficacy.

We have also examined the radiation-induced fibrosarcoma-1 (RIF-1) cell line and its photodynamic therapy-resistant variant, RIF-8A. The resistant variants have morphological and functional mitochondrial alterations compared with the parental cells.[16,17] Furthermore, the RIF-8A cells are cross-resistant to cisplatin relative to the RIF-1 cells.[17] Therefore, mitochondrial changes potentially contribute to cisplatin resistance.

In this paper we demonstrate using two techniques that the cisplatin-resistant C13* cells have elevated membrane potentials compared with the 2008 and RH4 cells and that these altered membrane potentials can be exploited using the lipophilic cation Deca. We have further characterised the mitochondrial membrane potential alterations by examining the mitochondrial efflux of Rh123 and the effect of cisplatin on the mitochondrial membrane potential. We also investigated the possibility of increasing cisplatin's efficacy by combining it with Deca.

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Results

The accumulation of tetraphenylphosphorium bromide [^{3}H]TPP$^+$ (1 h) due to the plasma membrane potential, mitochondrial membrane potential or both the plasma and mitochondrial membrane potentials combined for 2008, C13* and RH4 cells is shown in Figure 1. We have shown that the C13* cells had approximately a 2.1 ± 0.07-fold increase in the whole cell accumulation of TPP$^+$ and a 2.0 ± 0.07-fold increase in the mitochondrial accumulation of TPP$^+$ compared with the 2008 cells and these data closely resemble those reported by Andrews and Albright.[6] The main difference in the two protocols was that Andrews and Albright[6] used RPMI media in which the NaCl had been replaced by equimolar KCl for selective depolarisation of the plasma membrane potential while we simply supplemented normal RPMI with 137 mM KCl. Andrews and Albright[6] found that the mitochondrial TTP$^+$ accumulation of the 2008 and C13* cells accounted for 57% and 53% of the total TPP$^+$ accumulation, respectively. Similarly, we found that the mitochondrial TPP$^+$ accumulation accounted for 56% and 53% of the total TPP$^+$ accumulation in 2008 and C13 cells respectively. The similarity in the results suggest that simply supplementing media with 137 mM KCl is sufficient to depolarise the plasma membrane potential.

To limit the use of radioactivity we developed a method using Rh123 to investigate plasma and mitochondrial membrane potentials. A time course for Rh123 accumulation was performed to determine when Rh123 accumulation reach a maximum in the cells. It was observed that Rh123 accumulates slower than TPP$^+$ reaching a maximum in the mitochondria after approximately 2 h. The accumulation of Rh123 (2 h) in response to the plasma membrane potential, mitochondrial membrane potential or both the plasma and mitochondrial membrane potentials combined for 2008, C13*, and RH4 cells is shown in Figure 2. The whole cell accumulation and the mitochondrial Rh123 accumulation was approximately two-fold higher in the C13* cells relative to the 2008 or RH4 cells. Therefore the data using Rh123 accumulation closely resemble the data using [^{3}H]TPP$^+$ accumulation.

In addition to the accumulation of Rh123, the differences in the retention of Rh123 in the three cell types was also examined. Figure 3 shows the mitochondrial retention of Rh123 after a 3 h exposure to 100 µM of Rh123. The amount of Rh123 per unit DNA at each time point is expressed relative to the amount of Rh123 per unit DNA immediately after the 3 h Rh123 incubation. The RH4 and C13* cells required 2.6 ± 0.9-fold and 8.5 ± 1.4-fold, respectively, more time than the 2008 cells to remove 50% of the Rh123 from their mitochondria.

The effect of a 1 h exposure to various concentrations of cisplatin on the mitochondrial Rh123 accumulation in 2008, C13* and RH4 cells is shown in Table 1. The cisplatin concentrations used represent an IC_{50} and an IC_{90} (as determined 5 days after a 1 h cisplatin concentration using the DNA fluorochrome method) and an equal cisplatin dose of 50 µM for each cell type. These data indicate that there is a significant increase in the mitochondrial Rh123 accumulation at the two highest cisplatin doses in the 2008 cells after 48 h whereas none of the cisplatin concentrations at any of the time points significantly increased the mitochondrial Rh123 accumulation of either C13* or RH4 cells. The amount of Rh123 fluorescence per unit DNA of cisplatin-treated cells at each time point was expressed relative to the amount of Rh123 fluorescence per unit DNA of cells that were not treated with cisplatin.

We investigated whether the increased mitochondrial

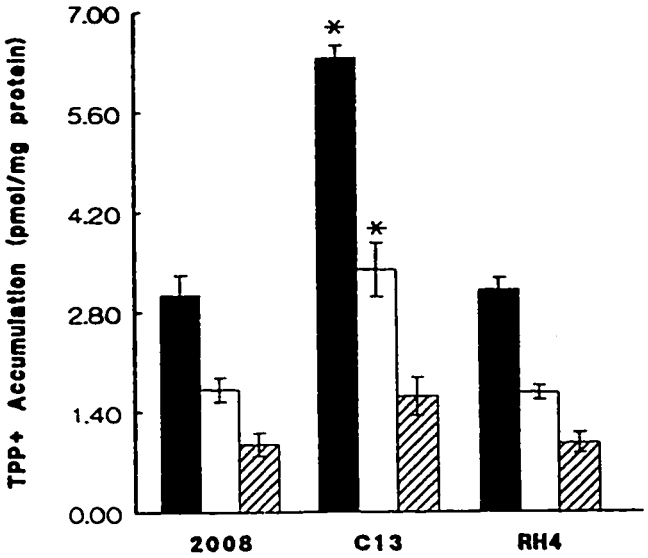

Figure 1 [^{3}H]TPP$^+$ accumulation after a 1 h incubation in 2008, C13* and RH4 cells. [^{3}H]TPP$^+$ accumulation was determined in normal RPMI media (■), RPMI media + 137 mM KCl (□) and RPMI medium + 5 µM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (▨) in order to determine whole cell, mitochondrial and plasma membrane [^{3}H]TPP$^+$ accumulation, respectively. Columns, mean (n = 3); *bars*, s.e.m. * $P < 0.05$ for C13* *versus* 2008 and RH4 as determined by a Tukey test

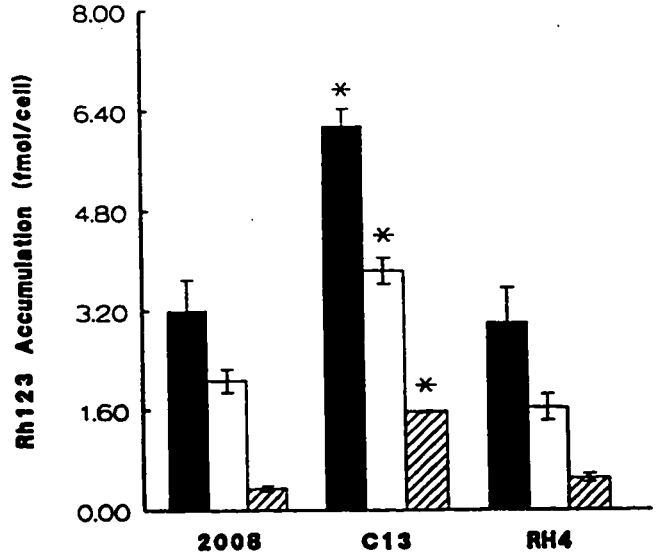

Figure 2 Rh123 accumulation after a 2 h incubation in 2008, C13* and RH4 cells. Rh123 accumulation was determined in normal RPMI media (■), RPMI media + 137 mM KCl (□) and RPMI medium + 5 µM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (▨) in order to determine whole cell, mitochondrial and plasma membrane [^{3}H]TPP$^+$ accumulation, respectively. The amount of Rh123 was determined using a fluorescent plate scanner. Columns, mean (n = 3); *bars*, s.e.m. * $P < 0.05$ for C13* *versus* 2008 and RH4 as determined by a Tukey test

membrane potential in the C13* cells could be exploited using Deca. Deca is a lipophilic cation like Rh123 and thus it accumulates in the mitochondria in response to the mitochondrial membrane potential and it dissipates this membrane potential. Deca was used as the cytotoxic agent instead of Rh123 because Deca is a divalent cation and thus requires lower concentrations to dissipate the mitochondrial membrane potential. The cytotoxicity of a 3 h Deca exposure in 2008, C13*, and RH4 cells is shown in Figure 4. The RH4 and C13* cells were approximately 4.4 ± 1.3-fold and 7.1 ± 1.7-fold, respectively, more sensitive to Deca than the 2008 cells.

In addition to the toxicity of Deca alone we investigated the interaction of Deca with cisplatin to determine if this combination resulted in synergistic cell kill in any of the cell lines. The combination indexes for the interaction of cisplatin and Deca at the 50% effect level for 2008, C13* and RH4 are shown in Table 2. More importantly, using a dose of 0.625 μM Deca reduced the amount of cisplatin required to kill 50% of the C13* cells by approximately 93-fold (cisplatin IC_{50} without Deca was 25.3 μM while with Deca 0.625 μM the cisplatin IC_{50} was 0.27 μM). This concentration of Deca reduced the cisplatin IC_{50} in the 2008 cells only approximately three-fold and a Deca concentration of 1.25 μM only reduced the cisplatin IC_{50} in the 2008 cells approximately four-fold.

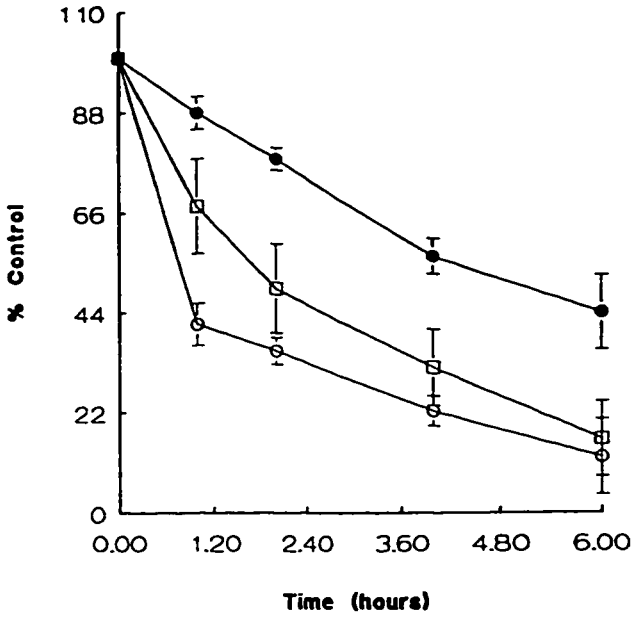

Figure 3 Rh123 efflux from the mitochondria of 2008 (○), C13* (●), and RH4 (□) cells. The cells were exposed to 100 μM of Rh123 for 3 h in RPMI media + 137 mM KCl followed by the removal of the Rh123-containing media and replacement with Rh123-free RPMI media + 137 mM KCl for the appropriate time interval. The amount of Rh123 was determined using a fluorescent plate scanner. Mean ($n = 4$); *bars*, s.e.m.

Table 1 Mitochondrial Rh123 accumulation following a 1 h exposure to cisplatin

Time after a 1 h cisplatin exposure	Mitochondrial Rh123 accumulation (% control) 2008	C13*	RH4
8 h			
IC_{50}	107.2 ± 2 3[b]	96.0 ± 3.2	95.4 ± 5.6
IC_{90}	111.4 ± 1.9	104.2 ± 4.1	94.6 ± 4.1
50 μM	109.9 ± 2.6	102.5 ± 4.0	94.7 ± 4.9
24 h			
IC_{50}	105.4 ± 2.4	102.7 ± 4.2	104.2 ± 3.3
IC_{90}	119.3 ± 4.8	112.4 ± 7.5	103.6 ± 6.4
50 μM	137.7 ± 10.3*	107.2 ± 7.8	103.5 ± 5 9
48 h			
IC_{50}	134.5 ± 14.4	113.8 ± 4.1	114.4 ± 9.4
IC_{90}	204.4 ± 14.4*	133.2 ± 16.1	134.1 ± 26.5
50 μM	259.2 ± 47.5*	124.6 ± 10.9	139.0 ± 23.2

[a]The cisplatin concentrations which represent the IC_{50} and IC_{90} were 5 and 21 μM respectively for 2008 and RH4 cells and 21 and 100 μM, respectively for C13* cells
[b]Values are expressed as a percentage of a control population of cells which received no drug treatment ($n \geq 3$)
* $P < 0.05$ as determined by a Dunnett's test

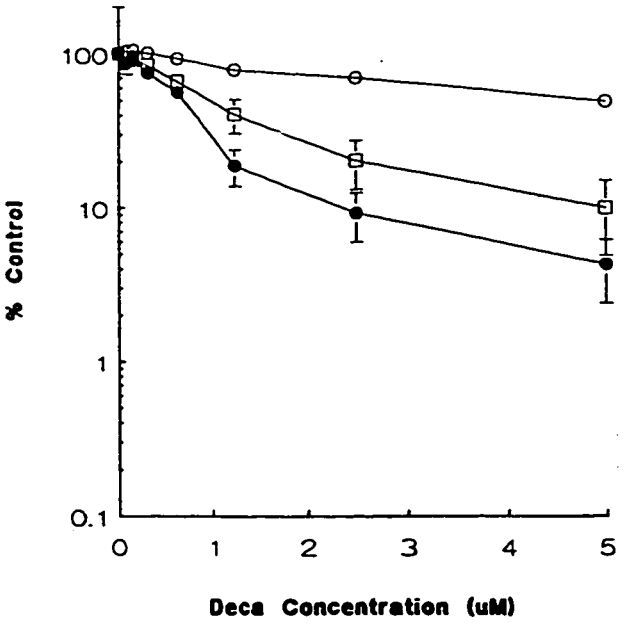

Figure 4 Effect of Deca on 2008 (○), C13* (●) and RH4 (□) survival. Cytotoxicity was assessed by a DNA fluorochrome assay after a 3 h exposure to Deca. Values are expressed as percentage of control ± s.e.m. (*bars*); ($n = 4$)

Table 2 Combination indexes for the interaction of cisplatin and deca at the 50% effect level for different cisplatin concentrations

	Cisplatin concentration Dose 1	Dose 2	Dose 3	Dose 4[b]
2008	0.51 ± 0.09*	0.52 ± 0.09*	0.81 ± 0.18	1.14 ± 0.20
C13*	0.66 ± 0.10*	0.67 ± 0.06*	0.91 ± 0.08	1.40 ± 0.11
RH4	0.71 ± 0.17	0.96 ± 0.20	1.92 ± 0.45	3.44 ± 0.83*

[a]Combination indexes were calculated using the formula for mutually non-exclusive drugs
[b]Dose 1, dose 2, dose 3 and dose 4 represent the cisplatin concentrations of 0.39, 0.78, 3.125 and 6.25 μM, respectively, in 2008 and RH4 cells and 1.56, 6.25, 12.5 and 25 μM, respectively, in C13* cells
* $P < 0.05$ as determined by a Dunnett's test

Discussion

The mechanism(s) of cisplatin resistance in C13* cells are multifactorial.[6] Although there appears to be increased levels of glutathione and metallothioneins as well as decreased cisplatin accumulation in the C13* cells relative to the 2008 cells, these differences do not exist between the C13* cells and the cisplatin-sensitive RH4 cells.[7] In addition, there is no apparent difference in the repair of cisplatin intrastrand adducts in any of the three cell types.[8,11] Therefore, it appears that the mitochondrial or plasma membrane potential changes in the C13* cells are an important component that contribute to its cisplatin-resistant phenotype and thus we focused on the mitochondrial alterations in these cells.

Using the accumulation of [^{3}H]TPP$^+$ we have confirmed that the C13* cells have increased whole cell accumulation of [^{3}H]TPP$^+$, as well as elevated mitochondrial and plasma membrane accumulation of [^{3}H]TPP$^+$ relative to either the 2008 or RH4 cells. Moreover, we have shown that the addition of 137 mM KCl to normal media provides results similar to those using media in which the NaCl has been replaced with an equimolar amount of KCl. Therefore, customised media is not required to selectively dissipate the plasma membrane potential. Furthermore, it has been shown that Rh123 accumulation can replace [^{3}H]TPP$^+$ accumulation to investigate membrane potentials and thus the use of radioisotopes can be avoided.

The increase in mitochondrial [^{3}H]TTP$^+$ or Rh123 accumulation in the C13* cells could result from either an increase in either mitochondrial number or size. This is unlikely as (1) Andrews and Albright[6] found no qualitative differences in the number of mitochondria of the 2008 and C13* cells at the electron microscopy level and using 10-n-Nonyl Acridine Orange, a indicator of mitochondrial mass,[18] we found that the C13* cells did not have an increase in mitochondrial mass compared with either the 2008 or RH4 cells (data not shown). Therefore, the increase in mitochondrial [^{3}H]TPP$^+$ or Rh123 accumulation by the C13* cells appears to reflect an increase in mitochondrial membrane potential relative to the 2008 and RH4 cells rather than an increase in mitochondrial number or size.

In addition to Rh123 accumulation we examined its efflux in the 2008, C13* and RH4 cells. The mitochondria of the C13* cells retained Rh123 for an extended period of time compared with the mitochondria of the 2008 cells while the mitochondrial Rh123 retention in the RH4 cells is intermediate with respect to those of the 2008 and C13* cells. These results suggest that Rh123 efflux is influenced by factors other than the membrane potential (that is, the mitochondria of the 2008 and RH4 cells accumulate Rh123 to a similar extent but differ in its retention) and further investigation is required to identify these factors.

Regardless of the factors influencing Rh123 retention, the duration of Rh123 retention appears to predict the toxicity of another lipophilic cation, Deca, in these cells. C13* cells which retain Rh123 for prolonged periods of time compared with 2008 or RH4 cells are the most sensitive to Deca's toxicity; 2008 cells which retain Rh123 for the least amount of time are also the least sensitive to Deca. Although Deca has also been reported to affect protein kinase C,[19,20] calmodulin[21,22] and DNA,[20] and thus a target other than the mitochondria may contribute to the differences in the sensitivity of the three cell types to Deca, the Rh123 retention data appears to provide an indicator of Deca sensitivity in these cell types. Therefore, Rh123 retention may provide a way to predict the cells that may be candidates for Deca sensitisation.

Some of the early experiments demonstrating that Rh123 was preferentially accumulated and retained in carcinoma cells compared with normal cells used the normal monkey kidney epithelial cell line CV-1.[23,24] The CV-1 cell line has recently been shown to contain high levels of P-glycoprotein,[25] a pump for which Rh123 is a substrate.[26–28] Therefore, the low accumulation and retention of Rh123 in the CV-1 cells was a result of a high P-glycoprotein level which may not be indicative of a 'normal' control cell line. It is unlikely that the differences in Rh123 accumulation and retention in the 2008, C13* and RH4 cells are a result of altered P-glycoprotein contents in the cells as doxorubicin (another chemotherapeutic agent that is a classical substrate for P-glycoprotein) cytotoxicity is similar in all three cell types.[7]

We also determined whether cisplatin treatment induced any membrane effects on the mitochondria by examining mitochondrial Rh123 accumulation following cisplatin treatment. Only the 2008 cells significantly increased their mitochondrial Rh123 accumulation following cisplatin treatment and this increase in mitochondrial Rh123 accumulation did not occur until 24–48 h after the 1 h cisplatin exposure. A possible explanation for the increase in mitochondrial Rh123 accumulation following cisplatin treatment in the 2008 cells but not the C13* or RH4 cells is that the 2008 cells attempt to maximise their mitochondrial membrane potential following cisplatin treatment and this characteristic provides protection against cisplatin cytotoxicity. The C13* cells, on the other hand, may have maximised their mitochondrial membrane potential as a result of the cisplatin selection protocol that was used to generate them. Support for this hypothesis is derived from the experiment of Andrews and Albright[6] in which they hyperpolarised the mitochondrial membrane potential in the 2008 and C13* cells using nigericin. This experiment suggested that the mitochondrial membrane potential contributed approximately 33% of the mitochondrial electrochemical gradient in the 2008 cells and 83% in the C13* cells. Because the RH4 cells were generated from the C13* cells it is possible that the mitochondrial membrane potential is the primary contributor to the mitochondrial electrochemical gradient in the RH4 cells but the electrochemical gradient has been reduced. Hyperpolarisation of the RH4 mitochondria has not been investigated.

Alternatively, the 2008 cells with inherently lower mitochondrial membrane potentials are killed early while those cells with higher mitochondrial membrane potentials have a survival advantage following cisplatin treatment. This would result in the measurement of the cells that have survived cisplatin treatment (that is, those cells with elevated mitochondrial membrane potentials). As the C13* and RH4 cells may have maximised their mitochondrial membrane potentials this selection process would not occur and thus no increase in mitochondrial Rh123 accumulation would be apparent. Independent of the explanation for the increase in mitochondrial membrane potential in the 2008 cells following cisplatin treatment, the results suggest that

increasing the mitochondrial membrane potential provides some sort of survival advantage when challenged with cisplatin in these cells. Shinomiya *et al*[29] have observed a similar response to cisplatin treatment (increased Rh123 accumulation) in EL-4 lymphoma cells. They propose that the increase in Rh123 accumulation arises due to an increase in cell volume as a result of a blockade in the cell cycle at the G_2 + M phase. The cell cycle profiles following cisplatin treatment were not investigated in the 2008, C13* and RH4 cells and thus a G_2 + M blockade remains a possible explanation for the increase in Rh123 accumulation in our cells.

One of the proposed mechanisms for an increased mitochondrial membrane potential providing protection against cisplatin toxicity is that the mitochondria serve as a sink and sequester cisplatin and prevent it from interacting with critical targets such as DNA.[6] Once cisplatin enters the cell the low chloride environment favours the conversion of cisplatin to a positively charged aquated species.[30–32] Therefore, it is conceivable that an increase in mitochondrial membrane potential (increase in the negative charge inside the mitochondria) could more efficiently sequester the charged cisplatin species. Alternatively, the increased mitochondrial membrane potential could increase the ability of the cell to produce ATP that could be used in detoxification or removal of cisplatin.

To investigate if the mitochondrial alterations in the C13* cells could be exploited, we examined the interaction of Deca and cisplatin. In each of the cell types the trend in the interaction of cisplatin and Deca moves from synergy at low cisplatin concentrations to antagonism for higher cisplatin concentrations. Deca can be used to greatly reduce the amount of cisplatin required to kill the C13* cells and thus reverse cisplatin resistance. Using a concentration of Deca of 0.625 μM, approximately 93-fold less cisplatin is required to kill 50% of the cells. Therefore, the use of Deca, alone or in combination with cisplatin, can be used to effectively kill cisplatin-resistant cells in our study. Other timing protocols using cisplatin and Deca may prove more effective but alternative protocols were not investigated in this study.

In conclusion, both [^{3}H]TPP$^+$ and Rh123 accumulation indicates that the C13* cells have elevated mitochondrial and plasma membrane potentials compared with either the 2008 or RH4 cells. The mitochondria of the C13* cells also retain lipophilic cations like Rh123 for extended periods of time compared with those of the 2008 and RH4 cells which renders the C13* cells hypersensitive to other lipophilic cations such as Deca. The increase in mitochondrial membrane potential in the 2008 cells following cisplatin treatment suggests that some cell types respond to cisplatin by altering their mitochondrial activity. Finally, Deca may be used alone or in combination with cisplatin to effectively treat cisplatin-resistant tumours that have alterations in their mitochondrial membrane potential and these mitochondrial alterations can be identified using simple Rh123 assays.

Materials and methods

Materials

cis-Diamminedichloroplatinum(II), rhodamine 123, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and dequalinium chloride were obtained from Sigma Chemical (St. Louis, MO). H33258 was obtained from Calbiochem (La Jolla, CA). Tetraphenylphosphonium bromide, [Phenyl-^{3}H]- was obtained from DuPont NEN Research Products (Mississauga, Ontario, Canada). The Micro BCA protein assay reagent kit and the ACS scintillation fluid were obtained from Pierce (Rockford, IL) and Amersham Canada (Oakville, Ontario, Canada), respectively.

Cell lines and culture conditions

The human ovarian carcinoma cell line 2008 its cisplatin-resistant variant, C13*, and the Rh123-resistant variant selected from C13* cells, RH4, were generously provided by Dr Paul Andrews, Georgetown University, Rockville, MD. The cells were grown in RPMI media supplemented with 5% foetal bovine serum, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B and maintained at 37°C and 5% CO_2 in a humidified incubator.

Membrane potential

Membrane potentials were determined using either [^{3}H]TPP$^+$ or Rh123. A modification of the method of Andrews and Albright[6] was used to examine membrane potential using [^{3}H]TPP$^+$. Briefly, 2008, C13* and RH4 cells in log-phase growth were seeded overnight in 6-well plates at a density of 5×10^5 cells/well. The media was removed and replaced with normal RPMI media, RPMI media supplemented with 137 mM KCl (for selective depolarisation of the plasma membrane) or RPMI media supplemented with 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (for selective depolarisation of the mitochondrial membrane potential) each of which contained 5 μM [^{3}H]TPP$^+$ (0.2 μCi ml^{-1}). Following the appropriate incubation time at 37°C, the plates were washed four times in ice-cold phosphate-buffered saline. The cells were then digested overnight in 1 N NaOH and an aliquot was mixed with 7 ml of acidified scintillation fluid (ACS scintillation fluid acidified with 12 ml of glacial acetic acid per litre of ACS). The amount of radioactivity was determined on a beta counter (Beckman Instruments, Mississauga, Ontario, Canada). The radioactivity of each sample was normalised per milligram of cellular protein as determined using Micro BCA spectrophotometric protein assay.[33]

For determination of membrane potentials using Rh123, cells in log-phase growth were seeded overnight in 24-well plates at a density of 1.5×10^5 cells/well. The media was removed and replaced with normal RPMI media, RPMI media supplemented with 137 mM KCl or RPMI media supplemented with 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone each of which contained 50 μM of Rh123. Following Rh123 removal at the appropriate times, the wells were washed twice with phosphate-buffered saline, and then deionised water was added (300 μl per well). The Rh123 fluorescence and DNA content of each well was determined as previously described.[17]

Rh123 efflux

Cells in log-phase growth were seeded overnight in 24-well plates at a density of 1.5×10^5 cells/well. The cells were then incubated with 100 μM of Rh123 for 3 hours at 37°C in either normal RPMI media or RPMI media supplemented with 137 mM KCl. The Rh123-containing media was then removed and Rh123-free media, either normal RPMI or RPMI + 137 mM KCl was added. At the appropri-

ate times the media was removed, the cells were washed twice with phosphate-buffered saline and 300 µl of deionised water was added per well. The Rh123 fluorescence and DNA content of each well was determined as previously described.[17]

Cisplatin-induced Rh123 accumulation

Cells in log-phase growth were seeded overnight in 24-well plates at a density such that approximately 1.5×10^5 cells/well were present at the appropriate time interval (used a doubling time of 24 h to determine initial seeding density). The cells were then exposed to various concentrations of cisplatin for 1 h followed by the replacement of drug-containing media with drug-free media. At the appropriate time point, the drug free media was removed and cells were incubated with media supplemented with 137 mM KCl and 50 µM of Rh123 for 2 h at 37°C. After the 2 h incubation the cells were washed twice with phosphate-buffered saline and 300 µl of deionised water was added to each well. The Rh123 fluorescence and DNA content of each well was determined as previously described.[17]

Survival assays

Cells in log-phase growth were seeded overnight in 24-well plates at a density of 5000, 2008 cells/well or 2000, C13* and RH4 cells/well. The cells were exposed to cisplatin for 1 h or Deca for 3 h after which the drug-containing media was removed and the cells were incubated in drug-free media for 5 days at 37°C. At this time the media was removed, the cells were washed once in phosphate-buffered saline, and 300 µl of deionised water was added to each well. The cells were subjected to one freeze-thaw cycle (–80°C) to lyse the cells and the amount of DNA in each well (used to indicate cell survival) was determined by the addition of 100 µl of the DNA fluorochrome, H33258 (40 µg ml^{-1} in TNE buffer; 10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4).[34] The plates were read on a Cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ontario, Canada) using excitation/emission wavelengths of 360/460 nm.

The treatment protocol used to investigate the interaction of cisplatin and Deca was Deca for 2 h, cisplatin and Deca for 1 h followed by 5 days in drug-free media. Cell growth was assessed on a Cytofluor 2350 fluorescent plate scanner using the H33258 DNA fluorochrome method as described above. The nature of the interaction of cisplatin and Deca was assessed using the combination index formula for mutually non-exclusive drugs at the 50% effect level.[35]

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Discussion

Cells maintain a plasma membrane potential of approximately 60 mV and a mitochondrial membrane potential of approximately 180 mV (165). Rh123 accumulates in cells as a result of the combined plasma and mitochondrial membrane potentials. In order to measure mitochondrial Rh123 accumulation, the plasma membrane potential must be dissipated. Plasma membrane potentials are generated primarily by the actions of the Na^+/K^+-ATPase which pumps three sodium ions out of the cells and two potassium ions into the cell (262). The net effect of this pump is the creation of a negative charge inside of cells compared to the extracellular medium. The actions of the Na^+/K^+-ATPase cause cells to contain high levels of potassium and low levels of sodium relative to the extracellular medium. Potassium ion concentration within cells is approximately 137 mM while media normally contains approximately 5 mM of potassium (165). Therefore, one way of dissipating the plasma membrane potential is to incubate cells in media in which the NaCl has been replaced by an equimolar KCl concentration. This media dissipates the potassium ion concentration gradient across the plasma membrane and thus the plasma membrane potential (152).

Rather than replacing NaCl with an equimolar concentration of KCl in the medium we examined whether supplementing normal media with 137 mM KCl was capable of dissipating the plasma membrane potential. After obtaining the 2008, C13*, and RH4 cells from Dr. P. Andrews, $[^3H]TPP^+$ accumulation was

investigated in these cells using the protocol provided by Dr. Andrews except normal media was supplemented with 137 mM KCl rather than altering sodium and potassium concentrations of the media. When our results were compared to those previously published by Andrews and Albright (152) it was found that supplementing media with 137 mM KCl provided similar reductions in the plasma membrane potential as substituting equimolar concentrations of potassium for sodium. Thus, customized media is apparently not required to dissipate the plasma membrane potential.

Another objective of this study was to develop a method for measuring mitochondrial membrane potentials in monolayer cells utilizing a fluorescent probe rather than [^{3}H]TPP$^+$. Although non-radioactive methods for measuring mitochondrial membrane potential using flow cytometry have been described (189,190), the potential influence that trypsinizing and suspending cells has on the mitochondrial membrane potential has not been extensively examined. Therefore, a method for measuring mitochondrial Rh123 accumulation, in cells grown as monolayers, was developed. This method provided results similar to those obtained using [^{3}H]TPP$^+$ and thus was used to measure plasma and mitochondrial membrane potentials in this study and subsequent studies.

Deca sensitivity was examined in 2008, C13*, and RH4 cells to determine whether mitochondrial Rh123 accumulation predicted Deca sensitivity in these cells. It was observed that C13* cells, which contain mitochondria that accumulate more Rh123 than the mitochondria of either 2008 or RH4, were

more sensitive to Deca than either 2008 or RH4 cells. What was unexpected was the increased sensitivity of RH4 cells to Deca compared to 2008 cells since the mitochondria of these two cell types accumulated similar amounts of Rh123. The difference in Deca sensitivity between 2008 and RH4 cells may be explained by the mitochondrial retention of lipophilic cations in these cells. Although RH4 mitochondria accumulated similar amounts of Rh123 as the mitochondria in 2008 cells, RH4 mitochondria retained this Rh123 for longer periods of time. Thus, it appears that mitochondrial retention of lipophilic cations may provide a better predictor of lipophilic cation sensitivity than mitochondrial accumulation. Measuring Rh123 retention may provide a relatively simple test to predict which tumours would respond to lipophilic cation-based therapy.

Mitochondrial membrane potentials following cisplatin treatment in 2008, C13*, and RH4 cells were also examined to determine the response of these cells to cisplatin exposure. While cisplatin treatment had no effect on the mitochondrial membrane potentials in either C13* or RH4 cells, 2008 cells responded to cisplatin with a significant increase in their mitochondrial membrane potential. An increase in mitochondrial accumulation of Rh123 following cisplatin treatment had been reported in EL-4 lymphoma cells (263).

At least two potential explanations exist for the observed increase in mitochondrial membrane potential in 2008 cells following cisplatin treatment. The 2008 cells may elevate their mitochondrial membrane potential, when treated with cisplatin, as a protective mechanism against cisplatin cytotoxicity.

This elevated mitochondrial membrane potential may augment ATP production that could then be utilized to remove cisplatin and/or repair cisplatin damage. An elevated mitochondrial membrane potential may also increase the capacity of mitochondria to scavenge positively charged cisplatin species since an elevated mitochondrial membrane potential indicates a greater negative charge within mitochondria. An alternative explanation for the observed increase in mitochondrial membrane potential in 2008 cells following cisplatin treatment is that populations of 2008 cells are heterogeneous with respect to their mitochondrial membrane potentials and cisplatin selectively kills 2008 cells with lower mitochondrial membrane potentials. This selective elimination of 2008 cells with low mitochondrial membrane potentials would result in only 2008 cells with elevated mitochondrial membrane potentials surviving the cisplatin treatment. Since Rh123 fluorescence is expressed per cell, measuring Rh123 fluorescence in 2008 cells that survived the cisplatin treatment (i.e. 2008 cells with elevated mitochondrial membrane potentials), would give a higher fluorescence per cell than the control population (cells that vary with respect to their mitochondrial membrane potentials). The concentrations of cisplatin employed did reduce cell viability.

It is unclear why C13* or RH4 cells did not elevate their mitochondrial membrane potential in response to cisplatin. It is possible that the selection protocol for generating C13* cells has maximized their mitochondrial membrane potential. Thus, as previously proposed, 2008 cells represent a heterogeneous

population, with respect to mitochondrial membrane potential and cisplatin selectively kill cells, within that population, with low mitochondrial membrane potential, leaving cells with higher mitochondrial membrane potentials. Several cisplatin selection cycles may eliminate cells with low mitochondrial membrane potentials and result in a homogenous population of cells with high mitochondrial membrane potentials, the C13* cells. This idea of heterogeneity/homogeneity is supported by flow cytometry data which shows that for a given cell size there is a large range of Rh123 staining in 2008 cells while Rh123 staining in C13* cells is more homogeneous (153). It remains unclear why RH4 cells do not elevate their mitochondrial membrane potential in the presence of cisplatin since they appear to have similar heterogeneity with respect to Rh123 staining as 2008 cells (153). Some aspect of the selection process through which RH4 cells were generated may have altered the ability of RH4 cells to elevate their mitochondrial membrane potential.

Another objective of this study was to determine the interaction between cisplatin and Deca. In particular, it was investigated whether an elevated mitochondrial membrane potential in our cisplatin-resistant variant could be exploited. Our data indicated that, in both 2008 and C13* cells, the interaction between Deca and cisplatin is synergistic at several of the doses examined. More significant may be the observation that the addition of Deca can reduce the amount of cisplatin required, by as much as 93-fold, without compromising cell kill in the cisplatin-resistant variant. Since cisplatin-based therapy is associated

with numerous side effects, these results suggest that combining cisplatin with lipophilic cations could reduce cisplatin-mediated side effects without conceding tumour cell kill. Therefore, combinations of Deca and cisplatin may not only be useful for treating tumour cells but this combination may also be efficacious against cisplatin-resistant tumours. If the concentrations of Deca (or other lipophilic cations) prove relatively non-toxic, then their addition to cisplatin-based therapies may improve the treatment of certain cisplatin-resistant tumours.

Investigations into the usefulness of lipophilic cations in clinical treatment protocols are still ongoing. Since the publication of this manuscript a series of studies have been performed on a new lipophilic cation, MKT-077, which is currently being examined in Phase I/II clinical trials (264-266). MKT-077 has been shown to inhibit tumour cell growth both *in vitro* and *in vivo*, and appeared relatively non-toxic to normal tissues (265,266). In culture, this agent selectively inhibited respiration in tumour cell mitochondria but not mitochondria in normal cells. In addition, it was observed that there was a loss of mtDNA, but not nuclear DNA, in tumour cells, an effect that was not observed in normal cells (264). If these new lipophilic cations become useful clinically, our study suggests that treatment modalities which combine these lipophilic cations with cisplatin may improve tumour cell kill and/or improve the response of cisplatin-resistant tumours.

