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HYDROGEN PEROXIDE PRODUCTION AND AUTOCRINE PROLIFERATION CONTROL

By

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ABSTRACT

An ovarian carcinoma culture model is the focus of this investigation based on a unique association between an elevated mitochondrial membrane potential and resistance to the chemotherapeutic agent cisplatin. The model consists of the 2008 parental cell line, the C13* subline that has acquired stable resistance to cisplatin and the RH4 revertant line that has regained sensitivity to cisplatin following selection with the anti-mitochondrial agent rhodamine 123. The cumulative data for resistance mechanisms that operate in this model suggest that increased tolerance to damage is a major determinant of the overall cytotoxic response to cisplatin. We have adopted the general hypothesis that mitochondria play a role in the mediation of resistance to cisplatin, and have furthered the investigation of these cells in terms of their energetic and redox balance characteristics. No significant differences exist between 2008, C13* and RH4 cells in terms of glycolytic capacity but when assayed for mitochondrial function both the cisplatin-resistant C13* and the cisplatin-sensitive RH4 cells have a significantly reduced capacity for mitochondrial respiration. This common characteristic indicated that alterations in energy production do not influence resistance, but perhaps other associated mitochondrial activities like reactive oxygen species production could have an impact. Measurement of extracellular hydrogen peroxide (H_2O_2) production revealed a significant increase in the C13* versus 2008 cell population, which is the result of

contributions by multiple intracellular sources including mitochondria and potentially novel flavoproteins. The exact relationship between increased H_2O_2 production and cisplatin resistance is not yet defined but one major implication is the evidence for extracellular H_2O_2 as a required autocrine growth factor for various cultured cell lines including 2008 and C13* cells. The pro-proliferation role of H_2O_2 suggests it could influence the balance of survival versus death effector signals that may have an impact on the threshold of apoptosis initiation in these cells.

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LIST OF ABBREVIATIONS

ADED	anti-apoptotic death effector domain
AIF	apoptosis inducing factor
ADP	adenosine diphosphate
APC	adenoma polyposis coli
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSO	L-buthionine-[S,R]-sulfoximine
BrDU	bromodeoxyuridine
°C	degrees Celsius
CDDP	cisplatinum (II) diammine dichloride (cisplatin)
Cys	cysteine
$cPLA_2$	cytosolic phospholipase A_2
DCC	deleted in colon cancer
2-DG	2-deoxy-D-glucose
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPI	diphenylene iodonium
DTNB	5,5′ dithiobis(2-nitrobenzoic acid)
EC	endothelial cell
EDTA	ethylene diamine tetraacetate
EGFR	epidermal growth factor receptor
ERCC	excision repair cross complementation
ERK	extracellular signal regulated kinase
ETC	electron transport chain
FIGO	International Federation of Gynecologists and Obstetricians
FITC	fluorescein
FBS	fetal bovine serum
gadd	growth arrest and DNA damage
γGCS	γ-glutamylcysteine synthetase
GF	growth factor
Glu	glutamine
Gly	glycine
GOX	glucose oxidase
GPX	glutathione peroxidase
GS	glutathione synthetase
GSH	glutathione
GSSG	glutathione disulfide
GR	glutathione reductase
γGT	γ-glutamyl transpeptidase
HIF	hypoxia-inducible factor

hMLH1	human, Mut L homologue-1
HSF-1	heat shock factor-1
hsp	heat shock protein
HRP	horseradish peroxidase
IC_{50}	inhibitory concentration for 50% of the population
IC_{90}	inhibitory concentration for 90% of the population
IAP	inhibitor of apoptosis protein
JNK	c-Jun NH_2 terminal kinase
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
M	molar
MAPKK	mitogen activated protein kinase kinase
αMEM	α minimum essentials medium
mM	millimolar
MMP	matrix metalloproteinase
MnSOD	manganese superoxide dismutase
MPT	mitochondrial permeability transition
mRNA	messenger ribonucleic acid
MtDNA	mitochondrial deoxyribonucleic acid
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NAO	10N-nonyl acridine orange
NER	nucleotide excision repair
NFκB	nuclear factor-κB
nM	nanomolar
PAK	protein kinase
PAO	phenylarsine oxide
PARP	poly-ADP-ribose polymerase
PBS	phosphate buffered saline
P_i	orthophosphate
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PKC	protein kinase C
Rh123	rhodamine 123
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SA	sulfosalicylic acid
-SH	sulfhydryl group
TNB	5-thio-2 nitrobenzoic acid
TPP+	tetraphenyl phosphonium ion
μl	microliter
μM	micromolar

VSMC	vascular smooth muscle cell
XPA	Xeroderma pigmentosum, complementation group A
Y-P	tyrosine phosphorylation

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1.0 INTRODUCTION

1.1 OVARIAN CANCER AS A MODEL OF DISEASE

Cancer cells are generally described in text books as having fundamental common properties related to abnormal proliferation, lack of differentiation and increased invasive potential (Ruddon 1995). Despite these generalities, the modern approach to cancer therapeutics is to consider cancers as site-specific diseases. Thus individual discoveries in cancer biology and therapeutics are usually based on a single disease site, followed by tests for commonality among other disease sites. Ovarian cancer has been a popular focus of study in the past few decades, providing a significant background for scientists regarding the gross pathology of the disease, effective treatment strategies, tumour recurrence rates and most recently, molecular pathology. On its own, the literature pertaining to the treatment of ovarian cancer is extensive enough to show that current therapies do not accomplish much in terms of a cure, primarily due to the development of therapeutic resistance in recurrent disease. The long history of clinical data has been matched by *in vitro* studies of isolated ovarian cancer cell lines to determine the mechanisms involved in treatment resistance. In this investigation we have chosen an ovarian carcinoma culture system that represents the scenario of clinical therapeutic resistance, to take advantage of the

extensive history that is available as a guideline for the generation of new hypotheses regarding the mechanisms of treatment resistance.

1.2 BIOLOGY OF OVARIAN CANCER

Ovarian cancer is sometimes named "the silent killer of women" since approximately 80% of patients will have advanced disease that has already spread from the ovaries at first diagnosis, a characteristic that translates into a poor chance of long term survival (Appendix 1) (Ozols 1992). Inadequate detection of tumours at earlier stages results from the fact that patients are asymptomatic until the disease is widespread and subsequent abdominal discomfort or distension due to the development of ascites sends them to a physician (Ozols 1992). As a result, oncologists are faced with a more aggressive tumour cell population at the time of initial therapy, which may account for the poor long-term (>5 year) survival rates of patients that present themselves with disseminated (28%) versus localized (96%) disease (Landis *et al.* 1999). Although ovarian cancer is not the most common cancer afflicting women - representing an estimated 4.0% of new cancer cases in women of Canada and the United States in 1998 - the probability of mortality resulting from ovarian cancer (1 chance in 95.2) is very near the lifetime probability of having this cancer (1 chance in 66.7), giving it high priority for the development of curative therapy (NCIC Canadian Cancer Statistics 1998; Landis *et al.* 1999).

Over 90% of ovarian cancers arise from the surface epithelium of the ovaries (Friedlander 1998). Ovarian epithelial cells behave like a generative stem cell population such that the daughter cells of each generation do not commit to a terminal differentiation program (Hamilton 1992) (Appendix 2). In this scenario any DNA mutations will be inherited by each subsequent generation, thus increasing the number of cells with accumulated mutations and the subsequent risk of cancer development (Hamilton, 1992). One suggestion put forward by M. Fathalla to explain the etiology of ovarian cancer is the "incessant ovulation hypothesis" (1971). This theory suggests that the proliferation of epithelial cells during post-ovulation wound repair is a risk factor for transformation, and that an increased number of ovulation cycles increases ovarian cancer risk. Epidemiological data supporting this hypothesis shows that factors which reduce the number of ovulations - oral contraceptive use, multiple pregnancies and lactation – also reduce the lifetime risk of developing ovarian cancer (Auersperg *et al.* 1998; Daly and Obrams 1998). This same data also supports an alternate hypothesis for ovarian cancer etiology related to the excessive hormonal stimulation of epithelial cells that are not involved in post-ovulation wound repair but present in inclusion cysts (Appendix 2). Each of these factors – multiparity, oral contraceptive use and lactation – regulates the production of several hormonal classes including gonadotropins, estrogens and androgens, suggesting that reduced hormonal

stimulation of these cells may be the protective factor (Risch 1998). At this time it is unclear whether the incessant ovulation or hormonal etiology of ovarian cancer predominates (Risch 1998).

Epithelial ovarian tumours can be subtyped into 5 categories according to their histology: the most common serous carcinomas are histologically similar to the fallopian tube, mucinous carcinomas are like the endocervix, endometroid like the endometrium, clear cell like granulosa cells, and an unclassified, undifferentiated type which is the rarest case (Appendix 3) (Hamilton 1992). Although there is some degree of association between histologic subtype and 5 year survival rates, cellular differentiation state (grade) and tumour stage (FIGO I-IV) are the major prognostic indicators for clinicians making decisions about subsequent therapy (Appendix 3) (Friedlander 1998). Presentation with disease classified as Stage III or IV, and the presence of a poorly-differentiated tumour cell population are strong indicators of a poor outcome in terms of survival, and thus dictate the need for an aggressive therapy (Friedlander 1998).

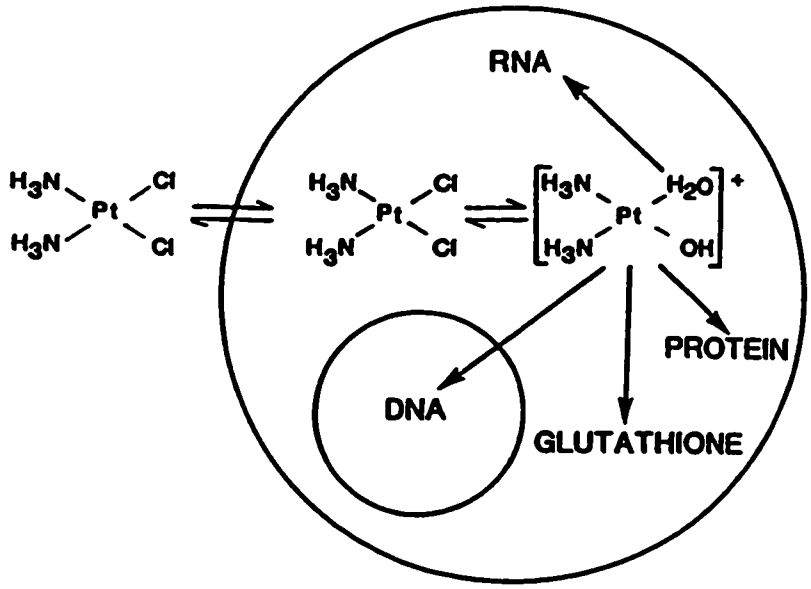
1.3 CISPLATIN CHEMOTHERAPY IN THE TREATMENT OF ADVANCED OVARIAN CANCER

At present, the most effective therapeutic strategy for advanced ovarian cancer is cytoreductive surgery to remove the majority of tumour bulk followed

by chemotherapy with a platinum-based agent like *cis*-platinum(II)diammine dichloride (CDDP, cisplatin) (Boente, Chi and Hoskins 1998; McGuire and Ozols, 1998). Cisplatin is a charged electrophilic molecule in the intracellular environment that reacts with nucleophilic sites in proteins, RNA and DNA (Figure 3) (Rosenberg 1985; Pinto and Lippard 1985). *In vitro* studies have suggested that the formation of nuclear DNA adducts and subsequent inhibition of DNA synthesis is the critical mechanism responsible for cisplatin's anticancer activity (Rosenberg 1985; Pinto and Lippard 1985). Recent evidence also shows that the individual cellular response to DNA damage also has an impact on the overall cytotoxic effect of cisplatin (Mamenta *et al.* 1994; Johnson *et al.* 1997). Ovarian cancer is generally considered a cisplatin-sensitive disease since 60-80% of patients achieve a clinical response to therapy, so defined by the absence of tumour progression for a period of 6 months following therapy (Thigpen, Vance and Khansur 1993; Auersperg *et al.*, 1998). The data gathered over the last 20 years shows that this translates into an improved long term (>5 year) survival rate for 50% of patients receiving cisplatin therapy versus 37% of those who do not receive cisplatin-based treatment (Aabo *et al.* 1998; Landis *et al.* 1999). A very common and disturbing feature of ovarian cancer, however, is the recurrence of disease in 40-60% of initially responsive patients with phenotypically distinct tumours that are resistant to second-line therapy with cisplatin (Thigpen, Vance and Khansur 1993). The choices for subsequent salvage therapy are limited by

the existence of a major degree of cross-resistance to other modalities like radiation and alkylating agents, suggesting that cellular changes associated with cisplatin resistance are responsible for this multiple modality resistant phenotype (Hamaguchi *et al.* 1993). The number of patients with recurrent tumours that have acquired resistance combined with 20-30% of patients that had tumours innately refractory to first line cisplatin therapy represents a large population for which there is currently no effective treatment (McGuire and Ozols 1998). Overall, the number of patients with advanced ovarian cancer that survive more than 10 years is less than 20% (Thigpen, Vance and Khansur 1993). Salvage therapy options include the topoisomerase inhibitor topotecan, the microtubule polymerization agent paclitaxel and the pyrimidine analogue gemcitabine based on the distinct primary mechanism of action of these agents versus cisplatin (McGuire and Ozols 1998). The response rate to any of these agents is only 20-30%, indicating that the majority of patients are refractory to all current salvage therapy options (Ozols 1998). Given the major impact of cisplatin resistance to subsequent therapy options in recurrent disease, much consideration has been given to the mechanisms involved in cisplatin resistance and the feasibility of reversing resistance as a means of improving overall survival in ovarian cancer patients.

Figure 1: Intracellular Targets of Cisplatin
(Reprinted from Eastman 1990)



Initial investigations into the mechanisms of cisplatin resistance in ovarian cancer deliberated over the contributions of altered host-tumour interactions versus alterations in drug sensitivity at the cellular level. In terms of host influence, the effective concentration of cisplatin reaching the tumour site could be reduced by general pharmacokinetic changes (reduced absorption or increased excretion) or the isolation of tumour cells at non-perfused sites (Perez *et al.* 1993). In the case of solid ovarian tumours, the bulk is surgically removed to improve drug access to residual disease, but given the abnormal

et al. 1993). In the case of solid ovarian tumours, the bulk is surgically removed to improve drug access to residual disease, but given the abnormal vascularization patterns of solid tumours, some cells may not receive sufficient exposure to effective concentrations of intravenously administered cisplatin (Boente, Chi and Hoskins 1998; Jain 1994). Despite the potential influence of such host factors, several studies suggested that an acquired cellular mechanism was involved in clinical cisplatin resistance: Wilson *et al.* (1987) showed that ovarian cancer cells from primary patient tumours tested for cisplatin chemosensitivity *in vitro* was predictive of the patient's response to cisplatin. In addition, isolated cells from recurrent tumours were shown to have reduced cisplatin sensitivity compared to cells assayed prior to chemotherapy (Inoue *et al.* 1985; Wolf *et al.* 1987; Masazza *et al.* 1991; Kern 1998). This evidence for a cellular mechanism led to the hypothesis that innately resistant cells selected from the residual population are the progenitors of the recurrent tumour, giving it a phenotypic advantage when cisplatin-based therapy is introduced for a second time (Inoue *et al.* 1985). The connection between tumour recurrence and cisplatin resistance is not absolute, however, and there is a measurable difference in response rates to salvage cisplatin therapy depending on the rapidity of recurrence. Markman *et al.* (1991) showed that if the duration of non-progression is >2 years from the time of initial therapy, the response rate to second-line cisplatin therapy is greater (59%) than if the tumour recurred within

5-12 months (27%). There have also been case reports of individual patients responding to multiple rounds of cisplatin-based therapy (Markman *et al.* 1997). A possible explanation for this phenomenon has come from recent molecular fingerprinting data comparing primary and late (>2 year) recurrent tumours. Buller *et al.* (1998) has shown that 77% of paired primary and late-recurrent tumours are genetically distinct, suggesting that the majority of "late" recurrences are actually second primary tumours. This evidence for a polyclonal origin of ovarian cancer also challenges the conclusions of Jacobs *et al.* (1992) that ovarian cancers have a monoclonal origin. These findings have direct clinical relevance for therapeutic decision making since they offer a biological basis for the improved cisplatin response rates of patients with "late-recurrent" disease. Undoubtedly, molecular typing of individual tumours represents the future of prognosis determination and patient stratification for clinical trials. Nevertheless, the phenomenon of cisplatin resistance is still an existing problem for most ovarian cancer patients who belong to the non-responsive group (tumour progression <6 months from therapy) and the rapid progression group (5-12 months from therapy). The immediate need for more effective therapy in advanced ovarian cancer has led to clinical trials using combinations of first-line agents that are not expected to have cross-resistance such as cisplatin and paclitaxel (McQuire and Ozols 1998). The earliest data suggests an improvement in 5 year survival rates with this combination versus

cisplatin alone although the data for recurrence frequency and resistance phenotype is not yet available (McGuire and Ozols 1998). Overall, the cisplatin-based strategies that have been implemented for the last 20 years seem to have reached their therapeutic limit, which translates into a loss of life for most patients with advanced ovarian cancer. It is clear that an improved understanding of drug resistance is the most logical route for the development of more effective therapies. Advances in molecular biology and genetic techniques in combination with the use of *in vitro* culture models have been fundamental to the identification of cellular changes involved in resistance to drugs like cisplatin. Interestingly, *in vitro* investigations of the mechanism of cisplatin resistance have also revealed information about the basic biology of this disease, highlighting the concept that a cell's sensitivity to therapy is intimately linked to its tumourigenic phenotype.