Chapter 2. Characterization of cells resistant to Photofrin II-mediated photodynamic therapy (PDT).

The previous chapter characterized mitochondrial alterations associated with cisplatin resistance and demonstrated that these altered mitochondria could be selectively inhibited by lipophilic cations. To determine whether mitochondria were capable of modulating the sensitivity of cells to cancer therapies, other than cisplatin-based chemotherapy, the mitochondria in radiation-induced rat fibroblast 1 (RIF-1) cells and their PDT-resistant variant, were characterized. Two observations prompted us to examine mitochondrial changes associated with PDT resistance; (i) mitochondria appear to be the primary target of Photofrin II-mediated PDT and (ii) preliminary characterization of mitochondria in the PDT-resistant variant showed similar ultrastructural changes as those described in the cisplatin-resistant variant, C13* (152).

The use of PDT as a cancer treatment can be traced back to 1903 when topically-applied eosin and sunlight were used to treat patients with skin cancer (267). However, PDT was initially used primarily as a means of detecting and localizing tumours. The antitumour activity of PDT was not appreciated until 1975, when it was observed that tumours transplanted into mice could be destroyed, without appreciable toxicity to surrounding tissue, using hematoporphyrin (Hpd) as a photosensitizer and red light (268). Subsequently, in 1976, the first full clinical report of PDT appeared using Hpd as the

photosensitizer (267). Hpd is a mixture of various porphyrin species and is a relatively poor sensitizer (269,270). Fractionation of Hpd lead to the identification of a fraction containing most of the photosensitizing properties and this fraction was termed porfimer sodium or Photofrin II (268,271). Photofrin II has since been investigated in clinical trials for bronchus, esophagus, and bladder tumours (267).

PDT requires the simultaneous interactions of a sensitizer, an appropriate wavelength of light, and oxygen (272). In the absence of oxygen, photosensitizers such as Photofrin II, have no effect. Even lowering the amount of oxygen from 5% to 1% reduces Photofrin II's effect by 50% (273). Photosensitizers are also relatively inert until they are activated by a specific wavelength of light. Absorption of a specific wavelength of light causes a sensitizer to adopt an excited state (274). This excited state is a short-lived triplet state that can interact with oxygen through two types of reactions. Type I reactions involve the transfer of hydrogen atoms or electrons to a solvent or substrate which, in turn, interacts with oxygen to produce ROS. Type II reactions involve the direct transfer of energy from the sensitizer to oxygen resulting in the generation of the highly reactive oxygen species, singlet oxygen. The lifetime of singlet oxygen has been estimated to range between 0.6×10^{-6} - 100×10^{-6} seconds depending on the environment (275).

Studies using [^{14}C]Photofrin II in mice found tissue distribution (normal tissue) to be as follows; liver, adrenal gland, urinary bladder > pancreas, kidney,

spleen > stomach, bone, lung, heart > skin > muscle >>> brain (274). Similar studies using [^{14}C]Photofrin II in mice demonstrated that 24 hours after injection only 1% of the Photofrin II remained in the circulation. Within seven to eight hours following an injection of Photofrin II, most of the compound is thought to bind to albumin, low density lipoprotein and high density lipoprotein. Photofrin is primarily found bound to high density lipoprotein at later time points (eight days) (276).

Unlike other cancer treatments, PDT does not rely on selective accumulation or cytotoxicity of tumour cells compared to normal cells. For example Hpd accumulates to greater extents in normal liver and kidney tissue compared to tumour cells but unless these organs are exposed to light, toxicity does not occur. Higher concentrations of Hpd have been observed in tumours compared to skin or muscle in mice yet, the mechanisms through which this selective accumulation occurs remains unclear (277). Although PDT's dependence on light provides a certain amount of selectivity, this property also limits the use of PDT to areas accessible to light. Porphyrins absorption spectrum is maximal at 400 nm with four other absorption bands between 500-650 nm with 630 nm being the weakest absorption band (278,279). Although 630 nm is the weakest absorption band, it is the wavelength normally used clinically because higher wavelengths of light provide greater tissue penetration (280). Tissues components such as hemoglobin limit the effective penetration of

light. Tissue penetration of light at 630 nm has been reported to range from 1-15 mm depending on tissue type and illumination source (267).

Patients receiving Photofrin II must avoid sunlight for 2-8 weeks and thus skin photosensitivity, rather than drug toxicity, has limited the maximum tolerated dose (281,282). Studies performed in mice with [^{14}C]Photofrin II indicate that elimination of Photofrin from the circulation appears to be explained by triexponential elimination with half-lives of 4 hours, 9 days and 36 days. This elimination profile results in approximately 1% of the photosensitizer in the circulation after 24 hours and 0.01% after 75 days. The primary route excretion appears to be the feces with little or no drug metabolism (274).

It is unclear how Photofrin enters cells but uptake may be mediated, in part, via an active transport mechanism (283). Once in cells, Photofrin II appears to accumulate in, and induces damage to, cellular membranes such as the plasma, mitochondrial, lysosomal, endoplasmic reticulum and nuclear membranes (274). Photosensitizer localization appears dependent on the duration of incubation; short incubations of approximately 1 hour lead to primarily plasma membrane accumulation while longer incubations (16-18 hours) leads to a redistribution into intracellular organelles like mitochondria and lysosomes (284).

PDT-mediated damage is thought to result from preoxidation of phospholipids and cholesterol leading to alterations in membrane permeability and fluidity. Disruption of membrane-associated enzymes and receptors has

also been reported (285). Of the membrane-associated enzymes that are disrupted, mitochondrial membrane enzymes may represent a key target that leads to cell death (286,287). Several groups have implicated mitochondria as being the primary targets of hematophorphyrin-derived photosensitizers (287-289). Specifically within mitochondria, PDT has been reported to effect cytochrome *c* oxidase (290), succinate dehydrogenase (290,291), ATP production (292), transport of intermediates across the inner membrane (289,293), calcium transport (288), coupling of respiration and oxidative phosphorylation (288,290,294), and Rh123 accumulation (290). Mitochondria also appear to be the major site of Photofrin II localization following redistribution of the drug from the plasma membrane (287).

Other investigators argue that inhibition of plasma membrane-associated transport systems and damage to DNA repair enzymes, may precede, mitochondrial disruption during PDT (274). DNA repair enzymes represent an unlikely target for PDT since PDT induced similar amounts of cytotoxicity in normal and repair-deficient fibroblasts (295). When myocardial cells treated with Hpd were specifically irradiated in the nucleus, hyaloplasm or mitochondria, targeting mitochondria resulted in cell death at lower light doses than the other regions (296). Often the earliest detectable change occurs in the mitochondria as soon as 10-15 minutes after PDT (297). Therefore, the direct cellular cytotoxicity of porphyrin-mediated PDT appears to be mediated by disruption of mitochondria.

In vivo, PDT may also kill tumours indirectly. Some observations indicate that tumour vasculature is the primary target for PDT. Studies have shown that tumour cells harvested from animals immediately after PDT do not have altered *in vitro* clonogenic survival (298). In addition, photosensitizer concentration have been reported to be higher in vascular stromal cells than in tumour cells (299,300) and PDT decreases tumour blood flow and oxygen tension (301). Thus, it appears that direct tumour cell kill, vascular shutdown and possibly an inflammatory response all contribute to tumour destruction *in vivo* by PDT (274,302).

In an attempt to identify mechanism(s) through which Photofrin II-mediated PDT induces direct tumour cell death, our laboratory (303) and Luna and Gomer (304) generated variants of murine radiation-induced fibroblast 1 (RIF-1) cells resistant to Photofrin II-mediated PDT. In our laboratory, a PDT-resistant variant named, RIF-8A, was generated by subjecting RIF-1 cells to 8 selection cycles of Photofrin II followed by re-growth of single surviving colonies. The first four cycles involved treating RIF-1 cells with increasing concentrations of Photofrin II for 18 hours followed by a fixed light dose. The last four cycles involved treating cells with increasing concentrations of Photofrin II for 4 hours followed by a fixed light dose. This selection protocol produced RIF-8A cells which were approximately 2-logs resistant to Photofrin II compared to RIF-1 cells. Similarly, Luna and Gomer (304) generated PDT-resistant variants after 10 selection cycles of RIF-1 cells treated with Photofrin II for either one hour or

16 hours. Characterization of these variants indicated that PDT-resistance was not a result of increased scavenging of ROS by glutathione or superoxide dismutase. Furthermore, neither elevated P-glycoprotein expression nor decreased drug accumulation were observed in the PDT-resistant variants (303,304). These results suggest that some cellular process other than altered drug accumulation or ROS scavenging was responsible for mediating the decrease in PDT sensitivity. The following manuscript further characterizes the PDT-resistant variant, RIF-8A, in an attempt to identify potential mechanisms that cause these cells to become resistant to PDT.

In this manuscript various mitochondrial assays such as measurement of ATP content, succinate dehydrogenase activity and oxygen consumption were conducted by R.A. Moorehead. All other assays were performed by S.M Sharkey. In the discussion of this manuscript I have focused on the functional alterations observed in the mitochondria of the PDT-resistant variant, RIF-8A.

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1. Mitochondrial Alterations in Photodynamic Therapy-Resistant Cells. Sheila M. Sharkey, Brian C. Wilson, Roger Moorehead, and Gurmit Singh. *Cancer Research* **53**:4994-4999, 1993.

2. Cross-Resistance to Cisplatin in Cells Resistant to Phótofrin-Mediated Photodynamic Therapy. Roger A. Moorehead, Steven G. Armstrong, Brian C. Wilson, and Gurmit Singh. *Cancer Research* **54**:2556-2559, 1994.

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[CANCER RESEARCH 53, 4994–4998, October 15, 1993]

Mitochondrial Alterations in Photodynamic Therapy-resistant Cells[1]

Sheila M. Sharkey, Brian C. Wilson, Roger Moorehead, and Gurmit Singh[2]

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ABSTRACT

The characterization of radiation-induced fibrosarcoma cells (RIF-8A) which have been selected for resistance to Photofrin-mediated photodynamic therapy (PDT) is detailed in this report. Morphological and functional assessment of mitochondria in both the resistant RIF-8A and parental RIF-1 cells show distinct differences. Electron micrographs show that the mitochondria in the RIF-8A cells are relatively smaller, stain more densely, and display a higher cristae density than RIF-1 cells. P. A. Andrews *et al.* (Cancer Res., *52:* 1895–1901, 1992) reported similar mitochondrial differences between a human ovarian carcinoma cell line, 2008, and its cisplatin-resistant counterpart (C13*). Dose-response curves demonstrate that these cisplatin-resistant C13* cells show cross-resistance to Photofrin-mediated PDT.

Functionally, the RIF-8A cells produce more ATP and demonstrate higher succinate dehydrogenase activity than do the RIF-1 cells, but the rates of oxygen consumption do not differ between the two cell types. The PDT-sensitive RIF-1 cells demonstrate a significantly higher susceptibility to inhibition of glycolytic activity as determined by 2-deoxy-*d*-glucose survival curves. These findings suggest differences in the efficacy and/or mode(s) of energy production in the RIF-1 and RIF-8A cells. Since mitochondria are sensitive targets for porphyrin-mediated PDT, the observed changes in mitochondrial structure and/or function may be involved in the PDT resistance seen in RIF-8A cells.

INTRODUCTION

Photodynamic cancer therapy uses the localized delivery of light to activate photosensitizing compounds which are selectively retained by tumor tissues. Although this treatment modality is undergoing clinical trials, primarily with the hematoporphyrin derivative Photofrin (QuadraLogic Technologies Inc., Vancouver, British Columbia, Canada), the mechanisms of action of PDT treatment are not fully understood.

Resistant cell lines have been used by several investigators in order to explore the characteristics of PDT cytotoxicity. This has been achieved by looking at either the PDT susceptibility of cells resistant to various other treatment modalities or at the nature of PDT[3]-induced resistance. The use of various cell lines, photosensitizers, induction protocols, and light/dark applications has made it difficult to reach general conclusions. Most commonly, MDR cells have been tested for cross-resistance to PDT, but these results have been conflicting. Decreased drug uptake has been shown to account for resistance to the photocytotoxicity of the anthrapyrazole, PD 110095 (1) and varying degrees of cross-resistance to rhodamine 123 plus light in murine melanoma MDR+ cells (2) and without light in P388/ADR leukemia cells (1) and to Photofrin or hematoporphyrin derivative in Chinese hamster ovary MDR+ cells (3, 4). However no such cross-resistance was seen in P388/ADR cells to mesoporphyrin plus light (5) or in K562 erythroleukemic MDR cells tested for PDT sensitivity using sulfonated aluminum phthalocynaine as the photosensitizer (6). Heat-resistant Chinese hamster fibroblasts (3012) and temperature-resistant RIF cells (TR-4, TR-5, TR-10) did not show cross-resistance to Photofrin-mediated PDT (7). The assessment of the sensitivity to PDT of cells with altered repair capabilities has also yielded variable findings. Evans *et al.* (8) showed increased sensitivity to the cytotoxic effects of sulfonated aluminium phthalocynaine-mediated PDT in leukemic cells deficient in the repair of UV-induced damage (LY-R) but not LY-S cells which have enhanced sensitivity to ionizing radiation (8). When testing of xeroderma pigmentosum and ataxia telangiectasia cells for Photofrin-mediated PDT sensitivity, Gomer *et al.* (9) found no such difference.

The information available regarding PDT-induced resistance is somewhat more limited. We (3) and Luna *et al.* (10) reported the isolation of RIF-1 cells resistant to Photofrin-mediated PDT.

The protocol for the isolation of our PDT-resistant RIF-1 cell variant and its initial characterization were published previously (3). Briefly, single surviving colonies were harvested after subjecting RIF-1 cells to successive courses of PDT with increasing concentrations of Photofrin. Incremental increases in resistance were observed throughout the selection procedure, and the final, most resistant variant, designated RIF-8A, was obtained after eight cycles of PDT. The average ratio, D_{10} (RIF-8A)/D_{10} (RIF-1), for 2.7×10^3 J/m^2 of light dose was 1.8 ± 0.4 (mean $\pm$ SD) (3). The magnitude of resistance is similar to that observed clinically and, thus, is relevant. However, classical resistance is much greater than that obtained in our cells and may not be relevant in human disease.

Earlier investigations showed that the resistance displayed by RIF-8A was not a result of diminished drug uptake, nor was it consistent with a multidrug-resistant phenotype in which p-glycoprotein mediates enhanced drug efflux (3, 11). Analysis of the karyotypes revealed that the majority of parental RIF-1 cells were diploid or tetraploid (40 and 80 chromosomes) and contained some abnormal chromosomes. The RIF-8A cell karyotypes were inconsistent, the most frequently observed being polyploidies of 120 chromosomes (conducted by Dr. D. Miller, Wayne State University). Further characterization of the parental and resistant cells has, therefore, been undertaken in order to determine structural and/or functional differences which may provide insight into the mechanisms underlying altered susceptibility to PDT and, hence, its mechanism of cytotoxicity.

Mitochondria have repeatedly been implicated as primary targets of porphyrin-mediated PDT (12–14). Lipophilic porphyrins concentrate intracellularly in association with mitochondria (14, 15). Enhancing this mitochondrial localization through substitutions in the side chain structures of the tetrapyrrole ring enhances the phototoxicity of these compounds (15). Furthermore, Moreno and Salet (16) demonstrated that, after hematoporphyrin incubation, irradiation of subcellular areas rich in mitochondria results in cell death, whereas microirradiation of the nucleus or hyaloplasm did not do so. The earliest damage after porphyrin PDT occurs in the mitochondria and is associated with impairment of a number of mitochondrial enzymes involved in the oxidative phosphorylation pathway (12–17). If mitochondria are primary targets of porphyrin PDT, then differences in these organelle may account for differences in susceptibility to this treatment. For comparison, morphometric and functional analyses of RIF-1 and

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[3] The abbreviations used are: PDT, photodynamic therapy; MDR, multidrug resistant; RIF, radiation-induced fibrosarcoma; D_{10}, drug dose for 10% survival; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; 2-DG, 2-deoxy-*d*-glucose.

RIF-8A mitochondria were undertaken. Electron micrographs were used to investigate structural alterations in these organelles. Also, sensitivity to PDT was determined in a human ovarian carcinoma line (2008) and its cisplatin-resistant counterpart C13* which does not demonstrate characteristics of multidrug resistance (18, 29). The mitochondria in these cells demonstrate striking similarities to those of the RIF-1 and RIF-8A cells, respectively.

Biochemical determinations of succinate dehydrogenase activity, an enzyme in the electron transport chain, and susceptibility to the inhibition of glycolytic activity were also performed to investigate the bioenergetics of the parental RIF-1 and resistant RIF-8A cells.

This is the first study that shows a possible association between mitochondrial alterations and resistance to PDT; preliminary results have been presented previously as a work in progress (11). Here, we report the results of the completed study.

MATERIALS AND METHODS

Materials. Fetal calf serum, 10% trypsin/EDTA, α-minimal essential medium, RPMI, glutamine, and penicillin-streptomycin were purchased from Gibco BRL (Burlington, Ontario, Canada). Ribonucleosides and deoxyribonucleosides, 2-deoxy-*d*-glucose, luciferin/luciferase, and MTT, as well as the Bioluminescent somatic ATP assay kit, were from Sigma (St. Louis, MO). Photofrin was from Quadralogic Technologies Inc. (Vancouver, British Columbia, Canada). The bicinchoninic acid protein assay was obtained from Pierce (Rockford, IL).

Cells. RIF-1 cells were obtained from Dr. B. Henderson, Roswell Park Memorial Institute, Buffalo, NY. RIF-8A cells were derived as previously described (3). Cells were grown as monolayers in α-minimal essential medium containing ribonucleosides and deoxyribonucleosides, supplemented with 10% fetal calf serum, 2 mM glutamine, 1% penicillin, and streptomycin in 75-cm^2 culture flasks. Doubling times for these cells were obtained from the linear portion of their growth curves. The values presented are the means of eight independent determinations. Human ovarian carcinoma cells 2008 and C13* were provided by Dr. Paul Andrews, Georgetown University, Rockville, MD (18), and were grown in RPMI supplemented with 5% fetal calf serum with 1% penicillin and streptomycin. All cells were maintained at 37°C and 5% CO_2/95% air in a humid environment.

Morphometric Analysis. Preconfluent RIF-1 and RIF 8A cells were fixed in 2% glutaraldehyde, dehydrated in a graded alcohol series, and fixed in Spurr's resin (19). Thin sections (70 nm) were stained with uranyl acetate and lead citrate and transferred to grids. Electron micrographs were obtained at magnifications of ×1500 for cellular and nuclear analysis and at ×10,000 for mitochondrial analysis. A Bioquant automated image analysis system (R & M; Biometrics Inc., Nashville, TN) was used to determine cellular, nuclear, and mitochondrial cross-sectional areas. Mitochondrial areas were determined as a fraction of the cytoplasmic area present in each ×10,000 photomicrograph. The values presented in Table 2 have been converted from mitochondrial area per unit area cytoplasm to a per cell basis using the cellular minus nuclear cross-sectional areas presented in Table 1. A minimum of 20 micrographs were evaluated for each cell type at each magnification.

Cellular Protein Determination. Preconfluent RIF-1 and RIF-8A cells were harvested using trypsin-EDTA and counted. Varying cellular concentrations were analyzed using a bicinchoninic acid microassay with bovine serum albumin as a standard (20). Ninety-six-well plates, with triplicates of each sample, were analyzed at 570 nm on a Biokinetics Reader (Bio-Tek Instruments).

PDT Survival Curves. Preconfluent 2008 and C13* cells were removed from culture flasks by 10 min of incubation in 5 ml of 1× trypsin. After the cells were spun at 1000 rpm for 5 min, they were resuspended and counted, and appropriate dilutions were made to obtain 2×10^3 cells/ml. One ml of cell suspension was added to 8 ml of medium in culture plates, and cells were allowed to adhere for 4–6 h, at which time the appropriate Photofrin dose in 1 ml of medium was added to the plates; control plates received 1 ml of medium only. After 18 h of incubation, the drugged medium was removed and replaced with fresh medium alone. Plates were then irradiated on a 100- x 50-cm light-diffusing surface illuminated by a bank of fluorescent tubes (Philips type TL/83), filtered with red acetate filters (No. 19, Roscolux, Rosco, CA), to give wide band illumination above 585 nm. The energy fluence rate was 9.2 W/m^2 in the wavelength band 623–634 nm, representing 12% of the total filtered output. Irradiation for 5 min resulted in an incident energy fluence of 2.7×10^3 J/m^2 in this photoactive band. All procedures were carried out in minimal ambient light conditions after the plating of cells. After 5 days of undisturbed growth in the dark, the plates were stained with methylene blue, and colonies containing >20 cells were counted.

2-Deoxy-*d*-Glucose Survival Curves. Preconfluent cells were removed from culture flasks using 0.5 ml of 10× trypsin, suspended in 10 ml medium, centrifuged at 1000 rpm for 5 min, and resuspended in medium. Cell counts were performed and dilutions were done to reach a final cell concentration of 2×10^3 cells/ml. One ml of the cell suspension was added to 8 ml of medium in culture plates and allowed to adhere for 4 h. One ml of a 10-fold concentration of 2-DG was added to each of the triplicate plates per dose. Control plates received 1 ml of medium. Cells remained in the various concentrations of 2-DG for 5 days, at which time the plates were stained with methylene blue and colonies with >20 cells were counted. Survival is expressed as a percentage of control plate counts.

ATP Content. Total cellular ATP content was assayed using a modified luciferin/luciferase assay (21, 22). RIF-1 or RIF-8A cells were removed from 35-mm wells and suspended in medium. Half of each aliquot was used to quantitate cell number, and the remaining suspension was added to an equal volume of acetonitrile and sonicated to disrupt cellular membranes. Serial dilutions were done to achieve cell concentrations ranging from 20 to 300 cells/sample. In each measurement, triplicate samples of RIF-1 and of RIF-8A were used. The chemiluminescence of 4 dilutions/sample was measured in order to ensure that the ATP concentrations obtained fell within the linear range of the curve. An ATP standard curve was used to determine total ATP content per sample. Results shown are the means from 6 separate experiments, each containing dilutions from at least 3 cell samples.

Succinate Dehydrogenase. Mitochondrial activity of succinate dehydrogenase was measured using a modified colorimetric assay in which a tetrazolium compound is converted into a blue formazan precipitate (MTT assay) (23). Quadruplicates of cell dilutions ranging from 1×10^3 to 5×10^4 in 96-well plates were incubated with 20 μl of MTT in phosphate-buffered saline for 4 h, at which time 100 μl of extraction buffer was added to dissolve the precipitate (24). Twenty-four h later, the absorbance of the dissolved formazan precipitate was measured at 570 nm. The results are expressed as the means of 2 independent experiments, wherein quadruplicate measurements were done on at least 4 cell dilutions.

Oxygen Consumption. Oxygen utilization in whole cells was measured using a Clarke oxygen electrode (YSI Inc., Yellow Springs, OH). The oxygen solubility in the chamber was calibrated at 234 nmol/ml and 35°C. Cell dilutions ranged from 2×10^5 to 3×10^6 cells/ml. Linear oxygen consumption in the cells was measured in phosphate-buffered saline. The microchamber (0.6 ml volume) was used to estimate the basal rate of oxygen consumption. Values used to calculate the average oxygen consumption/10^6 cells/min fell within the linear range of the rate curve for oxygen consumption.

Statistical Analysis. All values reported are means ± SEM. Student's *t* tests were used to determine statistical significance. Calculations were performed using Minitab Inc. data analysis software.

RESULTS

Cellular Characteristics. Gross morphological assessment of the RIF-1 and RIF-8A cells shows an increase in total cell size of the RIF-8A cells (Fig. 1). Nuclear size is also increased, resulting in an equivalent nuclear to cytoplasmic area ratio of 0.3 in the two cell types (Table 1). Total cellular protein content is also double that of the parental cells. However, the cell doubling time for both cell types are similar.

Mitochondrial Characteristics. Electron micrographs of mitochondria from RIF-1 and RIF-8A cells show clearly their morphological differences (Fig. 2). The mitochondria in the parental RIF-1 cells are relatively larger than RIF-8A cells and of low density with sparse cristae. The mitochondria of the resistant RIF-8A line are smaller, more electron dense, and display a higher cristae density than the RIF-1 line. The morphometric measurements shown in Table 2

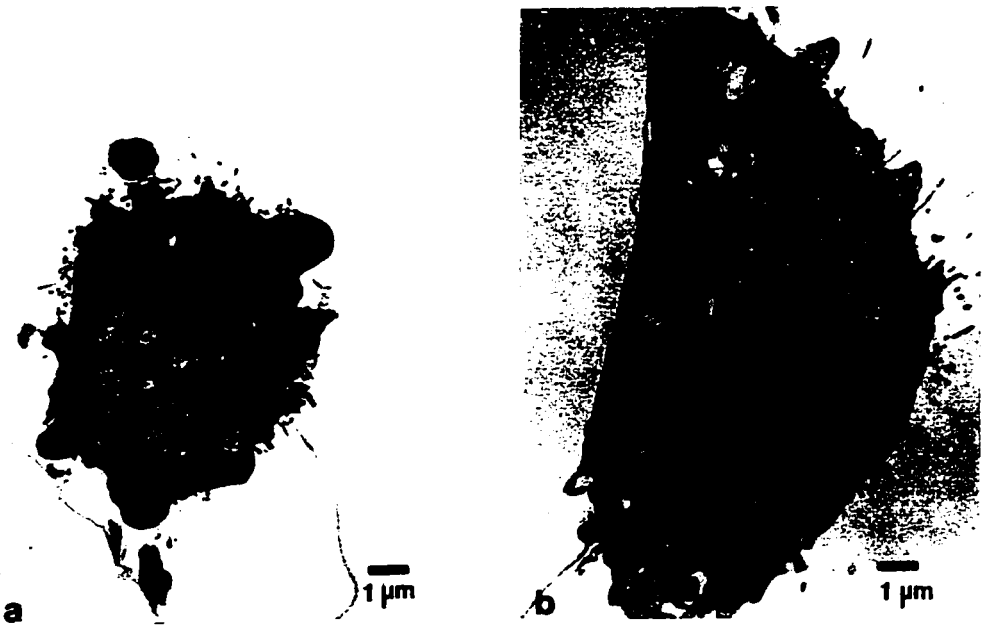

Fig. 1. Electron micrographs of RIF-1 (*a*) and RIF-8A cells (*b*).

Table 1. *Cellular measurements*

	RIF-1	RIF-8A
Cellular area (μm^2)	64.8 ± 2.7	166.0 ± 14.5[a]
Nuclear area (μm^2)	22.8 ± 1.5	59.1 ± 5.8[a]
Protein content (pg/cell)	142 ± 43	259 ± 18[a]
Doubling times (h)	19 ± 2	21 ± 1

[a] $P < 0.05$ for difference between RIF-1 and RIF-8A.

demonstrate the greater cross-sectional areas of the RIF-1 mitochondria. Total mitochondrial area per cell in RIF-8A is double that of RIF-1.

The total cellular ATP content and succinate dehydrogenase activity of the resistant RIF-8A cells are significantly elevated relative to the RIF-1 cells. However, no significant difference was found in oxygen consumption rates per cell between the resistant and parental cells (Table 2).

Cell Survival Assays. The representative survival curves shown in Fig. 3 demonstrate that the RIF-1 cells are significantly more sensitive to the inhibition of glycolysis by 2-DG than are the PDT-resistant RIF-8A cells.

Fig. 4 shows the clonogenic survival curves for the 2008 and C13* human ovarian carcinoma cells as a function of the Photofrin concentration after 18 h incubation and a fixed light dose. The C13* cells originally selected for resistance to cisplatin show greater PDT survival at higher Photofrin concentrations than do the parental 2008 cells.

DISCUSSION

The purpose of this study was to characterize the PDT-resistant RIF-8A cells and to compare them to the parental RIF-1 cells, in order to investigate the possible mechanism(s) of induced resistance. The striking differences in mitochondrial appearance of RIF-1 and RIF-8A are intriguing in this regard. The RIF-8A cells contain smaller mitochondria with more electron-dense matrices. Although the total mitochondrial area in these PDT-resistant cells is double that of the parental line, when the greater cell size is considered the ratios of mitochondrial area per unit cytoplasmic area are comparable, RIF-8A:RIF-1 ≈ 0.8 ± 0.1 which implies that there is not a significant alteration in cellular energy requirement per unit cell mass. Owing to the large size difference between RIF-1 and RIF-8A cells, increases in ATP content and succinate dehydrogenase activity per cell in RIF-8A were not unexpected. Of interest is the absence of an accompanying significant increase in oxygen consumption in these cells. That is, for an equivalent rate of oxygen consumption, RIF-8A cells contain a greater intracellular ATP pool, suggesting an altered energy metabolism when compared to the RIF-1 cells.

Similar mitochondrial alterations have been noted in cells selected for by various conditions, including growth in glucose-free media (25), diminished susceptibility to trypsin detachment (26), and, more recently, cisplatin resistance (11). Because mitochondria are targets of porphyrin-mediated PDT, it was of interest to assess the relative sensitivities of the 2008 and C13* cells to PDT, since their mitochondria appeared so similar to those of the parental RIF-1 and resistant RIF-8A cells, respectively. These studies determined that the C13* cells are indeed cross-resistant to PDT. Cisplatin survival curves were also determined for the PDT-sensitive and -resistant cells (data not shown). The ratio of concentrations at which there is 10% survival after 1 h of cisplatin exposure for RIF-8A:RIF-1 was approximately 1.7 (C13*:2008 ≈ 6). These findings are not interpreted as necessarily implying equivalent mechanisms of photodynamic cytotoxicity of resistance for the RIF and ovarian cells but are of interest because of the resemblance of the mitochondria in the corresponding pairs of sensitive and resistant cells.

Mitochondrial variations have been reported between normal and malignant cells and between parental cell lines and related cells which have been exposed to various selective pressures (25, 27–29). Generally, as tumor growth rates increase and levels of differentiation decrease, mitochondrial differences become more pronounced, with altered cristae structure, more dilute matrices, and changes in organelle shape. Associated with these morphological changes are alterations in cellular bioenergetics (27). Depending on the cell lines analyzed, an enhanced capacity for glycolysis may or may not be associated with impaired oxidative phosphorylation (30, 31). Whether this elevated glucose utilization occurs in order to meet increased cellular demands for energy beyond that which can be supplied by oxidative phosphorylation or is a component of a more global alteration in metabolic activity has yet to be determined (27, 32).

In order to explore possible differences in the bioenergetics between RIF-1 and RIF-8A cells, survival assays in the presence of 2-DG, an inhibitor of glycolysis, were performed. [2-DG is phos-

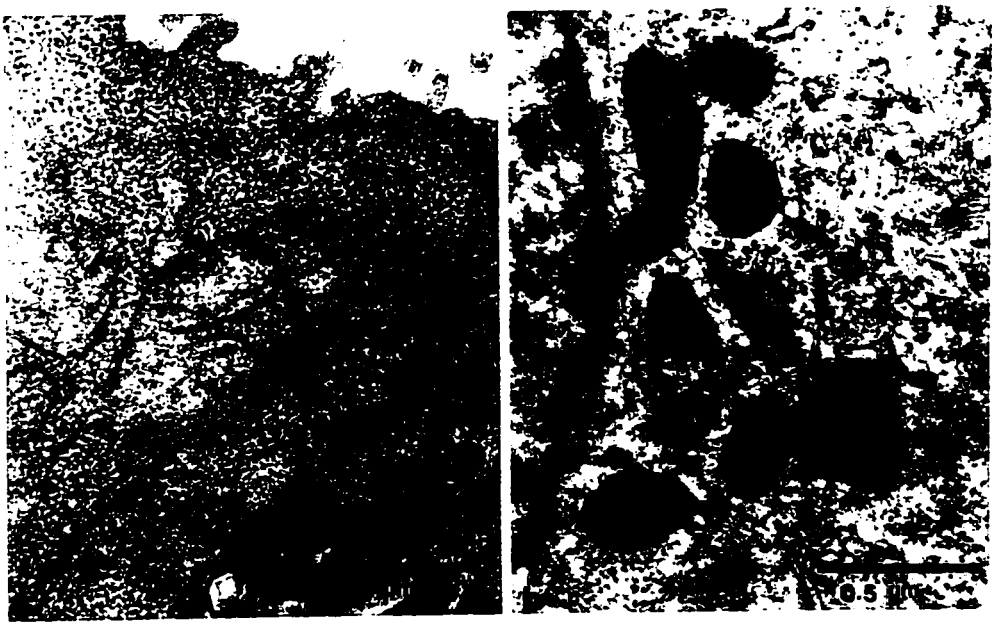

Fig. 2. Electron micrographs of RIF-1 (*a*) and RIF-8A mitochondria (*b*).

Table 2. *Morphometric and biochemical analysis of mitochondria*

	RIF-1	RIF-8A
Average cross-sectional area (μm^2)	0.110 ± 0.003	0.081 ± 0.002[a]
Total mitochondrial area (μm^2/cell)	2.7 ± 0.3	5.3 ± 0.8[a]
No. of mitochondria/cell	24 ± 3	65 ± 10
ATP content (fM/cell)	9.2 ± 2.2	15 ± 1.5[a]
Succinate dehydrogenase[b]	2.1 ± 0.3	3.9 ± 0.6[a]
Oxygen consumption ($\mu l/10^6$ cells/min)	0.13 ± 0.02	0.16 ± 0.02

[a] $P < 0.05$ for difference between RIF-1 and RIF-8A.
[b] A_{570} per 10^6 cells.

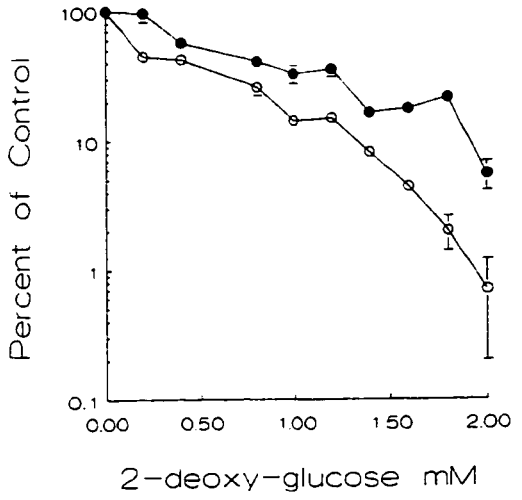

Fig. 3. Percentage survival of RIF-1 (○) and RIF-8A (●) cells after 5 days of continuous incubation in medium containing various concentrations of 2-DG. Results are from a representative experiment and are the means (*points*) ± SEM (*bars*) of colonies from triplicate plates, expressed as percentages of control values. Error bars not shown are smaller than the symbol size. Triplicate plates were done for each data point in each of the three experiments performed.

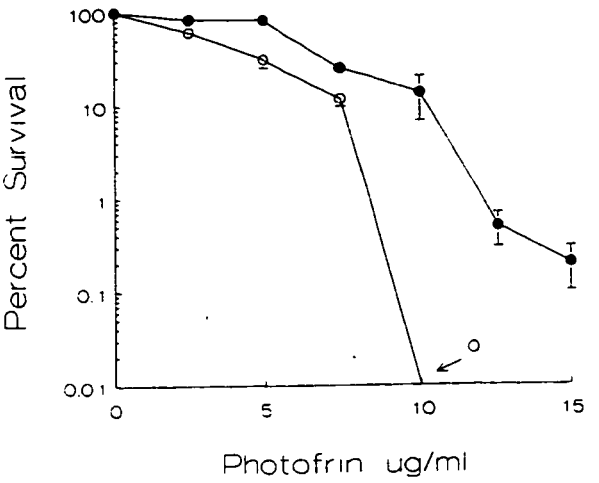

Fig. 4. PDT dose-response curves of 2008 (○) and C13 (●) cells and survival, expressed as percentages of control plates. Results are the means (*points*) ± SEM (*bars*) of triplicate plates from a representative experiment. Triplicate plates were done for each data point in each of the three experiments performed, and survival is expressed as a percentage of the no-drug controls.

phorylated by hexokinase to 2-deoxy-glucose-6-phosphate, which cannot be further processed (33).] The significantly lower survival of the RIF-1 cells in the presence of 2-DG indicates a greater dependence on glucose utilization in these cells compared to the RIF-8A cells. RIF-1 cells were also more profoundly affected by the inhibitory effects of 2-DG. Along with decreased survival, RIF-1 cells displayed greater morphological alterations with distinct reduction in cell size, diminished cell spreading, and fewer cells per colony than the RIF-8A cells (data not shown). This greater sensitivity to 2DG in RIF-1, together with the morphological and functional differences of the mitochondria in the two cell types, is consistent with previous reports which showed that other cell lines containing enlarged mitochondria with few cristae (like RIF-1) also display increased levels of glycolytic activity (25, 32).