1.4 MECHANISMS OF ACQUIRED CISPLATIN RESISTANCE: DATA OBTAINED FROM *IN VITRO* MODELS OF OVARIAN CANCER

Cultured ovarian carcinoma cell lines isolated from primary ovarian tumours have been the backbone of research into the potential mechanisms of cisplatin resistance. Multiple groups have developed cisplatin-resistant sublines of these cells following single step or continuous selection with cisplatin in culture. This approach assumes the validity of the acquired model of resistance

development such that clones selected at each round of dosing have either innate or up-regulated mechanisms that allow survival in the presence of cytotoxic concentrations of cisplatin. The method of selection and resultant resistance ratios between parent cells and their sublines varies greatly from <10 fold (low-level resistance) to >100 fold (high-level resistance) (Table 1). This discrepancy has initiated the debate as to what level of resistance is clinically relevant, since the mechanisms involved in super-resistant lines may be very different from those pairs with low level resistance ratios (Scanlon *et al.* 1989). Cisplatin-resistant tumour cells isolated from recurrent tumours show a relatively low level of resistance (<10 fold), suggesting that this magnitude of resistance is most relevant (Wolf *et al.* 1987). As Hamilton astutely summarizes, however, most of the mechanisms suggested by *in vitro* studies have not yet been validated in the clinical scenario, so there is no basis for excluding any potential factor (1992). Since this is still primarily true in 1999, the data gathered from *in vitro* studies can be viewed as potentially relevant until the clinical data catches up to verify or nullify their mechanistic relevance. Generally, the mechanisms described *in vitro* can be grouped into two categories based on their pharmacologic dependence (Table 1). Under the pharmacologic heading comes all the data regarding the reduced access of cisplatin to its target DNA including reduced cellular accumulation of drug, enhanced de-activation of drug, or enhanced DNA repair. The alternate mechanism that is somewhat independent of the

classic dose-response relationship has been referred to as "increased cellular tolerance", and generally involves the absence of an appropriate cell death (apoptotic) response following cisplatin-induced DNA damage (Kerr, Winterford and Harmon 1994; Desoize 1994; Hickman 1996). The most significant advance in the understanding of cisplatin resistance to-date is the realization that multiple mechanisms are likely to be involved, even in relatively homogeneous culture models, and that the observed changes in cisplatin-resistant cells are not independent but interdependent factors.

Table 1: Contributing Mechanisms to Acquired Cisplatin Resistance in Ovarian Carcinoma Culture Models

Ovarian Carcinoma Culture Models of Acquired Cisplatin Resistance	Reported Resistance Ratio (RR)	Intracellular Accumulation of Cisplatin	Detoxification Reactions	Cisplatin-DNA Adduct Levels/ DNA Repair	Tolerance to Cisplatin-DNA Lesions
A2780 parental A2780DDP resistant	3-fold (Goto *et al.* 1995)	↑ Efflux of GSH-Cisplatin Conjugates	↑ γGCS protein ↑ GSH content	NA	NA
	8-fold (Schmidt and Chaney 1993)	↓ Accumulation of cisplatin	↑ GSH content	No difference in genomic cisplatin-DNA adduct levels	Increased replicative bypass (Mamenta *et al.* 1994)
A2780 parental CP70 resistant C30 resistant C200 resistant	- 13-28 fold 270-500 fold 735-1000 fold (Hamilton, Young and Ozols 1984)	↓ Accumulation of cisplatin in CP70 cells caused by ↑ efflux (Parker *et al.* 1991)	↑ GSH content (in proportion with RR) (Godwin *et al.* 1992) ↑ γGCS (heavy subunit) mRNA (Yao *et al.* 1995)	↑ Genomic adduct removal (Parker *et al.* 1991) (Johnson *et al.* 1994) ↑ Gene-specific repair of interstrand cross links (Zhen *et al.* 1992) ↑ ERCC1 expression (Li *et al.* 1998)	Increased replicative bypass in CP70 (resulting from defective mismatch repair) (Vaisman *et al.* 1998)
PE01 parental PE04 resistant	- 3 fold (Wolf *et al.* 1987)	NA	↑ GSH content ↑ GST, ↑ γGT and ↑GPX activities (Lewis, Hayes and Wolf 1988)	NA	NA

(NA= Not Assessed)

2008 parental 2008/DDP resistant C13* resistant	- 3 fold (Andrews *et al.* 1988a) 8-10 fold (Andrews and Albright 1992)	↓ Accumulation of cisplatin in 2008/DDP cells caused by ↑ efflux (Mann, Andrews and Howell 1990)	↑ GSH content in 2008/DDP and C13* No difference in GSTπ Expression in C13* (Parekh and Simpkins, 1996)	↓ Genomic cisplatin-DNA adduct levels & ↑ Gene-specific repair of ICL in C13* (Zhen *et al.* 1992) No increase in NER capacity in C13* (Moorehead *et al.*1996) No defects in C13* mismatch repair (Aebi *et al.* 1996)	C13* cells do not halt DNA synthesis following cisplatin treatment (Nefedova and Singh, 1996) Increased replicative bypass in C13* (Mamenta *et al.* 1994)
AovC-M parental AovC-R resistant	- 5 fold (De Pooter *et al.* 1996)	↓ Accumulation of cisplatin	NA	No difference in genomic cisplatin-DNA adduct levels, or adduct type	NA
CH1 parental CH1cisR resistant	- 3–6 fold (Kelland *et al.* 1992)	No difference in accumulation	No difference in GSH	No difference in total genomic cisplatin-DNA adduct levels (Kelland *et al.* 1992) ↑ repair of interstrand cross links (O'Neill *et al.* 1992)	NA
IGROV-1 parental IGROV-$1_{Pt\,as}$ IGROV-$1_{Pt\,1.0}$ Resistant	- 6 fold 14 fold (Perego *et al.* 1996)	NA	↑ Metallothionein IIa expression ↑ GSH content (in proportion with RR)	↓ Cisplatin-DNA adducts in IGROV-$1_{Pt\,1.0}$ cells	↓ susceptibility to cisplatin-induced apoptosis, via ↓ bax expression (Perego *et al.* 1996)
SKOV-3 parental SKOV-3 CDDP/R resistant	- 6-fold (Husain *et al.* 1998)	NA	NA	↑ wild type BRCA1 protein, associated with enhanced DNA repair capacity	NA

Significance of Reduced Accumulation and Elevated Glutathione Levels in Cisplatin-Resistant Cells

The earliest change noted in cisplatin-resistant variants of ovarian cancer cell lines was the reduced intracellular accumulation of detectable platinum (Table 1). This observation furthered investigations into the mechanism of cisplatin uptake and efflux from sensitive and resistant cells, in favour of the hypothesis that reduced accumulation was a prominent factor in resistance. It is generally accepted that cisplatin uptake is not a carrier-mediated process, but proceeds via passive diffusion with a linear (first order) rate of accumulation from low to saturating concentrations of drug (Andrews and Howell 1990; Gately and Howell 1993). The rate of uptake is partially energy- and membrane potential-dependent and can be influenced by bilayer fluidity, but little emphasis has been placed on these properties in resistant cells (Andrews and Howell 1990). Increased cisplatin efflux in resistant cells is considered the major contributing factor to the overall reduced accumulation in culture models (Table 1). The efflux of platinum from cells is modeled as a biphasic response, with a fast and slow effluxing pool (Mann, Andrews and Howell, 1990). The proportion of platinum that is effluxed quickly is believed to be in aqueous form (non-reacted), whereas the slowly effluxed pool is believed to be bound to macromolecules such as proteins, RNA and DNA (Mann, Andrews and Howell, 1990). Cisplatin is not considered a substrate for the xenobiotic efflux pump P-

thus P-glycoprotein expression in culture models or tumour specimens is not believed to be factor in cisplatin resistance (Veneroni *et al.* 1994; Sharp *et al.* 1998). There is some evidence for an assisted efflux mechanism for cisplatin-glutathione conjugates, which may account for reduced cisplatin accumulation in some resistant lines (Goto *et al.* 1995; Kurokawa *et al.* 1997).

As an aquated electrophile in the intracellular environment, cisplatin reacts with nucleophilic sites such as the reduced sulfhydryl residues of amino acids like cysteine or methionine (Dedon and Borch 1987). Metallothioneins and the tripeptide glutathione (γGlu-Cys-Gly) are the most abundant intracellular sources of cysteine and both metallothioneins and glutathione (GSH) can react with cisplatin spontaneously or, in the case of GSH, via enzymatically catalyzed-reactions (Dedon and Borch 1987; Andrews and Howell 1990). The consistent appearance of elevated intracellular GSH concentrations in cisplatin-resistant sublines suggested that GSH was a prominent factor involved in resistance (Table 1). It was subsequently proposed that enhanced scavenging of cisplatin species by GSH was a detoxification mechanism that spared other critical targets such as DNA (O'Dwyer *et al. 1995;* Schröder *et al.* 1996). GSH is maintained primarily in the reduced form at high concentration (1-10mM) by *de novo* synthesis and salvage pathways (Figure 2). Enzymes involved in GSH synthesis include γglutamylcysteine synthetase (γGCS) and GSH synthetase in the *de novo* pathway and γ-glutamyltranspeptidase (γGT) of the salvage pathway. GSH

peroxidase (GPX) and GSH reductase (GR) maintain GSH in the reduced form, while GSH transferases (GST-α, -μ, -π) and the more recently described GS-X pump are involved in GSH-xenobiotic conjugate efflux from cells (Morrow and Cowan 1990; Kurokawa *et al.* 1997). *In vitro* studies verified that physiological concentrations of GSH were sufficient to reduce the rate of platinum-DNA adduct formation, suggesting that the scavenging effect of GSH was a significant factor determining cisplatin sensitivity (Dedon and Borch, 1987). Elevated GSH content has also been recorded in recurrent tumours suggesting that mechanisms involving elevated GSH in culture models are also relevant in the resistant disease of patients. (Table 2) The means by which elevated GSH associates with resistance *in vivo* has not been explained, however, and efforts to associate cisplatin response with the expression of GSH-related enzymes such as glutathione-S transferases have not yet shown any significant degree of correlation (Table 2).

Experiments designed to examine the effects of GSH depletion on cisplatin resistance in culture models utilized the agent buthionine sulfoximine (BSO) as a selective inhibitor of GCS, the rate-limiting enzyme involved in *de novo* GSH synthesis (Griffith 1982). Several groups utilized BSO to deplete cellular GSH prior to cisplatin treatment, and observed that ovarian cancer cells were sensitized to cisplatin with this combination (Fojo *et al.* 1987 Andrews *et al.* 1988b; Hirata *et al.* 1993). In response to the need for immediate improvements

in the therapy of cisplatin-resistant ovarian cancer, combination drug trials with BSO have been initiated in patients with recurrent tumours to evaluate the clinical efficacy of GSH depletion as a chemotherapeutic sensitizer (O'Dwyer *et al.* 1996; Bailey *et al.* 1997).

Figure 2: *De novo* and Salvage Pathways Involved in the Maintenance of Intracellular GSH

(Reprinted from Schröder *et al.* 1996)

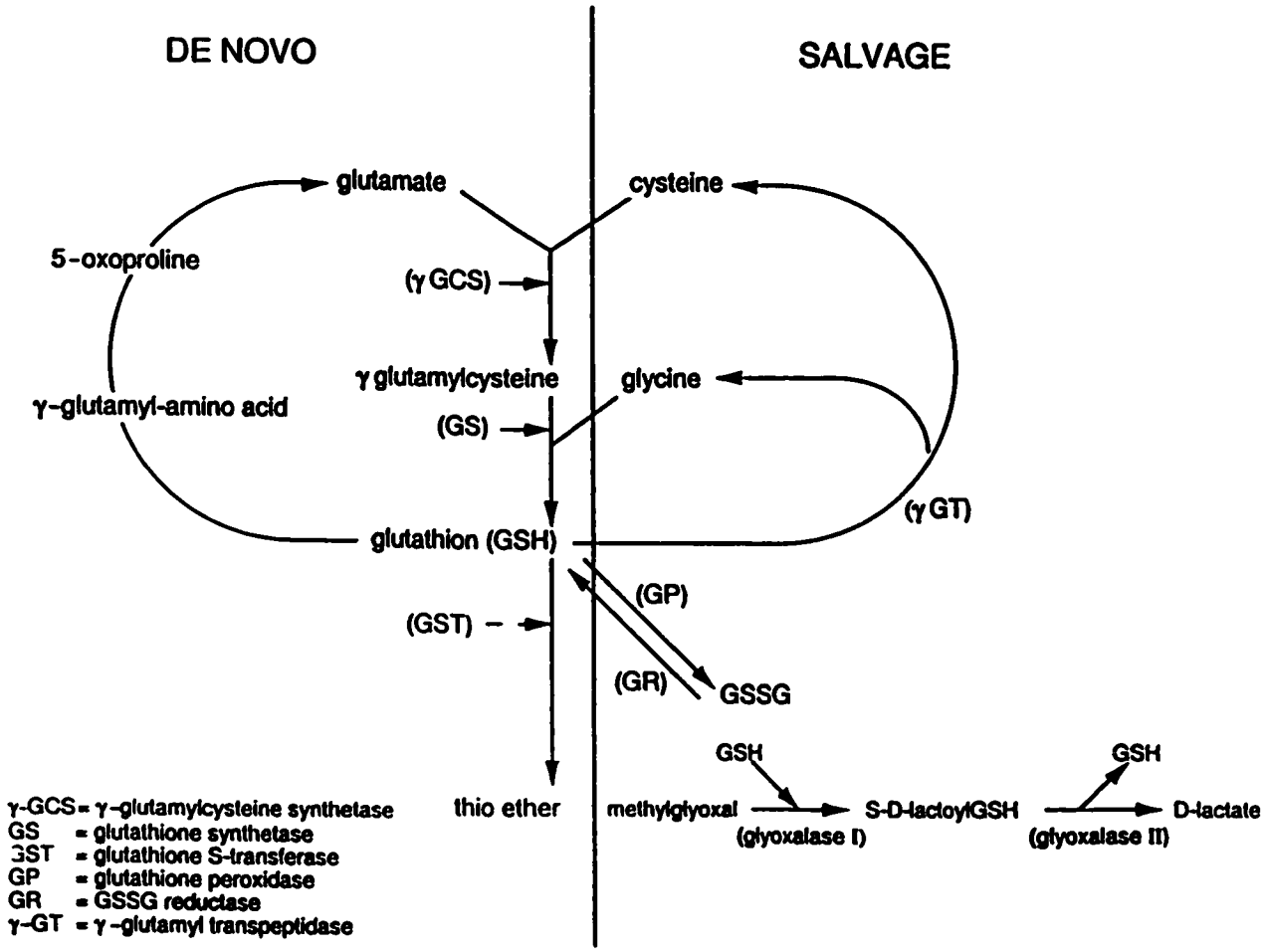


Table 2: *In Vivo* Correlations Between the Expression and Enzymatic Activity of Proteins with the Cisplatin-Resistant Phenotype of Ovarian Tumours

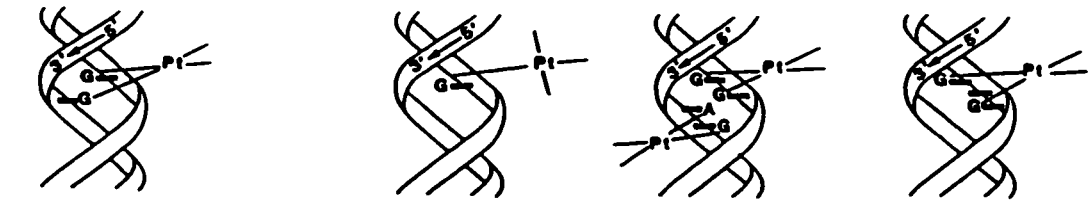
Study Design	Protein of interest	Results
(Djuric *et al.* 1990): Comparative study (primary versus residual disease post platinum therapy, unmatched samples)	GSH, GSH transferase(GST), GSH peroxidase (GP), Superoxide dismutase (SOD), Catalase, DT diaphorase, Aldehyde dehydrogenase(AD)	Residual disease had reduced levels of non-protein thiols, reduced GST and GP activity versus primary tumours.
Britten *et al.* 1992: Comparative study (primary versus recurrent disease post platinum therapy, mostly unmatched samples)	GSH GSH transferase (GST)	GSH levels are significantly elevated in recurrent disease but no difference in GST activity.
Murphy *et al.* 1992: Comparative study (primary versus residual disease post platinum therapy, matched samples)	GSH transferase (GST)	No difference in GST activity or isoenzyme distribution in residual disease.
Van der Zee *et al.* 1992: Comparative study (primary versus residual disease post platinum therapy, mostly unmatched samples)	GSH transferase (GST)	No difference in GST activity or GSTπ distribution in residual disease.
Dabholkar *et al.* 1992: Predictive study (mRNA expression versus clinical response to platinum therapy)	ERCC1 mRNA ERCC2 mRNA	Increased expression of ERCC1 mRNA but not ERCC2 mRNA correlated well with patient resistance.
Dabholkar *et al.* 1994: Predictive study (mRNA expression versus clinical response to platinum therapy)	ERCC1 mRNA XPA mRNA	Increased expression of the full length mRNA for ERCC1 and XPA correlated well with patient resistance.
Righetti *et al.* 1996: Predictive study (gene mutations versus clinical response to platinum therapy	p53 accumulation and mutation	Increased expression of mutant p53 proteins correlated well with patient resistance to cisplatin.
Buttitta *et al.* 1997: Predictive study (gene mutations versus clinical response to platinum therapy	p53 accumulation and mutation	Increased expression of mutant p53 proteins correlated well with patient resistance to cisplatin.
Fink *et al.* 1998: Comparative study (primary versus recurrent disease post platinum therapy, matched samples)	hMLH1	Reduced expression of hMLH1 correlated well with a majority (66%) of patients with recurrent disease.

Significance of Enhanced DNA Repair in Cisplatin-Resistant Cells

Cisplatin can form multiple species of adducts with DNA including intrastrand adducts and interstrand cross links (Figure 3). The most frequently observed lesions are intrastrand adducts between adjacent guanines or guanine-adenine bases, representing 65% and 25% of the total lesions, respectively (Crul *et al.* 1997). Monofunctional adducts and interstrand cross links (ICL) occur less frequently; although it is still a matter of debate as to which lesion is most significant for subsequent cytotoxicity (Bubley *et al.* 1994; Crul *et al.* 1997). Cisplatin-DNA adducts are repaired primarily by the nucleotide excision pathway (NER) which involves multiple proteins and enzymes for the purpose of DNA damage recognition, recruitment and subsequent repair (Appendix 4) (Crul *et al.* 1997; Reed 1998). Analysis of DNA repair capacity in terms of total genomic lesions and gene-specific lesions in *in vitro* models has revealed increased NER capacity as a positively associated factor in some cisplatin-resistant lines (Table 1). The over-expression of two key damage-recognition proteins (ERCC1 and XPA) involved in the NER pathway also has predictive value for clinical outcome in patients receiving platinum-based therapy, suggesting that enhanced NER capacity is a contributing mechanism to cisplatin resistance *in vivo* (Table 2). The most recent data, however, from pairs of resistant and parental lines that have been fully characterized for all recognized resistance mechanisms suggests that increased tolerance to DNA damage may

play a fundamental role in determining the cellular response to cisplatin (Mamenta *et al.* 1994; Vaisman *et al.* 1998). This tolerance is expressed by the ability of resistant cells to continue DNA synthesis even in the presence of a cisplatin-DNA adduct load that is sufficient to halt synthesis in the parental cells. The mechanism has been termed "enhanced replicative bypass" and refers to the fact that the DNA synthesizing machinery is somehow able to bypass replication blocks such as cisplatin-DNA adducts (Mamenta *et al.* 1994; Vaisman *et al.* 1998). Johnson *et al.* (1997) also performed a comprehensive analysis of all known factors involved in cisplatin resistance - accumulation, GSH levels, cisplatin-DNA adduct formation and repair rates – in 12 unrelated ovarian carcinoma cell lines with a wide range of intrinsic cisplatin resistance. These authors also concluded that increased tolerance to DNA damage (defined as the ratio of Pt-DNA load versus cisplatin IC_{30}) was the most significant factor associated with resistance. The agreement that DNA damage tolerance is a relevant factor in both intrinsic and acquired resistance mechanisms of cells in culture suggests it may have relevance to both the intrinsic and acquired resistance phenotypes of patient's tumours. Most importantly, the concept of increased tolerance of DNA damage challenged the original concept of a strict dose-response relationship between cisplatin-DNA adduct formation, DNA synthesis inhibition and the anticancer activity of cisplatin (Sorenson and Eastman 1988).

Figure 3: Species of Cisplatin-DNA Adducts
(Reprinted from Eastman 1990)



Significance of Apoptosis Avoidance to Cisplatin-Resistance

Cell tolerance to cisplatin-mediated DNA damage is a complex phenomenon that is poorly understood. Vaisman *et al.* (1998) has suggested that defective DNA mismatch repair is involved in this process, even though mismatch repair itself is not directly involved in the repair of cisplatin-DNA adducts (Appendix 4) (Crul *et al.* 1997). If defective mismatch repair confers protection from cisplatin-mediated toxicity, it may be the explanation for the apparent selection of cells with defective mismatch repair under conditions of acquired resistance to cisplatin (Fink *et al.* 1998). More indirect evidence that

cisplatin-resistant cells have defective mismatch repair comes from the acquisition of genetic instability concurrently with resistance, since mismatch repair is generally involved in the maintenance of genomic integrity (Wasenius *et al.* 1997). A positive correlation between defective mismatch repair and cisplatin resistance has also been reported in a survey of recurrent ovarian tumours, so defined by the reduced expression of the mismatch repair protein hMLH1 (Table 2). One possible explanation is that the DNA damage recognition proteins of the mismatch repair system participate as initiating signals in the cell death program called apoptosis (Crul *et al.* 1997). As opposed to the unregulated cellular breakdown that occurs during necrosis, apoptosis is a controlled mechanism of cell deletion that is morphologically characterized by chromatin condensation and the formation of apoptotic bodies (Figure 4). Several groups have confirmed that cisplatin-induced cytotoxicity proceeds via initiation of apoptosis (Eastman 1990; Barry, Behnke and Eastman 1990; Ormerod *et al.* 1994; Meyn *et al.* 1995; Raaphorst *et al.* 1998). Cisplatin-induced cell death is usually preceded by arrest in the G_2 phase of the cell cycle, the gap phase that precedes mitosis, and although the exact mechanism in uncertain, mismatch repair proteins may be involved in this process (Hawn *et al.* 1995).

Figure 4: Morphological Characteristics of Apoptosis versus Necrosis
(Reprinted from Kerr *et al.* 1994)

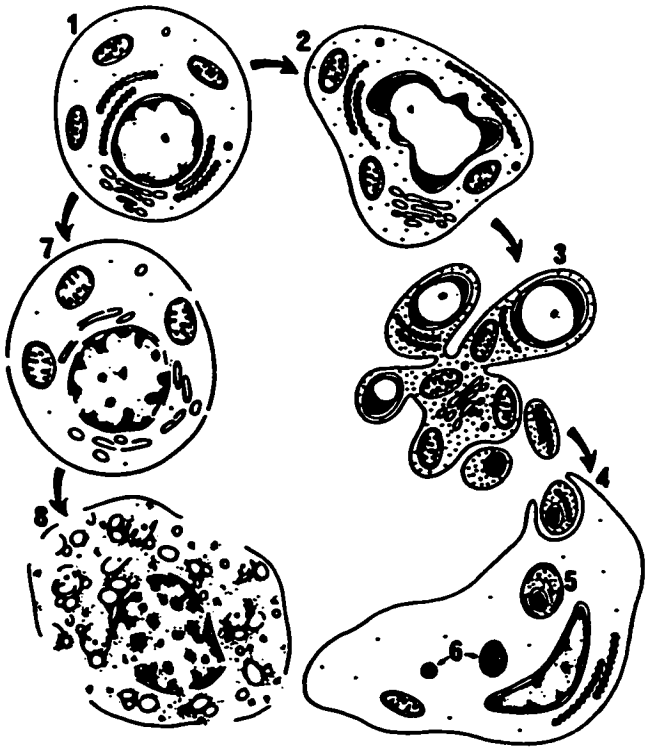


Diagram illustrating sequence of ultrastructural changes in apoptosis (2–6) and necrosis (7 and 8). (1) Normal cell. Early apoptosis (2) is characterized by compaction and margination of nuclear chromatin, condensation of cytoplasm, and convolution of nuclear and cell outlines. (3) At a later stage, the nucleus fragments, and protuberances that form on the cell surface separate to produce apoptotic bodies, which (4) are phagocytosed by nearby cells and (5 and 6) degraded within lysosomes. (7) The development of necrosis is associated with irregular clumping of chromatin, marked swelling of organelles and focal disruption of membranes. (8) Membranes subsequently disintegrate, but the cell usually retains its overall shape until removed by mononuclear phagocytes.

Apoptosis is biochemically regulated by a balance of pro- and anti-apoptotic factors that are known to have a significant impact on cellular

sensitivity to chemotherapeutic agents like cisplatin (Figure 5) (Kerr, Winterford and Harmon 1994; Desoize 1994; Hickman 1996; Coukos and Rubin 1998; Reed 1999). Apoptosis avoidance has been described in the IGROV ovarian cell culture model of acquired cisplatin resistance, so mediated by the reduced expression of the pro-apoptotic protein bax (Perego *et al.* 1996). The generality of this resistance mechanism in other culture models of cisplatin resistance is not yet known; however, given the complexity of apoptosis regulation, it seems likely that multiple mechanisms of apoptosis avoidance may exist (Figure 5) (Reed, 1999).

Resistance to apoptosis is also expected to make a significant contribution to drug resistance *in vivo* since key proteins like p53 that regulate apoptosis initiation are commonly mutated in many cancer types, including advanced ovarian cancer (Henriksen *et al.* 1994; Milner *et al.* 1993; Teneriello *et al.* 1993; Mowat 1998). p53 is a pivotal player in the regulation of cell cycle arrest and apoptosis initiation following DNA damage, and several groups have suggested that p53 mutations are predictive of clinical resistance to cisplatin in ovarian cancer patients (Table 2). Apoptosis avoidance may also explain the broad cross-resistance to various chemotherapeutic drugs that is observed in cisplatin-resistant cell lines (Hickman 1996). The overall significance of apoptosis avoidance highlights the need for a clear understanding of the anti-apoptotic factors that operate in cisplatin-resistant cell lines. One promising lead is the

observation that most cells that are considered cisplatin resistant are not infinitely resistant to apoptosis. This indicates that the machinery for apoptosis is intact and these resistant cells merely have a raised threshold for initiation that may be amenable to therapeutic intervention. To increase our knowledge about cellular factors that may influence the cisplatin resistance threshold, we have chosen to investigate the significance of reported mitochondrial alterations in a well-characterized culture model of acquired cisplatin resistance.

Figure 5: Apoptosis Initiation is Regulated by a Balance of Pro- and Anti-Apoptotic Factors

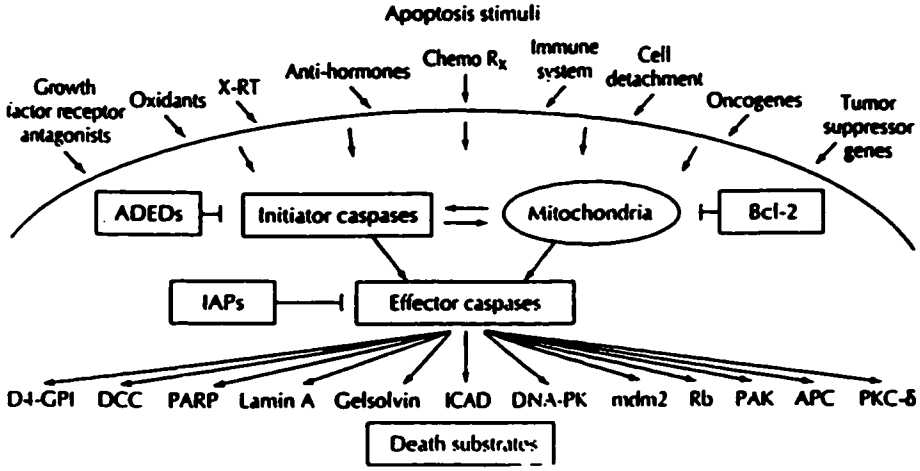


Figure 5: ADED – anti-apoptotic death effector domain proteins, IAPs – inhibitor of apoptosis proteins, Reprinted from Reed, 1999.

1.5 THE 2008/C13* MODEL OF ACQUIRED CISPLATIN RESISTANCE

Mitochondrial Alterations Associated with Cisplatin Resistance in C13 Cells*

The 2008 cell line was originally established from a patient with serous carcinoma of the ovary (Disaia *et al.* 1972). The C13* cells are a subline of 2008 cells that have acquired resistance to cisplatin following an *in vitro* exposure schedule of 13 consecutive monthly selections with 1μM cisplatin. The surviving population was subsequently exposed to continuous cisplatin starting at a concentration of 0.25μM and followed by incremental increases to 5.25μM (Andrews and Albright 1992). The cisplatin resistance ratios for C13* versus 2008 cells are calculated from the ratio of the 50% or 90% inhibitory concentrations (IC_{50} or IC_{90}) following either a 1h pulse with cisplatin or a continuous exposure. The reported IC_{50} resistance ratios from different groups examining this culture pair vary from 4-15 fold depending on the length of cisplatin exposure -pulse versus continuous- and the type of assay used to measure toxicity (Table 3). Irrespective of the resistance ratio value, each group reports that cisplatin resistance was stable in C13* cells without the requirement of further selection with cisplatin. This suggests that permanent cellular alterations contribute to resistance in C13* cells.

Table 3: Cisplatin Resistance Ratios Reported for the 2008/C13*Ovarian Carcinoma Culture Model

Research Group	Cisplatin Exposure Period	Toxicity Assay (endpoint in days)	Resistance Ratio	
			IC_{50}	IC_{90}
Andrews and Albright 1992	Continuous	Clonogenic assay (10d)	15	-
Zhen *et al.* 1992	1h pulse	Clonogenic assay	10	-
Brooks *et al.* 1995	1h pulse	Clonogenic assay (14d)	-	3
Parekh and Simpkins 1996	Continuous	Cell number assay (3d)	12	-
Moorehead *et al.* 1996	1h pulse	Cell number assay (4d)	4	5
Delmastro *et al.*1997	1h pulse	Cell number assay (3d)	12	-

The 2008/C13* model has been extensively characterized for the classical mechanisms of cisplatin resistance including alterations in drug accumulation, detoxification pathways and DNA repair capacity (Table 1). The data suggests that the cisplatin resistant phenotype of C13* cells is associated with increased tolerance to DNA damage, mediated in part by enhanced replicative bypass (Mamenta *et al.* 1994). This concept of reduced tolerance undermines the significance of other contributing mechanisms such as reduced cisplatin accumulation, increased detoxification, or increased gene-specific repair in this model system. The data also suggests that as yet unidentified mechanisms are operating to mediate this increase in DNA damage tolerance. Identification of

the molecular participants is required to improve our understanding of this mechanism and to reveal novel therapeutic targets to reverse resistance. The 2008/C13* model has been the subject of several investigations for alternative mechanisms that contribute to cisplatin resistance. The appearance of mitochondrial alterations concurrently with cisplatin resistance in C13* cells have implicated mitochondria as novel mediators of the resistant phenotype.