The structural and functional dynamics of mitochondria are complex, and detailed reviews are available (34, 35). Those issues relevant to the present findings and their possible associations with resistance to porphyrin-mediated PDT will now be described. Originally, Hackenbrock (34) linked ultrastructural transformations in mitochondria to changes in energy metabolism. Assessing changes within a given cell type, he demonstrated that smaller, more electron-dense (condensed) mitochondria displayed higher levels of oxidative phosphorylation when compared to larger, sparsely staining (orthodox) mitochondria

(35). However, it is not clear that these morphologies may be used to infer specific bioenergetic differences when comparing two cell types. Further structural alterations associated with functional changes have been demonstrated by Knoll and Brdiczka (36), who demonstrated that actively phosphorylating mitochondria have an increased frequency of inner-outer mitochondrial membrane contact sites. Also pertinent is the finding that, in general, tumor cell hexokinase is preferentially bound to the outer mitochondrial membrane at these contact sites (37, 38). The binding site for the enzyme appears to be a voltage-dependent anion channel protein, which spans the outer mitochondrial membrane and associates with the ADP/ATP translocator forming these contact points (36, 39). This interaction may allow hexokinase preferential access to mitochondrially generated ATP in association with the rapid restitution of intramitochondrial ADP (40). Therefore, mitochondrial morphology reflects aspects of the bioenergetic status of cells, and this may alter susceptibility to PDT.

Porphyrin-mediated PDT has been shown to cause inactivation of numerous mitochondrial enzymes (12), inhibition of ATPase (41), and uncoupling of oxidative phosphorylation (13). The available evidence suggests that damage to the ADP/ATP translocator may be a critical event in PDT disruption of oxidative phosphorylation (41; for review see Ref. 14).

There is evidence that the functional state of mitochondria may alter the number of inner-outer mitochondrial membrane contact sites (35), that this regulation may be disrupted in tumor cells of varying glycolytic capacity (31), and that porphyrin compounds bind to these same sites which contain targets that are sensitive to the damaging effects of porphyrin-mediated PDT (14, 41, 42).

Given that many other *in vitro* studies of Photofrin-mediated PDT have shown mitochondria to be sensitive targets for PDT damage, the observed differences in mitochondrial structure and function between the RIF-1 and RIF-8A cells could well be involved in the PDT-induced resistance which we have observed. Mechanisms such as altered photosensitizer targeting or binding, reduced sensitivity to oxidative damage, or enhanced capacity for repair of such damage could result from either or both of the altered structural and metabolic states. Whether or not the observed changes in mitochondrial morphology and cellular bioenergetics are central to the expression of photodynamic resistance has yet to be determined. Precisely how these findings may relate to changes in sensitivity to porphyrin-mediated PDT is presently being investigated.

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Discussion

Although previous studies demonstrated that Photofrin II accumulated in mitochondrial, lysosomal, nuclear and plasma membranes, the critical target for cell death remained unresolved (274). One approach used to identify the mechanism of action of a drug is to develop a variant that is resistant to the drug of interest. Once a resistant variant has been generated, comparison of the variant to the parental line often reveals changes in the variant that allowed it to become resistant to a particular agent. These changes may reveal clues as to the critical targets within a cell. With Photofrin II-mediated PDT our laboratory (290) and others (292,296,305-307) observed that mitochondrial function was disrupted after PDT. Therefore, a variant resistant to PDT was generated and its mitochondrial characteristics were compared to the parental cell line to investigate whether mitochondrial alterations were associated with PDT resistance. This was the first report to demonstrate that resistance to Photofrin II-mediated PDT may arise from changes in mitochondria.

Initially, it was observed that the ultrastructure of mitochondria in RIF-8A cells was different from RIF-1 mitochondria. RIF-8A mitochondria were smaller with more densely staining cristae. An increase in staining density of mitochondrial cristae had previously been reported to be associated with increased mitochondrial respiratory chain activity (187,188). Therefore, assays of mitochondrial function were performed to investigate whether the structural

changes in the mitochondria of RIF-8A were associated with altered mitochondrial activity.

Since mitochondria are the primary producers of ATP within most cells (164) we examined the total cellular ATP content in RIF-1 and RIF-8A cells. RIF-8A cells contained approximately two-fold more ATP than RIF-1 cells. Mitochondria of RIF-1 and RIF-8A cells were further characterized by examining succinate dehydrogenase activity and total cellular oxygen consumption, both of which are indicators of mitochondrial oxidative phosphorylation. Succinate dehydrogenase is an enzyme in complex II of the electron transport chain but is also found, to a lessor degree, within the cytosol (210). Therefore, although succinate dehydrogenase activity may not provide the most specific method for determining electron transport chain activity, large alterations in its function may indicate changes in electron transport chain activity. In contrast, oxygen is used as the terminal acceptor for electrons passed down the electron transport chain and almost all of the oxygen consumed by the cell is used for this purpose (210). It was observed that succinate dehydrogenase activity was approximately 2-fold higher in RIF-8A cells compared to RIF-1 cells. This increase in succinate dehydrogenase activity in RIF-8A cells was similar in magnitude to the increase in ATP content in RIF-8A cells compared to the parental line. However, oxygen consumption was not doubled in RIF-8A cells compared to RIF-1 cells and thus provided an intriguing result.

An approximately doubling of cellular ATP and succinate dehydrogenase activity may simply reflect the fact that RIF-8A cells are approximately twice as large and contain roughly double the mitochondrial area compared to RIF-1 cells. The lack of increase in oxygen consumption may reflect an increase in ATP production efficiency in RIF-8A cells. To maximize ATP production efficiency, all of the hydrogen ions pumped across the inner mitochondrial membrane would return through the F_1F_0 ATPase and be used to generate ATP, yet, this is not the case. Hydrogen ions can also leak across the inner mitochondrial membrane (down their concentration gradient) at sites other than the F_1F_0 ATPase and thus do not generate ATP (308). These hydrogen ions still required the movement of electrons down the electron transport chain to pump them out of the mitochondrial matrix and thus oxygen is still consumed even though ATP is not produced. A classic example of this phenomenon occurs following treatment of cells with ionophores like FCCP. FCCP alters the permeability of the inner mitochondrial membrane allowing hydrogen ions to leak back into the mitochondrial matrix (242,243). In an attempt to maintain the electrochemical gradient, electrons are still passed down the electron transport chain and oxygen is still consumed but little or no ATP is produced. Therefore, since the increase in oxygen consumption is less than the increase in either electron transport chain activity or ATP produced, these data suggest that RIF-8A mitochondria are more efficient at utilizing the hydrogen ions that are pumped out to generate ATP.

These first two chapters described mitochondrial alterations associated with resistance to cisplatin and PDT. Similarities in the mitochondrial ultrastructure in the PDT-resistant and cisplatin-resistant variants suggested that the mitochondrial alterations associated with the PDT-resistant variant, RIF-8A, may also render this cell type resistant to cisplatin. The cisplatin sensitivity in RIF-1 and RIF-8A, as well as further mitochondrial characterization in these cells, was investigated in the next manuscript.

Chapter 3. Cells resistant to PDT are cross-resistant to cisplatin.

In the previous manuscript, characterization of a PDT-resistant variant showed that several mitochondrial characteristics differed between the PDT-resistant variant and the parental line. These alterations included increased cristae staining and elevated capacity to produce ATP. Intriguingly, a similar increase in cristae staining was observed in a cisplatin-resistant variant (152). As shown in Chapter 1 and by Andrews and Albright (152) this cisplatin-resistant variant also had an elevated mitochondrial membrane potential compared to its parental line. Therefore, it appear that increased cristae staining density may be associated with cisplatin resistance and an increase in mitochondrial membrane potential. These observation suggest that RIF-8A cells may have an increased mitochondrial membrane potential and/or decreased cisplatin sensitivity compared to RIF-1 cells.

Another interesting observation from the previous manuscript was that C13* cells were cross-resistant to Photofrin II-mediated PDT. PDT resistance can result from an overexpression of P-glycoprotein which reduces intracellular Photofrin II concentrations as shown in a multi-drug resistant variant of Chinese hamster ovary (CHO) cells (309). It is improbable that C13* cells are resistant to PDT as a result of P-glycoprotein overexpression since adriamycin sensitivity in 2008 and C13* cells is similar. Adriamycin is a substrate for P-glycoprotein and cells overexpressing P-glycoprotein are resistant to this drug (303). Thus, it is

possible that the mitochondrial changes in C13* cells render them resistant to Photofrin II-mediated PDT. Similarly, the mitochondrial changes associated with PDT resistance in RIF-8A cells may decrease cisplatin sensitivity in these cells compared to their parental line.

Since cisplatin is thought to induce cell death through binding to nuclear DNA and inhibiting DNA synthesis and transcription, it remains unclear how mitochondrial alterations could contribute to cisplatin resistance. It has been suggested that an increase in the internal negative charge within mitochondria, due to an elevated mitochondrial membrane potential, may sequester positively charged cisplatin species from interacting with more critical targets (152). Alternatively, intracellular calcium concentrations and thus calcium-mediated signal transduction may be altered in the cisplatin-resistant variant. Calcium can be accumulated in mitochondria and this accumulation is dependent on the magnitude of the mitochondrial membrane potential (207-209). Therefore, alterations in the mitochondrial membrane potential may influenced intracellular calcium concentrations. Regardless of the mechanism through which mitochondria contribute to drug resistance, changes in their activity associated with drug resistance may provide alternative strategies for treating drug-resistant tumours.

In this manuscript, the mitochondrial membrane potential and cisplatin sensitivity of RIF-8A cells were investigated to examine whether the mitochondrial structural changes associated with PDT-resistance resulted in an

elevated mitochondrial membrane potential and/or decreased cisplatin sensitivity.

All assays in this manuscript were performed by R.A. Moorehead except for the quantitation of DNA-bound cisplatin by atomic absorption spectroscopy which was performed by Dr. S.G. Armstrong (postdoctoral fellow in our laboratory).

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2. Cross-Resistance to Cisplatin in Cells Resistant to Phótofrin-Mediated Photodynamic Therapy. Roger A. Moorehead, Steven G. Armstrong, Brian C. Wilson, and Gurmit Singh. *Cancer Research* **54**:2556-2559, 1994.

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[CANCER RESEARCH 54, 2556–2559, May 15, 1994]

Advances in Brief

Cross-Resistance to Cisplatin in Cells Resistant to Photofrin-mediated Photodynamic Therapy[1]

Roger A. Moorehead, Steven G. Armstrong, Brian C. Wilson,[2] and Gurmit Singh[3]

Ontario Cancer Treatment and Research Foundation, Hamilton Regional Cancer Centre, and Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Abstract

This study shows that a Photofrin-induced photodynamic therapy-resistant variant (RIF-8A) of a radiation-induced fibrosarcoma-1 cell line (RIF-1) is cross-resistant to *cis*-diamminedichloroplatinum(II) (cisplatin). This is the first study to show cross-resistance to cisplatin in photodynamic therapy-resistant variants *in vitro*. Resistance does not appear to be the result of elevated glutathione levels since neither the resistant variant (RIF-8A) nor the parental line (RIF-1) varied in total glutathione levels. However, cisplatin-DNA adduct levels differed significantly between the two cell types. Immediately following a 1-h exposure to cisplatin (50 μM), RIF-1 cells contained 44.6 ± 2.0 (SEM) pg platinum/μg DNA while RIF-8A cells contained 24.8 ± 6.3 pg platinum/μg DNA. In addition, the resistant variant had decreased plasma and mitochondrial membrane potentials. The plasma and mitochondrial membranes of the resistant variant accumulated 3- and 3.6-fold less rhodamine 123, respectively. The difference in rhodamine 123 accumulation could not be attributed to elevated P-glycoprotein expression because both the parental line and the variant contained similar amounts of P-glycoprotein. In conclusion, alterations in the plasma and/or mitochondrial membrane potentials may provide cells with a survival advantage when challenged with either photodynamic therapy or cisplatin *in vitro*. This appears to be a novel mechanism of resistance.

Introduction

Cisplatin[4] is considered one of the most effective antitumor agents for a large number of solid tumors, but its usefulness is limited by the frequent growth of cisplatin-resistant cell populations (1). Due to the difficulties of investigating drug resistance *in vivo*, the mechanisms involved in cisplatin resistance have traditionally been studied *in vitro* in variants selected through a process of incremental drug exposure over an extended period of time (1). These cisplatin-induced resistant variants are often 10–30 times more resistant to cisplatin than their parental lines. Such selection procedures have been shown to induce multiple changes that contribute to cisplatin resistance including altered drug transport, increased metallothionein or glutathione levels, and altered DNA adduct formation and/or repair, thereby making it difficult to determine the relative importance of each mechanism (2). Alternatively, variants that possess a more modest level of resistance to cisplatin may provide a more appropriate model system to study mechanisms of cisplatin resistance, since resistance greater than about 5-fold may not be clinically relevant (3).

In this paper, we present a novel model system to study cisplatin resistance *in vitro*, utilizing cells resistant to PDT and which demonstrate cisplatin cross-resistance. Previously, we characterized RIF-1 and its PDT-resistant variant (RIF-8A) and demonstrated morphological and functional mitochondrial differences (4). The structural changes in the mitochondria of RIF-8A cells strongly resemble those observed in C13* cells (5), a cisplatin-induced resistant variant of the human ovarian carcinoma cell line (2008), in that the mitochondria in the resistant variants appeared to be in a condensed state with densely staining cristae compared to their respective parental line. The mitochondrial changes in C13* cells contributed to cisplatin resistance through an undetermined mechanism (5, 6). Therefore, RIF-8A cells were further investigated to determine if the mitochondrial structural alterations provided similar functional changes as observed in C13* cells (5) and if the mitochondrial alterations contributed to the cisplatin cross-resistance.

Materials and Methods

Materials. *cis*-Diamminedichloroplatinum(II), 5-sulfosalicylic acid, NADPH, 5,5′-dithiobis(2-nitrobenzoic acid), glutathione reductase, and reduced glutathione were obtained from Sigma Chemical Co., St. Louis, MO. Rhodamine 123 and H33258 were obtained from Eastman Kodak, Rochester, NY, and Calbiochem, La Jolla, CA, respectively. Alkaline phosphatase conjugate, α-minimal essential medium, molecular weight standards, and Mycotect kits were purchased form Gibco BRL, Burlington, Ontario, Canada. The monoclonal antibody for P-glycoprotein (P-glycoMAb C219) was purchased from Cantocor, Malvern, PA.

Cell Lines and Culture Conditions. RIF-1 cells were obtained originally from Dr. B. Henderson, Roswell Park Memorial Institute, Buffalo, NY. The PDT-resistant variant (RIF-8A) was generated in our laboratory by eight selection cycles with Photofrin and light as described previously (7). CHO cells and CHO-MDR were provided by Dr. V. Ling, Ontario Cancer Institute, Toronto, Ontario, Canada. The CHO-MDR line, containing the P-glycoprotein gene (CH^RC5), was derived from the AUX B1 parent line as described by Ling and Thompson (8). RIF-1, RIF-8A, CHO, and CHO-MDR cells were grown in α-minimal essential medium medium containing deoxyribonucleosides and ribonucleosides and supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. All cultures were maintained at 37°C and 5% CO_2 in a humidified incubator. RIF-1 and RIF-8A were tested with a Mycotect kit and were shown to be *Mycoplasma* negative.

Survival Assays. Cells in log-phase growth were seeded overnight at a cell density of approximately 100 cells/well in 6-well plates and were treated, in triplicate, for 1 h with various concentrations of cisplatin (prepared fresh for each experiment in PBS, pH 7.2). At this point, the drug-containing medium was removed and replaced with drug-free medium and the cells were incubated at 37°C for 5 days. Surviving colonies were stained with methylene blue and colonies containing 20 or more cells were counted.

P-glycoprotein Determination. Cells in log phase growth were trypsinized, centrifuged at 400 × *g* for 5 min, and washed once with phosphate-buffered saline. Plasma membranes from these cultures were prepared using the method of Gerlach *et al.* (9). Thirty μg total protein, as determined by the Pierce Micro BCA spectrophotometric protein assay (10), and

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[4] The abbreviations used are: cisplatin, *cis*-diamminedichloroplatinum(II); Rh123, rhodamine 123; PDT, photodynamic therapy; RIF-1, radiation-induced fibrosarcoma-1; RIF-8A, PDT resistant variant of RIF-1; CHO, Chinese hamster ovary; CHO-MDR, multidrug-resistant variant of CHO; TNE buffer, 10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4.

prestained protein molecular weight standards were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9) and transferred to nitrocellulose filters (11). The protein was detected with biotinylated primary antibody specific to P-glycoprotein (1:10,000 dilution of P-glycoMAb in Tris-buffered saline with 0.05% Tween 20) using a modification of the method of Blake *et al.* (12). The blots were photographed and analyzed using an optical densitometer (Bio-Rad Laboratories, Richmond, CA).

Glutathione Assay. RIF-1 and RIF-8A cells were prepared as described previously (13). The total glutathione content was determined by the method of Akerboom and Sies (14) at a wavelength of 412 nm on a Beckman DU-7 spectrophotometer. Data are expressed as nmol/mg protein.

Quantification of Platinum-DNA Adducts. Cells in log-phase growth were seeded overnight in 150- x 25-mm dishes at a density of 8×10^6 cells/plate. Following a 1-h exposure to cisplatin (50 μM in PBS, pH 7.2), cells were washed with drug-free media and harvested by cell scraping with a rubber policeman. The cells were centrifuged at 150 × *g* for 5 min, resuspended in PBS, and again centrifuged at 150 × *g* for 5 min. The supernatant was discarded and the cell pellets were stored at −70°C. DNA was isolated from the cell pellets by resuspending them in digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA (pH 8), 0.5% sodium dodecyl sulfate, 100 μg/ml proteinase K, and 2.5 units/μl RNase T1). Cell digests were incubated at 37°C for 2 h followed by an 18-h digestion at 50°C. Samples were extracted twice with phenol:chloroform (1:1) and centrifuged at 1700 × *g* for 10 min. The aqueous phase was reextracted with chloroform:isoamyl alcohol (25:1) and dialyzed against H_2O for 24 h at 4°C. The samples were lyophilized and reconstituted in PBS (calcium and magnesium free), and DNA content was determined by UV absorbance at 260 nm. Platinum-DNA adducts were determined by flameless atomic absorption spectroscopy with Zeeman background correction, using a Perkin-Elmer 5100 ZL atomic absorption spectrophotometer. For each sample, 250 μg of DNA were digested with nitric acid: perchloric acid (3:1) at ambient temperature for 18 h. After acid digestion, samples were evaporated to dryness and reconstituted in 250 μl of 10% nitric acid, and 25-μl aliquots were injected into the atomic absorption spectroscopy unit. Platinum-DNA adduct levels are expressed as pg platinum/μg DNA as determined from a platinum standard curve prepared in 10% nitric acid. The lower limit of detection was 250 pg of platinum (10 pg platinum/μg DNA).

Rhodamine 123 Uptake. RIF-1 and RIF-8A cells in log-phase growth were seeded overnight in 24-well plates at a density of 5×10^4 cells/well and treated with Rh123 (100 μM) for 1 h. The Rh123 accumulation was determined in normal potassium media (whole cell uptake), media supplemented with 137 mM potassium chloride (for selective depolarization of the plasma membrane potential), or normal potassium media supplemented with 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (for selective depolarization of mitochondrial membrane potential) (5). Following Rh123 removal, the wells were washed twice with phosphate-buffered saline, and deionized water was added (300 μl/well). The plates were then stored, protected from light, at room temperature for a minimum of 2 h. In order to determine the DNA content in each well, 100 μl of the DNA fluorochrome, H33258 (40 μg/ml in TNE buffer), were added to each well (15). The plates were read on a Cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ontario, Canada) using excitation/emission wavelengths of 485/530 nm for Rh123 and 360/460 nm for the DNA fluorochrome (H33258). Cell number/DNA and Rh123 standard curves were generated to enable the fluorescent readings to be converted to fmol of Rh123/cell.

Statistics. All values report the mean ± SEM unless otherwise specified. Statistical analysis was carried out using an unpaired Student's *t* test; $P < 0.05$ was considered statistically significant.

Results and Discussion

Clonogenic survival studies were performed to compare cisplatin sensitivity in RIF-1 and RIF-8A cells (Fig. 1). The 90% inhibitory concentrations for a one h cisplatin exposure were 9.0 μM for RIF-1 cells and 14.6 μM for RIF-8A cells. The resistant variant was approximately 1.6-fold resistant to cisplatin. Since this is the first time cross-resistance to cisplatin in a PDT-induced resistant variant *in vitro* has been demonstrated, the focus of this study was to investigate possible mechanism(s) involved in the cross-resistance to cisplatin.

It has been demonstrated previously that elevated levels of glutathione, a known scavenger of cisplatin, can contribute to cisplatin resistance (16). However, there was no apparent difference in total cellular glutathione (oxidized glutathione and reduced glutathione) content inasmuch as RIF-1 contained 5.1 nmol/mg protein while the variant RIF-8A contained 5.4 nmol/mg protein (Table 2). Our observations are consistent with those of Luna and Gomer (17), who found no differences in reduced glutathione and glutathione peroxidase in RIF-1 cells compared to their PDT-resistant variants.

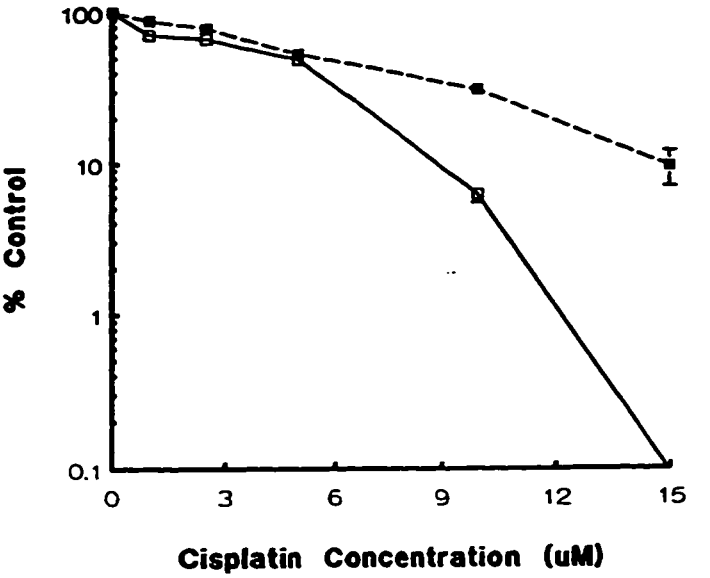

Fig. 1. Effect of cisplatin on RIF-1 and RIF-8A survival. Cytotoxicity was assessed by clonogenic survival after a 1-h cisplatin exposure. (□) RIF-1; (■) RIF-8A. Values are expressed as percentage of control ± SEM (*bars*). Representative experiment, experiment repeated at least twice.

Table 1 *P-glycoprotein content in RIF-1, RIF-8A, CHO, and CHO-MDR cells*

	CHO	CHO-MDR	RIF-1	RIF-8A
P-glycoprotein[a]	0.75 ± 0.02	2.29 ± 0.22[b]	0.60 ± 0.12	0.70 ± 0.07

[a] Values are expressed as arbitrary absorbance units/mg protein ($n = 3$).
[b] $P < 0.05$ for CHO *versus* CHO-MDR.

Another common form of drug resistance in tumor cells involves MDR resistance. The main component of MDR is the M_r 170,000 plasma membrane protein, P-glycoprotein, which is an energy-dependent efflux pump with an affinity for a variety of chemotherapeutic agents (18). We have shown previously that there is no difference in the survival of RIF-1 and RIF-8A cells following Adriamycin administration (7), and the present study demonstrates that there are no significant differences in P-glycoprotein content between RIF-1 cells and the variant RIF-8A. Western blots were used to quantitate P-glycoprotein levels in four different cell types: RIF-1; RIF-8A; CHO; and CHO-MDR cells (Table 1). CHO-MDR cells were used as a positive control and contained approximately 3 times the amount of P-glycoprotein found in CHO cells; however, there was no significant difference in P-glycoprotein content between RIF-1 and RIF-8A. Although P-glycoprotein (M_r 170,000) is not believed to play a role in cisplatin resistance, Rh123 has been shown to be actively extruded by this protein (19). Since Rh123 was used as an indicator of membrane potential in this study, it was crucial to demonstrate that a P-glycoprotein-dependent mechanism did not influence the Rh123 results.

Mitochondrial alterations have been shown to be associated with cell transformation, but little is known about their role in drug resistance (20). Our previous characterization of RIF-1 and RIF-8A cells revealed structural changes in the mitochondria of the resistant variant

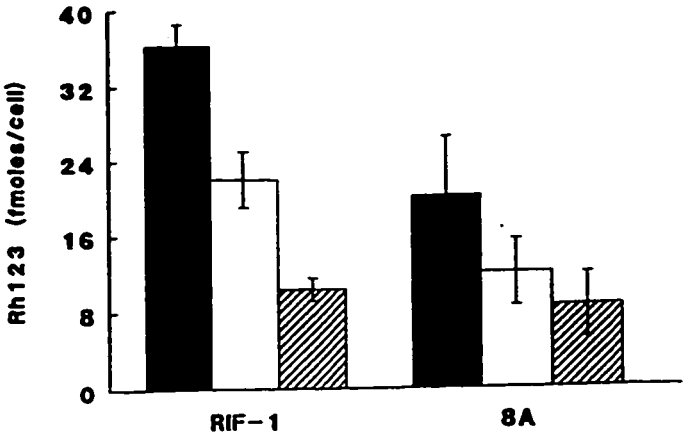

Fig. 2. Rh123 accumulation in RIF-1 and RIF-8A cells. Rh123 accumulation was tested in (□) normal α-minimal essential media, (▨) α-minimal essential medium + 137 mM KCl, and (▩) α-minimal essential medium + 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in order to determine whole cell, mitochondrial, and plasma membrane Rh123 accumulation respectively. *Columns*, mean (n = 4); *bars*, SEM.

(4). Interestingly, the mitochondrial structure of RIF-8A was strikingly similar to that of a human ovarian cisplatin-induced resistant variant, C13* (4, 5). Furthermore, we have shown C13* cells to be cross-resistant to Photofrin-mediated PDT (4). Thus, the observation that RIF-8A cells are cross-resistant to cisplatin suggests possible common mechanisms of resistance related to the mitochondrial alterations. However, the ultrastructural changes of the mitochondria in RIF-8A cells were not accompanied by an increase in mitochondrial membrane potential, in contrast to that observed in C13* cells (Fig. 2). Rh123, which accumulates in response to membrane potential, was used to compare both the plasma and mitochondrial membrane potentials in RIF-1 and RIF-8A cells (21, 22). The mitochondrial and plasma membranes of RIF-8A cells accumulated 3.6- and 3-fold less Rh123, respectively, relative to RIF-1 cells (Table 2). As a result of a cell size and mitochondrial area difference between RIF-1 and RIF-8A cells, Rh123 accumulation is expressed as amol of Rh123/mm^2 of mitochondrial area and amol of Rh123/mm^2 of cytoplasmic area, since a doubling of mitochondrial area could increase Rh123 accumulation independent of a membrane potential difference (5). Mitochondrial and cytoplasmic areas of RIF-1 and RIF-8A were published previously by Sharkey *et al.* (4).

Even though an increase in mitochondrial membrane potential is believed to indicate an enhanced rate of ATP production via oxidative phosphorylation (22), diminished mitochondrial membrane potential in RIF-8A cells did not compromise total cellular ATP content (4). This observation could be interpreted to suggest that RIF-8A cells have an increased dependence on ATP production via glycolysis; however, both electron microscopy and 2-deoxyglucose sensitivity (4) suggest that this is not the case. Ultrastructural changes associated with RIF-8A mitochondria appear similar to those described by Hackenbrock *et al.* (23) as being in a condensed state indicative of elevated oxidative phosphorylation activity, in contrast to the "orthodox-like" mitochondria in RIF-1 cells. Also, RIF-1 cells are more sensitive than RIF-8A cells to 2-deoxyglucose toxicity, an inhibitor of glycolytic ATP production, suggesting that RIF-8A cells have a reduced relative dependence on glycolysis (4).

Another possible explanation for the observed decrease in mitochondrial membrane potential in RIF-8A without a corresponding decrease in total cellular ATP is that the mitochondria in RIF-8A cells express a greater portion of their electrochemical gradient as a pH gradient rather than as a membrane potential. The driving force behind ATP synthesis is the mitochondrial electrochemical gradient, which consists of both a membrane potential and a pH gradient (22). Thus, the mitochondria in RIF-1 and RIF-8A cells may have similar electrochemical gradients but vary in the relative contribution of the two aforementioned components. A final possible explanation is that the mitochondria of RIF-8A are more tightly coupled than those of RIF-1 and thus have increased their ATP production efficiency. Not all protons extruded by the electron transport chain return via the F_1F_0ATPase, thus producing ATP; some protons leak across the inner mitochondrial membrane reducing the efficiency of ATP synthesis (24). The hypothesis of an increased efficiency is supported by previous work which demonstrated that RIF-8A cells utilize less oxygen than RIF-1 cells to maintain similar intracellular ATP pools (4).

Several investigators have suggested that mitochondria may play a role in determining cisplatin sensitivity. Cisplatin has been shown to bind to mitochondrial DNA with greater affinity than nuclear DNA (25). In addition, combination chemotherapy using cisplatin and agents that alter mitochondrial function (*i.e.*, lipophilic cations) have been shown to enhance cisplatin cytotoxicity *in vitro* (26) and *in vivo* (27). Furthermore, Shinomiya *et al.* (28) demonstrated that Rh123 fluorescence intensity in cells increased following exposure to cisplatin. Finally, a human ovarian cancer cell line (2008) and its resistant variant (C13*) have been shown to respond to cisplatin by elevating their ATP production (29).

Cisplatin-DNA interactions also differed between RIF-1 and RIF-8A cells. Cisplatin-DNA adduct levels were determined by atomic absorption spectometry immediately following a 1-h exposure to cisplatin (50 μM) (Table 2). RIF-8A cells contained 24.8 ± 6.3 pg platinum/μg DNA which was significantly less than the 44.6 ± 2.0 pg platinum/μg DNA found in RIF-1 cells. Since the cytotoxic actions of cisplatin are believed to arise through its interaction with DNA, a decreased amount of DNA damage could contribute to cisplatin resistance (2). Thus, the 2-fold difference in adduct levels could account for the cisplatin resistance observed (1.6-fold). A number of other studies examining either intra- or interstrand cross-links in cisplatin-resistant variants have also shown adduct levels to be decreased in their resistant variants (30–32), including C13* cells (33). If platinum-DNA adduct levels reflect a net balance between adduct formation and repair, the lower number of adducts in the cisplatin-resistant variants may suggest an up-regulated platinum-DNA adduct repair capacity.

In this regard, the structurally and functionally altered mitochondria in RIF-8A (and C13*) may influence the efficiency of DNA repair. For example, the enzymes involved in DNA synthesis and/or repair may increase the energy requirements of the cell and thus trigger a demand for ATP production. This increased energy demand could result, in turn, in an elevation in mitochondrial activity, similar to the increase in mitochondrial activity observed during normal cell proliferation (20, 28). Therefore, enhanced capacity to produce ATP in response to cisplatin damage may provide the energy required by the enzymes involved in the repair of cisplatin adducts, even though basal

Table 2 *Comparison of parental and PDT-resistant variant*

	RIF-1	RIF-8A
Glutathione (nmol/mg protein)[a]	5.1	5.4
Pt-DNA adducts[b,c] (pg Pt/μg DNA)	44.6 ± 2.0	24.8 ± 6.3[d]
Mitochondrial Rh123 accumulation[e] (amol/mm^2 mitochondrial area)	8188 ± 1120	2275 ± 640[d]
Plasma membrane Rh123 accumulation[e] (amol/mm^2 cytoplasmic area)	156 ± 20	52 ± 20[d]

[a] n = 2.
[b] n = 3.
[c] Adduct levels immediately after a 1-h exposure to cisplatin (50 μM).
[d] $P < 0.05$ for RIF-1 *versus* RIF-8A.
[e] n = 4.

ATP levels, in the absence of cisplatin, were equivalent in RIF-1 and RIF-8A (4).

In summary, we have demonstrated that a PDT-resistant variant is cross-resistant to cisplatin. One of the mechanisms that apparently contributes to the resistance is a decrease in platinum-DNA adduct levels in the resistant variant. The Rh123 accumulation experiments suggest that both the plasma and mitochondrial membrane potentials are reduced in the cisplatin-resistant variant. This study suggests a novel mode of resistance.

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Discussion

RIF-8A cells were found to be approximately 1.6-fold resistant to cisplatin compared to the parental RIF-1 cells as determined by a colony forming assay at an IC_{50} concentration. Although the amount of resistance is not that large this is the first report to demonstrate that PDT-resistant cells are cross-resistant to cisplatin. Furthermore, this level of resistance may represent a clinically relevant amount of cisplatin resistance (28).

Since RIF-8A cells were resistant to cisplatin and contained mitochondria that appeared structurally similar to the mitochondria in C13* cells, the mitochondrial membrane potential in RIF-8A cells was examined. Rh123 accumulation by the whole cell, mitochondria, and plasma membrane of RIF-1 and RIF-8A cells was assessed. On a per cell basis, RIF-1 mitochondria accumulated slightly more Rh123 than RIF-8A mitochondrial but the difference was not significant. An increase in mitochondrial Rh123 accumulation could reflect either an increase in mitochondrial membrane potential without an increase in mitochondrial mass or an increase in mitochondrial mass without an increase in mitochondrial membrane potential. Andrews and Albright (152) observed no significant increase in mitochondrial mass between 2008 and C13* cells as determined at the electron microscopy level. Therefore, the elevation in mitochondrial Rh123 accumulation in C13* cells presumably reflects an elevation in mitochondrial membrane potential. When electron micrographs of RIF-1 and RIF-8A cells were compared in the previous manuscript, it was

observed that RIF-8A were approximately twice as large as RIF-1 cells and contained an approximate 2-fold increase in mitochondrial area. Normalizing the mitochondrial Rh123 accumulation per mitochondrial area indicated that RIF-8A cells had significantly lower mitochondrial membrane potentials than RIF-1 cells. Similarly, when plasma membrane accumulation of Rh123 was normalized using the average cross-sectional area of the two cell types, RIF-8A cells had significantly lower plasma membrane potentials than RIF-1 cells.

These data are not consistent with the observed increase in mitochondrial membrane potential associated with cisplatin resistance in the human ovarian model system. Before it could concluded that Rh123 accumulation was lower in RIF-8A cells than in RIF-1 cells, P-glycoprotein content had to be determined in the two cell types. P-glycoprotein is a plasma membrane pump that actively pumps out cytotoxic agents and thus lowers the intracellular concentration of several drugs. More recently, Rh123 has been shown to be pumped out of cells by P-glycoprotein and accumulation and efflux of Rh123 are used to determine the presence of increased P-glycoprotein expression (310-313).