Kimura *et al.* (1993a) applied the technique of subtractive hybridization to identify differentially expressed genes in C13* versus 2008 cells. This approach identified that the mitochondrially-associated heat shock protein-60 (hsp60) is over-expressed in cisplatin resistant C13* cells compared to the parental 2008 line. Hsp60 is a member of the heat-shock protein family that functions as a protein chaperone to ensure appropriate protein-folding in the mitochondrion, an especially critical function when cells are under the stress of elevated temperatures (Black and Subjeck 1991; Pardue, Ballinger and Hogan 1992). Hsp60 protein is induced in 2008 cells following cisplatin exposure, but both the mRNA and protein levels of hsp60 are constitutively elevated in C13* cells in the absence of cisplatin stimulation (Kimura *et al.* 1993a). One explanation may be provided by the fact the C13* subline has multiple abnormalities (both gains and losses) of chromosomal copy number compared to 2008 cells (Wasenius *et al.* 1997). Thus it is possible that increased hsp60 expression does not necessarily contribute to resistance but is merely the result of genomic amplification of the

hsp60 gene. Although this theory remains to be tested, the idea that elevated hsp60 expression is involved cisplatin resistance is strengthened by the fact that this mitochondrially-associated stress protein is also up-regulated in the CP70 cisplatin-resistant subline of A2780 ovarian cancer cells (Yamamoto *et al.* 1999). Furthermore, there exists a positive clinical correlation between high levels of hsp60 expression and reduced long-term survival in ovarian cancer patients (Kimura *et al.* 1993b). Although this particular study did not correlate hsp60 expression with therapeutic resistance, it could be hypothesized that hsp60 is involved in cisplatin resistance since reduced long-term survival is generally associated with a lack of response to standard first-line therapies that include cisplatin. In addition to the expression of a mitochondrially-associated stress protein, other mitochondrial properties have been associated with resistance as shown by the research group that developed the C13* subline.

As part of an investigation to explain reduced cisplatin accumulation in C13* versus 2008 cells, Andrews and Albright (1992) identified that C13* cells had both an increased plasma and mitochondrial membrane potential based on the accumulation of the cation tetraphenylphosphonium (TPP^+). The plasma membrane potential is maintained primarily by sodium and potassium gradients across the plasma membrane but in mitochondria the membrane potential is composed of both cationic and proton gradient contributions (Skulachev 1992). The proton gradient is generated by the activities of complexes I, III and IV of

the electron transport chain (ETC) as they transfer electrons to oxygen (respiration) and simultaneously pump protons into the intermembrane space (Figure 6). Since the inner mitochondrial membrane is highly impermeable to the passive diffusion of protons, their accumulation in the intermembrane space generates a strong electrical and concentration-dependent driving force (proton-motive force) for the flow of protons back into the matrix. Normally, this flux of protons is tightly coupled with the generation adenosine triphosphate (ATP) at the site of the ATP synthase (Complex V), with only minimal leakage of protons across the bilayer (Figure 7). A follow-up investigation using ^{31}P nuclear magnetic resonance showed that the steady-state concentration of phosphorylated metabolites like ATP and its derivatives were not different between C13* and 2008 cells (Berghmans *et al.* 1992). Thus the alteration of membrane potential does not compromise the energetic equilibrium of C13* cells. The investigators did realize that an increase in mitochondrial membrane potential could sensitize C13* cells to the toxic effects of high concentrations of lipophilic cations like rhodamine 123 (Rh123). These molecules induce toxicity following selective accumulation in mitochondria as a function of the membrane potential magnitude, resulting in gradient dissipation and inhibition of ATP synthesis leading to cell death (Modica-Napolitano and Aprille 1987; Singh and Shaughnessy 1988; Chen 1988). Using Rh123-induced toxicity as a selection pressure, Zinkewich-Péotti and Andrews (1992) were subsequently able to select

revertants from the C13* population which did not have elevated mitochondrial membrane potential. Of significant interest was the observation that these revertants (RH4 cells) with reduced mitochondrial membrane potential were much more similar to 2008 cells in terms of their cisplatin sensitivity (3-fold resistant) than the C13* cells (15-fold resistant). The authors confirmed that RH4 cells were not identical to 2008 cells since they still possessed other characteristics of C13* cells including elevated GSH content, and reduced cisplatin accumulation. Thus, in this model system, the unique conclusion was made that an elevated mitochondrial membrane potential was independently associated with cisplatin resistance, although the exact mechanism of resistance conferral was not known.

Figure 6: Mitochondria are Multi-functional Organelles that Participate in Energy Production, Calcium Homeostasis, Redox Balance and Apoptosis Induction

(Reprinted from Wallace 1999)

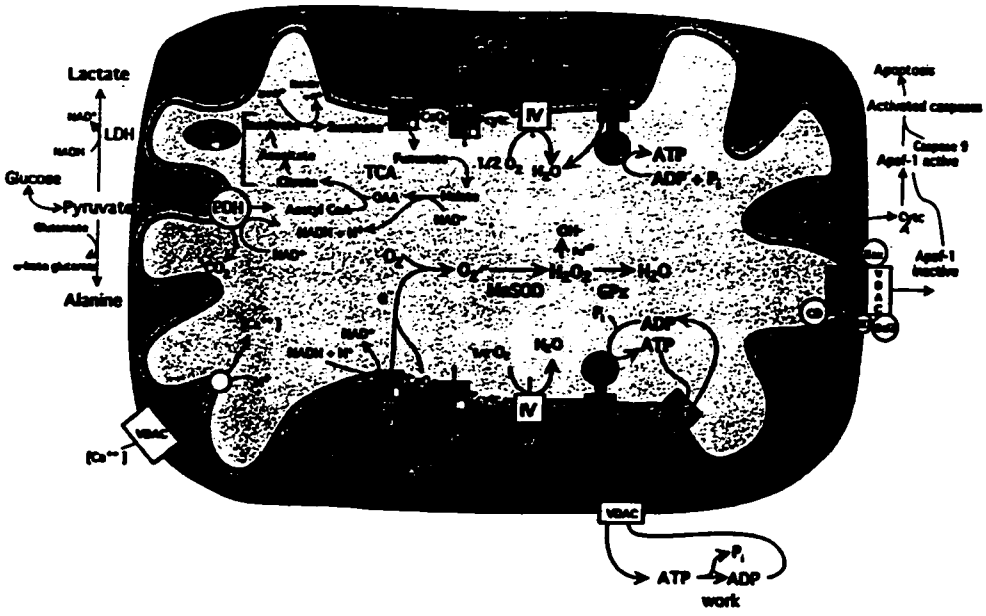
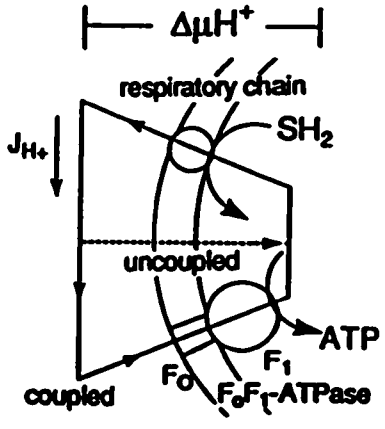


Figure 7: Proton Flux is Tightly Coupled to the Production of ATP by the Minimization of Proton Leak

(Reprinted from Terada 1990)



Mitochondria are Multi-Purpose Organelles that Participate in Calcium Homeostasis, Apoptosis Induction and Redox Balance: Implications for Cisplatin Resistance

Although mitochondria are primarily recognized for their capacity to generate the majority of intracellular ATP, they also participate in other cellular processes that are dependent on maintenance of the mitochondrial membrane potential. Mitochondria can uptake free calcium ions (Ca^{2+}) by a uniporter mechanism that operates as a function of their negative membrane potential and have two distinct efflux mechanisms (Gunter *et al.* 1994). The uptake of Ca^{2+} by mitochondria plays a role in the regulation of metabolism since the activity of several matrix enzymes is stimulated by increased Ca^{2+}; these include the ATP synthase and the dehydrogenases of the citric acid cycle and the electron transport chain (Gunter *et al.* 1994). The capacity of mitochondria to sequester large amounts of intracellular calcium in the form of an insoluble calcium phosphate complex ($Ca_3(PO_4)_2$) in the matrix is also believed to serve a protective function against toxic Ca^{2+} loading of the cytosol (Gunter *et al.* 1994). As yet there have been no studies to suggest that altered mitochondrial Ca^{2+} deposition, uptake or efflux is a mediator of cisplatin resistance.

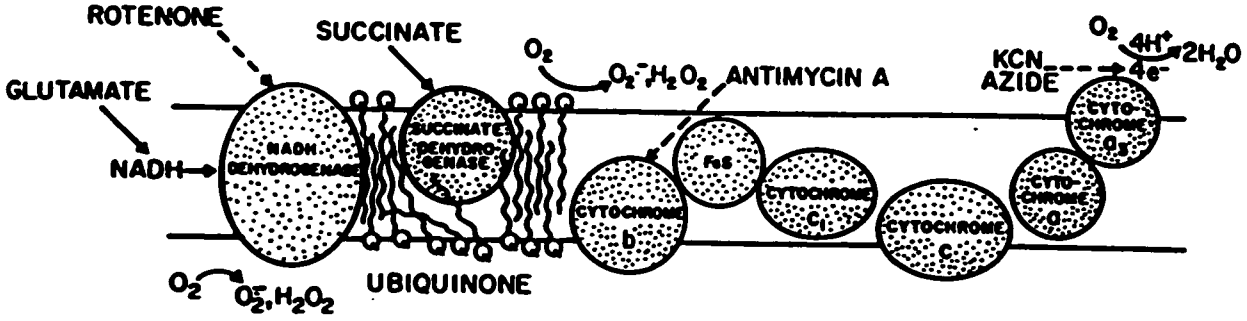
Mitochondria have also been identified as important participants in apoptosis initiation through the release of pro-apoptotic factors such as cytochrome c and the protease AIF (apoptosis-inducing factor) (Kroemer, Dallaporta and Resche-Rigon 1998; Green and Reed 1998). These molecules are involved in the activation of caspases, the downstream protease executors of

apoptosis (Figure 5). Mitochondrial participation in apoptosis seems to be intimately associated with the opening of a non-specific channel called the mitochondrial permeability transition (MPT) pore. Opening of the pore leads to membrane depolarization, the uncoupling of oxidative phosphorylation and the uncontrolled passage of low molecular weight solutes (<1500 Daltons) including cytochrome c and AIF (Lemasters *et al.* 1998). In several model systems MPT is a required event for the apoptotic response to occur following various chemical or biological apoptogenic signals, thus implicating mitochondria as a key regulator of this process (Lemasters *et al.* 1998). Various factors that influence the probability of MPT have been identified: conditions that promote MPT include elevated free Ca^{2+} or inorganic phosphate (P_i) concentrations in the matrix, pyridine nucleotide oxidation, or oxidation of vicinal thiol groups in the pore protein complex. Conditions that deter MPT include elevated concentrations of Mg^{2+} or ADP, an acidic matrix pH and a high membrane potential (Lemasters *et al.* 1998). Given the evidence for cisplatin treatment as an apoptogenic signal, it is feasible that the increased mitochondrial membrane potential observed in C13* cells could have an impact on apoptosis resistance. As yet, no studies have been conducted to record the details of apoptotic events in 2008 and C13* cells in response to cisplatin, and what role mitochondrial membrane potential or MPT plays in this model system.

Another mitochondrial activity that may impact on MPT and cellular redox balance in general is the generation of reactive oxygen species (ROS) as a by-product of electron transport activity (Turrens 1997; Lemasters *et al.* 1998). It is currently accepted that mitochondria are the most prominent source of ROS production in the cell since 1-2% of total oxygen (O_2) consumption is incompletely reduced to superoxide (O_2^-) anion during respiration (Figure 8) (Boveris, Oshino and Chance 1972; Boveris and Chance 1973). The major sites of O_2^- production are the NADH dehydrogenase (Complex I) or semiubiquinone (Complex III) electron carriers, although the prominent contributor may vary in a given cell type (Kwong and Sohal 1998). Since the production of O_2^- is not an enzymatic process, the rate of generation follows the principles of the law of mass action. Thus any condition that leads to increased reduction of the participating electron carriers (chemical blockade of ETC, lack of ADP substrate, downstream defect in respiratory components) or increases the concentration of oxygen (ischemia/reperfusion, hyperroxia) can enhance O_2^- generation.

Figure 8: Sites of Superoxide Generation in the Mitochondrial Electron Transport Chain

(Reprinted from Freeman and Crapo 1982)



Superoxide can dismutate spontaneously or enzymatically into hydrogen peroxide (H_2O_2) by the activity of mitochondria-specific manganese superoxide dismutase (MnSOD). H_2O_2 can be neutralized to H_2O in the mitochondrion or cytosol by the action of glutathione peroxidase, or it can diffuse through the bilayers to react with catalase in peroxisomes (Chance, Sies and Boveris 1979; Reed 1990). Mitochondrial production of H_2O_2 is believed to have an impact on cellular aging and carcinogenesis since it is a substrate for hydroxyl radical ($OH^{\cdot}$) formation, a highly reactive radical that is an efficient DNA mutagen (Imlay and Linn 1988; Barja *et al.* 1994; Giulivi *et al.* 1995; Herrero and Barja 1998). Since mitochondria contain DNA that encodes for several components of the respiratory chain, it is suggested that accumulated mutations will eventually reduce the efficiency of respiration and increase ROS production to ultimately induce nuclear DNA mutations (Papa 1996; Dreher and Junod 1996). Although

this may be a relevant factor in aging and carcinogenesis, the basic concept that ROS are simply toxic by-products of respiration has been recently up-dated to include a positive function. There is substantial evidence accumulated for a physiological role of ROS like H_2O_2 as intracellular signalling intermediates at low concentrations ($\approx 10^{-9}$M) (Baeuerle, Rupec and Pahl 1996; Poyton and McEwen 1996; Suzuki, Forman and Sevanian 1997). Multiple investigators using many different cell types have shown that exogenously applied H_2O_2 can influence both gene expression and proliferation (Table 4). As a result, there is significant interest in the signalling potential of endogenously generated ROS by non-phagocytic cell types like fibroblasts, endothelial cells and tumour cells (Table 5). In these cells the exact source of $O_2^{\cdot}$ and H_2O_2 generation is not well defined, but several intracellular sources are recognized including the mitochondrion and novel plasma-membrane oxidase assemblies that are similar to the phagocytic complex (Figure 9).

The impact of H_2O_2 as a second messenger has not been investigated in the context of cisplatin resistance, but a related investigation has shown that cells with acquired resistance to exogenous oxidative stress also become cross-resistant to cisplatin (Spitz *et al.* 1993). This raises the possibility that endogenous oxidative stress arising from mitochondrially-derived ROS could have a similar influence on cisplatin resistance.

Table 4: Evidence for H_2O_2 Involvement in Intracellular Signalling Pathways

Reference	Model System	Exogenous/ Endogenous [H_2O_2] Supply	Signalling/Gene Expression Influence
Courgeon *et al.* 1988	Drosophila cells	Exogenous Addition	↑ hsp 70, hsp 23 expression
Sullivan *et al. 1994*	HER 14 cells (NIH 3T3 with EGFR)	Exogenous Addition	Inhibition of phosphatases
Sundaresan *et al. 1995*	Rat VSMC	Endogenous Generation	Second messenger for PDGF
Gamou and Shimizu 1995	NA (squamous carcinoma) cells	Exogenous Addition	↑ Y-P of EGFR
Timblin Janssen and Mossman1995	Tracheal epithelial cells	Exogenous Addition	↑ transcriptional activity at AP-1
Chen, Olashaw and Wu 1995	HeLa (cervical carcinoma) cells	Exogenous Addition	↑ MAPKK activity
Guyton *et al.* 1996a	NIH3T3, HeLa, Rat1, PC12, VSMC	Exogenous Addition	↑ ERK2 activity ↑ fos, jun mRNA
González-Rubio *et al.* 1996	Rat mesangial cells	Exogenous Addition	↑ Y-P of PDGFR
Rao 1996 Rao 1997	VSMC	Exogenous Addition	↑ Y-P of EGFR ↑ Y-P of FGFR
Jacquier-Sarlin and Polla 1996	Pre-monocytic U937 cells	Exogenous Addition	↑ transcriptional activity of HSF-1
Leclerc *et al.* 1997	Spermatozoa	Exogenous Addition	↑ Y-P content of total protein
Irani *et al.* 1997	NIH3T3, Ras or Raf transformed	Endogenous Generation	GF-independent Cell growth
Bae *et al.* 1997	A431 (epidermoid carcinom) cells	Endogenous Generation	↑ Y-P of EGFR
Tournier *et al.* 1997	Rat astrocytes	Exogenous Addition	↑ ERK, JNK, $cPLA_2$ activity
Belkhiri *et al.* 1997	Human umbilical vein EC	Exogenous Addition	↑ MMP-2 protein
Brenneisen *et al.* 1997	Human dermal fibroblasts	Exogenous Addition	↑ MMP-1 mRNA
Chandel *et al.* 1998	Hep3B (hepatoma) cells	Endogenous Generation	↑ transcriptional activity of HIF

Abbreviations: EC – endothelial cells, EGFR – epidermal growth factor receptor, ERK – extracellular signal regulated kinase, HIF – hypoxia-inducible factor, hsp- heat shock protein, HSF-1 – heat shock factor-1, JNK – c-Jun NH_2 terminal kinase, MAPKK – mitogen activated protein kinase kinase, MMP – matrix metalloproteinase, PDGF – platelet-derived growth factor, PDGFR – platelet-derived growth factor receptor, $cPLA_2$ – cytosolic phospholipase A_2 VSMC – vascular smooth muscle cell, Y-P, tyrosine phosphorylation

Figure 9: Cellular Sources of Reactive Oxygen Species
(Reprinted from Freeman and Crapo 1982).