Using western analysis, CHO cells and multi-drug resistant CHO (CHO-MDR) cells verified that our western protocol was capable of detecting differences in P-glycoprotein content. However, P-glycoprotein levels in RIF-1 and RIF-8A cells did not differ significantly. Indirect assays have also been used to suggest that P-glycoprotein levels are similar in RIF-1 and RIF-8A cells. Our lab has shown that while CHO-MDR cells are resistant to adriamycin (a P-

glycoprotein substrate) and accumulate less Photofrin II than CHO cells there is no significant difference in adriamycin sensitivity or Photofrin II accumulation between RIF-1 and RIF-8A cells (303). Therefore, the decreased Rh123 accumulation appears to reflect a decrease in mitochondrial and plasma membrane potentials in RIF-8A cells relative to RIF-1 cells.

A decreased mitochondrial membrane potential in RIF-8A cells compared to RIF-1 cells is somewhat surprising considering the previous manuscript suggested that electron transport chain activity was elevated in RIF-8A cells compared to RIF-1 cells. It is possible that RIF-8A cells express their mitochondrial electrochemical gradient primarily as a pH gradient rather than a membrane potential. Andrews and Albright (152) showed that 2008 and C13* cells have similar mitochondrial electrochemical gradients but C13* cells express their electrochemical gradient primarily as a membrane potential while 2008 cells express theirs primarily as a pH gradient. The significance of the relative contributions of a membrane potential and a pH gradient to the overall electrochemical gradient are unknown (165).

The fact that RIF-8A cells produce more ATP than RIF-1 cells while maintaining a lower mitochondrial membrane potential and consuming less oxygen may indicate that RIF-8A cells are more dependent on glycolysis for ATP production. This possibility appears improbable considering the previous manuscript showed that RIF-1 cells were more sensitive to the competitive glycolytic inhibitor 2-deoxyglucose than RIF-8A cells.

Other cellular characteristics that could contribute to cisplatin resistance in RIF-8A cells were also investigated. Glutathione is an intracellular protein capable of scavenging cisplatin and thus prevents cisplatin from interacting with critical intracellular components (69). Glutathione content was found to be similar in RIF-1 and RIF-8A cells suggesting that sequestration of cisplatin by glutathione does not contribute to the cisplatin resistance in RIF-8A cells. The amount of DNA-bound cisplatin as measured by atomic absorption spectroscopy indicated that RIF-8A cells contained approximately 2-fold fewer cisplatin-DNA lesion than RIF-1 cells immediately after a one hour cisplatin incubation. The mechanism responsible for decreased cisplatin-DNA lesions in RIF-8A compared to RIF-1 cells is unclear and requires further investigation. After the publication of this manuscript, the number of cisplatin-DNA lesions following cisplatin treatment in 2008 and C13* was reported. It was observed that cisplatin resistance in C13* cells was also associated with a decreased number of cisplatin-DNA lesions compared to 2008 cells (126).

Therefore, in this manuscript, it was observed that RIF-8A cells were resistant to cisplatin compared to RIF-1 cells. Although the previous manuscript showed that RIF-8A mitochondria were similar in structure to mitochondria in C13* cells, this manuscript showed that the structural changes in RIF-8A mitochondria were not associated with an increase in mitochondrial membrane potential. This study suggests that the density of mitochondrial cristae staining does not necessarily reflect the mitochondrial membrane potential. Additional

support for this observation comes from RH4 cells. Although the mitochondria of C13* and RH4 cells appear similar at the electron microscopy level, RH4 mitochondria accumulate less Rh123 than C13* mitochondria (153). It should be noted that since the pH gradient of RIF-8A cells was not examined a decrease in mitochondrial membrane potential does not necessarily reflect a decreased rate of oxidative phosphorylation.

Although the decreased formation of cisplatin lesions is capable of accounting for the cisplatin resistance in RIF-8A cells it is difficult to exclude the mitochondrial alterations as a contributing factor in drug resistance because, (i) PDT resistance has been associated with mitochondrial alterations, (ii) cisplatin resistance has been associated with mitochondrial alterations, (iii) cisplatin-resistant variants containing mitochondrial alterations are cross-resistant to PDT, and (iv) PDT-resistant variants containing mitochondrial alterations are cross-resistant to cisplatin. Since it is unclear how mitochondrial alterations could contribute to drug resistance, the next manuscript examined whether changes in mitochondria can alter the capacity of cells to repair cisplatin lesions from their DNA.

Chapter 4 The repair of cisplatin-damaged DNA in human ovarian carcinoma cells resistant to cisplatin.

Repair of cisplatin-damaged nuclear DNA has been implicated as an important characteristic in cisplatin resistance as a result of several findings; (i) cisplatin can covalently bind to DNA and can inhibit DNA synthesis and transcription, (ii) mammalian cell lines deficient in nucleotide excision repair (NER) are hypersensitive to cisplatin, (iii) agents that inhibit DNA repair can potentiate cisplatin's cytotoxicity, and (iv) several cisplatin-resistant variants generated *in vitro* have enhanced repair of cisplatin-DNA lesions compared to their parental line.

Cisplatin preferentially binds to the N7 position of purines to form a number of monofunctional and bifunctional DNA adducts (4). Although only about 1% of the total intracellular cisplatin eventually interacts with nuclear DNA, it is this interaction that is thought to be critical for cytotoxicity (25). Cisplatin can form both intrastrand adducts and interstrand cross-links with DNA. Intrastrand adducts occur with the relative abundance of approximately 40-75% d(GpG)Pt, 10-20% d(GpNpG)Pt, 15-25% d(ApG)Pt. Monoadducts, DNA-protein crosslinks, and interstrand crosslinks comprise less than 5% of the total cisplatin-DNA lesions (21-24). Cisplatin has been demonstrated to inhibit DNA replication and transcription but controversy remains as to which cisplatin-DNA lesion is critical in interfering with these processes (314-317).

Several lines of evidence have demonstrated the importance of NER in determining cisplatin sensitivity including the use of cells deficient in NER.

Xeroderma pigmentosum (XP) is a human disease characterized by UV hypersensitivity and an increased incidence of skin cancer and neurological abnormalities (318-321). Seven different complementation groups, A to G, have been identified in XP (322,323). XP-A and XP-E, have been implicated in DNA damage recognition (318). XP-B and XP-D are components of the TFIIH complex and appear to function as DNA helicases that unwind DNA during transcription and repair (318,324-326) . XP-F (complexed with ERCC1) and XP-G have been reported to have DNA nuciease activity, while the function of XP-C is unknown (318).

Approximately 20% of XP patients do not fall within one of the seven complementation groups and have been classified as XP variants or XP-V. These XP patients present similar clinical characteristics as other XP patients but fibroblasts from XP-V patients repair UV-DNA damage at rates similar to control fibroblasts. It appears that unlike normal cells, XP-V cells are less efficient at bypassing UV lesions during DNA replication (327). In normal cells, UV lesions can be by-passed during DNA replication. These by-passed lesions are eventually repaired by a process termed 'post-replication' repair (318,327).

NER involves: detection of damaged DNA, incision 3' and 5' of the damage, excision of the damaged DNA strand, DNA synthesis, and ligation of the newly synthesized DNA (134,319,320,328). A simplified version of the role of XP proteins in NER may then occur as follows, XP-A and XP-E recognized damaged DNA. XP-A is capable of binding the XP-F/ERCC1 complex and the

replication protein HSSB (RPA) (329,330). XP-A also appears to recruit the TFIIH complex which may contain XP-C (331). XP-B and XP-D, components of the TFIIH complex, unwind the DNA using their DNA helicase activity which is required because incisions must take place on single-stranded DNA. Once unwound, XP-G, recruited by TFIIH, at the 3' end and possibly XP-F/ERCC1 complex, recruited by XP-A, at the 5' end, incise the DNA (332-334). Incision of damaged DNA usually occurs at the 5th phosphodiester bond (ranges from 3rd to 8th phosphodiester bond) 3' and the 24th phosphodiester bond (ranges from 20th to 26th phosphodiester bond) 5' of the DNA lesion (335,336). The incised strand is then displaced presumably by TFIIH (337). The DNA gap is filled in by DNA polymerase δ or ε and DNA ligation completes the repair process (320,331,338). Both DNA unwinding and incision require ATP hydrolysis (319,336).

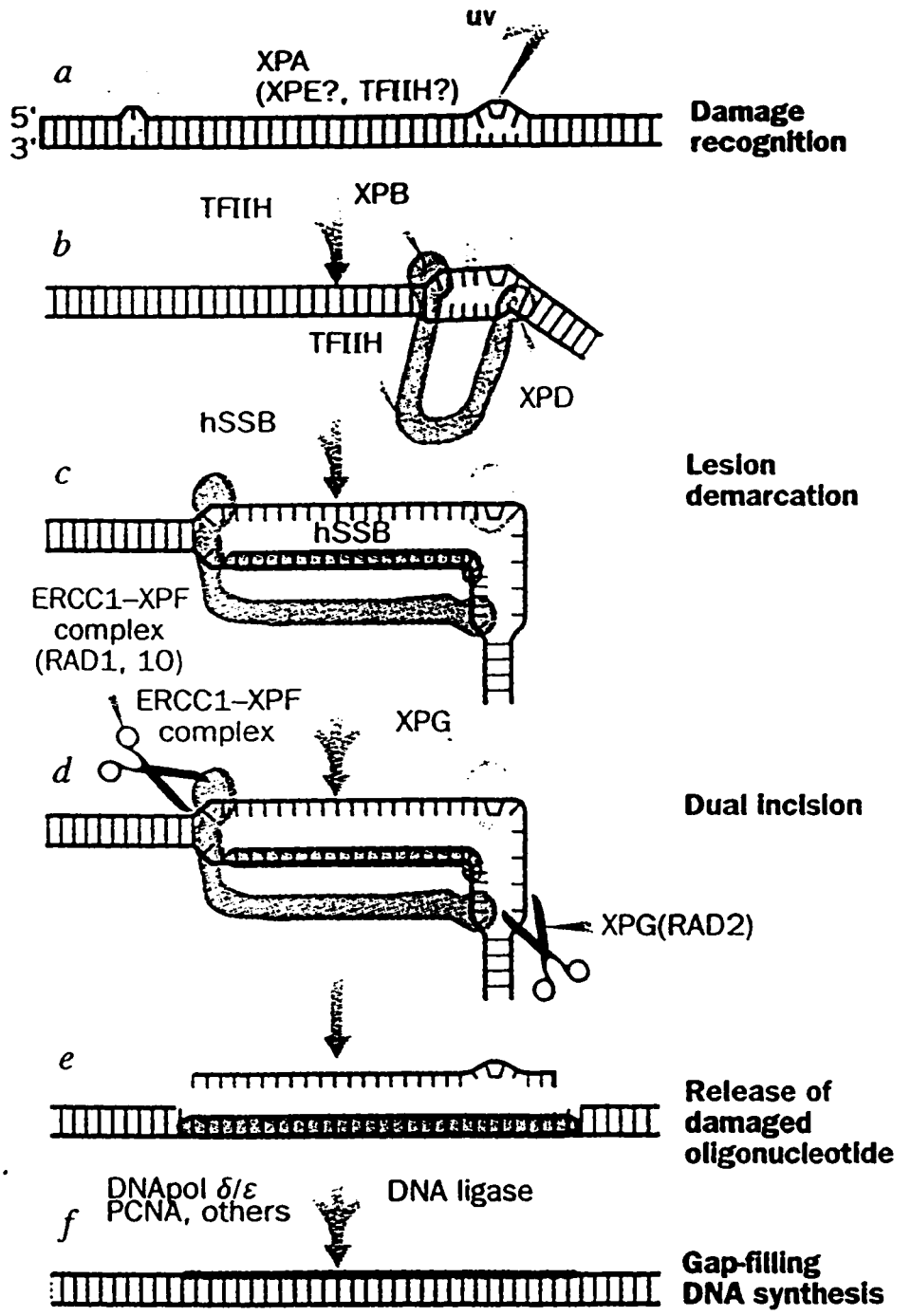

Figure 4. A schematic of NER. This figure was adapted from (320).

As a result of their deficiency in NER, fibroblasts from patients with XP have been shown to be hypersensitive to UV due to a decreased capacity to repair UV-induced DNA damage (111,339-342). Increased cisplatin sensitivity and decreased capacity to repair cisplatin-DNA lesions have also been observed in fibroblasts from patients with XP (110-112). These results suggest that cisplatin-induced DNA damage is repaired via NER and that defects in NER render cells more sensitive to cisplatin.

Rodent cells deficient in NER have also been used to study DNA repair and 11 complementation groups have been identified (318). Most of the rodent complementation groups correspond to complementation groups of human XP and another human disease characterized by a deficiency in DNA repair, namely Cockayne's syndrome (CS) (319). In fact, some of the human XP genes were cloned by virtue of complementing (correcting the repair defect) in repair-deficient rodent cell lines. Because of their ability to complement the repair defect in repair-deficient rodent cells these genes are also referred to as excision repair cross complementation (ERCC) genes (343,344). ERCC2, ERCC3, ERCC5 and ERCC6 have been shown to be equivalent to XP-D, XP-B, XP-G and CS-B respectively (318,345-348).

Most of the research on repair-deficient rodent cell lines, with respect to cisplatin, has focused on the ERCC1 gene. As previously mentioned, this gene has been proposed to complex with XP-F and perform an incision 5' of a DNA lesion. Studies have shown that mutants defective in ERCC1 are hypersensitive

to cisplatin and transfection of rodent complementation group one mutants, with human ERCC1, can restore some of the cisplatin resistance apparently by increasing repair of cisplatin lesions (113,114). In addition, human ovarian carcinoma patients resistant to cisplatin-based therapy were found to overexpress ERCC1 (349). However, the importance of ERCC1 in cisplatin-resistance remains unclear since overexpression of ERCC1 in normal CHO cells sensitized these cells to cisplatin (350) and no patients defective in ERCC1 have yet been identified (320).

In addition to cell mutants deficient in NER, the importance of DNA repair in determining cisplatin sensitivity has been demonstrated using agents capable of inhibiting different components of DNA synthesis and/ or repair. Inhibitors of DNA polymerase α and δ (115,118,122), topoisomerase I (351), topoisomerase II (352), and DNA synthesis (119,120,162,353,354) have all been shown to potentiate cisplatin's cytotoxicity. Several of these studies observed a decrease in the rate of repair of cisplatin lesions following the combination of cisplatin and repair inhibitor compared to that when cisplatin was administered alone (119,162,351-353,355). A potentially more important finding is that repair inhibitors were observed to increased cisplatin cytotoxicity in cisplatin-resistant cells but not in cisplatin-sensitive cells (115,122,353,354). Therefore, eliminating the DNA repair advantage in cisplatin-resistant variants may render these cells hypersensitive to cisplatin.

Repair of cisplatin lesions has also been directly examined in variants resistant to cisplatin. Initially, repair of cisplatin-DNA damage was investigated at the level of the entire genome. Enhanced repair of total genomic DNA was correlated with decreased cisplatin sensitivity in many of the studies (25,37,58,87,115,121,127) but not all (57,63,66,107,126,131). Some studies have also specifically examined the repair of either cisplatin intrastrand adducts or interstrand crosslinks and have observed a correlation between enhanced repair and cisplatin resistance (58,65,87,356). However, not all of the studies were able to demonstrate this correlation (57,61,62,132,356). More recently, preferential repair of UV-induced DNA damage in actively transcribed genes in Chinese hamster ovary (CHO) cells was described (21,133-136). It appears that UV-induced DNA damage is not only repaired faster in actively transcribed genes compared to the total genome but within an active gene the transcribed strand is repaired faster than the non-transcribed strand (135).

A possible explanation as to why DNA damage is repaired faster in transcribed genes than in non-transcribed genes is that transcription and NER are coupled. This coupling has been identified in yeast where a DNA helicase can bind to a RNA polymerase complex that has been stalled by DNA damage and initiate NER (357). In mammalian cells, the DNA helicase involved in transcription-coupled NER may be ERCC6, the gene mutated in CS (331,348). It has also been proposed that the DNA of active genes is more unwound and thus more accessible to DNA repair proteins (318). Another possible explanation for the enhanced rate of repair

in actively transcribed genes is that certain repair proteins may be localized within the transcription complex. This localization may lead to an increased number of repair proteins already in active genes and thus expedite the rate of assembly of the repair complex (318).

Since actively transcribed genes represent less than 1% of the total genome, enhanced repair of actively transcribed genes would not be detected by assays that evaluate total genomic DNA repair (134). In studies that have evaluated the repair of cisplatin-induced DNA damage in actively transcribed genes, there appears to be a correlation between enhanced repair of cisplatin interstrand crosslinks and resistance (37,126,127). However inconsistencies within these reports should be taken into consideration when evaluating the data. Two studies by the same group, in the same cells, reported different results. An increased rate of repair of cisplatin interstrand crosslinks from the ribosomal RNA gene compared to a non-coding region was observed in one of their studies (37) but not in the other (127). In addition, Zehn *et al.* (126) did not observed an increase in total genomic repair in the cisplatin resistant variant, A2780CP70, compared to its parental line while other investigators have reported an increased rate of repair of cisplatin-DNA lesions from the total genome in A2780CP70 cells compared to their parental line (25,37,115). Therefore, more studies in which the repair of cisplatin intrastrand adducts and interstrand crosslinks, at the level of the total genome and within specific genes, are required to determine the importance of DNA repair in cisplatin resistance.

Inconsistencies with respect to the repair of cisplatin-damaged DNA can be attributed to a number of factors; (i) a number of different mechanisms can lead to cisplatin resistance and in variants with high levels of resistance more than one mechanism is likely to be present, (ii) the presence of more than one cisplatin-resistant mechanism makes it difficult to obtain similar amounts of DNA damage in cisplatin-sensitive and cisplatin-resistant cells. This factor is important since the initial amount of DNA damage may influence repair rates (358,359), (iii) many studies only examine one type of cisplatin-induced lesion , (iv) different methods for measuring DNA repair are used, and (v) some studies have examined repair of the entire genome while others have examined repair within actively transcribed genes.

To avoid some of the aforementioned limitations, a host cell reactivation (HCR) of an adenovirus type 5 (Ad 5) was used to provide an indication of DNA repair in this manuscript. HCR measures the ability of cells to repair damaged viral DNA which is interpreted to reflect the cell's ability to repair its own DNA. This technique has been used to examine repair capacities in DNA repair-deficient syndromes such as XP (318,339,341,342). In addition, the HCR assay, using either a plasmid or a virus has been used to examine the ability of cells to repair damage induced by a number of agents including cisplatin (25,110,112,121,360,361). The HCR assay also permits the exposure of cisplatin-sensitive and cisplatin-resistant cells to the same amount of cisplatin-damaged DNA and high cisplatin concentrations are not required. In fact, the

cells are not exposed to cisplatin at all which also eliminates other potential mechanisms of cisplatin resistance such as altered accumulation or scavenging, from influencing the results.

In the following manuscript, an association between an elevated mitochondrial membrane potential in C13* cells and an increased capacity to repair cisplatin-damaged DNA compared to either 2008 or RH4 cells, was investigated. It was proposed that an elevated mitochondrial membrane potential may contribute to the cisplatin resistance by supplying energy needed to increase repair efficiency. A correlation between mitochondrial membrane potential and DNA repair was examined in an attempt to identify a mechanism to explain how an increase in mitochondrial membrane potential could contribute to cisplatin resistance in C13* cells. Since NER has been shown to be an energy-requiring process as both the unwinding of DNA and DNA incision require ATP hydrolysis, it is conceivable that DNA repair rates may depend on ATP availability (319,331,336). Furthermore, it has been suggested that novobiocin inhibits NER through inhibiting ATP production by mitochondria rather than a direct effect on topoisomerase (362).

All assays in this manuscript were conducted by R.A. Moorehead except DNA adduct measurements and aphidicolin sensitivity which were performed by Dr. S.G. Armstrong (postdoctoral fellow in our laboratory).

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ORIGINAL ARTICLE

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Nucleotide excision repair in the human ovarian carcinoma cell line (2008) and its cisplatin-resistant variant (C13*)

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Abstract Repair of cisplatin-damaged DNA was investigated in a human ovarian carcinoma cell line (2008) and its cisplatin-resistant variant (C13*) using a host-cell reactivation (HCR) assay. The HCR of cisplatin-damaged adenovirus (Ad) was not significantly different in C13* cells compared to 2008 cells. The cisplatin concentrations required to reduce the amount of viral DNA replicated to 50% were 0.12 ± 0.02 μM and 0.10 ± 0.01 μM after 48 h of repair in 2008 and C13* cells respectively. Similarly, the cisplatin concentration required to reduce the expression of a reporter gene inserted in the viral DNA was not significantly altered in C13* cells compared to the parental line (IC_{50} values were 0.28 ± 0.04 μM in 2008 cells and 0.17 ± 0.06 μM in C13* cells after 48 h of repair). Pretreatment of the cells with cisplatin, immediately prior to Ad infection, did not significantly alter the HCR of cisplatin-damaged Ad in either cell type. In addition, a cisplatin-sensitive variant derived from the C13* cells, namely the RH4 cells, did not differ significantly from either the 2008 or C13* cells in their ability to reactivate cisplatin-damaged Ad. Furthermore, a component of the nucleotide excision repair (NER) pathway, DNA polymerase α, was investigated using the competitive inhibitor aphidicolin. The combination of cisplatin and aphidicolin resulted in similar synergistic growth inhibition in both the 2008 and C13* cells providing additional support to the HCR results which suggest that enhanced NER is not responsible for the cisplatin resistance in C13* cells.

Key words Host cell reactivation · Cisplatin · DNA repair

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Introduction

Cisplatin-induced cytotoxicity is generally believed to be mediated through its ability to form intrastrand adducts and interstrand crosslinks within the cellular genome which can inhibit the transcription of genes and the replication of DNA (reviewed in references 5, 7, 12, 28 and 34). Thus, investigations into the mechanism(s) underlying cisplatin resistance have concentrated on the repair of these potentially lethal lesions. However, a consensus on the role of DNA repair in cisplatin resistance has not been reached. The variability of tumor type, cisplatin dose and the type of lesion investigated (intrastrand or interstrand), has obscured the importance of DNA repair in cisplatin resistance. The multifactorial nature of cisplatin-induced resistance makes it difficult to delineate a single mechanism that contributes to the resistance. In addition to enhanced repair of cisplatin-damaged DNA, mechanisms including altered drug transport, increased metallothionein or glutathione levels, mitochondrial alterations, and altered DNA adduct formation have been reported to contribute to cisplatin resistance [1, 7, 44]. It has been shown that equivalent extracellular cisplatin concentrations can result in a difference in cisplatin-DNA adduct formation in cisplatin-sensitive and cisplatin-resistant cells (as a result of decreased

accumulation or increased scavenging of cisplatin) [43]. Since the induction of certain genes (i.e. DNA damage-inducible genes) may be dependent on the amount of DNA damage, as is the case with UV irradiation [11], the initial amount of cisplatin-DNA damage may influence the rate of DNA repair. Therefore, inducing similar amounts of DNA damage in cisplatin-sensitive and cisplatin-resistant cells may be more appropriate when comparing DNA repair capacities in different cell types.

Zhen et al. [43] investigated total genomic and gene-specific repair of cisplatin intrastrand adducts and interstrand crosslinks in human ovarian carcinoma cells and suggested that enhanced repair of gene-specific interstrand crosslinks, but not intrastrand adducts, contributes to cisplatin resistance. Their study used atomic absorption spectroscopy (AAS) to assess the repair of cisplatin-DNA damage and as a result of the detection limits of AAS, cells were treated with supralethal cisplatin concentrations (as high as 1000 times the IC_{50}); it remains unclear how drug concentrations in this range affect the relative distribution of lesions and cellular repair capacity [38, 41]. Also, different initial levels of cisplatin-DNA damage were induced in the parental line and the cisplatin-resistant variant and as previously mentioned, the amount of DNA damage may influence the rate of DNA repair [11]. Data further supporting the idea that the amount of DNA damage may influence DNA repair rates have been reported by Johnson et al. [17]. They found that when the initial DNA platination levels are similar, repair of cisplatin damage from total genomic DNA is greater in the cisplatin-resistant variant A2780/CP70 relative to the parental A2780 line, after 8 h of repair time, whereas Zhen et al. [43] found no differences in repair between these cells at that time point when initial adduct formation differed in the two cell types. Parker et al. [25] have also shown in A2780 cells that the initial cisplatin adduct level can influence the rate of adduct removal.

We confirmed using both AAS and a polyclonal antibody to cisplatin adducts that equivalent cisplatin concentrations resulted in decreased adduct formation in the cisplatin-resistant variant compared with the parental line. Furthermore, supralethal cisplatin concentrations were required to obtain detectable levels of DNA adducts in both assays. To avoid the complications of supralethal cisplatin concentrations or different initial levels of DNA damage, we used a host cell reactivation (HCR) assay to investigate the repair capacities of the cells in our study. In the HCR assay, adenovirus type 5 (Ad 5) is incubated with cisplatin in order to induce cisplatin-viral DNA adducts. The cells are then infected with the cisplatin-damaged virus and their ability to replicate or transcribe the damaged viral DNA serves as an indicator of the repair capacity of the cell. Since the virus is exposed to cisplatin prior to infection, the cells are not directly exposed to the drug and only nanomolar concentrations of cisplatin are required to produce sufficient amounts of damage to the viral DNA. In addition, each cell type is exposed to an equal amount of cisplatin-damaged DNA.

Ad 5 can be used to investigate the repair of cisplatin damage because Ad 5 DNA is a linear duplex and its repair and replication are dependent on the enzyme pathways that are used in the cellular DNA of the host [14, 27, 32]. HCR of Ad has been used to examine repair capacities of DNA repair-deficient syndromes such as xeroderma pigmentosum (XP) [32, 39]. Fibroblasts from patients with XP are extremely sensitive to UV irradiation as a result of a deficiency in one or more of the steps in the nucleotide excision repair (NER) pathway [35]. NER involves several steps: recognition of the damaged DNA, incision of the DNA near the damage, excision and degradation of the damaged DNA strand, replacement of the damaged DNA strand and ligation of the replaced DNA strand [3, 13]. NER is also believed to be responsible for the repair of cisplatin adducts (XP patients are also hypersensitive to cisplatin) [5, 30]. In addition, the HCR assay system using either a plasmid [6, 25, 36] or a virus [23, 26, 27, 30] has been used to examine the ability of cells to repair damage induced by a number of agents including cisplatin.

Therefore, we used two variations of the HCR assay, viral DNA replication and expression of a reporter gene, to examine the repair capacities of a human ovarian carcinoma cell line, 2008, its cisplatin-resistant variant, C13*, and a rhodamine 123 revertant, RH4. The role of DNA polymerase α, a component of the NER pathway, in cisplatin resistance was also investigated.

Materials and methods

Materials

Cisplatin (*cis*-Diamminedichloroplatinum(II)), aphidicolin, and chloroquine diphosphate were obtained from Sigma Chemical Co., St. Louis, Mo. The 5-dodecanoylaminofluorescein di-ß-D-galactopyranoside (C_{12}FDG) was obtained from Molecular Probes, Eugene, Ore. Pronase and Ad 2 DNA were purchased from Boehringer Mannheim, Laval, Quebec, Canada and Gibco BRL, Burlington, Ontario, Canada respectively. Wild-type Ad 5 and Ad 5 HCMVSp1lacZ were provided by Dr. Frank Graham, McMaster University, Hamilton, Ontario, Canada.

Cell lines and culture conditions

The human ovarian carcinoma cell line, 2008 and its variants, C13* and RH4 were a generous gift from Dr. Paul Andrews, Georgetown University, Rockville, Md. The 2008, C13*, and RH4 cells were grown in RPMI-1640 medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. The AA8 and UV20 cells were provided by Dr. Larry Thompson, Lawrence Livermore National Laboratory, Livermore, Calif., with help from Dr. Gordon Whitmore,

Ontario Cancer Institute, Toronto, Ontario, Canada, and were grown in α-minimal essential medium and supplemented with 10% fetal bovine serum, 2 m*M* glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. All cultures were maintained at 37°C and in an atmosphere of air containing 5% CO_2 in a humidified incubator.

Quantification of cisplatin-DNA adduct formation

Cells in log-phase growth were seeded overnight in 150 × 25-mm dishes. The cells were exposed to various concentrations of cisplatin (prepared in phosphate-buffered saline, pH 7.2) for 1 h for AAS or for 2 h for the competitive ELISA. AAS and ELISA were performed as previously described [2, 24].

Virus treatment

Wild-type Ad 5 and Ad-5-HMCVSp1lacZ were treated with various concentrations of cisplatin for 24 h at 37°C in low chloride phosphate-buffered saline (4 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4; pH 7.2). The virus was diluted tenfold in serum-free medium prior to infection.

Replication of cisplatin-damaged Ad DNA

Cells seeded at a density of 2×10^5 cells per well in 24-well plates were infected with cisplatin-treated virus at a multiplicity of infection of 40 plaque forming units per cell for 90 min (agitated gently every 15 min) at 37°C in a humidified incubator. Following the infection period, medium containing unabsorbed virus was removed and the cells were incubated in fresh growth medium (0.5 ml per well) for 2–4 h. At this time, 0.2 ml of a lysis buffer (4 mg/ml pronase, 40 m*M* Tris pH 8.0, 40 m*M* EDTA pH 8.0, 2.4% sodium dodecyl sulfate) was added to one set of wells for each cell line to determine the extent of viral infection at time zero. Approximately 2 h after the addition of the lysis buffer, the samples were collected and stored at − 20°C until extraction of the DNA was performed. The remaining samples received lysis buffer 48 or 72 h after infection with the damaged virus and handled as previously described.

Total DNA (cellular and viral) was extracted with phenol/chloroform (1:1) and centrifuged at 12000 *g* for 2 min. The aqueous phase was re-extracted with chloroform/isoamyl alcohol (25:1) and centrifuged at 12000 *g* for 1 min. The DNA was precipitated with 2 volumes of absolute ethanol and 65 m*M* NaCl (stored overnight at − 20°C) and centrifuged at 12000 *g* for 30 min at 4°C. The supernatant was removed and the DNA was allowed to dry. The DNA was then resuspended in TE buffer (10 m*M* Tris, 1 m*M* EDTA; pH 8.0) and stored at − 20°C. The DNA was slot-blotted and the amount of viral DNA was detected using a ^{32}P-labelled Ad 2 DNA probe. The viral DNA was quantitated using a the phosphoimager (Molecular Dynamic, Sunnyvale, Calif.).

Transcription of a reporter gene from cisplatin-damaged Ad DNA

The platination of the viral DNA and the infection procedure were identical to the procedures previously described except that the Ad-5-HMCVsp1lacZ virus which has the *lacZ* gene inserted into the E1a region of the viral genome was used and the cells were seeded at a density of 2×10^4 cells per well in 24-well plates. Following the 90-min infection period, the cells were incubated 48 h later with medium containing 300 μ*M* chloroquine diphosphate for 30 min at 37°C protected from light. The chloroquine was then removed and medium containing 25 μ*M* C_{12}FDG was added to each well and the cells were incubated at 37°C protected from light [42]. The protein transcribed from the *lacZ* gene, β-galactosidase, converts C_{12}FDG to a fluorescent substrate. The amount of fluorescence (an indicator of β-galactosidase activity) was determined at various time intervals using excitation and emission wavelengths of 485 nm and 530 nm, respectively, on a Cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ontario, Canada). The amount of fluorescence served as an indicator of the amount of gene transcription.

For the enhanced HCR assay, the procedure used was identical to that described for transcription of a reporter gene from cisplatin-damaged Ad DNA except that the cells were pretreated with cisplatin (IC_{50} or IC_{90} as determined from our survival assays) for 1 h immediately prior to infection with the cisplatin-damaged virus.

Survival assays

Cells in log-phase growth were seeded overnight at a cell density of 2000 and 1000 cells per well for 2008 and C13* cells, respectively, in 96-well plates and treated with cisplatin (prepared fresh for each experiment in phosphate-buffered saline, pH 7.2) alone for 1 h or aphidicolin (prepared in absolute ethanol and stored at − 20°C) alone for 26 h. The treatment protocol used to investigate the interaction of aphidicolin and cisplatin was aphidicolin for 1 h, cisplatin and aphidicolin for 1 h followed by a medium change, exposure to aphidicolin for an additional 24 h and concluding with 3 days in drug-free medium. Cell growth was assessed 4 days after the initiation of drug treatment on a Cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ontario, Canada) using the H33258 DNA fluorochrome method as previously described [31].

The nature of the interaction between cisplatin and aphidicolin was assessed using isobologram analysis at the 50% effect level. A combination index (CI) was calculated for the interaction of cisplatin and aphidicolin in both the 2008 and C13* cells. A CI of 1 indicates that the two drugs interact in an additive fashion while CIs of < 1 and > 1 indicate that the two drugs interact in a synergistic and antagonistic fashion, respectively [33].

Statistical analysis

All values are means ± SEM unless otherwise specified. $P < 0.05$ was considered statistically significant.

Results

Cisplatin-DNA adduct formation

The number of cisplatin-DNA adducts formed following treatment with different cisplatin concentrations was determined by both AAS and a competitive ELISA. A plot of cisplatin-DNA adducts versus dose was used to calculate the slope of adduct formation (data not shown). The 2008 cells had a significantly greater slope than the C13* cells (the 2008 cells had slopes that were 3.6-fold and 2.4-fold greater than the C13* cells as detected by AAS and ELISA, respectively) indicating that at equivalent cisplatin concentrations more cisplatin-DNA adducts were formed in the 2008 cells than in the C13* cells.

Replication of cisplatin-damaged Ad DNA

The Ad was exposed to cisplatin in a solution of low chloride phosphate-buffered saline in an attempt to mimic intracellular chloride concentrations [27, 45]. The use of the low chloride phosphate-buffered saline also permitted us to decrease the amount of cisplatin necessary to induce the cisplatin-viral DNA damage since it is the hydrated form of cisplatin that binds to DNA and hydration of cisplatin is favoured in the presence of low concentrations of chloride ions [27, 45]. It was also determined that temperature and duration of drug exposure could influence the amount of cisplatin-induced damage in the viral genome. Therefore, incubation of the virus at 37°C for 24 h in a low chloride environment with nanomolar cisplatin concentrations provided sufficient damage to the viral DNA.

The extent of viral infection was determined in each cell type by harvesting the cells 4 h after the end of the viral infection period. The virus used for determining the extent of infectivity was treated with the highest dose of cisplatin (0.4 μM) to ensure that the cisplatin treatment did not prevent infection by the virus. The amount of viral DNA was similar in all three cell types indicating that the cisplatin treatment did not inhibit infection by the virus and the extent of viral infection was similar in 2008, C13* and RH4 cells.