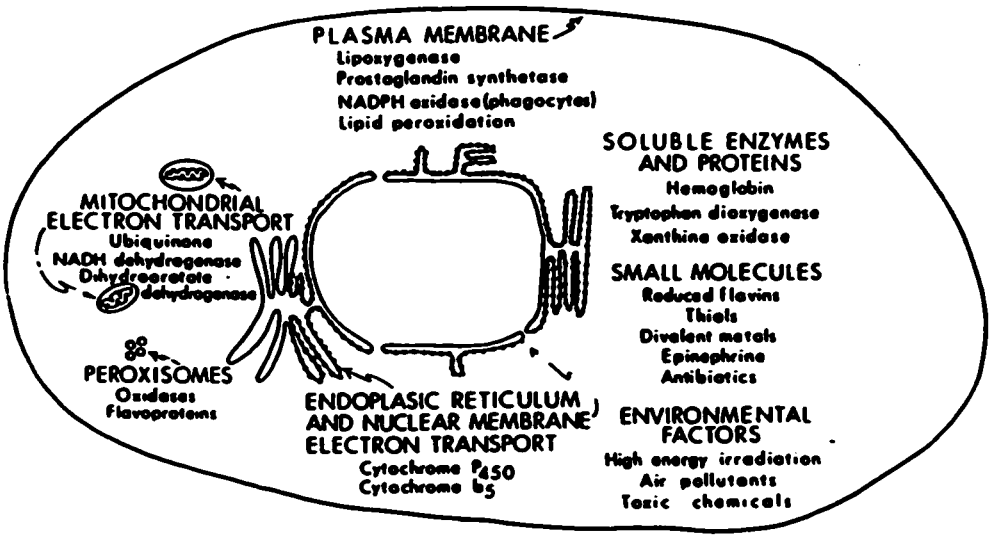


Table 5: Non-Phagocytic Cell Types Generate Extracellular $O_2^{\cdot}$ and H_2O_2

Cell Type	Detected Species	Stimulus	Reference
Endothelial cells (bovine or sheep, primary)	H_2O_2	Hyperoxia or hypoxia/reoxygenation	Kinnula *et al.* 1993 Sanders *et al.* 1993 Zulueta *et al.* 1995
Cardiac myocytes (bovine, primary)	H_2O_2	lactate or hypoxia/ reoxygenation	Mohazzab, Kaminski and Wolin 1997 Mohazzab *et al.* 1996
Adipocytes (human primary)	H_2O_2	Insulin	Krieger-Brauer and Kather 1995
Spermatozoa (human)	$O_2^{\cdot}$	exogenous NADPH	Aitken *et al.* 1997
Fibroblasts (human, primary)	$O_2^{\cdot}$	calcium ionophores/ tumour necrosis factor/ phorbol esters	Meier *et al.* 1993
Fibroblasts (human, normal IMR-90)	H_2O_2	Transforming growth factor β	Thannickal and Fanburg 1995
Fibroblasts (human, CCD-339sk)	$O_2^{\cdot}$	Constitutive	O'Donnell and Azzi 1996
Fibroblasts (SV40 transformed)	H_2O_2	Mechanical injury	Arbault *et al.* 1997
Thyroid cells (pig, primary)	H_2O_2	Calcium ion	Gorin *et al.* 1997
Tumour cell lines	H_2O_2	Constitutive	Szatrowski and Nathan 1991 Wang *et al.* 1996 Del Bello *et al.* 1999

Significance of Mitochondrial Alterations to Cisplatin Resistance in C13 Cells*

There are several reasons to suspect that the increased mitochondrial membrane potential of C13* cells is directly associated with an abnormality in mitochondrial function. Andrews and Albright (1992) noted that the proton gradient contribution to the overall membrane potential was reduced in C13* cells compared to 2008 cells. The authors suggested there was either a defect in the respiratory enzymes responsible for generating this gradient or a loss of proton coupling. Since cisplatin is an efficient mutagen, it is feasible that during the chronic cisplatin exposure schedule utilized to generate resistance in C13* cells, mutations accumulated in mitochondrial DNA (mtDNA) to initiate this process (Pinto and Lippard 1985; Rosenberg 1985). MtDNA itself is considered a sensitive target for mutagenesis by anticancer agents like cisplatin due to the absence of protective histone proteins and inefficient repair systems (Singh, Sharkey and Moorehead 1992). Cisplatin has also been shown to induce acute mitochondrial damage resulting in reduced respiration rates in cisplatin-sensitive cells of the S_3 segment of proximal tubule and in isolated mitochondria of mouse liver (Gordon and Gattone 1986; Singh 1989; Brady *et al.* 1990; Rosen, Figliomeni and Simpkins 1992). Acute cisplatin-mediated toxicity is believed to result from excessive oxidative stress generated by both direct production of ROS like H_2O_2 and the inactivation of antioxidant defenses (Kovacic *et al.* 1986; Sadzuka, Shoji and Takino 1992; Brady *et al.* 1993; Zhang and Lindup 1993;

Tsutsumishita *et al.* 1998). In this scenario, H_2O_2-derived $OH^{\cdot}$ could be the mutagenic factor that contributes to aberrant mitochondrial respiration in cells that survive the cisplatin selection process.

HYPOTHESIS

Alterations of mitochondrial physiology are involved in the mechanism of acquired cisplatin resistance in C13* ovarian carcinoma cells: increased generation of reactive oxygen species in the mitochondria of C13* cells initiates stress responses that contribute to their drug-resistant phenotype.

2.0 MATERIALS AND METHODS

Materials

All pharmacological reagents – allopurinol, antimycin A, L-buthionine-[S,R]-sulfoximine (BSO), *cis*-platinum(II)diammine dichloride (cisplatin), chloroquine, 2-deoxy-D-glucose, diphenyleneiodonium chloride (DPI), 8-methyl-N-vanillyl-6-noneamide (capsaicin), oligomycin B, pargyline, phenylarsine oxide (PAO), and rotenone were from Sigma Chemical Co. (St. Louis, MO). All cell culture reagents including RPMI 1640 medium, McCoy's 5A medium, Dulbecco's MEM, αMEM, fetal bovine serum (FBS) and the mycoplasma detection kit were from GIBCO Life Sciences (Burlington, ON). The modified Dulbecco's F12 medium was from Sigma Chemical Co. (St. Louis, MO).

Reagents required for extracellular H_2O_2 measurement and proliferation assays were from several sources: the fluorescent indicators 10-acetyl-3,7-dihydroxyphenoxazine (A6550), 7-hydroxy-6-methoxycourmarin (scopoletin) and 10N-nonyl acridine orange (NAO) were purchased from Molecular Probes (Eugene, OR). Bovine liver catalase, catalase and glucose oxidase from *Aspergillus niger*, horseradish peroxidase, hydrogen peroxide (H_2O_2 30% solution) and D-gluconolactone were from Sigma Chemical Co. (St. Louis, MO). The

DNA binding fluorochrome Hoechst 33258 (bisbenzimide) was from Calbiochem (La Jolla, CA).

For cell cycle analysis, propidium iodide (PI) and 5-bromo-2'-deoxyuridine (BrdU) were from Sigma Chemical Co. (St. Louis, MO). The monoclonal murine anti-BrdU antibody and fluorescein (FITC) conjugated goat anti-mouse secondary antibody were from Calbiochem (La Jolla, CA).

Cell Lines and Culture Conditions

2008 human ovarian carcinoma cells, the cisplatin resistant C13* subline and the RH4 revertants were a gift from Dr. P.A. Andrews (Georgetown U, MD). These cells were grown in RPMI medium supplemented with 5% FBS. The human colon carcinoma HT29 cell line (#38-HTB), the human ovarian carcinoma SKOV-3 cell line (# HTB-77) and murine 3T6 fibroblasts (# CCL-96) were obtained from the American Type Culture Collection (Rockville, MD). HT29 and SKOV-3 cells were grown in McCoy's 5A medium supplemented with 10% FBS. 3T6 fibroblasts and Hf172 human fibroblasts were grown in Dulbecco's MEM and αMEM, respectively, with 10% supplemented FBS. Normal ovarian surface epithelial cells (LLO) were a gift from Dr. H. Hirte (Hamilton Regional Cancer Centre). LLO cells were grown in modified Dulbecco's F12 medium supplemented with 12% FBS and were not maintained beyond 8 passages. All

cells were maintained as monolayer cultures at 37ºC in a humidified environment with 95% air and 5% CO_2.

Mycoplasma Detection Assay

All cells lines were confirmed mycoplasma negative with the GIBCO Mycotect kit for the detection of adenosine phosphorylase. Briefly, the assay is based on the sensitivity of indicator cells (3T6 mouse embryo fibroblasts) to the cytotoxic effects of the adenosine analogs 6-methylpurine and 6-methylpurine riboside (McGarrity and Carson 1982). These products are generated from the non-toxic substrate 6-methylpurine deoxyriboside by the activity of adenosine phosphorylase, an enzyme present in most species of mycoplasma. Cells that are infected with mycoplasma will release a significant amount of this parasite into their culture medium. The subsequent transfer of mycoplasma-contaminated culture medium to 3T6 cells would thus have the capacity to generate the toxic analogs when treated with the 6-methylpurine deoxyriboside substrate, and a positive result is recorded following 3T6 toxicity. A positive control is supplied with this kit in the form of the purified adenosine phosphorylase enzyme.

Protein Assay

Protein quantitation was based on the production of bicinchoninic acid (BCA) complexes with Cu^{1+} generated by the presence of peptide bonds of proteins under alkaline conditions (Lowry *et al.* 1951). The bimolecular complex of BCA and Cu^{1+} forms a water-soluble product that can be quantified by its absorbance at 562 nm. All reagents including the bovine serum albumin protein standard reagent were supplied in the micro BCA kit from Pierce Chemical Co. (Rockford IL): reagent A contains sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2N sodium hydroxide for the generation of alkaline conditions, reagent B contains 4% BCA in water and reagent C contains 4% cupric sulphate pentahydrate in water.

2008, C13* and RH4 cells were collected into pellets of 1.0, 2.5, 5.0, 7.5 and $10x10^5$ cells in microcentrifuge tubes following a triplicate count by hemocytometry. The cells were washed 2x in phosphate-buffered saline (PBS, pH 7.2) to remove non-cellular protein contamination and at the final step re-suspended into 100µl of 0.1% Triton X-100 detergent. Following a 10 minute incubation, 10µl aliquots (3 replicates) per sample were added to the wells of a 96-well microtitre plate containing 100µl of the mixed assay solution (50 parts reagent A, 48 parts reagent B, 2 parts reagent C). Protein standards in the range of 4 to 20 µg were taken from the kit supply of 2mg/ml ampules of bovine

serum albumin and the background absorbance was calculated by the addition of 10µl of 0.1% Trition X-100 alone to 100µl of assay solution. Following a 2h incubation at 37°C for full colour development, the absorbance at 570nm was measured on an EL340 microplate spectrophotometer (Bio-Tek Instruments Co., Winooski VT). Protein content (pg/cell) was calculated following the derivation of a standard curve (µg protein vs absorbance) and calculation of µg protein per various cell numbers ($1.0x10^6$-$1.0x10^5$).

Determination of Mitochondrial Mass

The fluorochrome 10-N-nonyl acridine orange (NAO) was used to measure mitochondrial mass based on its affinity for binding cardiolipin, a mitochondrial-specific phospholipid, found primarily in the inner mitochondrial membrane (Maftah *et al.* 1989; Petit *et al.* 1992; Schlame and Haldar 1993). Subconfluent populations of 2008, C13*, RH4, HT29 and SKOV-3 cells were collected with trypsin-EDTA (1x). Cells were spun (5 min, 1000 rpm) and the pellet re-suspended into their appropriate fully-supplemented medium, at a density of $5.0\text{-}7.5x10^5$ cells/ml. NAO was added to a final concentration of 5µM from a stock solution dissolved in 100% DMSO. Cell suspensions were rotated gently in minimal light for 45 min, following which cells were again pelleted and re-suspended in fresh medium to remove un-reacted NAO. The population

fluorescence profile (λ_{ex}480 nm, λ_{em}530nm) was determined on a Coulter Epics Profile II Flow Cytometer (Coulter, Burlington, ON).

Assay for Glycolytic Capacity

This assay determines the glycolytic capacity of a cellular homogenate by its ability to produce lactic acid in a controlled system (Bustamante, Morris and Pedersen 1981). Generally, in a sequential reaction lactic acid produced by a cellular homogenate or lactic acid standards were added to a buffer solution containing lactate dehydrogenase that generates NADH from NAD^+ during the conversion of lactic acid to pyruvate. The NADH generated is then quantified by recording the absorbance of the samples at 340nm.

Step 1: Lactic Acid Generation: Following collection by trypsinization and counting by hemocytometry, $1x10^7$ cells were washed in serum-free medium to remove extraneous protein and pellets re-suspended in phosphate-buffered saline (800µl). Two aliquots of 50µl were removed for protein determination as described previously. The cell suspensions were spun to form a pellet the cells and re-suspended in a 700µl volume of homogenization medium containing 220mM mannitol, 70mM sucrose, 2mM Hepes, 0.1% bovine serum albumin (BSA). The cell suspensions were sonicated and duplicate aliquots of 230µl were added to 70µl of reagent 1 (4mM NaCl, 10mM $MgCl_2$, 10mM K_2PO_4, 4mM ATP, 4mM ADP, 2.8mM NAD+, 4mM glucose) and incubated for 30min, 37°C for the

production of lactic acid. The assay was terminated by the addition of 700µl of chilled (4°C) 5% trichloroacetic acid. The precipitated proteins were pelleted at 10 000 rpm for 10 min, 4°C.

Step 2: Generation of NADH: The lactic acid concentration of the supernatant was determined by adding various sample volumes (50-200µl) to 800µl of reagent 2 (42mM hydrazine sulphate, 170mM glycyl glycine, 0.4mM EDTA, 0.85mM NAD^+, and 20 Units/ml of lactate dehydogenase) in a final volume of 1.0ml, and allowed to incubate at room temperature for 30 min. Lactic acid standards (5 to 100µM) were used to generate a standard curve. The glycolytic capacity of cells was calculated as µM of lactic acid produced per mg of cellular protein, and then converted to lactic acid produced per million cells using the calculated protein/cell values.

Cellular Respiration Assay with the Clark Oxygen Electrode

Mitochondrial respiration was determined from the steady-state oxygen consumption of permeabilized whole cells that were continuously stirred in a Clark-type oxygen electrode equipped with a micro-chamber (0.6ml) (YSI 3500, Yellow Springs, Ohio). Cells were collected by trypsinization, pelleted by centrifugation and resuspended in respiration medium (0.25M sucrose, 10mM $MgCl_2$, 20mM Hepes, 2mM KH_2PO_4, 1.0mM ADP). Cells were confirmed as permeabilized by the loss of their ability to exclude trypan blue within 2 minutes

of exposure to 0.005% digitonin solubilized in 100% dimethylsulfoxide. Respiration was assayed immediately following permeabilization by loading 8 to $10x10^6$ cells into the chamber. Following the measurement of a baseline rate of respiration in the absence of substrate, succinate was added via a Hamilton syringe to a final concentration of 5mM. Respiration rates were calculated as % oxygen consumption per min per mg protein (where air-saturated medium was arbitrarily defined as 100%) and converted to a rate per million cells based on the calculated protein/cell values.

Hoechst 33258 DNA Fluorochrome assay for the Quantification of Adherent Cell Number

The DNA binding bisbenzimidazole Hoechst 33258 (H33258) (Calbiochem, La Jolla, CA) is a fluorochrome used for the quantification of cell number and thus proliferation in microculture assays of adherent cells (Rago, Mitchen and Wilding 1990). H33258 has binding specificity for DNA but not RNA, which shifts its emission wavelength to the visible range (λ_{ex}360nm, λ_{em}460nm). For use as an endpoint of proliferation assays in 96 well microculture plates, evidence for a linear relationship between adherent cell number and fluorescent emissions was first confirmed for each cell line. Cells were seeded in the range of $1.0x10^3$ to $5.0x10^4$ per well and allowed to adhere for 4 to 5h in fully supplemented medium. Following adherence, the wells were emptied of medium and washed 1x with water and emptied again. The plates

were stored at -80°C until the time of assay when they were thawed and 100µl of water was added to each well to promote cell lysis. Following a 6h incubation with water, 100µl of 20µg/ml H33258 stock diluted in TNE buffer (10mM Tris HCl, 1mM EDTA, 2M NaCl, pH 7.4) was added to each well giving a final H33258 concentration of 10µg/ml. Incubation in minimal light for 30min preceded fluorescence recording in a Cytofluor 2350 Microplate Fluorescence reader (Millipore, Mississauga, ON).

Treatment Protocols for Cytotoxicity and Proliferation Assays

For proliferation assays extending over a period of 96h, cells were initially seeded in 96-well microculture plates at low density, ranging from $1x10^3$ to $5x10^3$ per well in fully supplemented medium. Cells were left undisturbed overnight to ensure adherence and logarithmic growth had resumed before drug treatment. Cisplatin was dissolved in fully-supplemented medium, sterilized through a 0.2µ syringe filter and administered as a 1h pulse. All other treatments were sterilized in a similar manner, but delivered as continuous exposures from initial dilutions in fully-supplemented medium to refresh the culture medium at time zero. No further drug or medium replacement was performed for the duration of the experiment.

Colony formation assays for 2008 and C13* cells were set up in 100mm dishes seeded with either $1.0x10^3$ 2008 cells or $2.5x10^2$ C13* cells in a total of

10mls of supplemented medium. Following 7 days of growth in the presence or absence of sterilized extracellular catalase, colonies were stained and fixed with methylene blue (0.05%) in 100% methanol, and counted under the microscope. In this assay a colony was defined a distinct cell cluster with >20 cells present.

Total Intracellular Glutathione Assay

Whole cell glutathione (GSH) was assayed spectrophotometrically using the 5,5′ dithiobis(2-nitrobenzoic acid) (DTNB) reductase recycling assay for reduced GSH and oxidized glutathione disulfide (GSSG) (Anderson 1985). Reduced GSH is oxidized by DTNB to produce GSSG and 5-thio-2-nitrobenzoic acid (TNB) which has a yellow colour that can be quantified at 405nm. Glutathione reductase in the reaction mixture utilizes NADPH to reduce GSSG to GSH and thus recycles GSH back into the reaction. The resulting rate of TNB formation is proportional to the sum of GSH and GSSG initially present in the sample.

Adherent 2008 and C13* cells were collected following treatment with 1x trypsin/EDTA. Cells were washed in PBS (2x) and cell number counted with a hemocytometer to provide a duplicate sample of $7.5x10^5$ cells in 1.5ml microcentrifuge tubes. Cells were pelleted at 2500 rpm and the supernatant discarded. The pellet was resuspended in 120µl of 10mM hydrochloric acid to reduce the enzymatic and non-enzymatic oxidation of GSH to GSSG. Cells were subsequently lysed by 3 freeze-thaw cycles, centrifuged at 13 000 rpm (10 000g)

for 5 min in a microcentrifuge and then 100µl of the supernatant collected into fresh tubes. A 50µl volume of 10% 5-sulfosalicylic (5-SA) acid was added to deproteinize the sample followed by centrifugation at 13000 rpm (5 min). Aliquots of 25µl of the resulting supernatant were subsequently assayed in comparison to 25µl aliquots of GSH standards equivalent to 0.5, 1, 1.5, 2.0, 2.5 and 3.0 nmoles diluted in 5% 5-SA. The complete reaction medium contained 25µl of GSH sample (standard or unknown), 175µl of water, 100µl of 6mM DTNB prepared in sodium phosphate (143mM) and Na_4 -EDTA (6.3mM) assay buffer (pH 7.5), plus 700µl of assay buffer supplemented with NADPH (0.248 mg/ml) daily. Following mixing, 100µl of each sample was aliquoted into duplicate wells of a 96 well plate. The reaction was initiated by the addition of 10µl of yeast GSSG reductase which had been diluted in assay buffer to a concentration of 2.2 activity units/ml such that a total of 0.022 units were added per well. Background rates of TNB conversion were monitored in the absence of added GSH (25µl of 5% 5-SA only) and presence of GSSG reductase. TNB absorbance was monitored at 405 nm in a 96 well plate spectrophotometer at 5 min intervals for a total of 30 min. The rate of TNB formation was calculated as a slope from linear regression analysis of absorbances recorded at 10, 20 and 30 min and the total nmole quantity of GSH present was calculated from a standard equation of the conversion rate of GSH standards.

Flow Cytometric Analysis: Cell Cycle Profiles and DNA Synthesis Activity

Quantification of the percent of cells in a heterogeneously cycling population that are in the DNA synthesis phase (S phase) of the cell cycle can be evaluated via incorporation of the modified nucleotide bromodeoxyuridine (BrdU). A pulse of this nucleotide is made available to cycling cells in their culture medium and is incorporated into the fraction of cells that are actively synthesizing DNA. The extent of incorporation is then evaluated via antibody-directed detection methods which amplifies a fluorescent signal that can be quantified on a per cell basis with flow cytometry.

Adherent cells ($5x10^5$-$1.0x10^6$) in 10ml of fully supplemented medium were dosed with BrdU at a concentration of 10μM for 30min for 2008 and SKOV-3 cells and for 60min with HT29 cells. At the appropriate time, the medium was removed and cells were collected following trypsinization into 15ml tubes. The cells were pelleted by centrifugation (1000 rpm for 5 min) and washed 2x with PBS (5mls). After the final spin, the supernatant wash was emptied from the tube and the pellet was suspended in approximately 100μl of PBS and transferred dropwise into a fresh tube containing 2mls of chilled 70% ethanol:water while vortexing. This fixation step proceeded on ice for 30min, after which time 2mls of 4N hydrochloric acid was added to the ethanol suspension and left at room temperature for 30 min more. The cells were then pelleted for 5 min at 2000rpm and the supernatant discarded to leave a dry

pellet. The cell pellet was resuspended into 1ml of 0.1M sodium tetraborate (Borax, pH 8.5) and transferred to a 1.5ml microcentrifuge tube. The cells were pelleted in the microcentrifuge (3 000rpm, 5 min) and the supernatant discarded. The pellet was then suspended either in 200µl of 70% ethanol:water for storage at -20°C or washed (2x) in 200µl of 0.5% Tween-20 in PBS in preparation for two-step antibody detection. Following the washing steps, the pellet was suspended in 50µl of Tween/PBS and incubated with 4µl of murine monoclonal anti-BrDU primary antibody for 30min at room temperature (100 ug/ml). Following incubation with primary antibody, the pellet was washed (2x) with 200µl of Tween/PBS and resuspended in 50µl prior to the addition of 4µl of secondary goat anti-mouse antibody that is conjugated to the fluorochrome fluorescein (FITC). Labelling proceeded for 30 min at room temperature followed by washing in Tween/PBS (2x, 200 µl) and a final resuspension into 1ml of PBS containing 5µg/ml of propidium iodide as a general label for cellular DNA. A minimum of $1x10^4$ cells were assessed for PI fluorescence (λ_{ex}493nm, λ_{em}636nm) and FITC fluorescence (λ_{ex}499nm, λ_{em}518nm) with an EPICS XL flow cytometer equipped with an argon laser (Coulter, Burlington, ON).

Extracellular Hydrogen Peroxide Production by Cultured Cell Lines

Two fluorescence-based assay systems were used to measure the net extracellular secretion of H_2O_2 by various cultured cell lines; one using the

fluorescent molecule 7-hydroxy-6-methoxycoumarin (scopoletin) and the other using the molecule *N*-acetyl-3,7-dihydroxyphenoxazine (A6550). Scopoletin is a fluorescent molecule that is oxidized to a non-fluorescent product by horseradish peroxidase (HRP) in the presence of H_2O_2 (Rest, 1994). The conversion of non-fluorescent A6550 into the fluorescent product 7-hydroxy-3H-phenoxazine-3-one (resorufin) is similarly dependent on the activity of HRP in the presence of H_2O_2 (Mohanty *et al.* 1997).

Cultured cells were plated at densities between $2.5x10^4$ and $7.5x10^4$ cells per well in fully supplemented medium followed by an adherence period of 4 to 5h. Following adherence, the wells were washed with assay buffer consisting of Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.1% calcium chloride and 0.1% magnesium chloride (pH 7.2-7.4) to remove all traces of medium. The wells were emptied again and 100µl of DPBS freshly supplemented with 0.1% gelatin (DPBSG) was added which contained 2 Units/ml of HRP. Subsequently, 100µl of DPBSG was added to each well containing either 100µM of A6550 or 20µM scopoletin to give final concentrations of 1Unit/ml HRP and 50µM A6550 or 10µM scopoletin in a 200µl volume. Wells containing both fluorochrome and HRP or fluorochrome alone in the absence of cells were used to measure background rates of oxidation as indicated by a loss of scopoletin fluorescence (λ_{ex}360nm, λ_{em}460nm) or a gain of A6550 fluorescence (λ_{ex}590nm, λ_{em}645nm). The changes in fluorescence were recorded continuously

at 30 min intervals for 3h in a Cytofluor 2350 fluorescence plate reader (Millipore, Mississauga, ON). For the A6550 probe, H_2O_2 production was calibrated against a standard curve of H_2O_2 in the final concentration range of 125 – 700 nM. For the less sensitive scopoletin probe, the standard curve was in the final concentration range of 0.5 - 3.0 μM hydrogen peroxide. The molar concentration of the 30% stock solution of H_2O_2 was calculated prior to use in these assays, based on the absorbance of H_2O_2 at 230 nm.

H_2O_2 production by glucose oxidase alone was measured using similar assay conditions but with replacement of the A6550 probe with the less sensitive scopoletin fluorochrome. Scopoletin fluorescence (λ_{ex}360nm, λ_{em}460nm) decreases following HRP catalyzed oxidation with H_2O_2 substrate as calibrated against an H_2O_2 standard curve (0.5 - 3μM).

Pharmacological Inhibition of H_2O_2 Production

Following the adherence period of 4h, the culture medium in each well of duplicate 96-well micro-culture plates was replaced with fully-supplemented medium containing the agent at its final desired concentration or suitable solvent controls. Following a 2h incubation period (37°C), one plate was immediately assayed for H_2O_2 production as described above. The matched plate was analysed for cell adherence using the DNA binding fluorochrome H33258

(λ_{ex}360nm, λ_{em}460nm) as previously described. The results for H_2O_2 production are reported only if the measured DNA content in inhibitor-treated wells fell within ± 5% of untreated controls.

Statistics

One way analysis of variance (ANOVA) was used to identify significant ($p<0.05$) differences between multiple group means when only one independent variable was involved, such as the 2008, C13* and RH4 bioenergetic characteristics and the pharmacological effects of oxidase inhibitors on extracellular H_2O_2 production. To follow a significant ANOVA result ($p<0.05$) and identify which group means were different, two post-hoc comparisons were employed depending on the situation: the Tukey test was used to make multiple pairwise comparisons or Dunnett's test was used to make comparisons to a single designated control group.

Each set of survival data obtained from single agent exposures was fitted to a quadratic equation for determination of the IC_{50} concentration, and the overall mean of these values is reported. The student's t-test was used to compare IC_{50} differences between 2008 and C13* cells.

The proliferation rescue data for catalase and glucose oxidase combinations was analysed by one-way ANOVA for glucose oxidase plus catalase combinations compared to the "control" data set of catalase treatment

alone. Dunnett's test for multiple comparisons to the control group was used as the post-hoc test following a positive result ($p<0.05$) with one-way ANOVA. All statistical analysis was performed using Minitab statistical software, (Academic Version 12).

3.0 RESULTS

3.1 CONFIRMATION OF CISPLATIN RESISTANCE

Cisplatin Cytotoxicity Profiles for 2008, C13 and RH4 Cells*

The DNA binding agent bisbenzimidazole (H33258) has been used in a fluorescence-based assay to calculate cell number as an endpoint for cytotoxicity assessment. The relationship between adherent cell number and DNA fluorescence was confirmed for each cell line used in this investigation. Figure 10 shows the data for 2008 and C13* cells as representative.

The reported resistance ratios for the 2008 cisplatin-sensitive and the C13* cisplatin-resistant cell line vary between groups of investigators using different cisplatin exposure protocols and methods of cytotoxicity assessment as shown in Table 3. The profiles for 2008, C13* and RH4 cell survival 4 days following a 1h cisplatin pulse are shown in Figure 11. The IC_{50} values for 2008, C13* and RH4 cells are equal to 3.2 ± 0.3 μM, 24.3 ± 3.1 μM and 3.8 ± 0.4 μM, respectively. The 7.5-fold IC_{50} resistance ratio for C13* versus 2008 cells is similar to the 8-fold resistance range reported by other investigators. Zinkewich-Péotti and Andrews (1992) reported low-level (3-fold) resistance of RH4 cells versus 2008 cells, but our data shows nearly equivalent sensitivity to cisplatin between these two cell type in this assay.

3.2 CELLULAR CHARACTERISTICS SUMMARY

Protein Content

A summary of some cellular and energetic characteristics of 2008, C13* and RH4 cells are presented in Table 6. The 2008 cells contain significantly more protein than either C13* or RH4 cells although there is no difference between the protein content of C13* and RH4 cells. Zinkewich-Péotti and Andrews (1992) used the evidence of consistent elevations of GSH content and altered mitochondrial localization in RH4 and C13* cells to prove that RH4 cisplatin-sensitive cells were a genuine revertant population of C13* precursors. The similarity of protein content between C13* and RH4 cells may then be used as further evidence for this conclusion.

Figure 10: Linear Relationship Between Cell Number and H33258 Fluorescence in 2008 and C13*Cells

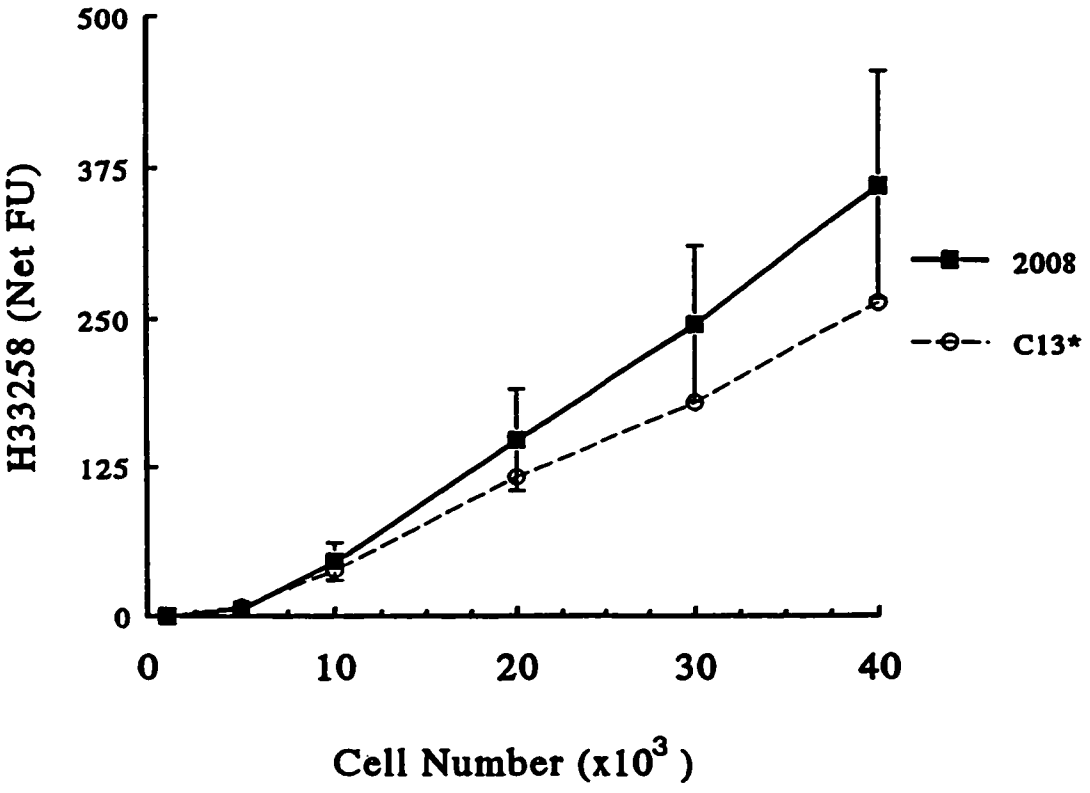


Figure 10: In 2008 and C13* cells a linear relationship is evident for the total DNA content of a specified adherent cell number versus fluorescence generated upon binding of the bisbenzimidazole molecule to DNA. Data represent mean ± SEM of n=3 independent experiments.