To determine the ability of the HCR assay to detect differences in the replication of cisplatin-damaged Ad DNA, AA8 and UV20 cells were used as controls. UV20 cells are UV repair-deficient mutants of the Chinese hamster ovary cell line AA8. The UV20 cells

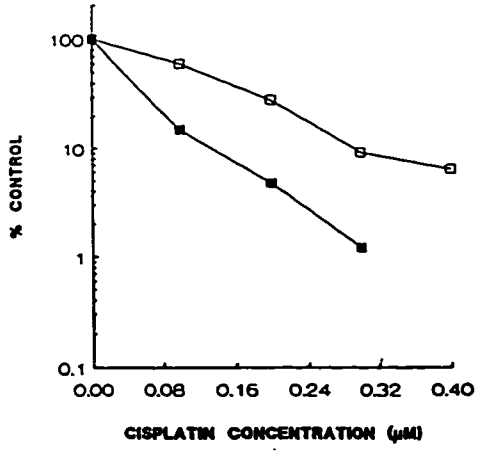

Fig. 1 Replication of cisplatin-damaged Ad DNA in AA8 (□) and UV20 (■) cells after 72 hours of repair. Cells were infected with virus treated with different concentrations of cisplatin and the ability of the cells to replicate the damaged viral DNA was assessed. The amount of viral DNA replication of cisplatin-treated virus is expressed relative to the amount of viral replication of undamaged virus. This is a representative experiment

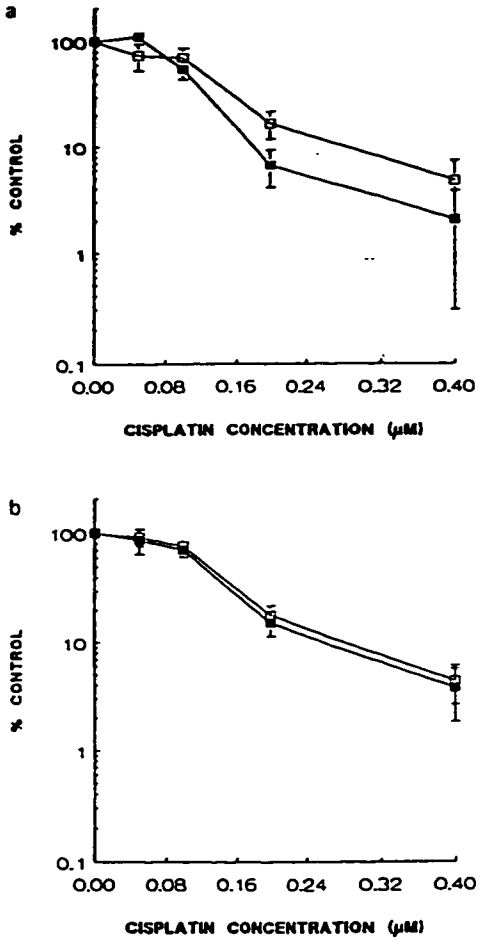

Fig. 2a,b Replication of cisplatin-damaged Ad DNA in 2008 (□) and C13* (■) cells after 48 h (a) and 72 h (b) of repair. Cells were infected with virus treated with different concentrations of cisplatin and the ability of the cells to replicate the damaged viral DNA was assessed. The amount of viral DNA replication of cisplatin-treated virus is expressed relative to the amount of viral replication of undamaged virus. Values are expressed as percentages of control ± SEM ($n = 3$)

are also hypersensitive to cisplatin because they lack the incision step of the excision repair pathway which is necessary for removal of cisplatin-DNA adducts [8]. Figure 1 shows a representative experiment indicating that at varying degrees of cisplatin-induced viral DNA damage, UV20 cells were less efficient than AA8 cells at replicating the cisplatin-damaged Ad DNA. Replication of the Ad DNA was assessed 72 h after the infection of the cells with damaged virus. Thus, the HCR assay can detect differences in the capacity of cells to replicate cisplatin-damaged Ad DNA. Figure 2 illustrates the ability of 2008 and C13* cells to replicate cisplatin-damaged Ad DNA. Replication was assessed at 48 and 72 h after viral infection and there was no significant difference in the capacities of the two cell types to replicate the damaged Ad DNA.

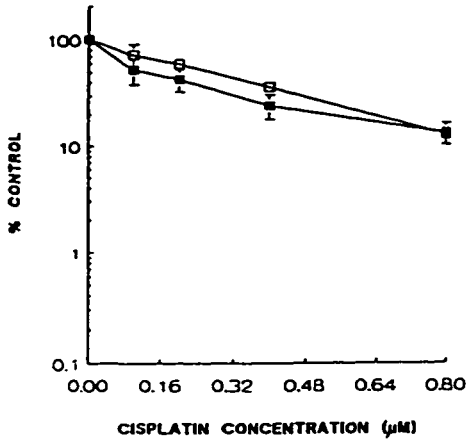

Fig. 3 Transcription of a reporter gene from cisplatin-damaged Ad DNA in 2008 (□) and C13* (■) cells. Cells were infected with virus treated with different concentrations of cisplatin and the ability of the cells to transcribed the *lacZ* gene was assessed after 48 h of repair. The amount of functional protein (ß-galactosidase) produced from cisplatin-treated virus was expressed relative to the amount of functional protein produced from undamaged virus. Values are expressed as percentages of control ± SEM ($n = 3$)

Transcription of a reporter gene from cisplatin-damaged Ad DNA

Some investigators have indicated that the repair of cisplatin damage is more efficient in actively transcribed gene regions than in the overall genome [3, 4, 19, 21, 22, 43]. In addition, some repair-deficient mutants (e.g. cells from patients with Cockayne's syndrome) repair damage from the total genome at levels similar to cells from normal subjects but are deficient in preferential repair of actively transcribed genes [3, 4, 23, 40]. Therefore, we examined the ability of 2008 and C13* cells to express a reporter gene from cisplatin-damaged Ad DNA after 48 h of repair. There was no significant difference between 2008 cells and its cisplatin-resistant variant, C13*, in their ability to express the *lacZ* gene in cisplatin-damaged Ad as shown in Fig. 3.

Enhanced HCR

Since the cells are not exposed to cisplatin in the standard HCR assay, we investigated whether cisplatin pretreatment affected the cells' ability to express the *lacZ* gene from cisplatin-damaged Ad. Each of the cell types was exposed to equitoxic cisplatin concentrations (IC_{50} and IC_{90}) and an equal cisplatin concentration (the IC_{50} dose for C13* cells and the IC_{90} dose for the 2008 cells were both 21 μ*M* of cisplatin) for 1 h immediately prior to virus infection. Figure 4 shows that pretreatment with cisplatin did not alter the ability of either 2008 or C13* cells to express a reporter gene from cisplatin-damaged Ad DNA relative to cells which received no drug pretreatment after 48 h of repair. However, pretreatment of the cells with an equal concentration of cisplatin (21 μ*M*) increased transcription of the *lacZ* gene from undamaged virus 5.24 ± 1.1-fold in the 2008 cells but only 1.64 ± 0.2-fold in the C13* cells (Table 1).

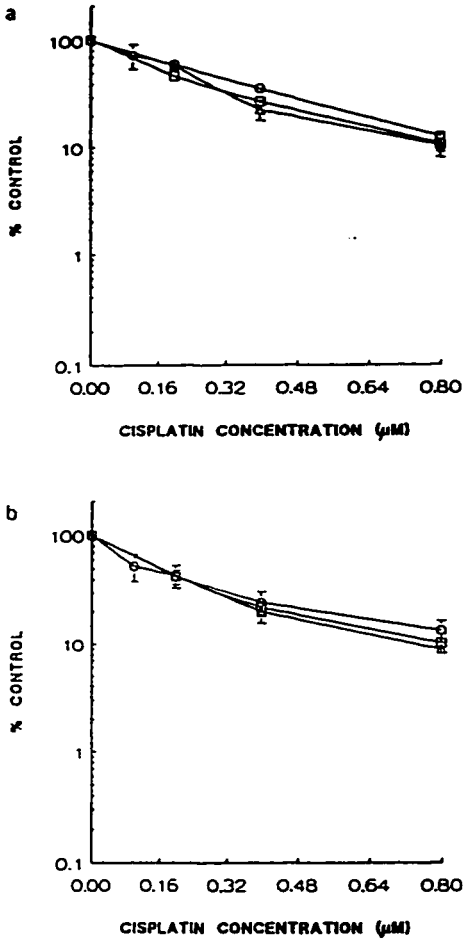

Fig. 4a, b Transcription of a reporter gene from cisplatin-treated Ad DNA after 48 h of repair in 2008 (a) and C13* (b) cells exposed to no cisplatin pretreatment (○), or pretreatment with the IC_{50} (□) or IC_{90} (△) concentration for 1 h immediately prior to virus infection. Values are expressed as percentages of control ± SEM ($n = 3$)

HCR of cisplatin-damaged Ad by RH4 cells

In addition to the 2008 and C13* cells, the HCR response of RH4 cells was investigated. RH4 cells were derived from C13* cells through selection for resistance to rhodamine 123 [44]. Not only did the selection procedure reduce the mitochondrial membrane potential, it also reverted the cisplatin sensitivity of the RH4 cells to a level comparable to the 2008 cells [44]. Table 2 shows that replication of cisplatin-damaged Ad DNA and the expression of a reporter gene from cisplatin-damaged Ad in RH4 cells was similar to both 2008 and C13* cells with or without cisplatin pretreatment.

Table 1 Induction of *lacZ* gene expression from undamaged virus in cells treated with cisplatin (-fold increase). The IC_{50} concentrations of cispaltin were 5 μ*M* for 2008 and RH4 cells and 21 μ*M* for C13* cells and the IC_{90} concentrations of cisplatin were 21 μM for 2008 and RH4 cells and 100 μM for C13* cells

Cisplatin dose	2008	C13	RH4
IC_{50}	2.11 ± 0.2	1.64 ± 0.2	1.51 ± 0.1
IC_{90}	5.24 ± 1.1	1.98 ± .03	1.86 ± 0.1

Interaction of aphidicolin and cisplatin

C13* cells were found to be resistant to cisplatin yet equally sensitive to aphidicolin relative to 2008 cells (Fig. 5). Isobologram analysis was used to determine the nature of the interaction between cisplatin and aphidicolin when used in combination and revealed a similar degree of synergy in both cell types; the CIs were 0.79 ± 0.06 and 0.77 ± 0.07 ($n = 4$) in 2008 and C13* cells, respectively, as determined at the 50% effect level.

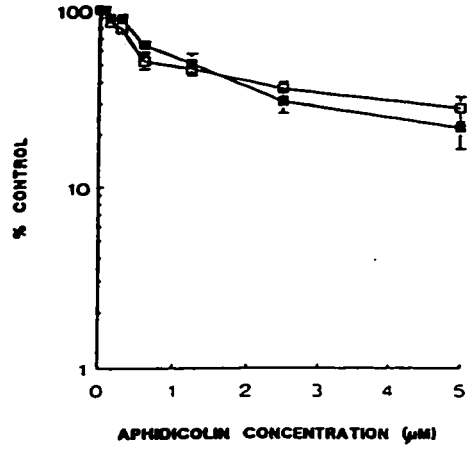

Fig. 5 Effect of aphidicolin on 2008 (□) and C13* (■) survival. Cytotoxicity was assessed by a DNA fluorometric assay 72 h after a 26-h aphidicolin exposure. Values are expressed as percentages of control ± SEM ($n = 3$)

Discussion

One of the many mechanisms that has been suggested as contributing to cisplatin resistance is the enhanced repair of cisplatin-induced DNA damage [5, 7, 12, 28, 34]. Owing to the limitations of the detection techniques used (i.e. AAS), supralethal doses of cisplatin are frequently used for repair studies. Since it remains unclear how a cell responds to damage beyond its capacity to repair [38, 41], we utilized the HCR assay to assess cellular repair capacity. In the HCR assay, cisplatin-DNA adducts are induced in a viral genome and the damaged virus is infected into the host cell. The ability of the host cell to replicate the cisplatin-damaged viral DNA or to express a reporter gene from cisplatin-damaged viral DNA is used as an indirect indicator of the cell's ability to repair cisplatin damage from its own DNA. This assay system permits repair to be assessed using low cisplatin concentrations and thus allows the comparison of our results with those reported by others using high cisplatin concentrations [27, 43].

The Chinese hamster ovary cell line AA8 and its UV-repair deficient mutant UV20 served as controls in the HCR protocol. UV20 cells are deficient in the incision step of excision repair and are hypersensitive to cisplatin in survival assays [8, 15]. This study demonstrated that UV20 cells were not as efficient at replicating the cisplatin-damaged viral DNA as AA8 cells, and thus the HCR assay can detect differences in the capacity of cells to replicate cisplatin-damaged Ad DNA. However, it is interesting to note that even though UV20 cells were 50-fold more sensitive to cisplatin than AA8 cells, they were only approximately 2.5-fold less efficient at replicating the cisplatin-damaged viral DNA. The lack of correlation between cisplatin sensitivity and viral replication capacity may reflect the inability of the HCR assay to detect differences in the repair of interstrand crosslinks. It has been shown that fibroblasts from patients with Fanconi's anemia (FA), which are thought to be defective in the repair of DNA interstrand crosslinks, replicate

Table 2. Cisplatin concentrations required to inhibit viral DNA replication or expression of a reporter gene by 50% in 2008, C13* and RH4 cells. Statistical analysis was performed using the Tukey test and $P < 0.05$ was considered significant. The IC_{50} cisplatin concentrations used were 5 μM for 2008 and RH4 cells and 21 μM for C13* cells and the IC_{90} cisplatin concentrations used were 21 μ*M* for 2008 and RH4 cells and 100 μ*M* for C13* cells

	2008	C13	RH4
Replication of cisplatin-damaged Ad DNA			
48 h	0.117 ± 0.20 μ*M*	0.101 ± 0.011 μ*M*	0.118 ± 0.004 μ*M*
72 h	0.160 ± 0.033 μ*M*	0.121 ± 0.009 μ*M*	0.134 ± 0.050 μ*M*
Expression of a reporter gene			
No pretreatment	0.276 ± 0.040 μ*M*	0.174 ± 0.059 μ*M*	0.189 ± 0.053 μ*M*
IC_{50} pretreatment	0.184 ± 0.009 μ*M*	0.164 ± 0.029 μ*M*	0.190 ± 0.038 μ*M*
IC_{90} pretreatment	0.235 ± 0.018 μ*M*	0.173 ± 0.042 μ*M*	0.182 ± 0.031 μ*M*

cisplatin-damaged viral DNA to the same extent as control fibroblasts even though FA cells are hypersensitive to cisplatin [9, 29, 30]. Therefore, reduced replication of cisplatin-damaged Ad DNA may only reflect the ability of cells to repair intrastrand adducts but not interstrand crosslinks from the total viral genome.

C13* cells were not more efficient in their ability to replicate cisplatin-damaged Ad DNA than 2008 cells. Similarly, preliminary results on the repair of UV-induced viral DNA damage indicated that C13* cells did not have an enhanced ability to replicate UV-damaged viral DNA compared with 2008 cells (data not shown). These results suggest that the ability of the NER system to repair intrastrand adducts from the total viral genome is not enhanced in the cisplatin-resistant variant relative to the parental cell line. Our results are consistent with those previously reported for 2008 cells and their cisplatin-resistant variant. Zhen et al. [43] and Jekunen et al. [16] have found that there are no significant differences in repair of total genomic intrastrand adducts between the parental line and its cisplatin-resistant variant as assessed by AAS and an isotopic analogue of cisplatin ([^{3}H]dichloro (ethylenediamine)platinum(II)), respectively.

Since it has recently been demonstrated that cells repair DNA damage, including cisplatin-DNA adducts, from actively transcribed gene regions more efficiently than from non-transcribed regions of DNA [3, 4, 19], we examined the cells' ability to repair cisplatin intrastrand adducts from a reporter gene within the viral genome. Using Ad 5 with the *lacZ* gene inserted into the E1a region of the viral genome, it was found that the cisplatin-resistant variant was not more efficient than the parental line at transcribing a reporter gene from cisplatin-damaged Ad DNA, suggesting that enhanced gene-specific repair of intrastrand adducts does not contribute to the cisplatin resistance in C13* cells. These results are consistent with those obtained by Zhen et al. [43].

To further examine repair capacities in 2008 and C13* cells an enhanced HCR assay was performed. Previous reports have indicated that pretreating cells with physical or chemical agents can enhance the replication of UV-damaged viral DNA (reviewed in reference 32). In this enhanced HCR assay the cells were exposed to cisplatin for 1 h immediately prior to their infection with cisplatin-damaged virus (a temporal relationship between cisplatin pretreatment and viral infection was not investigated in this study). This experiment was performed to determine whether C13* cells required cisplatin-induced cellular damage to stimulate their repair process. Neither of the cell types demonstrated any significant alteration in their ability to repair cisplatin damage following cisplatin pretreatment over the range of concentrations employed and thus there do not appear to be any significant differences in the NER process for cisplatin damage in 2008 and C13* cells.

We found that pretreatment with an equal cisplatin concentration increased the transcription of the *lacZ* gene in undamaged virus to a greater extent in 2008 cells than in C13* cells. This is consistent with the observation that DNA-damaging agents, such as UV radiation also increase the expression of a number of genes, termed damage-inducible genes (reviewed in reference 10). It has also been demonstrated that enhanced gene expression occurs at lower doses of a DNA damaging agent (i.e. UV radiation) in UV repair-deficient mutants compared with repair-proficient controls [10, 11]. Thus, it appears that induction of gene expression may be related to the amount of DNA damage (or the amount of unrepaired DNA damage). Our study demonstrated that the concentration of cisplatin required to enhance expression of a reporter gene was lower in 2008 cells than in C13* cells. This observation may indicate that 2008 cells respond to DNA damage in a manner similar to a repair-deficient cell type compared with C13* cells. However, a more likely explanation for the enhanced expression of the *lacZ* gene in 2008 cells compared with the C13* cells at equivalent cisplatin concentrations is that C13* cells require higher cisplatin concentrations than 2008 cells to induce similar amounts of cisplatin-DNA adducts. ([43]; this study). The low levels of *lacZ* gene induction in the RH4 cells (similar to C13* cells) may indicate that adduct formation in the RH4 cells occurs at levels similar to those observed in C13* cells. Since adduct formation has not been investigated in this cell type, further studies are required.

The repair capacity of RH4 cells was also determined. RH4 cells were derived from C13* cells through selection for resistance to rhodamine 123 [44]. This selection process not only decreased the mitochondrial membrane potential but also increased the cells' sensitivity to cisplatin to a level comparable to the parental 2008 cells. In each of the HCR experiments, the ability of RH4 cells to reactivate cisplatin-damaged Ad DNA was not significantly different from either 2008 or C13* cells. Therefore, there do not appear to be any significant differences in the ability to repair cisplatin-induced viral DNA damage between cisplatin-sensitive and cisplatin-resistant cells in our model system.

Further evidence to support a lack of a difference in NER capacities between 2008 and C13* cells was obtained using aphidicolin, an inhibitor of DNA polymerase α and δ [37]. DNA polymerase α has been proposed as one of the components involved in the repair of cisplatin-DNA adducts [34]. We found that 2008 and C13* cells were equally sensitive to aphidicolin. These results are not consistent with the survival data published by Katz et al. [20] who found that 2008 cells were 3.3-fold more sensitive to aphidicolin than their cisplatin-resistant variant (2008/DDP). It is possible that the different cisplatin selection protocols used to generate the cisplatin-resistant variants 2008/DDP and C13* influenced the

resistant mechanisms utilized by these variants. We also investigated the nature of the interaction between aphidicolin and cisplatin. Using isobologram analysis and assessing the interaction at the 50% effect level, it was found that the interaction was synergistic in both 2008 and C13* cells. Therefore, it appears that polymerase α and/or δ are important components of the cisplatin repair pathway in both 2008 and C13* cells, suggesting that these components of the NER pathway do not contribute to the cisplatin resistance in C13* cells.

Our aphidicolin and cisplatin results differ from those previously reported in which median effect analysis demonstrated synergy between aphidicolin and cisplatin in the cisplatin-resistant variant but only additivity in the parental line [20]. Although the drug treatment protocol and the parental cell line were the same, the formulation of aphidicolin, the cisplatin-resistant variant and the method used to determine the CI were different in our study compared to the study by Katz et al. [20]. It is unclear which of these factors contributed to the different results from the two studies.

Since our assay system appears unable to detect differences in the repair of cisplatin interstrand crosslinks [9, 29, 30], we are unable to comment on the suggestion by Zhen et al. [43] that enhanced repair of gene-specific interstrand crosslinks contributes to the cisplatin resistance in C13* cells. Johnson et al. [18] have also demonstrated a correlation between enhanced repair of cisplatin interstrand crosslinks and cisplatin resistance in a number of cisplatin-resistant variants of the parental A2780 cell line.

In conclusion, enhanced repair of total genomic or gene-specific cisplatin intrastrand adducts does not appear to contribute to cisplatin resistance in C13* cells. This study also suggests that RH4 cells repair intrastrand adducts from cisplatin-damaged Ad DNA with an efficiency similar to both 2008 and C13* cells. Furthermore, the similar synergistic growth inhibition mediated by the combination of cisplatin and aphidicolin in both the parental line and the cisplatin-resistant variant is consistent with the suggestion that enhanced NER does not contribute to the cisplatin resistance in C13* cells.

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Discussion

This manuscript examined whether a correlation between DNA repair and mitochondrial membrane potential existed. Since mitochondria are the primary source of ATP in most cells (164) and several steps in NER have been shown to be ATP-dependent (319,331,336), it is conceivable that NER may be influenced by mitochondrial ATP production. Thus, the synergy between lipophilic cations and cisplatin observed in Chapter 1, may reflect a decreased capacity to repair cisplatin-DNA lesions as a result of a disruption in mitochondrial function. Therefore, a correlation between an elevated mitochondrial membrane potential and enhanced capacity to repair cisplatin-induced DNA damage was investigated with the intention of examining whether DNA repair could be influenced by inhibiting mitochondrial function.

A HCR assay was used to provide a measurement of repair of cisplatin-damaged DNA in 2008, C13*, and RH4 cells. In this assay, Ad 5 was incubated with cisplatin in order to induce cisplatin-viral DNA lesions. Ad 5 can be used to investigate the repair of cisplatin damage because Ad 5 DNA is a linear duplex and its repair and replication are dependent on the enzyme pathways used to repair cellular DNA in the host (107,342,363). Cells are then infected with the cisplatin-damaged virus and their ability to replicate or transcribe damaged viral DNA serves as an indicator of the repair capacity of cells.

Our primary concern with many of the repair studies in the literature was that very high concentrations of cisplatin were required to produce an adequate

number of lesions for detection. In some instances, cells were exposed to cisplatin concentrations up to 1000 times their IC_{50}. This may be significant since it remains unclear how cells respond to damage beyond their capacity to repair and DNA damage profiles may differ depending on concentration of DNA damaging agent (358,359). An additional concern was that the initial number of cisplatin lesions induced in DNA, often differed in cisplatin-sensitive and cisplatin-resistant cells. Differential cisplatin lesion formation may arise as a result of the presence of other cisplatin resistance mechanisms such as decreased accumulation or increased scavenging of cisplatin. Since the initial amount of DNA damage may influence the rate at which the DNA is repaired, repair should be assessed following the induction of similar number of lesions in both cisplatin-sensitive and cisplatin resistant cells (25,364). The HCR assay overcomes both these limitations as viral DNA is incubated with cisplatin before cells are infected. Thus, cells are not exposed to cisplatin and both cisplatin-sensitive and cisplatin-resistant cells are exposed to the same amount of cisplatin-damaged DNA.

To summarize the results from this manuscript, HCR of cisplatin-treated Ad 5 DNA was capable of detecting a difference in the ability of normal CHO cells, AA8, to repair cisplatin-damaged DNA compared to UV20 cells which are deficient in NER. The difference in HCR between AA8 and UV20 cells was only about 2.5-fold. However, UV20 cells have been reported to be approximately 50-fold hypersensitive to cisplatin compared to AA8 cells (365). These results

may reflect the major limitation of the HCR assay; it has been suggested that HCR of viral DNA is unable to detect repair of interstrand crosslinks (110,111,340). It has been demonstrated that transfection of complementation group 1 repair-deficient mutants, like UV20, with ERCC1, increased cisplatin interstrand crosslink repair and reduced cisplatin sensitivity (113,114). Therefore, UV20 cells may be only 2.5-fold less efficient at repairing cisplatin intrastrand adducts in Ad 5 DNA compared to AA8 cells. An alternative explanation for the small difference in HCR of cisplatin-damaged Ad DNA between AA8 and UV20 cells is that only small differences in repair may be required for large differences in cisplatin sensitivity.

When the HCR of cisplatin-damaged Ad DNA in 2008, C13* and RH4 cells was examined, it was observed that C13* cells did not repair cisplatin-damaged Ad DNA more efficiently than either 2008 or RH4 cells. These results suggest that repair of cisplatin damage from total genomic DNA was not enhanced in C13* cells compared to 2008 or RH4 cells. Repair of cisplatin damage from actively transcribed genes also did not appear to be enhanced in C13* cells compared to either 2008 or RH4 cells since C13* cells did not have an increased HCR of a reporter gene in cisplatin-damaged Ad DNA compared to 2008 and RH4 cells. These results suggest that cisplatin resistance in C13* cells is not due to an increase in the repair of cisplatin-damaged DNA and an elevation in mitochondrial membrane potential does not influence the repair of cisplatin-DNA lesions. It should be noted that HCR of Ad DNA probably reflects

the ability of cells to repair cisplatin intrastrand adducts but not interstrand crosslinks (110,111,340). Therefore, enhanced repair of interstrand crosslinks in C13* cells and an association between enhanced repair of interstrand crosslinks and elevations in mitochondrial membrane potential remain possibilities.

The HCR assay permitted us to investigate whether pre-treating cells with cisplatin was capable of enhancing the repair of cisplatin-damaged DNA. As previously mentioned, only the virus and not the cells are exposed to cisplatin in the HCR assay. Although only one time point was examined, pre-treatment of cells with cisplatin did not appear to enhance their ability to repair cisplatin damage from a reporter gene in Ad DNA. Interestingly, pre-treatment of 2008 cells with cisplatin enhanced the expression of a reporter gene in undamaged Ad DNA to a greater extent than C13* cells. Enhanced gene expression following UV irradiation is observed in UV repair-deficient mutants but not in repair-proficient controls (364,366). This result may suggest that 2008 cells may be deficient in some aspect of DNA repair compared to C13* cells.

The importance of DNA repair in 2008 and C13* cells was further investigated using aphidicolin, an inhibitor of DNA polymerase α and δ. DNA polymerase δ has been implicated in DNA synthesis following excision of damaged DNA (320,331,338). Aphidicolin was observed to be equitoxic to 2008 and C13* cells and combinations of cisplatin and aphidicolin proved to be synergistic in both 2008 and C13* cells. These data support our HCR data in

suggesting that NER is important in cell survival following cisplatin treatment, but does not appear to contribute to cisplatin resistance in C13* cells.

Since DNA repair did not correlate with mitochondrial membrane potential, the effect of mitochondrial inhibitors on DNA repair capacity were not pursued. Instead, a potential regulator of mitochondrial function, namely c-*fos,* was examined in the next chapter.

Chapter 5 Expression of c-*fos* and mitochondrial membrane potential in cisplatin resistance

In the previous chapter a potential mechanism through which an elevated mitochondrial membrane potential contributed to cisplatin resistance was examined. In this chapter, a potential regulator of mitochondrial membrane potential, namely the c-*fos* proto-oncogene, is considered. Furthermore, correlations between c-*fos* gene expression and cisplatin resistance and correlations between elevated mitochondrial membrane potential and cisplatin resistance were investigated in this chapter.

The c-*fos* gene encodes a nuclear transcription factor, c-Fos, which interacts with members of the c-Jun and ATF/CREB families to induce the transcription of a number of other genes (367). c-*fos* is a member of a multi-gene family which also includes Fos B (368), ΔFos B (369-372), Fra-1 (373,374), Fra-2 (373,375,376), and R-*fos* (377). These family members are often expressed at the same time and may be redundant with respect to function (378). All Fos family members contain a bZip region that is required for dimerization with Jun family members. When members of the Fos family bind with members of the Jun family, they form a 'leucine zipper' motif which allows the proteins to bind to DNA (379-386). Human c-*fos* has been localized to chromosome 14q21-q31 and produces a 2.2 kb mRNA. The protein contains

380 amino acids and has a molecular weight of 55-62 kDa depending on the amount of phosphorylation (378,387).

The c-*fos* proto-oncogene was originally identified as the cellular homologue of the viral oncogene v-*fos* which was isolated from a spontaneous osteosarcoma in a CF1 mouse (388). There are two forms of v-*fos*, Finkel-Biskis-Jenkins (FBJ) and Finkel-Biskis-Reilly (FBR) both of which induce osteosarcomas in mice and rats (388,389). It was later demonstrated that the c-*fos* gene product was capable of transforming cells (390,391).

The v-*fos* retroviruses acquired components of the c-*fos* gene through genetic recombination and differ significantly from the c-*fos* gene (389,392). FBJ-MSV contains 4026 nucleotides of which only 1639 are derived from the c-*fos* gene (389). The protein product of FBJ-MSV has a molecular weight of 55 kDa with 5 amino acid changes (Ser^{14} -> Phe, Ile^{66} -> Thr, Gly^{109} -> Glu, Glu^{174} -> Lys, and Asp^{290} -> Asn) and a frame shift which alters the last 48 amino acids (C-terminal) of c-*fos* (389,393). The FBR-MSV contains 3791 nucleotides, 709 of which are derived from the c-*fos* gene (394). The protein encoded by FBR-MSV is a gag-fos-fox fusion protein which contains 236 amino acids of the c-Fos protein. Differences between c-*fos* and v-*fos* have identified regions within the gene that regulate the expression and function of c-*fos*.

Expression of c-*fos* mRNA is regulated by several response elements identified in the c-*fos* gene including, serum response element (SRE), *sis*-inducible element (SIE), and cyclic AMP response element (CRE) of which the

SRE appears to be the most important (378). These response elements have been implicated in mediating c-*fos* expression in response to serum, growth factors, phorbol esters (395-400), cAMP (401,402), growth hormone (403), several oncogenes (404-412), and physical stresses such as ROS, heat shock, and several DNA damaging agents including cisplatin (413-416).

Expression of the c-*fos* gene, at least following initiation from the SRE, seems to be autoregulated. It appears that the carboxy terminal portion, particularly three serine clusters (362-364, 368-369, 371-374) of the c-Fos protein regulate binding of c-Fos protein to the SRE and downregulation of its own expression through an incompletely understood mechanism (417-421). This autorepression is apparently dependent on phosphorylation as hypophosphorylated c-Fos protein or c-Fos protein mutated at these serine residues are unable to repress further c-*fos* expression (422). The importance of these serine clusters are demonstrated in the v-Fos protein which lacks these serines and thus does not inhibit its own expression (417,418,422-424). Other mechanisms are involved in negatively regulating c-*fos* expression but are beyond the scope of this thesis (reviewed in (378)).

Regulation of c-*fos* also occurs at the protein level through phosphorylation, oxidation/reduction, translocation of the protein from the cytoplasm to the nucleus and the availability of binding partners. c-Fos can be phosphorylated primarily on serine residues (and possibly threonine residues) that cause a shift in the protein's molecular weight from 55 kDa up to

approximately 65 kDa (425,426). Phosphorylation sites are clustered between amino acids 58-139 and 321-380. *In vitro* experiments have shown that cAMP-dependent protein kinase (PKA), protein kinase C (PKC), $p34^{cdc2}$ and a DNA-dependent kinase are able to phosphorylate the region between amino acids 58-139 while PKA and $p34^{cdc2}$ are able to phosphorylate the region between amino acids 321-380 (426). These phosphorylations have been implicated in regulating c-Fos's activity and/or stability (378,427).

In serum-starved cells, c-Fos protein accumulates in the cytoplasm with little or no c-Fos protein found within the nucleus. Upon continuous stimulation by growth factors, c-Fos protein is transported to the nucleus (423). Translocation of c-Fos appears to be mediated by a cAMP-dependent pathway since cytoplasmic accumulation of c-Fos protein can be reversed by elevated cAMP levels in the absence of serum (423). Other stimuli such as contacts with other cells or the extracellular matrix as well as physical stresses including pH and temperature, may also regulate the transport of c-Fos protein to the nucleus (378,428-431). In contrast, v-Fos proteins from either FBJ or FBR viruses evade this control and constitutively accumulate in the nucleus independent of serum stimulation (423).

Regulation of c-Fos activity by oxidation/reduction occurs at a single cysteine residue, Cys154 (432,433). Oxidation of this cysteine residue interferes with c-Fos's ability to bind DNA while binding is restored under reducing conditions (434). Substitution of Cys154 in c-Fos results in loss of

redox control and enhanced binding of c-Fos protein to DNA (435). Regulation of the oxidation/reduction status of Cys154 appears to be dependent on a ubiquitous 37-kDa nuclear protein called Ref-1 (436).

Since c-Fos protein (or other Fos family members) cannot homodimerize, their activity relies on the availability of binding partner such as members of the Jun family (378). Thus, the availability of binding partners can also influence c-Fos's activity.

In most cell types, c-*fos* gene expression is low or undetectable. Constitutive c-*fos* expression appears to be restricted to areas undergoing remodeling or terminal differentiation such as in the bone, skin, hair follicles and nail roots but may also play a role in programmed cell death (437,438). In most other cell types, c-*fos* expression can be induced by a variety of stimuli as outlined previously. When expressed, c-Fos protein can dimerize with members of the Jun family and bind to DNA. The major target for Fos/Jun dimers is the recognition element known as the AP-1 binding site or TPA-responsive element (TRE) which is located in the promoter regions of a number of genes (439,440). Once bound Fos/Jun dimers interact with other proteins to initiate transcription (441). Genes potentially regulated by c-Fos include; adipocyte aP2 (440,442,443), transin/stromolysin (444-446), α1(III) collagen gene (447), collagenase (448), tyrosine hydroxylation, (449), major histocompatibility class I and class II (450-452), IL-2 (453), osteocalcin (454,455), ovalbumin (456), and its own gene (417,419-421).

c-Fos has been implicated in regulating cellular replication and differentiation (457-461). Greenberg and Ziff (462,463) observed that the expression of c-*fos* is rapidly induced (within 5 minutes) in quiescent cells following stimulation with serum or growth factors. Moreover, microinjection of antibodies specific for c-Fos protein, inhibited cells from progressing from G_1-to-S phase of the cell cycle (464). Subsequent studies have demonstrated that other Fos family members as well as Jun family members are also involved in cell cycle progression (465,466).

Transgenic animals have provided a useful tool for investigating the role of c-*fos* *in vivo*, especially during development. These animals provide a system in which the c-*fos* gene can be overexpressed or eliminated. In normal mice, c-*fos* expression has been observed in bones and teeth during development, as well as in germ cells, hematopoeitic cells and cells in the central nervous system (438,467-471). In Fos transgenic animals, where the c-*fos* gene has been overexpressed alterations in bone, cartilage, and hematopoeitic cell development occur (472-475) and these animals frequently develop osteosarcomas (472,474,476,477).

c-*fos* knockout mice appear phenotypically normal for the first 10 days after which they grow slower and have shorter limbs than normal mice (478). Adult c-*fos* knockout mice are small in size, develop osteoporosis with defects in bone remodeling due to a lack of osteoclasts and have defects in tooth eruption and hematopoeisis (decreased numbers of thymocytes and splenocytes)

(478,479). These knockout mice support the c-*fos* overexpression experiments, in that c-*fos* expression regulates bone, cartilage and hematopoeitic development. Mice heterozygous for the c-*fos* mutation appear normal (478).

Since c-*fos* expression has been implicated in regulating cell proliferation and overexpression of c-*fos* can transform some cell types, modulating c-*fos* expression represents a potential strategy for inhibiting tumour cell growth. The effectiveness of targeting the c-*fos* gene using antisense oligonucleotides has been investigated *in vivo* using two experimental designs. Injection of MCF-7 cells containing a control vector, into nude mice, produced palpable tumours within two weeks and these tumours invaded skeletal muscle and lymphatics. In contrast, injection of MCF-7 cells containing the c-*fos* antisense vector produced smaller tumours that were not apparent until 10-12 weeks post-injection. Moreover these tumours were primarily encapsulated indicating a lower metastatic potential (480).

In the other system, MCF-7 tumuor cells were injected intraperitonealy and allowed to grow for 10 days. On day 10, either a control vector or the c-*fos* antisense vector were administered intraperitonealy. Administration of the c-*fos* antisense vector produced an 80% reduction in tumour mass compared to the control vector when examined 2 weeks after a single injection of the vectors (480). Furthermore, 9 out of 9 mice who received 5 daily doses of the c-*fos* antisense vector, 10 days after MCF-7 inoculation, survived for at least 55 days whereas all 9 mice receiving 5 daily doses of the control vector were dead by

day 45. One of the animals receiving the c-*fos* antisense vector remained tumour-free for 200 days (480). Based on these results a clinical trial evaluating the effectiveness of c-*fos* antisense in metastatic breast cancer has been proposed (481).

Overexpression of c-*fos* most likely contributes to cell transformation via its participation in cell cycle progression. However, c-*fos* expression may also be capable of elevating the mitochondrial membrane potential in cells, a common characteristic of tumour cells (165-167). Zarbl *et al.* (482,483) have observed that rat fibroblast cells transformed with v-*fos* but not a number of other viral oncogenes, possessed an elevated mitochondrial membrane potential as indicated by increased Rh123 accumulation and retention. Selection of revertants of the v-*fos*-transformed fibroblast cells with low mitochondrial membrane potentials lead to the identification of a *v-fos* effector gene termed *fte-1* (484). The *fte-1* gene shared significant homology with two genes from *Saccharomyces cerevisiae* one of which was *MFT-1*, a gene involved in mitochondrial protein import (485).

In addition to participating in cell transformation, c-*fos* expression may be involved in drug resistance. It has been observed that c-*fos* mRNA levels are elevated in samples from patients who have failed cisplatin-based therapy (6,145,486) and in established cisplatin-resistant variants *in vitro* (6,154-156,486,487). Furthermore, transfection of cells with a vector containing c-*fos* can result in cisplatin resistance (155) but an association between cisplatin resistance and c-*fos* overexpression cannot be demonstrated in all cell types (76). To examine

the importance of elevated *c-fos* mRNA in a cisplatin-resistant variant, Scanlon *et al.* (154-156,487) used two different protocols to reduce c-*fos* mRNA levels, treatment with cyclosporin A or transfection of cells with a c-*fos* ribozyme. The *c-fos* ribozyme is a segment of catalytic RNA that specifically cleaves *c-fos* mRNA. Under both conditions the decrease in *c-fos* mRNA correlated with an increased cisplatin sensitivity. It was speculated that cisplatin resistance resulted from a c-*fos*-mediated increase in the expression of proteins involved in DNA synthesis and repair such as thymidylate synthase, DNA polymerase β, topoisomerase I and topoisomerase II (154,155,487,488). The association between these enzymes was based on the temporal expression of the genes; c-*fos* mRNA was elevated approximately one hour after cisplatin treatment while the mRNA for the DNA synthesis and repair genes reached maximal levels approximately 2-3 hours after cisplatin treatment.

In this manuscript correlations between (i) c-*fos* expression and cisplatin sensitivity, (ii) mitochondrial membrane potential and cisplatin sensitivity, and (iii) c-*fos* expression and mitochondrial membrane potential are examined. Three different model systems used to investigate these correlations are summarized in Table 2. The first model system includes a rat fibroblast cell line that has been transfected with the c-*fos* oncogene. These cells have not been examined for either cisplatin sensitivity or mitochondrial characteristics. Our second model system is the human ovarian carcinoma cell line on which much of our previous work has focused. In this system, the cisplatin-resistant variant has

an elevated mitochondrial membrane potential compared to the parental line but c-*fos* expression has not been examined in these cells. Our final model system is another human ovarian carcinoma cell line in which the cisplatin-resistant variant has elevated expression of c-*fos* mRNA but mitochondrial membrane potentials have not been examined.

All data in this manuscript was obtained from experiments conducted by R.A. Moorehead.

Table 2. Model systems for examining the association between cisplatin resistance, c-*fos* expression, and mitochondrial membrane potential.

	2008/C13	A2780/A2780DDP	208F/CMVc-*fos*
Cisplatin sensitivity	C13 cells are resistant to cisplatin	A2780DDP cells are resistant to cisplatin	not determined
Mitochondrial membrane potential	elevated in cisplatin-resistant variant	not determined	not determined
c-*fos* expression	not determined	elevated in cisplatin-resistant variant	elevated in CMVc-*fos* cells

c-*fos* Expression and Mitochondrial Membrane Potential in Cisplatin Resistance[1]

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[5]Abbreviations used are: cisplatin, *cis*-diamminedichloroplatinum(II); CI, combination index; CMV, cytomegalovirus; Deca, dequalinium chloride; Fa, fraction affected; GSH, glutathione, IPTG, isopropyl ß-D-thiogalactopyranoside; MT, metallothionein; NAO, 10*N*-nonyl acridine orange; Rh123, rhodmaine 123;

ABSTRACT

Independent observations suggest that overexpression of c-*fos* or elevations in mitochondrial membrane potential may modulate cisplatin sensitivity but these characteristics require further investigation. We observed that rat fibroblast cells transfected with the c-*fos* gene were approximately 3-fold resistant to cisplatin compared to non-transfected controls. However, in a cisplatin-resistant variant, C13*, where cisplatin resistance was associated with an elevated mitochondrial membrane potential, there was no significant increase in either c-*fos* mRNA or protein levels, compared to the parental line, 2008. Furthermore, reduction of c-Fos protein levels in C13* and 2008 cells, using an antisense oligonucleotide for c-*fos* mRNA, did not sensitize these cells to cisplatin. Moreover, in two cisplatin-resistant variants which overexpressed c-*fos*, there was no increase in mitochondrial membrane potential compared to their respective parental lines. Similarly, reduction of c-*fos* expression did not lower the mitochondrial membrane potential in rat fibroblast cells. These data suggest that both c-*fos* overexpression and elevations in mitochondrial membrane potential are associated with cisplatin resistance but these factors are not interdependent.

INTRODUCTION

Cisplatin resistance has been associated with a number of cellular changes, several of which have been extensively investigated including; (i) enhanced scavenging of the drug through increased glutathione or metallothionein content, (ii) decreased accumulation and/or increased efflux of cisplatin, and (iii) increased repair of cisplatin-DNA lesions (1-4). However, no single mechanism has been consistently associated with cisplatin resistance.

In vitro, cisplatin resistance is often associated with a decrease in intracellular cisplatin concentration which can result from either decreased accumulation or increased efflux of the drug (5-10) but this characteristic is not always associated with cisplatin resistance (11-17). Another reported mechanism involved in cisplatin resistance is an elevation in scavenger molecules such as glutathione (GSH) and metallothionein (MT). Although GSH can form complexes with cisplatin (18) and elevated levels of GSH have been found in cisplatin-resistant cells (19-21) the lack of association in other cell types (22,23), the relatively slow formation of cisplatin-GSH complexes (24) and inconsistent results with the GSH depleting agent d,1-buthionine-S,R-sulfoximine (25,26) have raised questions as to the importance of elevated GSH in cisplatin resistance. MTs are a family of inducible proteins involved in heavy metal regulation such as controlling free intracellular zinc and copper concentrations (1,27). Like GSH, MT contains thiol groups that can interact with cisplatin (28,29). While transfection of cells with MT has been shown to induce

cisplatin resistance (30) and cells lacking MT are hypersensitive to cisplatin (31), cisplatin resistance has not been consistently associated with elevated MT expression in cultured cells (15,28,30,32,33). Enhanced repair of cisplatin-DNA lesions has been demonstrated in several cell culture systems for cisplatin intrastrand adducts (34-38) and cisplatin interstrand crosslinks (7,39,40) but increased repair in cisplatin-resistant variants is not always observed (14,41,42).

Clinically, the generation of cell populations resistant to cisplatin limits the effectiveness of this agent (43,44). The occurrence of cisplatin-resistant tumours approaches 60% in patients with ovarian carcinoma (44). Modulation of the above resistant mechanisms has not been capable of consistently sensitizing cisplatin-resistant tumours. Therefore, we investigated two other proposed cisplatin-resistant mechanisms namely, c-*fos* overexpression and increased mitochondrial membrane potential. Since c-*fos* expression and mitochondrial membrane potential can be modulated, they represent strategies through which cisplatin sensitivity may be restored.

c-*fos* is a nuclear proto-oncogene that can dimerize with members of the c-Jun and ATF/CREB families and enhance the transcription of a number of genes involved in regulating processes such as replication and differentiation (45). The role of c-*fos* in oncogenesis was identified when *v-fos*, the viral homologue of *c-fos*, was discovered as the gene in the Finkel-Biskis-Jinkins murine sarcoma virus responsible for cellular transformation (46). Since then, elevated expression of the

c-fos gene has been shown to transform fibroblasts *in vitro* and induce osteogenic sarcomas in transgenic mice (47-50).

Suppressing c-*fos* expression using an antisense oligonucleotide has been shown to inhibit tumour growth *in vivo*. Transfection of MCF-7, human breast carcinoma cells, with a vector containing an antisense oligonucleotide directed against c-*fos* mRNA, prior to their injection into nude mice, inhibited tumour cell growth and metastases compared to a control vector (51). Furthermore, administration of the c-*fos* antisense-containing vector to mice, 10 days after they had been inoculated with MCF-7 cells, prolonged survival compared to administration of a control vector (51). Clinical trials using c-*fos* antisense oligonucleotides in breast cancer patients have subsequently been proposed (52).

In addition to its role in cellular proliferation, *c-fos* expression has been implicated in contributing to cisplatin resistance in tumour cells. Kashani-Sabet *et al.* (53,54) have found elevated *c-fos* mRNA levels in tumour samples from patients who have failed cisplatin-based therapy and in established cisplatin-resistant variants *in vitro*. Furthermore, suppressing *c-fos* expression in the cisplatin-resistant variant using a c-*fos* ribozyme, restored cisplatin sensitivity while transfection of the parental line with the c-*fos* gene resulted in cisplatin resistance (53,55).

Mitochondrial alterations have also been implicated in cisplatin resistance (22,56). Andrews *et al.* (22,56) have shown that a cisplatin-resistant variant, C13*, had an elevated mitochondrial membrane potential compared to its parental human ovarian carcinoma cell line, 2008. Furthermore, revertants of C13* cells with low

mitochondrial membrane potential displayed a cisplatin sensitivity similar to the parental 2008 cells suggesting that the elevated mitochondrial membrane potential contributed to cisplatin resistance. Changes in the mitochondrial membrane potential may alter protein import into mitochondrial and/or ATP generation through oxidative phosphorylation, since these processes are dependent on the mitochondrial membrane potential (57-60).

Our previous work has demonstrated that the elevated mitochondrial membrane potential in C13* cells can be exploited using lipophilic cations (61). Lipophilic cations accumulate in mitochondria in response to the mitochondrial membrane potential and are used to measure the magnitude of the mitochondrial membrane potential at low doses (62-64). At high doses, lipophilic cations dissipate the mitochondrial membrane potential and thus disrupt mitochondrial function (63,65). We have shown that C13* cells are not only more sensitive to a lipophilic cation, dequalinium chloride (Deca), alone compared to their parental line, but the combination of Deca and cisplatin produced synergistic cell kill in C13* cells. Moreover, combinations of Deca and cisplatin have been shown to prolong survival of mice bearing ovarian carcinoma cells, beyond either agent alone (66).

Since modulation of c-*fos* expression or mitochondrial membrane potential may represent a strategy for treating cisplatin-resistant tumours, the correlations between (i) c-*fos* overexpression and cisplatin resistance and (ii) elevated mitochondrial membrane potential and cisplatin resistance were examined. We also

investigated whether an increase in mitochondrial membrane potential could be mediated by *c-fos* expression.

MATERIALS AND METHODS

Materials. *cis*-diamminedichloroplatinum(II), diethylpyrocarbonate, dithiothreitol, isopropyl ß-D-thiogalactopyranoside, phenylmethylsulfonyl fluoride, and rhodamine 123 were obtained from Sigma Chemical Co., St. Louis, MO. H33258 was obtained from Calbiochem, La Jolla, CA. 10*N*-nonyl acridine orange was purchased from Molecular Probes, Eugene, OR. Rabbit polyclonal c-*fos* and cyclin D1 primary antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Goat anti-rabbit horseradish peroxidase conjugated secondary antibody was obtained from Bio-Rad Laboratories Ltd, Mississauga, Ontario, Canada. Roswell Park Memorial Institute (RPMI) medium, Dulbecco's modified Eagle medium, lipofectin, and the Oligo$(dT)_{12\text{-}18}$ were obtained from Gibco BRL, Burlington, Ontario, Canada. GeneScreen Plus nylon filters were obtained from New England Nuclear Research Products, Boston, MA. Human *c-fos* oligonucleotide probe was obtained from Oncogene Science, Inc., Uniondale, NY. The phosphorothioate oligomer complementary to -6 to +14 of c-*fos* mRNA, FITC-labeled oligonucleotide, control phosphorothioate oligomer with no known complementary mRNA, and lipophilic cations were provided by Dr. B. Brown, Genta Incorporated, San Diego, CA.

Cell Lines and Culture Conditions. The rat fibroblast cell line 208F and its two *c-fos* transfectants, CMV*c-fos* and L1-3*c-fos*, were a generous gift from Dr. Tom Curran, St. Jude Children's Research Hospital, Memphis, TN. CMV*c-fos* cells

contained a vector in which the mouse *c-fos* gene expression was driven by the cytomegalovirus promoter and thus constitutively expressed high levels of c-Fos protein. L1-3*c-fos* cells contained a vector in which the mouse *c-fos* gene expression could be repressed by the addition of isopropyl ß-D-thiogalactopyranoside (IPTG) (67). 208F, CMV*c-fos* , and L1-3*c-fos* cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B and maintained at 37°C and 5% CO_2 in a humidified incubator.

The human ovarian carcinoma cell line, 2008, and its cisplatin-resistant variant C13*, were generously provided by Dr. Paul Andrews, Georgetown University, Rockville, MD. These cells were grown in RPMI medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B and maintained at 37°C and 5% CO_2 in a humidified incubator. The human ovarian carcinoma cell line A2780 and its cisplatin-resistant variant A2780DDP were generously provided by Dr. Kevin Scanlon, Berlex, CA. A2780 and A2780DDP cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B and maintained at 37°C and 5% CO_2 in a humidified incubator.

Western Blot Analysis of c-Fos Protein. Cells in log-phase growth were seeded overnight in 20 x 100 mm culture dishes. After two washes with phosphate buffered saline, pH 7.4, cells were lysed with 500 µl of RIPA lysis buffer (1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 1% phenylmethylsulfonyl fluoride, 3% aprotinin and 1% sodium orthovanadate in phosphate-buffered saline). Cells were scraped from cultures dishes and transferred to microcentrifuge tubes. Samples were sheared with a 21 gauge needle and phenylmethylsulfonyl fluoride (10 µl of 10 mg/ml stock in isopropanol) was added to each tube. Following a 45 minute incubation on ice, samples were spun at 12,000 x g for 20 minutes at 4°C . Supernatants were transferred to fresh microcentrifuge tubes and samples were stored at -80°C. Protein concentrations were determined using the Pierce Micro BCA spectrophotometric protein assay (68).

Proteins (10-20 µg/lane in 20-30 µl) were separated by polyacrylamide gel electrophoresis; 3% stacking gel and 7.5% separating gel for c-Fos and c-Shc proteins and 12% separating gel for cyclin D1 protein. Proteins were stacked at 30 V for 30 minutes and separated at 100 V for 65 minutes. Proteins were transferred to nitrocellulose membranes using the Mini Trans-Blot Cell (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) at 100 V for 45 minutes. Membranes were blocked overnight in 1% skim milk and 1% BSA in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). Western blot analysis was carried out using the appropriate rabbit polyclonal primary antibody and a horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody. Protein levels were detected

using ECL western blotting detection reagents (Amersham Canada Limited, Oakville, Ontario, Canada) and Kodak XAR 5 scientific imaging film (Eastman Kodak Company, Rochester, NY).

c-*fos* Antisense Treatment. 2008 and C13* cells were grown on coverslips placed at the bottom of the wells of a 6-well plate. To determine transfection efficiency, several lipophilic cations at different oligonucleotide:lipid ratios were tested. Cells were incubated for 4 hours in serum-free media containing a FITC-labelled all-phosphorothioate oligomer (250 nM) in the presence and absence of different amounts of various lipophilic cations (lipofectin, lipofectamine and two lipids generated by Genta Inc.). After the 4 hour incubation period the oligomer-containing media was removed and cells were place in supplemented media for approximately 24 hours. Cells were then visualized on a fluorescent microscope. We observed that a 1:9 ratio of oligonucleotide:lipofectin produced positive staining in the nuclei of approximately 80% of both 2008 and C13* cells while little or no nuclear staining was observed when cells were treated with the FITC-labelled oligonucleotide in the absence of lipophilic cation. In all subsequent experiments cells were treated with the c-*fos* antisense oligonucleotide in a 1:9 ratio with lipofectin in serum-free media for 4 hours.

Survival Assays. 208F, CMVc-*fos* or L1-3c-*fos* cells in log-phase growth were seeded overnight in 24-well plates at a density of 2000 cells/well while A2780 and A2780DDP cells in log-phase growth were seeded overnight in 6-well plates at a density of 10 000 cells/well. Cells were exposed to Deca for 3 hours or cisplatin for 1 hour, after which drug-containing media was removed and cells were incubated in drug-free media for 5 days at 37°C. At this time the media was removed, cells were washed once with phosphate-buffered saline, and 300 μl of deionized water was added to each well of a 24-well plate or 900 μl of deionized water was added to each well of a 6-well plate. DNA content of each well was then determined as previously described (23,61). To examine the effect of lowered c-*fos* expression on cisplatin sensitivity, L1-3c-*fos* cells in log-phase growth were seeded overnight in 24-well plates at a density of approximately 2000 cells/well in the absence or presence of 2 mM IPTG. Cell survival following a 1 hour treatment with cisplatin was assessed as described above.

Cisplatin sensitivity was also assessed in 208F and CMVc-*fos* using a colony forming assay. In this assay 208F and CMVc-*fos* cells in log phase growth were seeded in 6-well plates at a density of approximately 200 cells/well. Cells were treated with cisplatin for 1 hour and allowed to grow for 6 days in drug-free medium. Surviving colonies were stained with methylene blue and colonies containing 20 or more cells were counted.

To examine the interaction between cisplatin and c-*fos* antisense, 2008 and C13* cells were seeded overnight in 24-well plates at a density of approximately

5000 2008 cells/well and approximately 2500 C13* cells/well. Cells were then treated with c-*fos* antisense (concentrations ranging from 0.00975-0.3125 nmoles) in a 1:9 ratio with lipofectin in serum-free RPMI media for 4 hours. Cells were then returned to fully supplement RPMI media and various concentrations of cisplatin were added for 1 hour either immediately or 24 hours after c-*fos* antisense treatment. Cell survival was assessed 5 days after the initiation of c-*fos* antisense treatment using a DNA fluorochrome assay as described above. The nature of the interaction between cisplatin and c-*fos* antisense was assessed using median effect analysis. Combination indexes (CIs) were calculated and a CI of 1 indicates that the two agents interact in an additive fashion while CIs of < 1 and >1 indicate that the two agents interact in a synergistic and antagonistic fashion, respectively (69).

Rh123 Accumulation and Efflux. 208F and CMV*c-fos* cells in log-phase growth were seeded overnight in 24-well plates at a density of approximately 1.5 x 10^5 cells/well and Rh123 accumulation and efflux were determined as previously described (23,61). To examine the effect of lowered c-*fos* expression on mitochondrial membrane potential L1-3c-*fos* cells in log-phase growth were seeded overnight in 24-well plates at a density of approximately 1.5 x 10^5 cells/well in the presence or absence of 2 mM IPTG. Rh123 accumulation was determined as previously described (23,61).

A2780 and A2780DDP cells in log-phase growth were seeded overnight in 6-well plates at a density of approximately 5 x 10^5 cells/well. Media was removed and

replaced with normal Dulbecco's media or Dulbecco's media supplemented with 137 mM KCl both of which contained 50 μM of Rh123. Following Rh123 removal at the appropriate time interval, wells were washed twice with phosphate-buffered saline and deionized water (900 μl) was added to each well. After one freeze-thaw, 300 μl of the DNA fluorochrome, H33258 (40 μg/ml in TNE buffer; 10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4) was added to each well to determine DNA content/cell number in each well (70). Rh123 and DNA fluorochrome fluorescence was determined as previously described (23,61).

10*N*-Nonyl Acridine Orange (NAO) Staining. 208F, CMV*c-fos*, and L1-3c-*fos* cells in log-phase growth were seeded overnight in 24-well plates at known numbers (2×10^5 - 1.25×10^4 cells/well). A2780 and A2780DDP cells in log-phase growth were seeded overnight in 6-well plates at known numbers (2×10^6 - 1.25×10^5). The media was removed and replaced with media containing 10 μg/ml of NAO (a stock of 10 mg/ml NAO in DMSO was stored at -20°C) (71,72). Following a 30 minute incubation at 37°C, the NAO-containing media was removed and the cells were washed twice with phosphate buffered saline. Deionized water (300 μl for 24-well plates or 900 μl for 6-well plates) was added to each well and the plates were read on a Cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ontario, Canada) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

RNA Extraction. Cells in log-phase growth were seeded overnight in 150 x 25 mm culture dishes at a density of 1 x 10^7 cells/dish. Cells were then exposed to an IC_{90} cisplatin concentration for 1 hour. RNA extraction using the method of Chomczynski and Sacchi (73) was initiated immediately, 1 hour, or 2 hours after the cisplatin treatment. RNA was stored in diethylpyrocarbonate-treated water at -20°C.

Slot Blot Analysis of RNA. RNA was denatured and heated for 15 minutes at 60°C. Serial dilutions of RNA (maximum of 20 μg) in diethylpyrocarbonate-treated water were bound to GeneScreen Plus nylon filters using a slot-blot apparatus. Duplicate filters were generated; one was probed for *c-fos* mRNA while the other was probed with an oligo$(dT)_{18}$ to determine the amount of mRNA blotted. The oligo$(dT)_{18}$ was labelled with [γ-^{32}P]ATP by the method of Maxam and Gilbert (74) while the human *c-fos* oligonucleotide was labelled with [γ-^{32}P]ATP according to the manufacturer's protocol. Slot-blots probed with the oligo$(dT)_{18}$ were prehybridized in 15 ml containing 6x SSPE (20x SSPE contains 3 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA, pH 7.4), 5x Denhardt's (0.1 % Ficoll, 0.1 % polyvinylpyrrolidone, and 0.1 % bovine serum albumin), 1 % sodium dodecyl sulphate, and 0.1 % sheared salmon sperm DNA for 1 hour at 42°C. Hybridization was carried out in 15 ml containing 5x SSPE, 5x Denhardt's, 0.005 M Na_2HPO_4, and 2 μg of ^{32}P-labelled oligo$(dT)_{18}$ for 1 hour at room temperature. Blots were washed four times for 5 minutes each at room temperature in 2x SSC (0.3 M NaCl, 0.3 M sodium citrate). Slot-blots probed with the *c-fos* oligonucleotide were prehybridized in 15 ml containing 1 M NaCl, 50 mM

Tris-HCl pH 7.5, 10% dextran sulfate, 1% SDS, and 100 μg/ml of sheared salmon sperm DNA for 1 hour at 65°C. Labelled probe was then added to the prehybridization solution and the membranes were incubated overnight at 65°C. Membranes were washed, four times briefly in 2x SSC and 0.1 % SDS at room temperature, one time for 30 minutes in 2x SSC and 0.1 % SDS at 65°C, one time for 5 minutes in 2x SSC and 0.1 % SDS at room temperature followed by one brief rinse in 2x SSC at room temperature. Membranes were placed in phosphoimaging cassettes and the amount of radioactivity was determined on a phosphoimaging system (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Relationship Between c-*fos* Expression and Cisplatin Resistance. Western analysis confirmed c-Fos protein levels in 208F, CMV*c-fos*, and L1-3*c-fos* cells. Basal c-Fos protein levels were examined in logarithmically growing cells and were found to be elevated in the CMV*c-fos* cells compared to the parental 208F cells (Fig. 1a). We also confirmed that addition of 2 mM IPTG to the medium was capable of reducing c-Fos protein levels in L1-3*c-fos* cells as previously described (67) (Fig. 1a). IPTG, which should have no effect on the CMV promoter, did not alter c-Fos protein levels in CMV*c-fos* cells (Fig. 1a). To assess whether reduced c-Fos protein levels, following addition of IPTG to L1-3*c-fos* cells, altered c-Fos's function, we examined the levels of cyclin D1 in these cells since the levels of this protein appear to be regulated by *c-fos* expression (67). We found that cyclin D1 levels were also reduced in L1-3*c-fos* cells following an overnight incubation with 2 mM IPTG suggesting that c-Fos protein function is reduced (Fig. 1b).

To evaluate the effect of *c-fos* expression on cisplatin sensitivity, 208F and CMV*c-fos* cells were treated with cisplatin (Fig. 2a). CMV*c-fos* cells were 3.2 ± 0.6-fold (mean ± SEM; n=3) resistant to cisplatin compared to 208F cells at an IC_{50} as determined by a DNA fluorochrome assay. A colony forming assay also showed that CMV*c-fos* cells were 2.9-fold (n=2) resistant to cisplatin compared to 208F cells at an IC_{50}. In addition, L1-3*c-fos* cells were resistant to cisplatin compared to 208F cells. The IC_{50} for cisplatin was 5.6 ± 1.0 μM in the L1-3*c-fos* cells and 2.4 ± 0.1 μM

in the 208F cells (Fig. 2a). The correlation between c-*fos* expression and cisplatin sensitivity was further investigated in L1-*3c-fos* cells in the presence and absence of IPTG. We observed that lowering c-*fos* expression in L1-3c-*fos* cells, through the addition of IPTG, did not increase the sensitivity of these cells to cisplatin (Fig. 2b).

The correlation between c-*fos* expression and cisplatin sensitivity was also examined in a human ovarian carcinoma model system. This system contained a parental line, 2008, and a cisplatin-resistant variant, C13*. Since c-*fos* expression had not been determined in these cells we looked at both c-*fos* mRNA and protein levels. There was no significant difference in either the basal levels of c-*fos* mRNA (C13* cells contained 1.2 ± 0.3-fold (mean ± SEM; n=6) more c-*fos* mRNA than 2008 cells) or c-Fos protein (Fig. 3a) in C13* cells compared to 2008 cells. Furthermore, c-*fos* expression was not induced, at either the mRNA (Table 1) or protein level (Figs. 3b & 3c), in either cell type, following a one hour treatment with their respective IC_{90} concentration of cisplatin.

It is possible that c-*fos* expression is already high in both 2008 and C13* cells and thus, the effect of lowering c-*fos* expression on cisplatin sensitivity, using an antisense oligonucleotide for c-*fos* mRNA, was examined. Western analysis showed that c-Fos protein levels were reduced following c-*fos* antisense treatment. The c-*fos* antisense was capable of lowering c-Fos protein levels in a dose-dependent fashion by 24 hours and the levels remained low after 48 hours in C13* cells (Fig. 4a). To investigate whether the addition of any oligonucleotide to C13* cells would effect c-Fos protein levels, C13* cells were incubated with a control

oligonucleotide which has no known complementary mRNA. At a concentration equivalent to the highest concentration of c-*fos* antisense used (0.625 nmoles), the control oligonucleotide did not appear to alter c-Fos protein levels (Fig. 4b). c-*fos* antisense was also shown to be specific for c-*fos* mRNA, since there was no obvious reduction in c-Shc protein levels following c-*fos* antisense treatment (Fig. 4c). However, the levels of cyclin D1 protein, which have been reported to be regulated by c-*fos* expression (67), where decreased in C13* samples following c-*fos* antisense treatment (Fig. 4d). Similar results were observed in 2008 cells (Figs. 5a & 5b).

Cisplatin sensitivity was investigated in 2008 and C13* cells following treatment with c-*fos* antisense. When 2008 and C13* cells were exposed to c-*fos* antisense for 4 hours followed by a one hour cisplatin treatment either immediately after the antisense treatment (Figs 6a & 6b) or 24 hours after c-*fos* antisense treatment (Figs. 6c & 6d), a synergistic interaction between cisplatin and c-*fos* antisense was not observed. However, a reduction in c-*fos* expression was capable of lowering the amount of cisplatin required to obtain an equivalent decrease in cell survival, particularly in C13* cells.

Relationship Between Mitochondrial Membrane Potential and Cisplatin Resistance. There was no significant difference in the mitochondrial membrane potential in the human ovarian carcinoma cell line, A2780, compared to its cisplatin-resistant variant, A2780DDP as indicated by Rh123 accumulation in media

supplemented with 137 mM KCl (Fig. 7a). To ensure that an alteration in mitochondrial number had not occurred, A2780 and A2780DDP cells were probed with NAO, an indicator of inner mitochondrial membrane mass (71,72). There was no significant difference in NAO staining; A2780 cells had 1.3 ± 0.2-fold (mean ± SEM; n=3) more NAO staining than A2780DDP cells. We have shown that cells with elevated mitochondrial membrane potentials are hypersensitive to Deca (61) and thus we examined Deca sensitivity in A2780 and A2780DDP cells (Fig. 7b). A2780DDP did not display an increased sensitivity to Deca compared to A2780 cells further suggesting that cisplatin resistance in A2780DDP cells was not associated with an increase in mitochondrial membrane potential.

Since we had shown that CMVc-*fos* cells were resistant to cisplatin compared to 208F cells we also tested these cells for changes in their mitochondrial membrane potential. There was no significant difference in mitochondrial membrane potential between CMVc-*fos* and 208F cells as indicated by Rh123 accumulation in media supplemented with 137 mM KCl (Fig. 8a). Also, CMVc-*fos* cells did not retain Rh123 for longer periods of time compared to 208F cells (Fig. 8b) nor were CMVc-*fos* cells more sensitive to Deca than 208F cells (Fig. 8c). These data support the Rh123 accumulation data in suggesting that cisplatin resistance in CMVc-*fos* cells is not associated with an increase in mitochondrial membrane potential. In addition, 208F cells had 1.2 ± 0.2-fold (mean ± SEM; n=4) more NAO staining than CMVc-*fos* cells suggesting that the two cell types did not differ in their mitochondrial mass.

Relationship Between c-*fos* Expression and Mitochondrial Membrane Potential. We examined whether c-*fos* was capable of regulating mitochondrial membrane potential as was observed with v-*fos,* by Zarbl *et al.* (75,76). A correlation between an increase in c-*fos* expression and an elevation in mitochondrial membrane potential was not observed; (i) CMVc-*fos* cells, which overexpress c-*fos* compared to 208F cells, did not possess an elevated mitochondrial membrane potential compared to 208F cells (Fig. 8a), (ii) lowering c-*fos* expression in L1-3c-*fos* cells by treatment with IPTG did not alter the mitochondrial membrane potential (Fig. 9) or effect NAO staining (NAO staining in the presence of IPTG was 1.0 ± 0.2-fold (mean ± SEM, n=3) more than in the absence of IPTG), (iii) A2780DDP cells which have been reported to have elevated levels of c-*fos* mRNA compared to A2780 cells (53), did not have an elevation in their mitochondrial membrane potential relative to A2780 cells (Fig. 7a), and (iv) C13* cells which have an elevation in their mitochondrial membrane potential compared to 2008 cells (56,61) did not contain significantly more c-*fos* mRNA than 2008 cells. Similarly, there was no apparent difference in c-Fos protein levels between C13* and 2008 cells (Fig. 3a).

DISCUSSION

This study was designed to examined the correlations between (i) c-*fos* expression and cisplatin resistance and (ii) elevated mitochondrial membrane potential and cisplatin resistance in an attempt to identify novel approaches for treating cisplatin-resistant tumours. Elevated expression of the *c-fos* gene has been associated with cisplatin resistance in patient's tumours that did not respond to cisplatin-based chemotherapy and in established cisplatin-resistant cell lines (54,77). This study demonstrated that constitutive overexpression of the c-*fos* gene can lead to cisplatin resistance since CMVc-*fos* and L1-3c-*fos* cells were resistant to cisplatin compared to 208F cells. It remains unclear why addition of IPTG to L1-3c-*fos* cells, which lowers c-Fos protein levels, did not effect cisplatin sensitivity. This IPTG treatment has been shown to reduce c-Fos binding to AP-1 sites and inhibit c-Fos target gene expression in L1-3c-*fos* cells (67). It is possible that c-*fos* expression is rapidly inhibited, yet, some c-Fos target genes may have longer half lives and it is these target genes which may ultimately be responsible for determining cisplatin sensitivity. Alternatively, the reduced growth rate observed in L1-3c-*fos* cells treated with IPTG (67) may limit cisplatin cytotoxicity since cisplatin is more effective against rapidly proliferating cells (3).

Although a correlation between an increase in c-*fos* expression and cisplatin resistance has been observed in this study (CMVc-*fos* and L1-3c-*fos* cells) and in a human ovarian carcinoma cell line (53), overexpression of c-*fos* may not occur in all cases of cisplatin resistance. Cisplatin resistance, in the absence of c-*fos*

overexpression, was observed in C13* cells. C13* cells are resistant to cisplatin compared to 2008 cells, but C13* cells have similar levels of c-*fos* mRNA and protein as 2008 cells. Furthermore, the interaction between c-*fos* antisense and cisplatin did not produce synergistic cell kill in either 2008 or C13* cells. This finding is in contrast to observations previously reported in a different human ovarian carcinoma cell line. Scanlon *et al.* (53,55,78) showed that lowering c-*fos* expression using either cyclosporin A or a c-*fos* ribozyme, restored cisplatin sensitivity. The difference between this study and those by Scanlon *et al.* (53,55,78) is that we observed a dose-dependent toxicity of c-*fos* antisense treatment alone in both 2008 and C13* cells while cell survival following treatment with the c-*fos* ribozyme was not provided. It seems unlikely that the c-*fos* ribozyme would have no effect on cell growth/survival since we found that c-*fos* antisense treatment alone, decreased growth/survival in A2780 and A2780DDP cells (data not shown). We did find that a c-*fos* antisense concentration of 0.195 nmoles reduced the amount of cisplatin required to kill 50% of C13* cells by approximately 6-fold (n=2) but the interaction between cisplatin and c-*fos* antisense was additive at best. These results suggest that C13* cells have acquired cisplatin resistance through mechanisms independent of an increase in c-*fos* expression. A lack of an association between c-*fos* expression and cisplatin sensitivity has also been observed in NIH 3T3 cells transfected with the c-*fos* gene (79).

A mitochondrial membrane potential is essential for cellular processes such as protein import into mitochondria and production of ATP through oxidative

phosphorylation (57,59,60,80). Changes in mitochondrial characteristics have been associated with cell transformation (63,80) and drug resistance (22,23,56,81). Although an increase in mitochondrial membrane potential was associated with cisplatin resistance in C13* cells, we found no correlation between cisplatin resistance and an increase in mitochondrial membrane potential in either the 208F/CMVc-*fos* or A2780/A2780DDP model systems suggesting that cisplatin resistance can occur independent of an increase in mitochondrial membrane potential.

This study also examined the relationship between c-*fos* expression and mitochondrial membrane potential. Zarbl *et al.* (75) observed that transfection of rat fibroblast cells with v-*fos* induced an increase in mitochondrial membrane potential, as determined by Rh123 accumulation and retention, in v-*fos*-transfected cells compared to non-transfected controls. A number of other viral oncogenes including v-Ha-*ras* and v-*myc* were unable to alter the mitochondrial membrane potential (75). Furthermore, a gene termed *fte-1*, which shares considerable homology to a gene involved in mitochondrial protein import in yeast, was found to be essential for the elevation in mitochondrial membrane potential in the v-*fos* transformed cells (82). To our knowledge, the ability of c-*fos* to regulate mitochondrial activity has not been examined. We found no relationship between c-*fos* expression and mitochondrial membrane potential in any of our three model systems suggesting that c-*fos,* is unlike v-*fos,* in its ability to regulate mitochondria. Differences in protein products between of v-*fos* and c-*fos* and/or differences in the regulation of v-Fos and c-Fos

protein may contribute to the contrasting mitochondrial influence these two genes possess (83,84). It is possible that *c-fos* could alter some other mitochondrial characteristic since we only examined the mitochondrial membrane potential.

In summary, we have observed that while both overexpression of *c-fos* and elevations in mitochondrial membrane potential have been associated with cisplatin resistance these characteristics are not interdependent. Treatment of cisplatin-resistant tumours may be improved by modulating *c-fos* expression or mitochondrial function but it appears that pre-screening tumours would be required to determine the optimal approach to restore cisplatin sensitivity.

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FIGURE LEGENDS

Fig. 1. (a) c-Fos protein levels in 208F, CMV*c-fos*, and L1-3*c-fos* cells in the absence and presence of 2 mM IPTG and (b) cyclin D1 protein levels in L1-3c-*fos* cells in the absence and presence of 2 mM IPTG.

Fig. 2. Effect of cisplatin on (a) (O) 208F, (●) CMV*c-fos*, and (□) L1-3*c-fos* cell survival or (b) L1-3c-*fos* cell survival in the (O) absence or (●) presence of 2 mM IPTG. Cytotoxicity was assessed by a DNA fluorochrome assay 5 days after a 1-hour cisplatin exposure. Values are expressed as percentage of control ± SEM (*bars*), (n=4).