Figure 11: Cytotoxic Response of 2008, C13* and RH4 Cells to Cisplatin

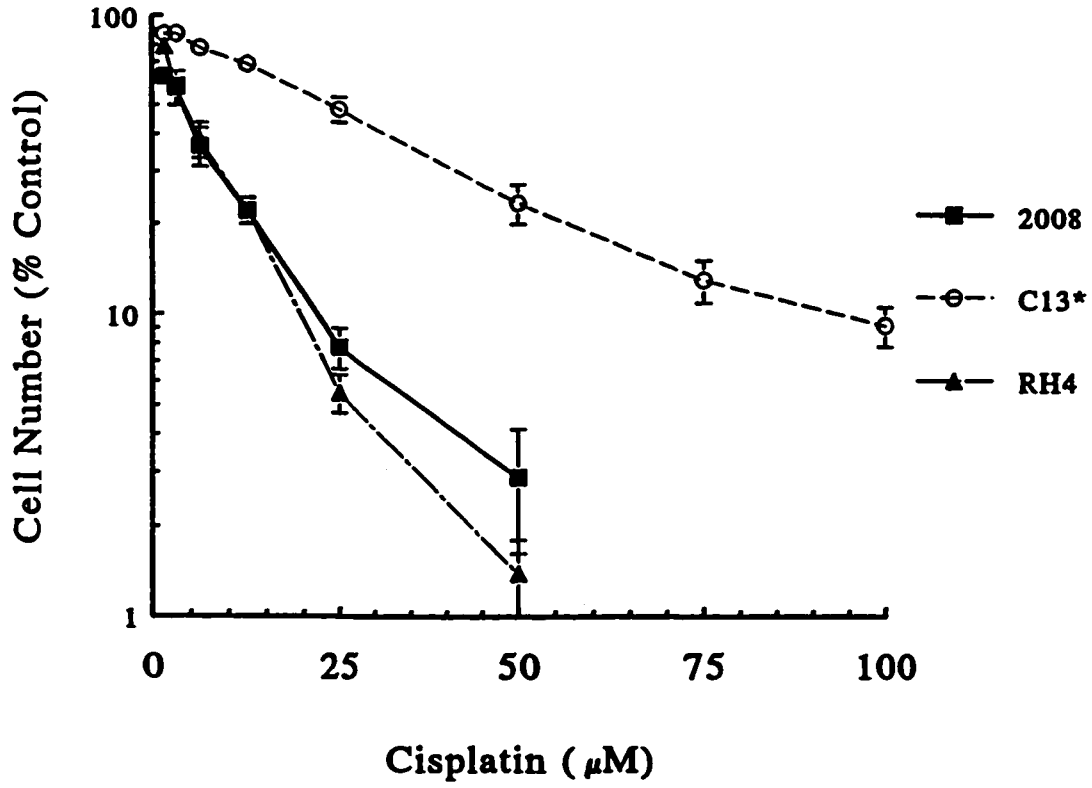


Figure 11: 2008, C13* and RH4 survival 96h following a 1h pulse of cisplatin. Data represent mean ± SEM of n=4 independent experiments.

Table 6: Cellular Characteristics of 2008, C13* and RH4 Cells

Cell type	Protein content (pg/cell)	NAO Fluorescence/ Cell	Glycolytic capacity (μM lactic acid/10^6 cells)	Cellular respiration rate (% O_2/ Min/10^6 cells)
2008	191.1 ± 2.8✣	116.8 ± 7.1	29.8 ± 5.4	2.41 ± 0.11†
C13*	141.0 ± 11.8	85.3 ± 7.5	26.9 ± 3.1	1.11 ± 0.24
RH4	160.8 ± 16.2	94.3 ± 13.0	37.9 ± 5.2	1.02 ± 0.04

Table 6: These data represent the mean ± SEM of n=3 independent assays.

(✣) Indicates that 2008 protein content per cell was significantly greater than either C13* or RH4 ($p<0.05$).

(†) Indicates that the cellular respiration rate of 2008 cells was significantly greater than either C13* or RH4 cells. ($p=0.001$).

Determination of Mitochondrial Mass

Mitochondrial mass was determined from the population fluorescence profiles generated following flow cytometric analysis of cells stained with nonyl-acridine orange (NAO). This molecule binds to the phospholipid cardiolipin that is predominantly located in the inner mitochondrial membrane, thus providing an estimate of inner mitochondrial membrane mass. The population means for NAO fluorescence were not significantly different between 2008, C13* and RH4 cells (Table 6). The capacity of the flow cytometer to collect data for each individual cell provides an overall distribution of fluorescence in the entire population, as shown in Figure 12 for 2008 cells. NAO fluorescence profiles for 2008, C13* and RH4 cells is a normally distributed characteristic within the population. The data is presented for an NAO incubation concentration of 5 μM, although the relative results for mean fluorescence were not different if this concentration was reduced to 0.5 or 0.1 μM NAO.

Figure 12: Distribution of NAO Fluorescence in the 2008 Cell Population

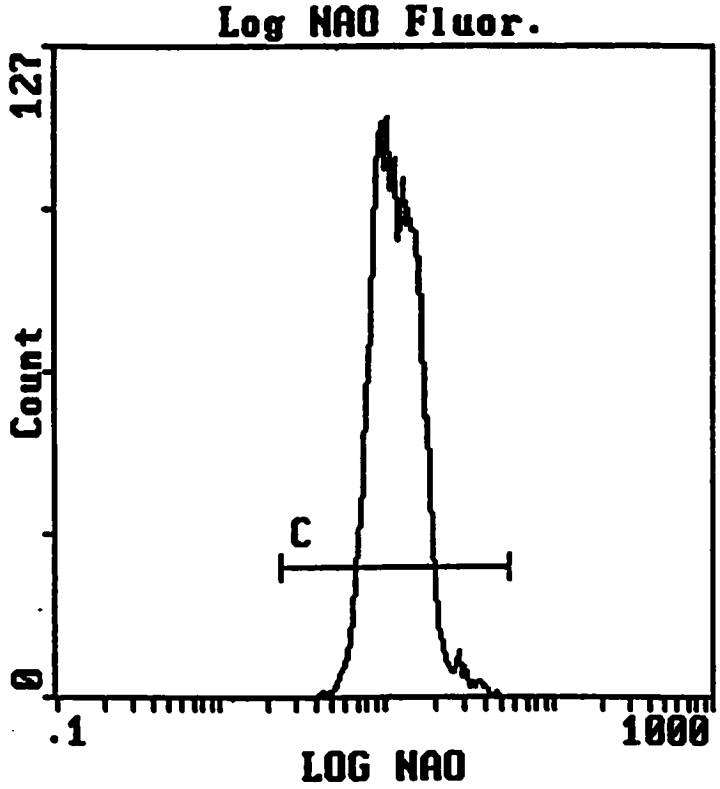


Figure 12: A representative NAO population fluorescence profile for 2008 cells, generated from a sample of 10 000 cells treated for 45 min with 1μM NAO.

Cellular Glycolytic Capacity

Total glycolytic capacity was measured by the ability of a cytosolic homogenate preparation to generate lactic acid under conditions of excess substrate. No significant differences in total glycolytic capacity were observed between 2008, C13* and RH4 cells (Table 6). It should be noted, however, that this assay does not account for differences in glycolytic efficiency *in situ,* as contributed to by the appropriate compartmentalization of glycolytic enzymes (Masters 1991).

Cellular Oxygen Consumption

A Clark-type oxygen electrode was used to measure the respiration potential of 2008, C13* and RH4 cells made permeable to an unlimited supply of respiratory substrates with digitonin. In the absence of added mitochondrial substrates, basal respiration rates were not substantial enough to be recorded by this apparatus. When supplied with excess ADP and succinate, the respiration rate of 2008, C13* and RH4 cells were stimulated immediately, presumably by the initiation of mitochondrial oxygen consumption, and so defined as State 3 respiration. Interestingly, the State 3 respiration rate of C13* and RH4 cells was approximately 50% of that recorded for an equal number of 2008 cells (Table 6). In the absence of any significant difference in mitochondrial mass, this suggests that C13* cells have acquired a respiratory defect related to the transfer of

electrons to oxygen, or oxygen consumption potential, concurrently with cisplatin resistance. The association of this defect to the mechanism of cisplatin resistance in C13* cells is questionable however, since this defect is maintained in the RH4 revertants despite their lowered mitochondrial membrane potential and returned cisplatin sensitivity. Overall, this implies that either the elevated mitochondrial membrane potential acts alone or in concert with this respiratory defect to mediate resistance.

Cellular Responses to Energetic Inhibitors

As a follow-up to the direct measurements for the energetic parameters of glycolytic capacity and mitochondrial respiration, the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) and the specific inhibitor of oxidative phosphorylation oligomycin were used to assess functional energetic differences between 2008, C13* and RH4 cells. The effect of these agents on 2008, C13* and RH4 cell growth are shown in Figures 13 and 14. There were no significant differences in the cytotoxic response to the glucose analogue 2-DG between 2008, C13* and RH4 cells, with respective IC_{50} values of 2.5 ± 0.4mM, 1.5 ± 0.4mM and 1.8 ± 0.3mM. This result corroborates the absence of measured differences in whole cell glycolytic capacity in these cell types.

In contrast, a continuous exposure to oligomycin revealed differences in the cell growth response of 2008, C13* and RH4 cells that supported the direct

mitochondrial respiration measurement data. Both C13* and RH4 cells were more severely affected by oligomycin exposure in terms of reduced cell growth than 2008 cells. The calculated IC_{50} values for 2008, C13* and RH4 cells were 2.2 ± 0.4 ng/ml, 0.63 ± 0.25 ng/ml and 0.89 ± 0.21 ng/ml respectively, and were significantly different overall (p=0.027). Thus both cell types that have a respiration defect were more sensitive to growth inhibition by an agent that shuts down mitochondrial production of ATP, suggesting they may be more dependent on ATP from this source, or have reduced reserve capacity for ATP generation versus 2008 cells.

Overall, the comparative data between the energetic characteristics of 2008 and RH4 cisplatin-sensitive and C13* cisplatin-resistant cells has been useful to direct the investigation as to what mitochondrial activities are most influential to cisplatin resistance. The presence of similar defects of mitochondrial respiration in the C13* and RH4 revertant line despite differences in mitochondrial membrane potential suggests that diminished mitochondrial ATP production may not be the influential factor for resistance. Instead it seems that the trait of an elevated mitochondrial membrane potential alone or in combination with the respiration defect is the influential factor in terms of resistance, with an as yet unknown impact on alternate mitochondrial or cellular functions.

Figure 13: Cytotoxic Effects of 2-Deoxy-D-Glucose in 2008, C13* and RH4 Cells

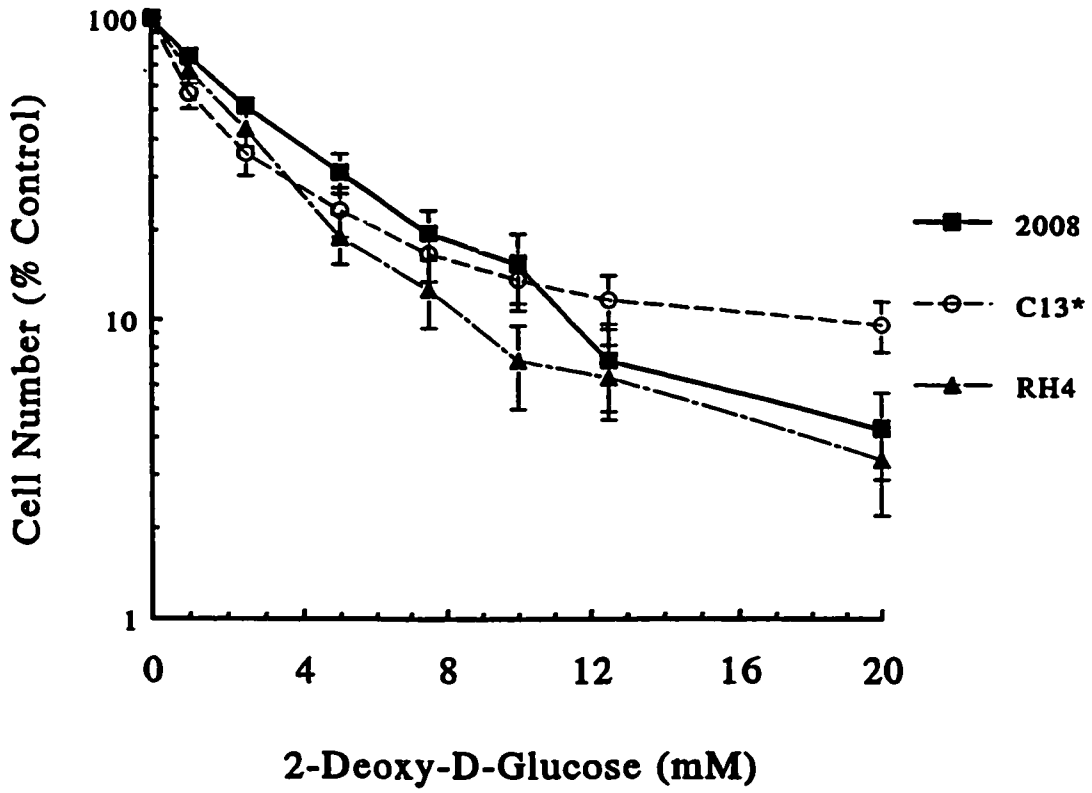


Figure 13: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

Figure 14: Cytotoxic Effect of Oligomycin in 2008, C13* and RH4 Cells

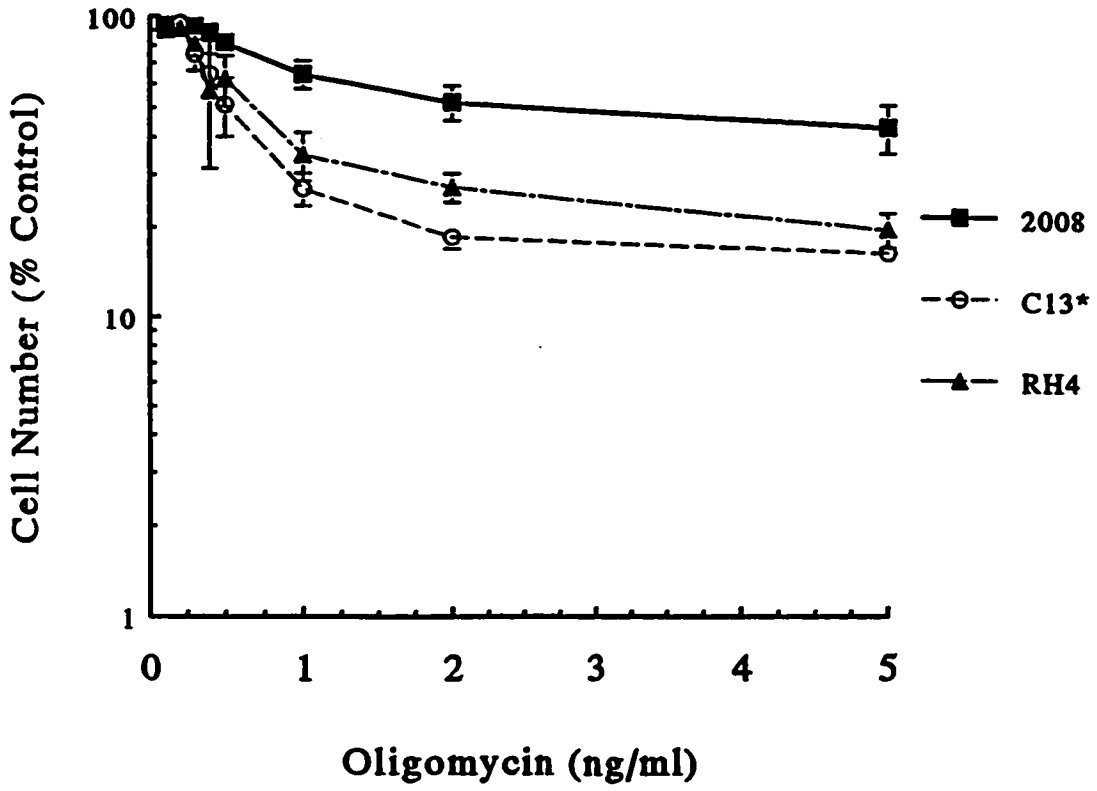


Figure 14: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

3.3 CHARACTERISTICS OF REDOX BALANCE

Total Glutathione Content in 2008 and C13 Cells*

The investigating group led by P.A. Andrews has reported elevated total GSH content in several sublines derived from the 2008 population with various levels of acquired cisplatin resistance, including the C13* cells (Table 1). An assay for total GSH was used to confirm this difference in the 2008/C13* pair cultured under our laboratory conditions (Student's t-test, $p<0.02$) (Table 7).