Fig. 3. Protein levels of c-Fos in (a) 2008 and C13* cells with no cisplatin pre-treatment, (b) 2008 cells after a 1 hour incubation with an IC_{90} concentration of cisplatin, and (c) C13* cells after a 1 hour incubation with an IC_{90} concentration of cisplatin

Fig. 4. Protein levels in C13* cells; (a) c-Fos protein levels 24 or 48 hours after c-*fos* antisense treatment, (b) c-Fos protein levels 48 hours after no treatment or treatment with a control oligonucleotide, (c) c-Shc protein levels 48 hours after c-*fos* antisense treatment, and (d) cyclin D1 protein levels 48 hours after c-*fos* antisense treatment.

Fig. 5. Protein levels of (a) c-Fos or (b) cyclin D1 in 2008 cells 48 hours after c-*fos* antisense treatment.

Fig. 6. Interaction of cisplatin and c-*fos* antisense in 2008 and C13* cells. Cells were treated with various concentrations of cisplatin for 1 hour either immediately after the 4 c-*fos* antisense treatment in (a) 2008 and (b) C13* cells or 24 hours after the c-*fos* antisense treatment in (c) 2008 and (d) C13* cells. Cell survival was determined using a DNA fluorochrome assay. Values are expressed as mean ± SEM (*bars*), (n=3).

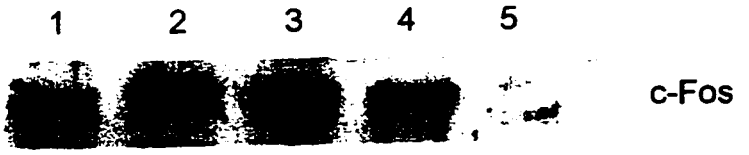
Fig. 7. Evaluation of mitochondrial membrane potential in A2780 and A2780DDP cells by measuring (a) Rh123 accumulation and (b) Deca sensitivity. Rh123 accumulation was determined in Dulbecco's media supplemented with 137 mM KCl in (O) A2780 and (●) A2780DDP cells. Deca sensitivity was assessed in (O) A2780 and (●) A2780DDP cells 5 days after a 3 hour treatment with Deca. Cell survival was determined using a DNA fluorochrome assay. Values represent mean ± SEM (*bars*), (n=3).

Fig. 8. Evaluation of mitochondrial membrane potential in 208F and CMVc-*fos* cells by measuring (a) Rh123 accumulation, (b) Rh123 retention, and (c) Deca sensitivity. Rh123 accumulation was determined in Dulbecco's media supplemented with 137 mM KCl in (O) 208F and (●) CMVc-*fos* cells. Rh123 retention was determined in

(O) 208F and (●) CMVc-*fos* cells at various time intervals following a 3 hour incubation with Rh123 in RPMI media containing 137 mM KCl. Following the 3 hour Rh123 incubation, cells were maintained in RPMI media + 137 mM KCl. Deca sensitivity was assessed in (O) 208F and (●) CMVc-*fos* cells 5 days after a 3 hour treatment with Deca. Cell survival was determined using a DNA fluorochrome assay. Values in all experiments represent mean ± SEM (*bars*), (n=3).

Fig. 9. Rh123 accumulation after a 3 hour incubation in L1-3c-*fos* in the absence or presence of 2 mM IPTG. Rh123 accumulation was determined in (■) normal Dulbecco's media or (□) Dulbecco's media supplemented with 137 mM KCl. Values represent mean (*columns*) ± SEM (*bars*), (n=3).

1a

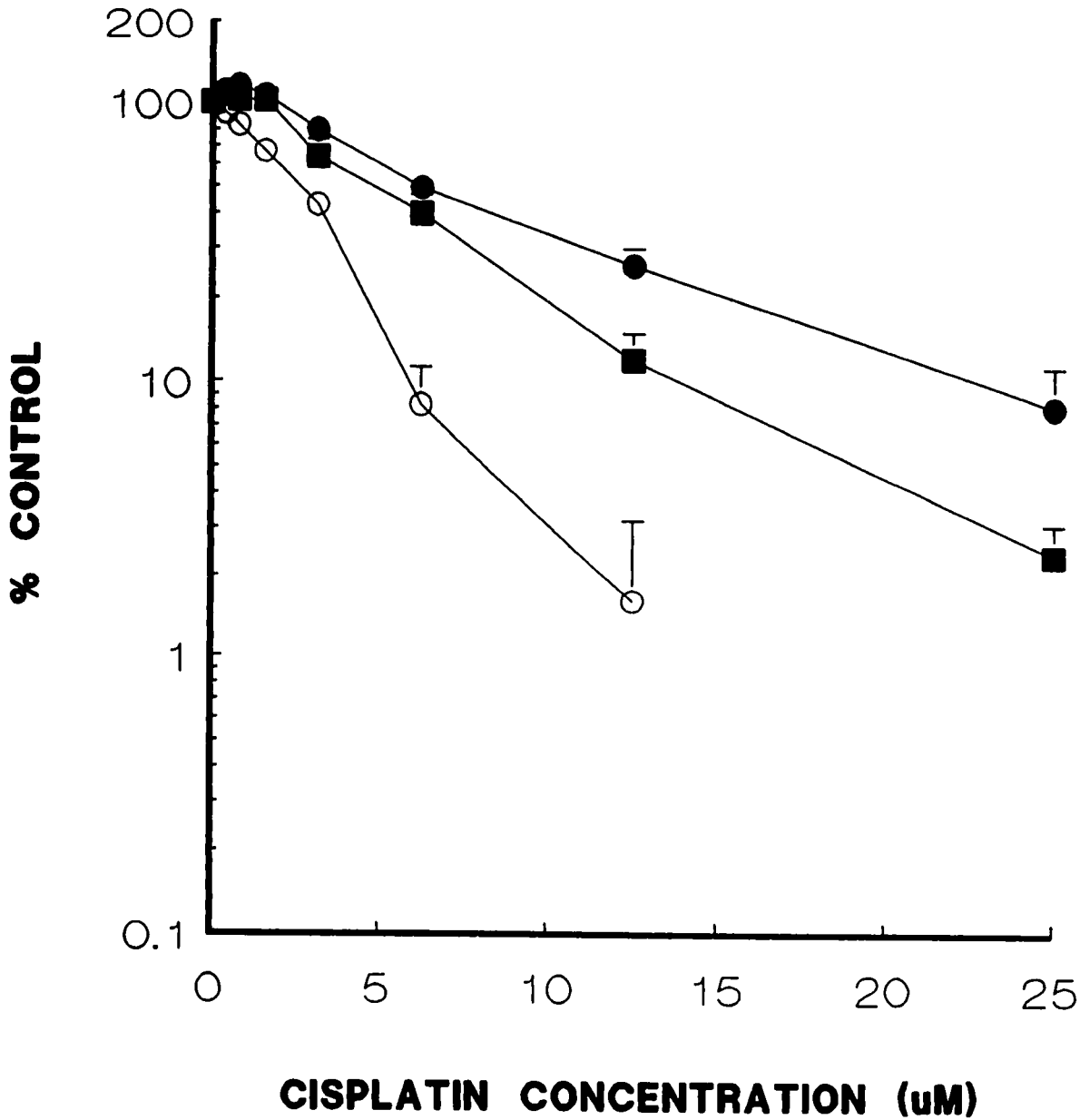


Lanes 1 208F
2 CMVc-*fos*
3 CMVc-*fos* + IPTG
4 L1-3c-*fos*
5 L1-3c-*fos* + IPTG

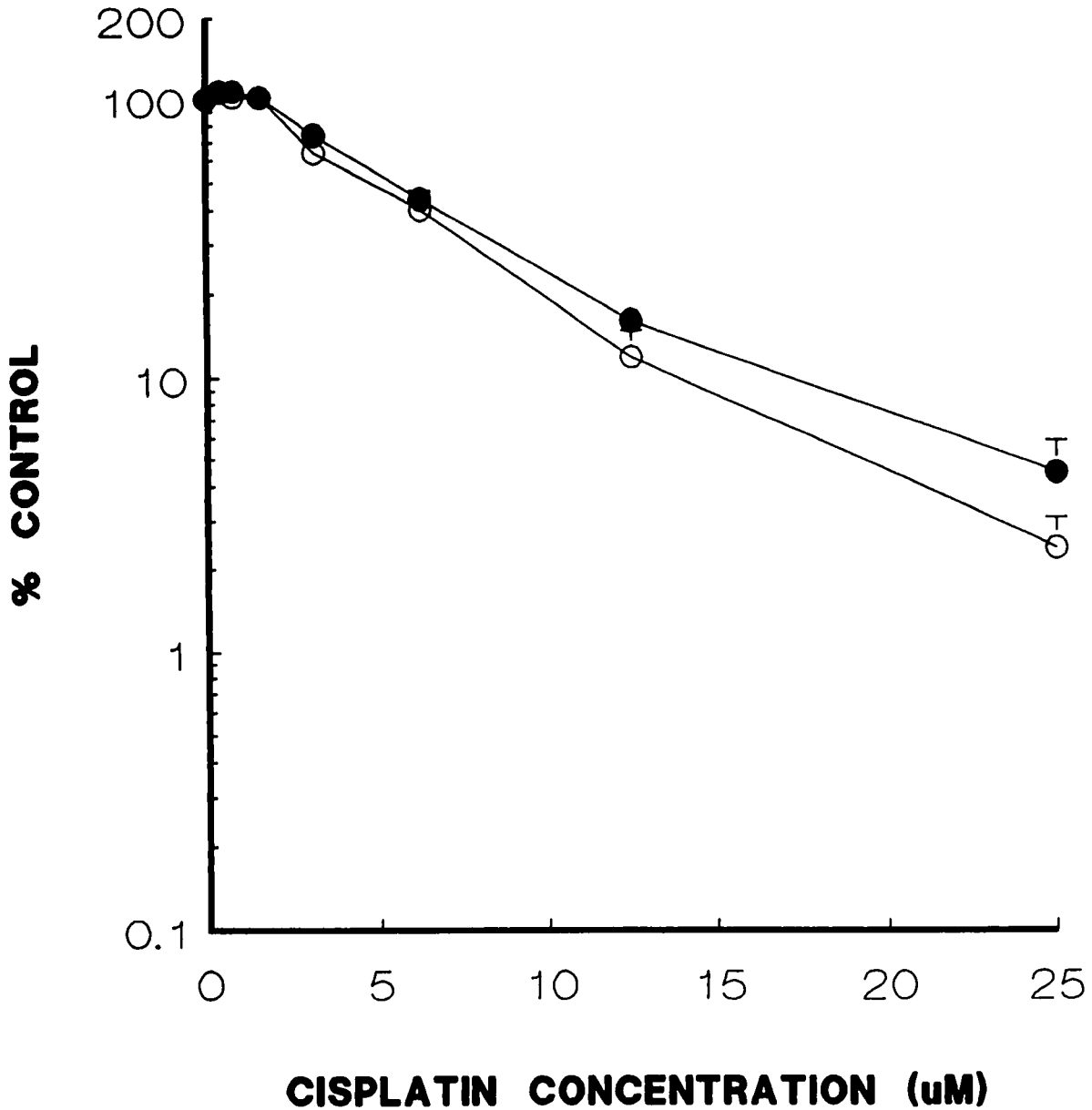
1b

Lanes 1 L1-3c-*fos*
2 L1-3c-*fos* + IPTG

2a


200
100
10
1
0.1
% CONTROL
0
5
10
15
20
25
CISPLATIN CONCENTRATION (uM)

2b


200
100
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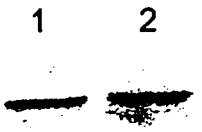
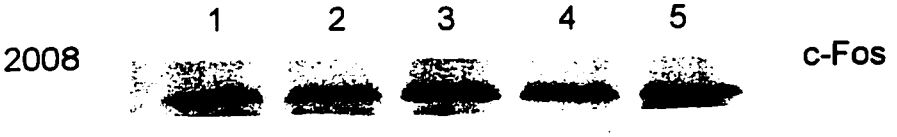
1
0.1
% CONTROL
0
5
10
15
20
25
CISPLATIN CONCENTRATION (uM)

3a

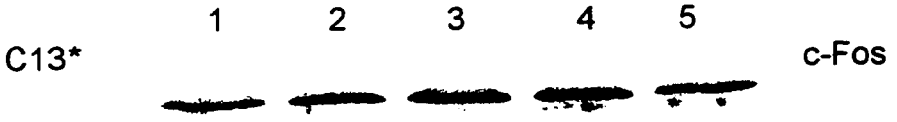


Lanes 1 2008
2 C13*

3b

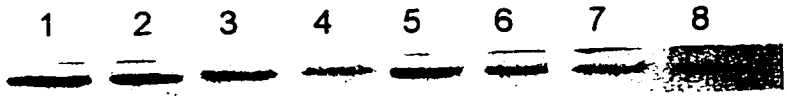


3c



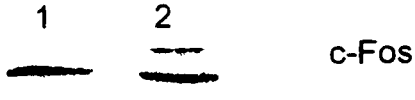
Lanes 1 no cisplatin treatment
2 2 hours after cisplatin treatment
3 4 hours after cisplatin treatment
4 6 hours after cisplatin treatment
5 12 hours after cisplatin treatment

4a



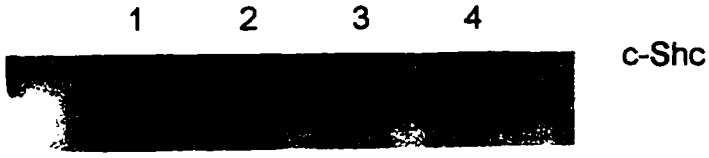
Lanes 1 24 hour control
2 24 hours after 0.156 nmoles of c-*fos* antisense
3 24 hours after 0.3125 nmoles of c-*fos* antisense
4 24 hours after 0.625 nmoles of c-*fos* antisense
5 48 hour control
6 48 hours after 0.156 nmoles of c-*fos* antisense
7 48 hours after 0.3125 nmoles of c-*fos* antisense
8 48 hours after 0.625 nmoles of c-*fos* antisense

4b

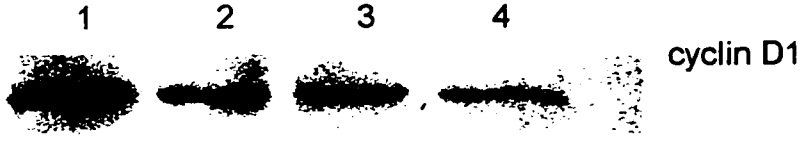


Lanes 1 48 hour control
2 48 hours after control oligonucleotide

4c

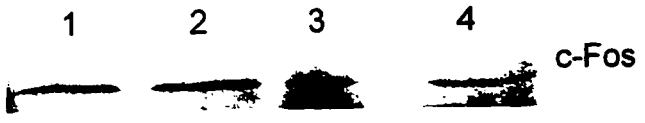


4d

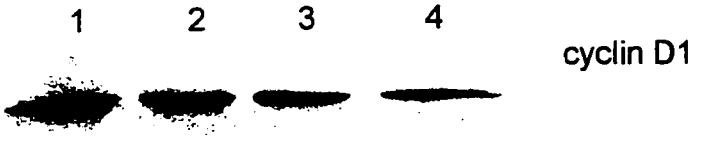


Lanes 1 48 hour control
2 48 hours after 0.156 nmoles of c-*fos* antisense
3 48 hours after 0.3125 nmoles of c-*fos* antisense
4 48 hours after 0.625 nmoles of c-*fos* antisense

5a

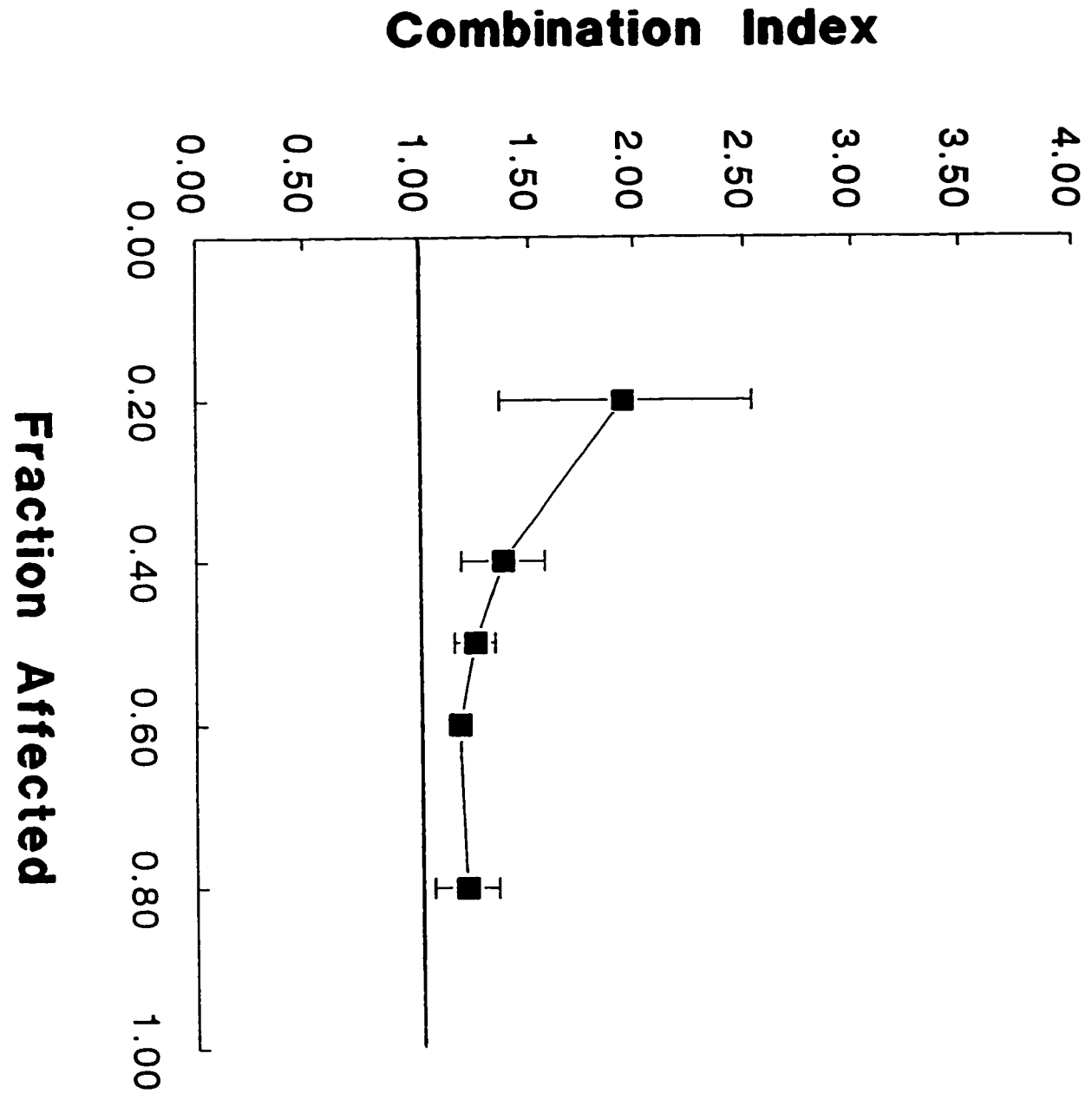


5b

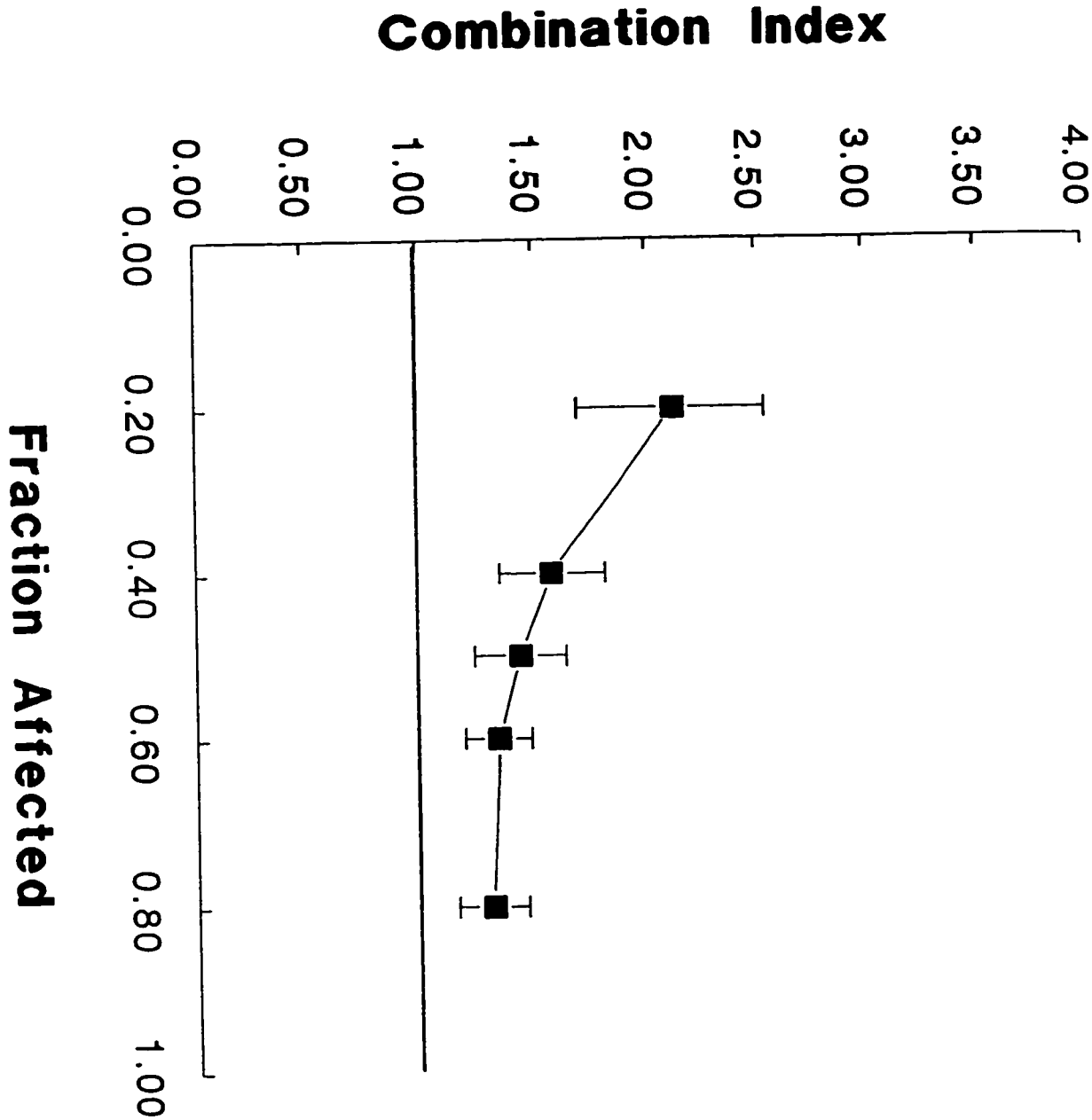


Lanes 1 48 hour control
2 48 hours after control oligonucleotide
3 48 hours after 0.3125 nmoles of c-*fos* antisense
4 48 hours after 0.625 nmoles of c-*fos* antisense

6a


Combination Index
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
Fraction Affected
0.00
0.20
0.40
0.60
0.80
1.00

6b


Combination Index
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0.50
1.00
1.50
2.00
2.50
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4.00
Fraction Affected
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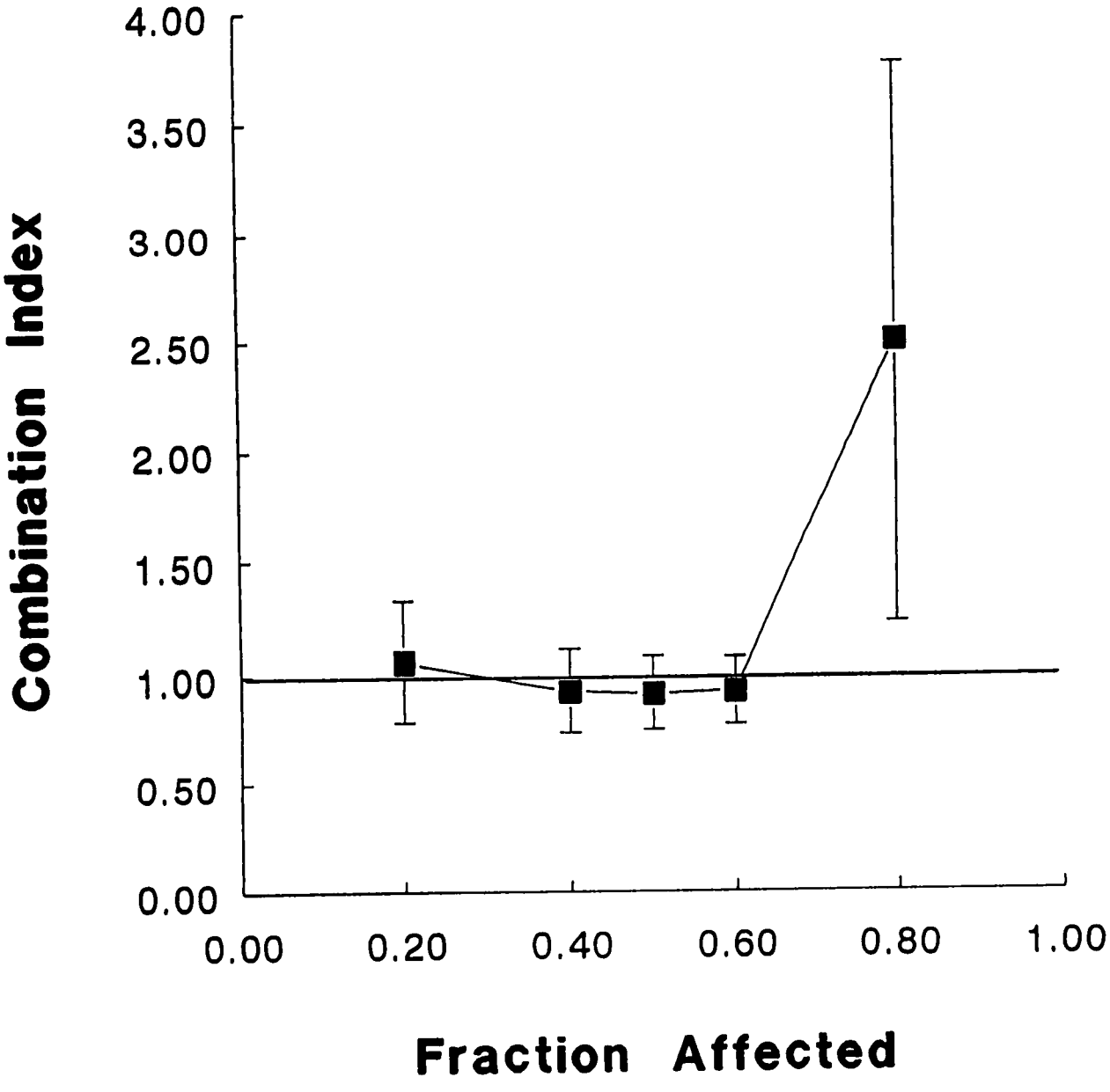
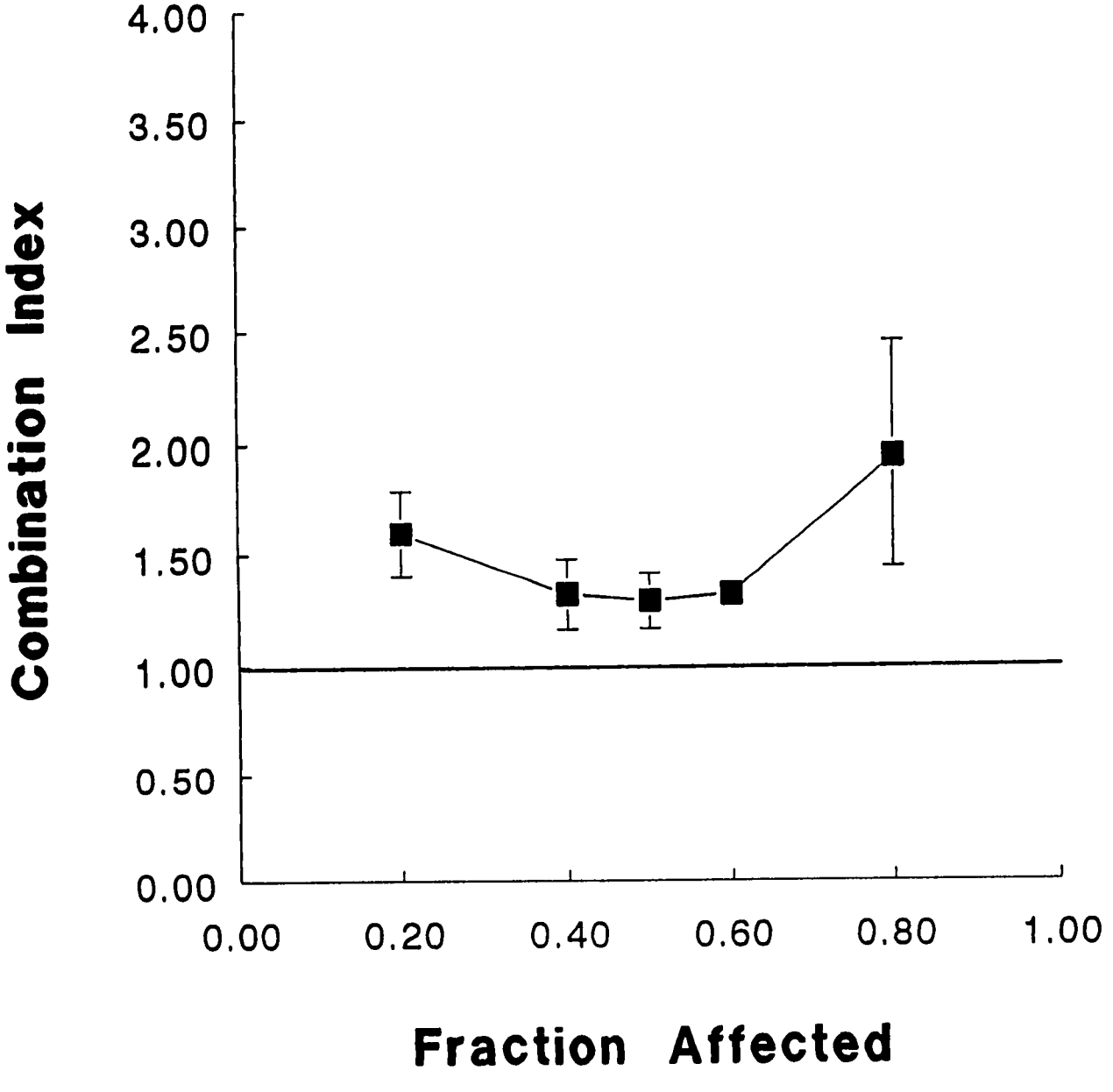
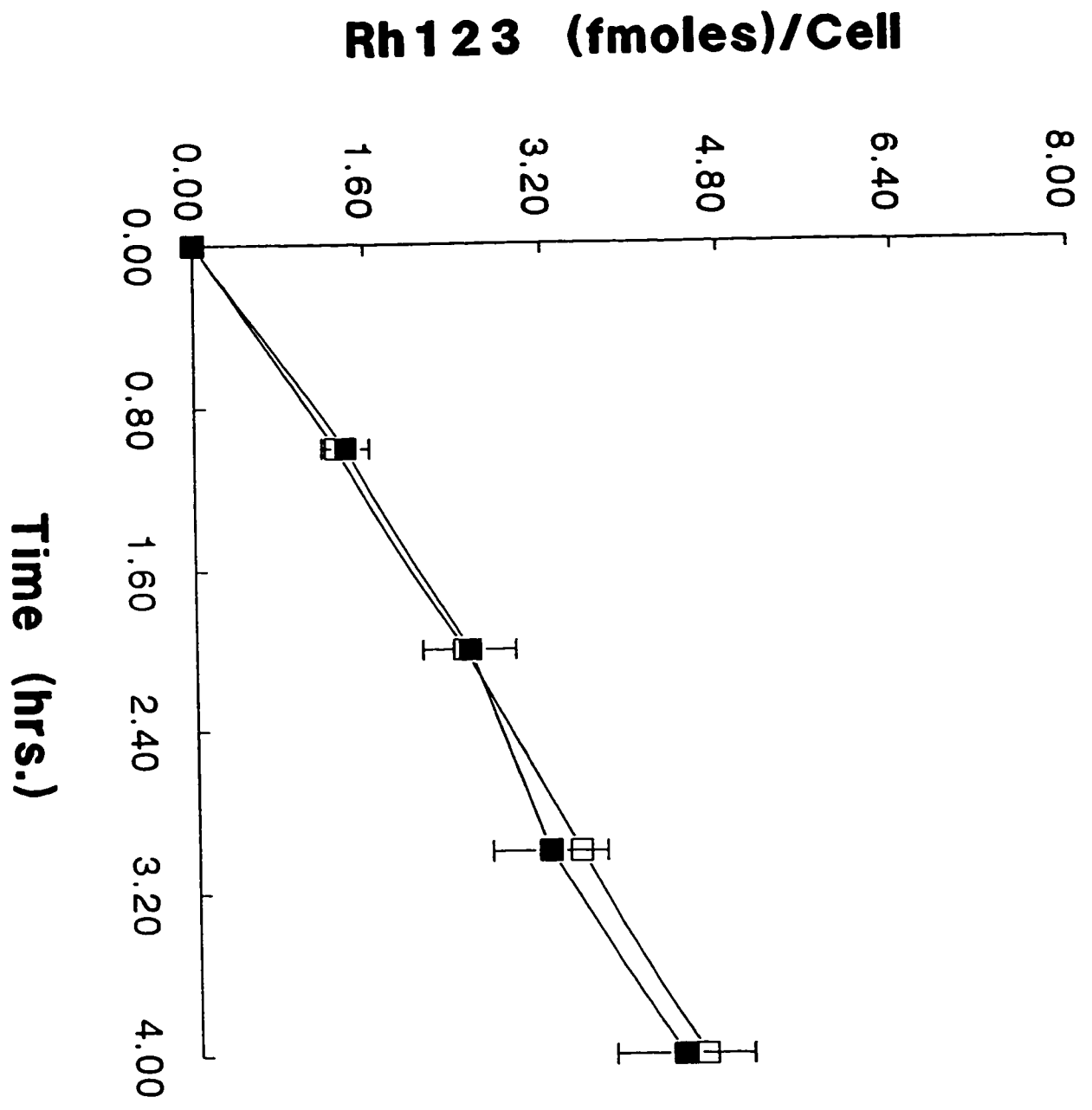
0.40
0.60
0.80
1.00

6c

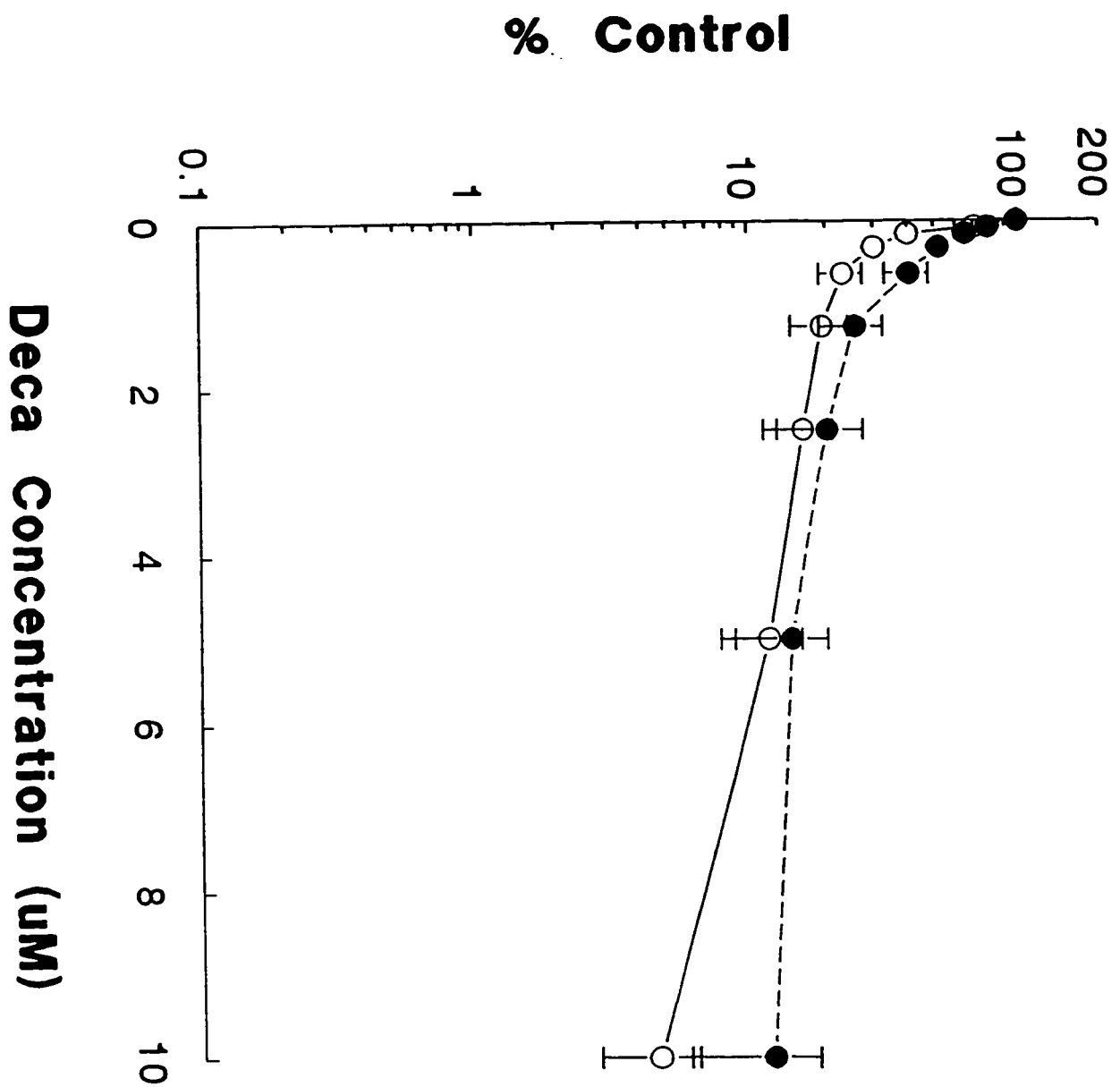

Combination Index
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.20
0.40
0.60
0.80
1.00
Fraction Affected

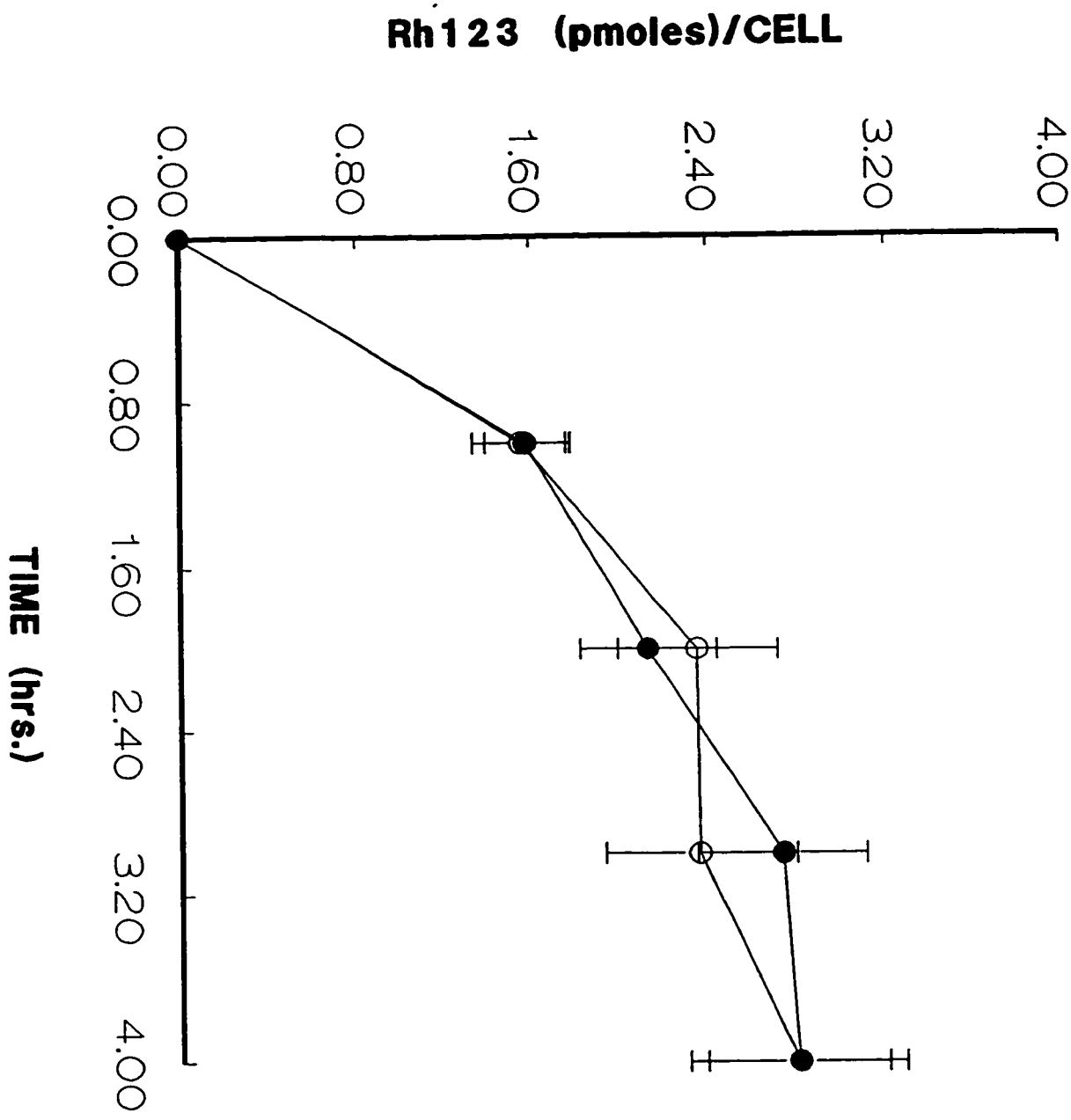
6d


Combination Index
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.20
0.40
0.60
0.80
1.00
Fraction Affected

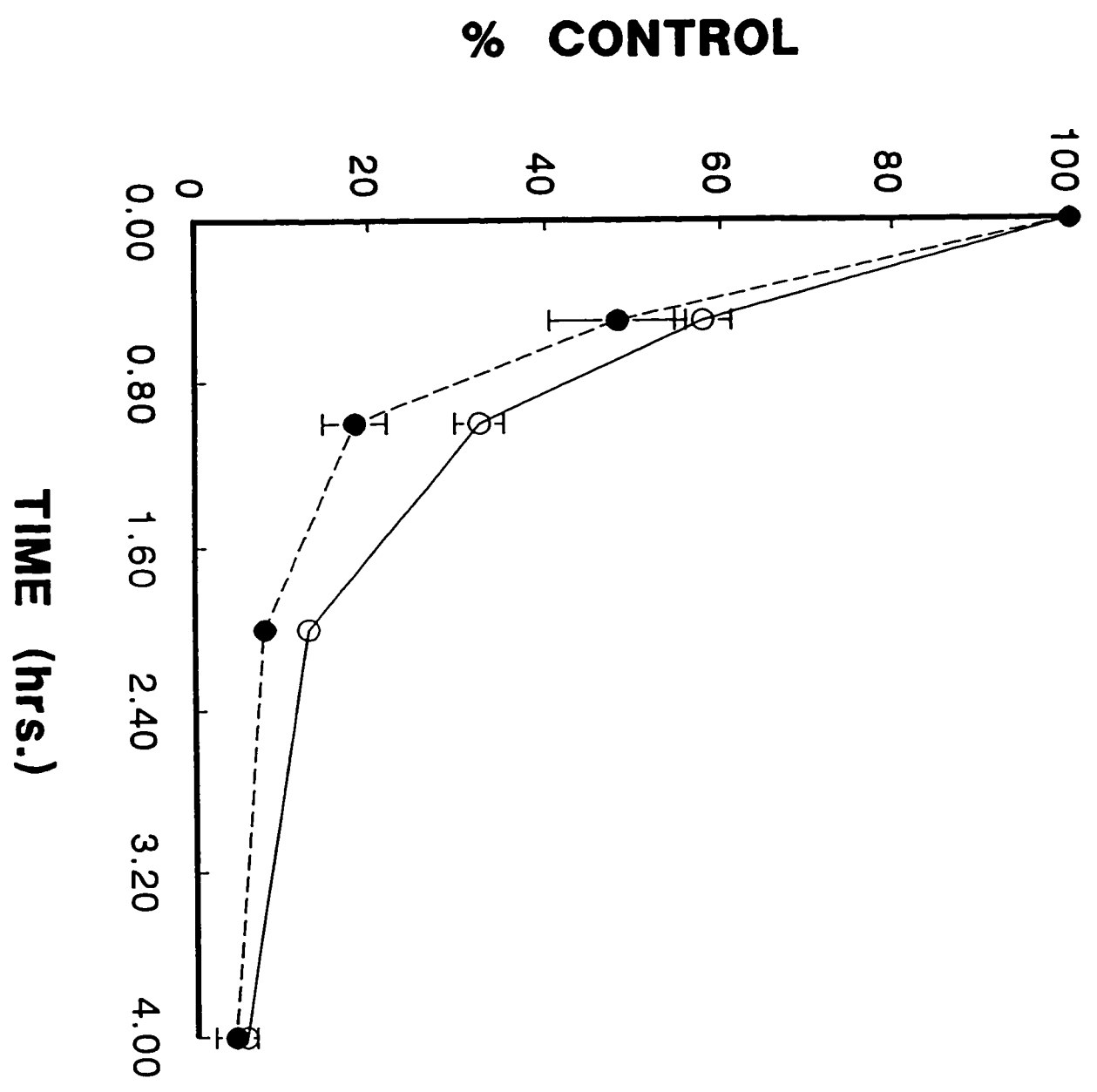

Rh123 (fmoles)/Cell
0.00
1.60
3.20
4.80
6.40
8.00
Time (hrs.)
0.00
0.80
1.60
2.40
3.20
4.00

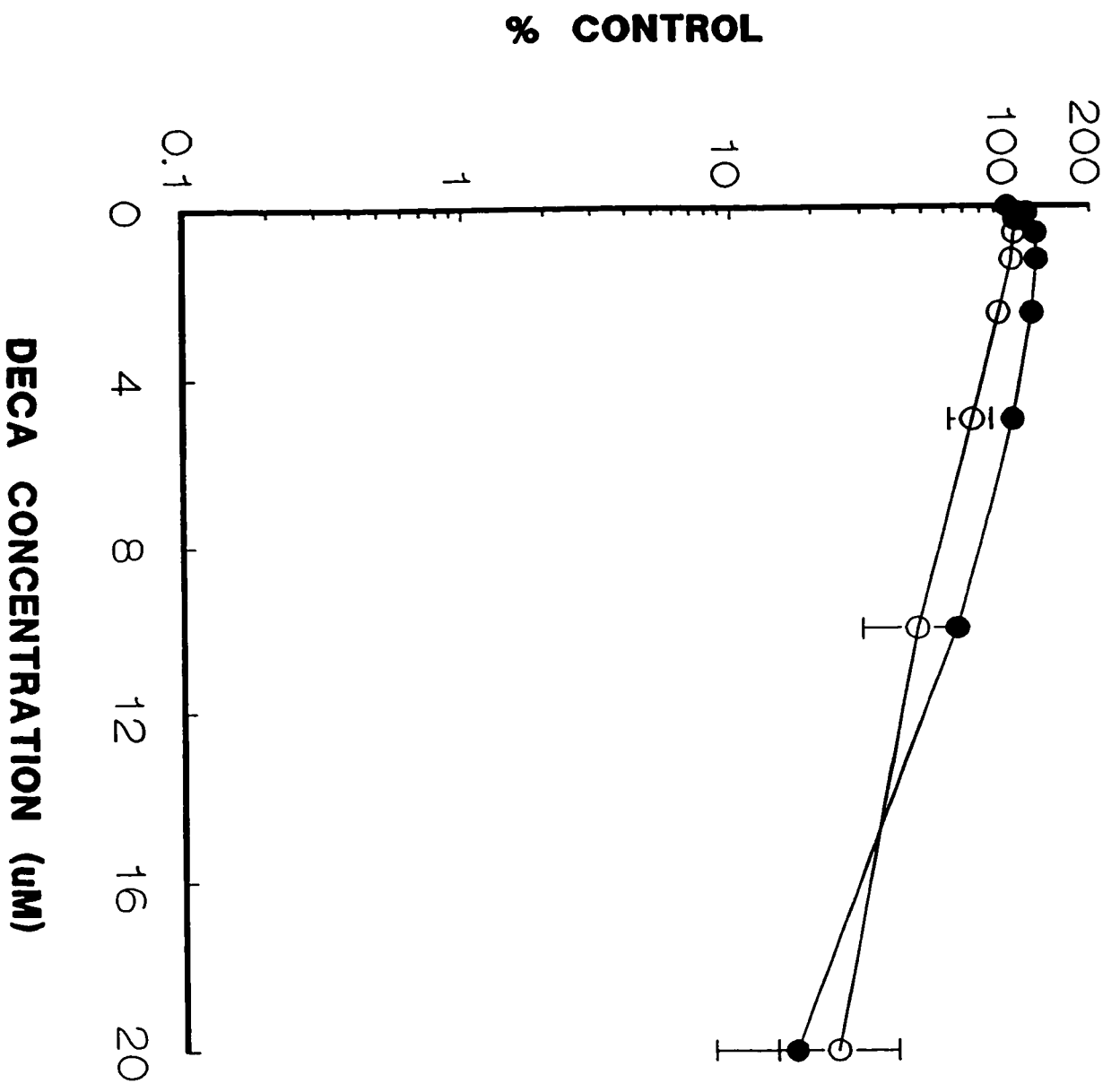
7a


% Control
0.1
1
10
100
200
0
2
4
6
8
10
Deca Concentration (uM)


Rh123 (pmoles)/CELL
0.00
0.80
1.60
2.40
3.20
4.00
0.00
0.80
1.60
2.40
3.20
4.00
TIME (hrs.)

8b




% CONTROL
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1
10
100
200
0
4
8
12
16
20
DECA CONCENTRATION (uM)

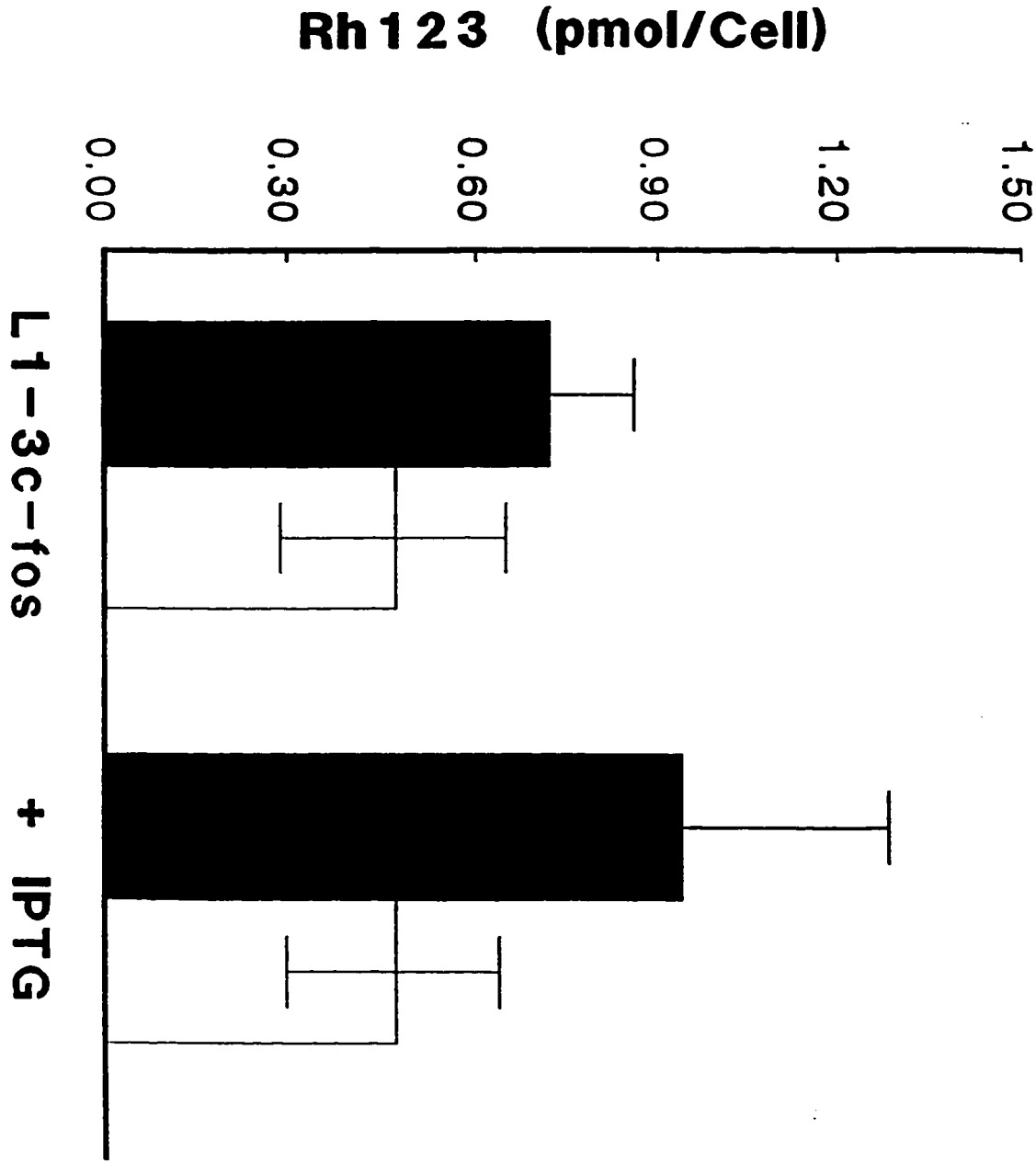
Rh123 (pmol/Cell)
0.00
0.30
0.60
0.90
1.20
1.50
L1-3c-fos
+ IPTG

Table 1 *c-fos mRNA levels following a 1 hour exposure to an IC_{90} cisplatin concentration*

	no cisplatin	Immediately after cisplatin treatment	1 hour after cisplatin treatment	2 hours after cisplatin treatment
2008	1.0	1.2 ± 0.2[a]	0.9 ± 0.2	1.0 ± 0.2
C13*	1.0	0.8 ± 0.2	0.9 ± 0.3	1.0 ± 0.3

[a] the amount of *c-fos* mRNA relative to no cisplatin treatment.

Discussion

Since little is known about the regulation of mitochondria, work by Zarbl *et al.* (482), in which transfection of rat fibroblasts with the v-*fos* gene resulted in an increase in mitochondrial membrane potential, provided intriguing information. This observation combined with the reported association between overexpression of c-*fos* and cisplatin resistance lead us to examine whether overexpression of the c-*fos* gene regulated mitochondrial function and in doing so rendered cells resistant to cisplatin.

Overexpression of c-*fos* has been associated with some cases of cisplatin resistance (145,154,155,487) and thus, the correlation between cisplatin resistance and c-*fos* expression was initially examined. Rat fibroblast cells transfected with the c-*fos* gene were observed to be resistant to cisplatin compared to non-transfected controls. However, reduction of c-*fos* expression in L1-3c-*fos* cells, with IPTG, was unable to restore cisplatin sensitivity. It is possible that even though c-Fos protein levels were reduced after an overnight IPTG treatment, proteins expressed from c-Fos target genes may still be present in adequate amounts. The observations of Miao and Curran (391) indicated that L1-3c-*fos* cells retained cell morphology which was consistent with a transformed phenotype for at least two days after c-Fos protein repression with IPTG. Therefore, adequate amounts c-*fos*-regulated protein may still have be present when cisplatin was added to these cells.

Another possible explanation for the lack of increased cisplatin sensitivity following a reduction in c-Fos protein levels is that IPTG somehow interfered with cisplatin cytotoxicity. Although reduced cisplatin sensitivity in the presence of IPTG has not been previously reported, it was observed that pretreatment of CMVc-*fos* cells with IPTG slightly reduced cisplatin sensitivity in these cells (unpublished data). Since IPTG had no effect on c-*fos* expression in CMVc-*fos* cells, these results suggest that IPTG may somehow effect cisplatin-mediated cell kill.

A third possible explanation for decreased cisplatin sensitivity in the presence of IPTG, is that the reduced growth rate of L1-3c-*fos* cells in the presence of IPTG limits cisplatin sensitivity (391). Cisplatin appears to be more effective on rapidly proliferating cells and thus, reduced proliferation rates may render cells less sensitive to cisplatin (26). To eliminate any potential interaction between IPTG and cisplatin, we attempted to lower c-*fos* expression using an antisense oligonucleotide against c-*fos* mRNA. However, reasonable transfection efficiencies could not be attained in these cells. Instead of pursuing the correlation between c-*fos* expression and cisplatin sensitivity in a rat fibroblast cell line, a more relevant, human ovarian carcinoma cell line was investigated.

In the human ovarian carcinoma cell line, 2008, and its cisplatin-resistant variant, C13* there was no apparent difference in the basal levels of either c-*fos* mRNA or protein. These results suggest that c-*fos* expression is not an

important determinant of cisplatin sensitivity in these cell types. To further investigate the validity of this statement, c-*fos* expression was lowered using an antisense oligonucleotide complementary to c-*fos* mRNA. An antisense approach was used to lower c-*fos* expression, rather than a ribozyme, for several reasons; (i) antisense therapy is more clinically relevant and thus transition from the laboratory to the clinical setting would be more rapid, (ii) time-consuming generation of stable transfectants is not required as transient transfection with c-*fos* antisense can be performed more rapidly, (iii) reduction of c-*fos* expression can be obtained in a dose-dependent manner using the c-*fos* antisense, and (iv) agents such as dexamethasone, which are used to induce ribozyme expression from a vector, are not required in this antisense protocol.

The effect of lowered c-*fos* expression on cisplatin sensitivity was determined by examining the interaction between the c-*fos* antisense and cisplatin on cell survival. It was reasoned that if c-*fos* expression regulated cisplatin sensitivity, then lowering c-*fos* expression should render cells hypersensitive to cisplatin. Although the amount of cisplatin required to achieve and IC_{50} in both 2008 and C13* cells was reduced by the addition of the c-*fos* antisense, the interaction was not synergistic. These results support the basal c-*fos* expression in these cell, which together, suggest that c-*fos* expression does not influence cisplatin sensitivity in these cells.

Our data contrasts that of Scanlon *et al.* (155) who observed that treatment of a cisplatin resistant variant, with a ribozyme specific for c-*fos*

mRNA, completely restored cisplatin sensitivity. However, we did observed that treatment with the c-*fos* antisense was capable of reducing the amount of cisplatin required to obtain a certain amount of cell kill compared to cisplatin alone, but this effect was due to the toxicity and/or reduction in cell proliferation, produce by the c-*fos* antisense itself. Effects of the c-*fos* ribozyme on cell proliferation/survival were not provided in the studies performed by Scanlon *et al.* (155). Our preliminary data showed that c-*fos* antisense treatment was capable of inhibiting cell proliferation/survival in both the parental and cisplatin-resistant variant that were used in the study by Scanlon *et al.* (155) (unpublished data). Therefore, restoration of cisplatin sensitivity may have been a result of a *fos* ribozyme-mediated decrease in cell growth/survival. Cells transfected with the c-*fos* ribozyme were also obtained from Dr. K. Scanlon but adequate expression of the c-*fos* ribozyme, as determined by Western analysis of c-Fos protein levels and cisplatin survival assays, could not be obtained.

In the cisplatin-resistant variant, A2780DDP, overexpression of c-*fos* was associated with elevated expression of enzymes involved in DNA synthesis and repair (155). Enhanced repair of cisplatin-damaged DNA has been observed in A2780DDP cells compared to their parental line and thus offers a potential mechanism through which c-*fos* overexpression can induce cisplatin resistance (25,37,115,126,127). The lack of c-*fos* overexpression in C13* cells supports our data in Chapter 4 in that C13* cells do not repair cisplatin-damaged DNA more efficiently than 2008 cells. One report has shown a slight increase in the

repair of interstrand crosslinks from actively transcribed genes in C13* cells compared to 2008 cells (126).

Our findings and those previously reported in the literature indicate that c-*fos* overexpression is associated with cisplatin resistance in some cell lines (145,154,155,487). Furthermore, c-*fos* has been shown to transform cells (390,391). These properties of c-*fos* have made it a potential target in cancer therapy. Although antisense therapy directed against c-*fos* has been demonstrated to prolong survival in mice following injection of the human breast cancer cell line MCF-7 (480), c-*fos* may not represent an ideal target. Several properties of c-*fos* may limit its usefulness for anticancer therapy; (i) expression of c-*fos* is involved in normal cell proliferation, (ii) redundancy of function within the Fos family may permit cell survival/proliferation independent of c-*fos* expression (465,466,478) and (iii) expression of c-*fos* may be required for differentiation and programmed cell death (437).

Although targeting c-*fos* itself may not be ideal, some of the cellular processes regulated by c-*fos* expression may prove more useful for destroying tumour cells. For this reason the ability of c-*fos* to regulate mitochondrial activity, particularly the mitochondrial membrane potential, was investigated. If c-*fos* overexpression is associated with an increased mitochondrial membrane potential, a way of treating c-*fos* overexpressing tumors exists. It was observed in Chapter 1 that cells with elevated mitochondrial membrane potentials can be effectively treated with lipophilic cations. Also, understanding the regulation of

mitochondrial membrane potential may lead to more effective therapies against tumours with elevated mitochondrial membrane potential. Since an increase in mitochondrial membrane potential is commonly associated with cell transformation (165-167), altering this property may represent a relevant antitumour strategy.

To our knowledge, this is the first report to examine whether c-*fos* overexpression, like v-*fos* overexpression, can result in an increase in mitochondrial membrane potential. Rat fibroblasts transfected with a murine c-*fos* gene did not have an elevated mitochondrial membrane potential compared to non-transfected controls. In addition, suppression of c-*fos* expression in L1-3c-*fos* cells did not alter the mitochondrial membrane potential in these cells. Moreover, human ovarian carcinoma cells resistant to cisplatin and possessing elevated expression of c-*fos* mRNA did not have an elevated mitochondrial membrane potential compared to the parental line. Finally, in C13* cells which have an elevated mitochondrial membrane potential compared to their parental line, no alteration in c-*fos* expression, at either the mRNA or protein level, was observed. In all of the cases, cells were probed with NAO and it was determined mitochondrial mass was unaffected by alterations in c-*fos* expression. Therefore, in the model systems examined, there is no apparent correlation between c-*fos* expression and mitochondrial membrane potential.

As outlined in the introduction of this chapter, several important differences exist between regulation and function of c-*fos* and v-*fos*. A potential

effector gene of v-*fos*, namely *fte-1*, has been implicated in mediating the v-*fos*-induced increase in mitochondrial membrane potential (484). It is possible that c-*fos* does not regulate this gene and is therefore unable to alter the mitochondrial membrane potential. Further investigations of the mechanisms through which v-*fos* regulates mitochondrial membrane potential have not been reported.

Finally, we examined the association between an elevated mitochondrial membrane potential and cisplatin resistance in our three cisplatin-resistant variants. In the two cisplatin-resistant variants in which there was an elevation in c-*fos* expression, CMVc-*fos* and A2780DDP cells, there was no alteration in the mitochondrial membrane potential compared to their respective parental lines. As previously mentioned, C13* cells in which there is an elevation in mitochondrial membrane potential, there is no increase in c-*fos* expression. Therefore it appears that if a cells has an elevated expression of c-*fos* it does not require an alteration in its mitochondrial membrane potential to become resistant to cisplatin.

In summary, both overexpression of c-*fos* and elevations in mitochondrial membrane potential have been associated with cisplatin resistance. These two characteristics appear to act independently since mitochondrial membrane potentials did not correlated with c-*fos* expression. These data suggest that c-*fos* is unlike v-*fos* in its ability to regulate mitochondrial membrane potential and further illustrates the multifactorial nature of cisplatin resistance.

OVERALL CONCLUSIONS

Changes in mitochondrial function have been associated with normal cellular activities such as proliferation and motility (189,197). Since cells modulate their mitochondria during times of activity, it is not surprising that transformed cells require an elevation in their mitochondrial membrane potential to maintain the increased metabolic activity of these cellular process which are characteristic of many types of tumour cells (165). Following this rationale, it is conceivable that resistance to certain chemotherapeutic agents would also require elevated mitochondrial activity. Several well characterized drug resistance mechanisms including, drug efflux via P-glycoprotein and DNA repair, require ATP. Since mitochondria are the primary source of ATP in most cells (164), elevations in mitochondrial function and/or content may be required to provide the energy necessary to maintain the activity of a resistant mechanism. The data provided in this thesis supports this concept.

Manuscripts in this thesis have shown that cells resistant to Photofrin II-mediated PDT possessed mitochondria which differ in structure and function from the parental cells. Moreover, these PDT-resistant variants were cross resistant to cisplatin, supporting the idea that mitochondrial alterations may modulate tumour cell sensitivity to a number of cancer therapies. However, it remains unclear whether changes in mitochondria are causal in the induction of PDT and/or cisplatin resistance. The datum in this thesis suggests that an elevated mitochondrial membrane potential does not render a cell less sensitive

to cisplatin by modulating DNA repair, since there was no association between mitochondrial membrane potential and repair of cisplatin-DNA lesions. Scavenging of positively charged cisplatin species and altering calcium-mediated signal transduction have been proposed as potential mechanisms through which mitochondria could decrease a cell's sensitivity to cisplatin and need to be evaluated (152).

This thesis also investigated a potential regulator of mitochondrial activity. It is thought that communication between the nucleus and mitochondria exists, but the mediators remain uncharacterized (171). The c-*fos* gene was considered a reasonable candidate for mediating nuclear-mitochondrial interaction. Expression of c-*fos* regulates cell cycle progression and mitochondrial activity has been shown to change throughout the cell cycle (190-192,464). Moreover, v-*fos*, the viral homologue of c-*fos,* has been implicated in regulating mitochondrial membrane potential (482,483). However, data generated in this thesis suggests that c-*fos*, does not regulate mitochondrial membrane potential. It was observed that cells which overexpressed c-*fos* or had an elevated mitochondrial membrane potential were resistant to cisplatin. These two characteristics were not co-expressed in any of the cisplatin-resistant variants examined, suggesting that c-*fos* overexpression and elevated mitochondrial membrane potential are not interdependent.

The most important finding in this thesis was the ability of lipophilic cations to sensitize cells to cisplatin. The interaction of Deca and cisplatin

resulted in synergistic tumour cell kill in both the parental cell line and cisplatin-resistant variant. It was also observed that Deca was capable of sensitizing cells to cisplatin thereby greatly reducing the amount of cisplatin required while maintaining similar levels of tumour cell kill. Sensitizing cells to cisplatin is clinically relevant since cisplatin's efficacy against cisplatin-resistant tumours is limited by host toxicity (7-11). Moreover, adding Deca or other lipophilic cations in chemotherapeutic regimens may take advantage of the selectivity of these agents for tumor cells (168,220,238,251,252). Furthermore, it appears that Rh123 retention in tumour cells, which can be examined within hours, can predict which tumours would be most responsive to lipophilic cations and thus which patients would benefit from having lipophilic cations included in their therapy. Renewed interest in mitochondria and the future use of "anti-mitochondrial" agents may well rest on the fate of ongoing clinical trials examining lipophilic cations with enhanced tumour selectivity (264-266).

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IMAGE EVALUATION
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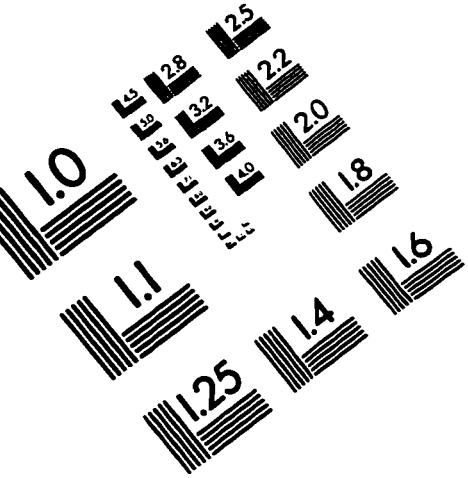

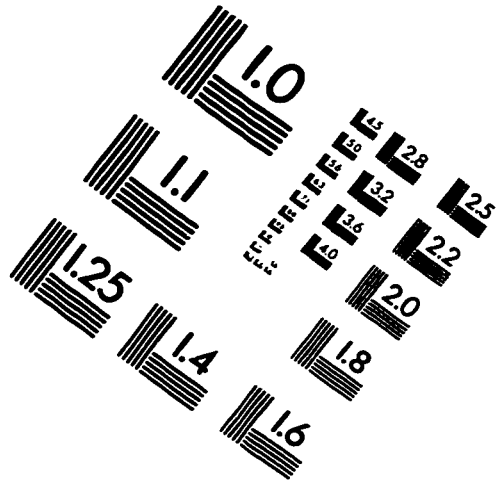

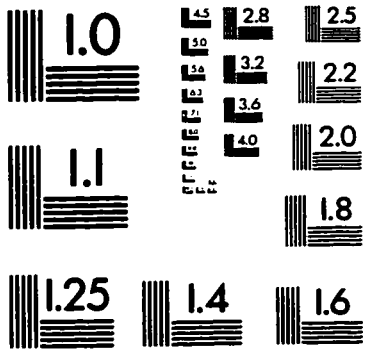

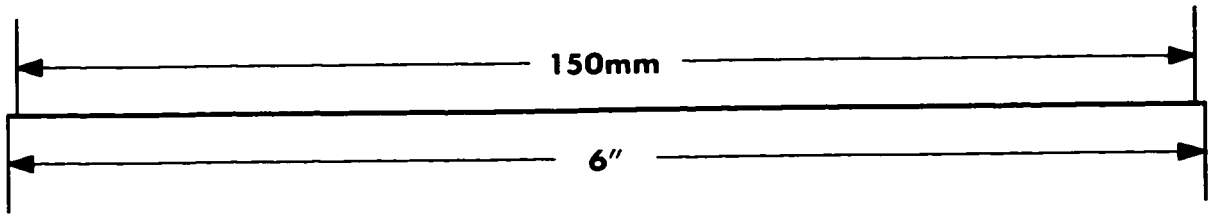

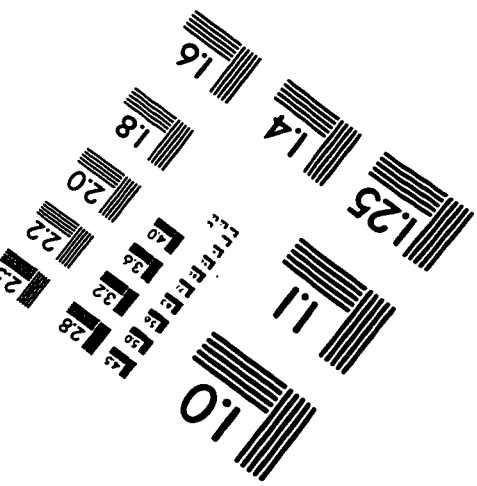

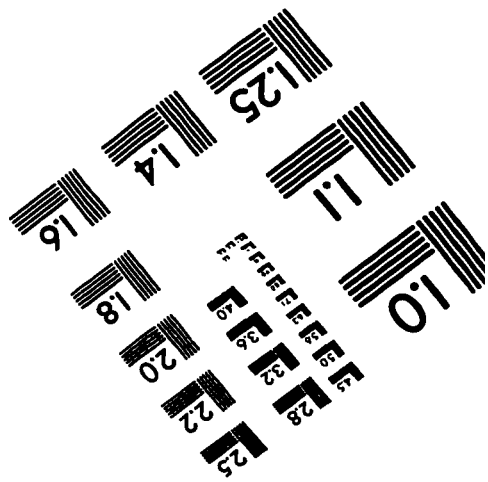

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