Table 7: Total GSH Content in 2008 and C13* Cells

Cell Type	Total GSH Content (nmole/10^6 cells)
2008	28.9 ± 2.4
C13*	53.3 ± 4.5

Sensitivity of C13 Cells to the Cytotoxic Effects of Glutathione Depletion*

The nearly two-fold increase in total GSH of C13* versus 2008 cells indicates a shift in the cellular redox balance. In terms of cisplatin resistance, enhanced rates of detoxification by GSH-consuming reactions was always considered to be the most relevant mechanism. Zinkewich-Péotti and Andrews

(1992) reported that elevated GSH was also present in the cisplatin-sensitive RH4 revertant, thus minimizing the hypothesis that GSH has a direct role in resistance via enhanced detoxification, at least in this model system. To examine the significance of elevated GSH to cell survival in general, 2008, C13* and RH4 cells were incubated with the agent buthionine sulfoximine (BSO), a specific inhibitor of γ-GCS, the rate-limiting enzyme for GSH synthesis (Griffith 1982). Figure 15 shows that C13* cells responded uniquely to BSO in terms of cell growth inhibition with an IC_{50} of 260μM BSO, while 2008 and RH4 cells were relatively unaffected. This suggests that elevated GSH plays a unique role in cisplatin-resistant C13* cells compared to RH4 cells, and that redox balance overall may be an influential factor in cisplatin resistance.

Figure 15: Cytotoxic Response of 2008, C13* and RH4 Cells to BSO

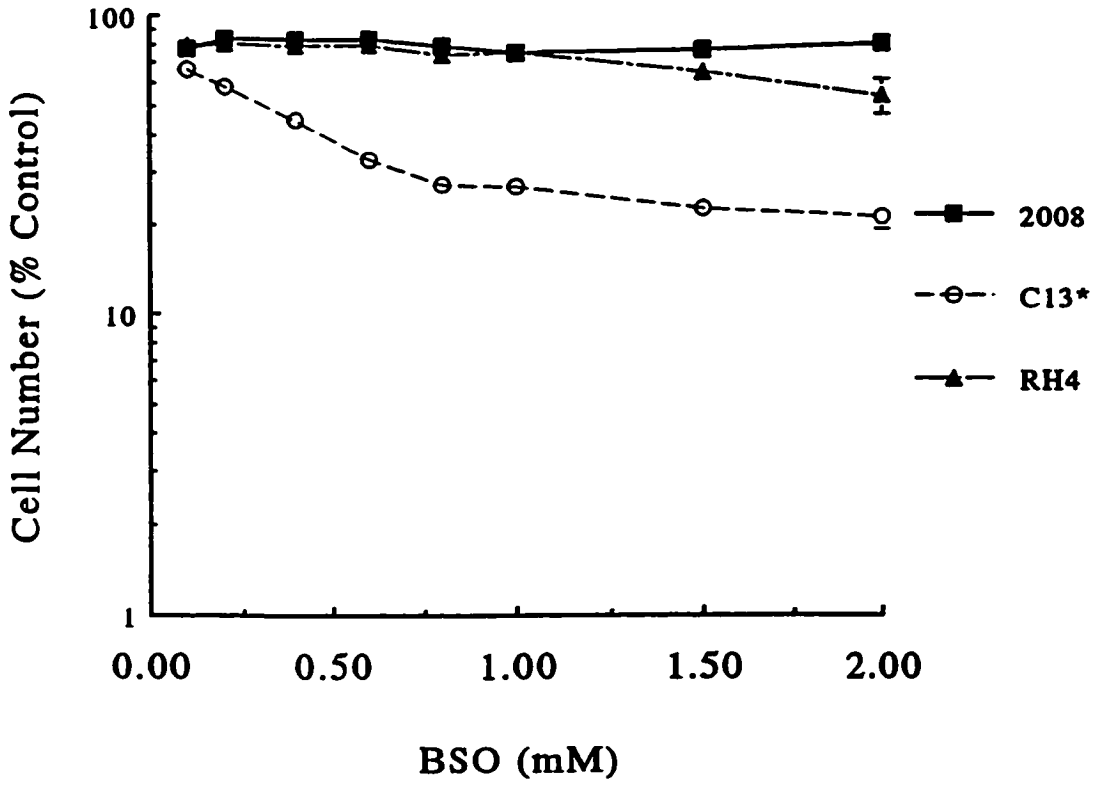


Figure 15: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

C13 Resistance to Oxidative Stress*

In order to assess the relative phenotype of C13* and 2008 cells in terms their capacity to resist oxidative stress, the enzyme glucose oxidase was utilized as a continuous H_2O_2-generating source. Incubations of this enzyme with its glucose substrate at a concentration equivalent to that present in the base RPMI medium used for 2008 and C13* cells was sufficient to stimulate the production of H_2O_2 as shown by the scopoletin fluorescence assay (Figure 16). Thus the addition of a filter-sterilized solution of the enzyme at similar concentrations to the growth medium of 2008 and C13* cells supplies an exogenous oxidative stress in the form of H_2O_2. Based on the cell adherence assay, C13* cells displayed significant resistance (2.4 fold) to exogenously applied oxidative stress (Figure 17).

Figure 16: Glucose Oxidase Generates H_2O_2 from Glucose

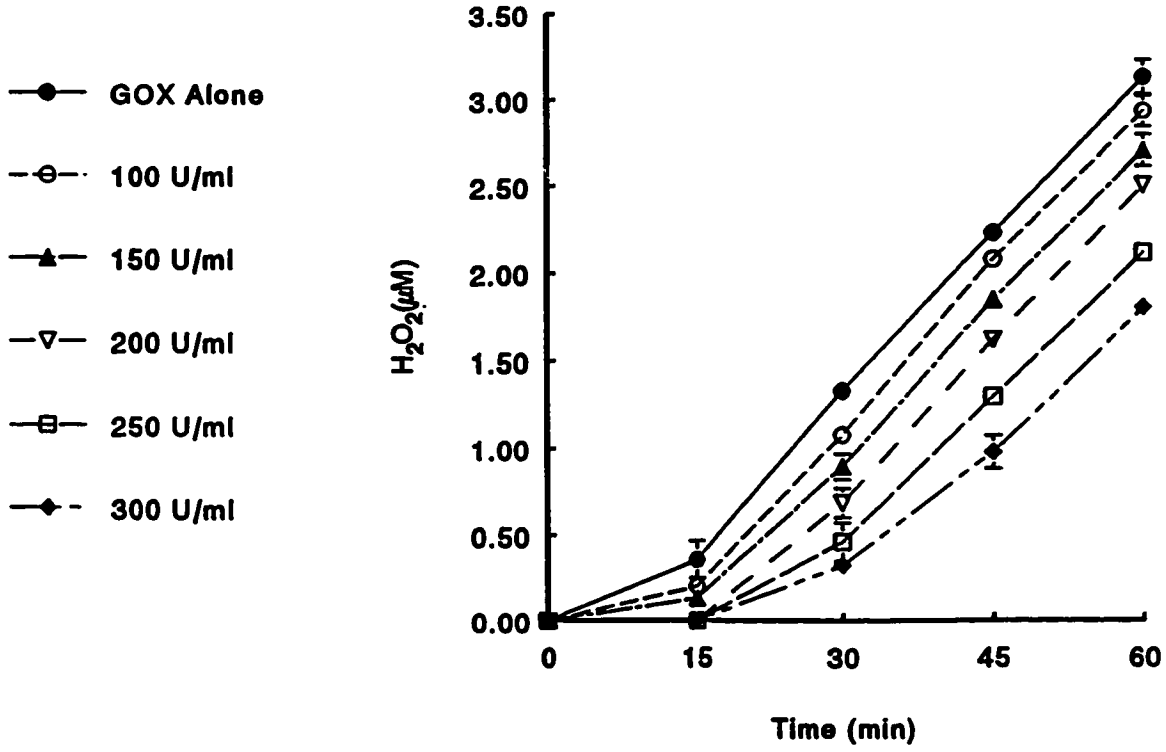


Figure 16: H_2O_2 production by $1.0x10^3$ U/ml glucose oxidase (GOX) supplied with 1g/litre of glucose as substrate and competition by increasing doses of catalase (100-300 U/ml). Data present mean ± SEM of n=3 independent experiments.

Figure 17: Cytotoxic Effect of Exogenous H_2O_2 Addition to 2008 and C13* Cells

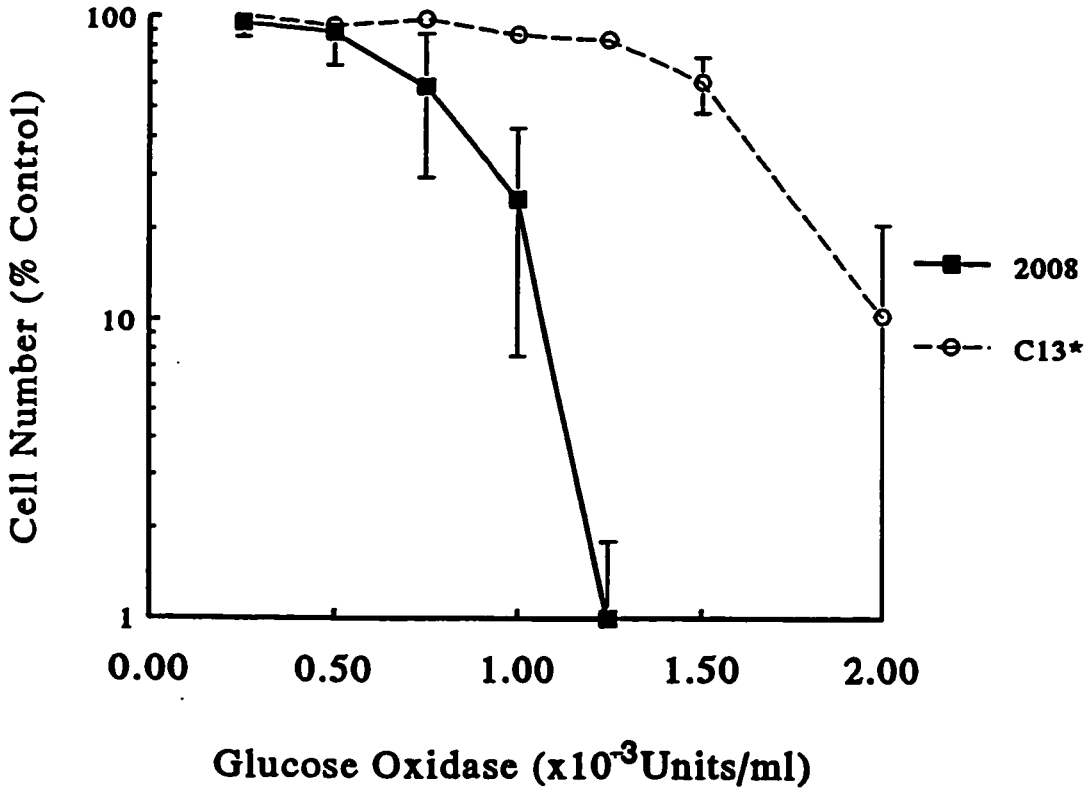


Figure 17: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

Constitutive H_2O_2 Production Profile of 2008 and C13 Cells*

In this investigation the sensitive fluorescent indicator A6550 was used to measure the net, constitutive production of extracellular H_2O_2 by 2008 and C13* cells. The H_2O_2 production phenotypes of these cells were compared to two other carcinoma cell lines - the HT29 colon carcinoma, and the SKOV-3 ovarian carcinoma - which have previously been reported to generate extracellular H_2O_2 by a similar assay protocol but with the indicator scopoletin (Szatrowksi and Nathan 1991). The net accumulated concentration of H_2O_2 (nM) was measured for $5.0x10^5$ adherent cells over a 3h assay period, as shown for 2008, C13*, HT29 and SKOV-3 cells in Figures 18 to 21, respectively. Confirmation that the fluorescent conversion of A6550 was H_2O_2-dependent is shown for each cell line by the capacity of increasing concentrations of catalase in the reaction medium to completely eliminate fluorescence (Figures 18-21). A summary of the H_2O_2 production phenotypes of these four lines according to the H_2O_2 concentration measured following 3h of accumulation is presented in Figure 22. We observed that in the SKOV-3 cell line net H_2O_2 production was significantly greater (5-10 fold) than in the other cell types. Net H_2O_2 production by C13* and HT29 cells was not significantly different but both C13* and HT29 cells produced significantly more (2 fold) H_2O_2 than 2008 cells.

Figure 18: Net Extracellular H_2O_2 Production by 2008 Cells

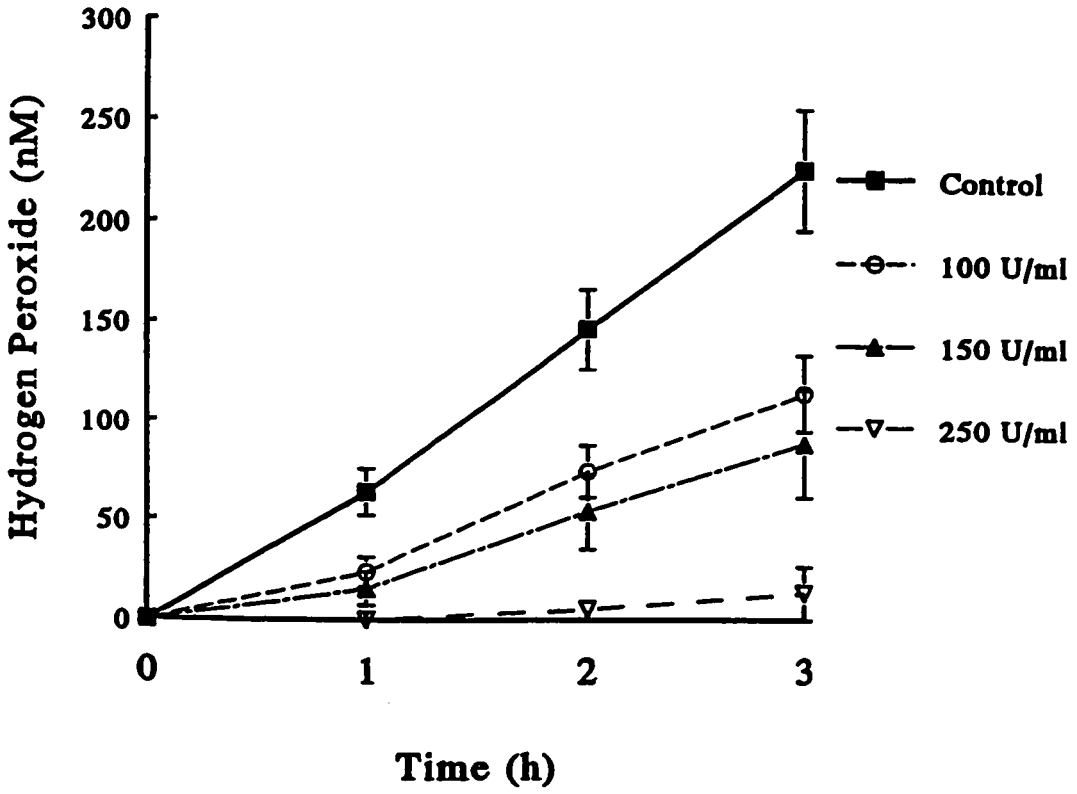


Figure 18: H_2O_2 generation by $5.0x10^4$ adherent 2008 cells as measured with the fluorescent indicator A6550. Increasing concentrations of catalase in the assay buffer eliminates fluorescent conversion of A6550 confirming the H_2O_2 specificity of the assay. Data represent mean ± SEM of n=3 independent experiments.

Figure 19: Net Extracellular H_2O_2 Production by C13* Cells

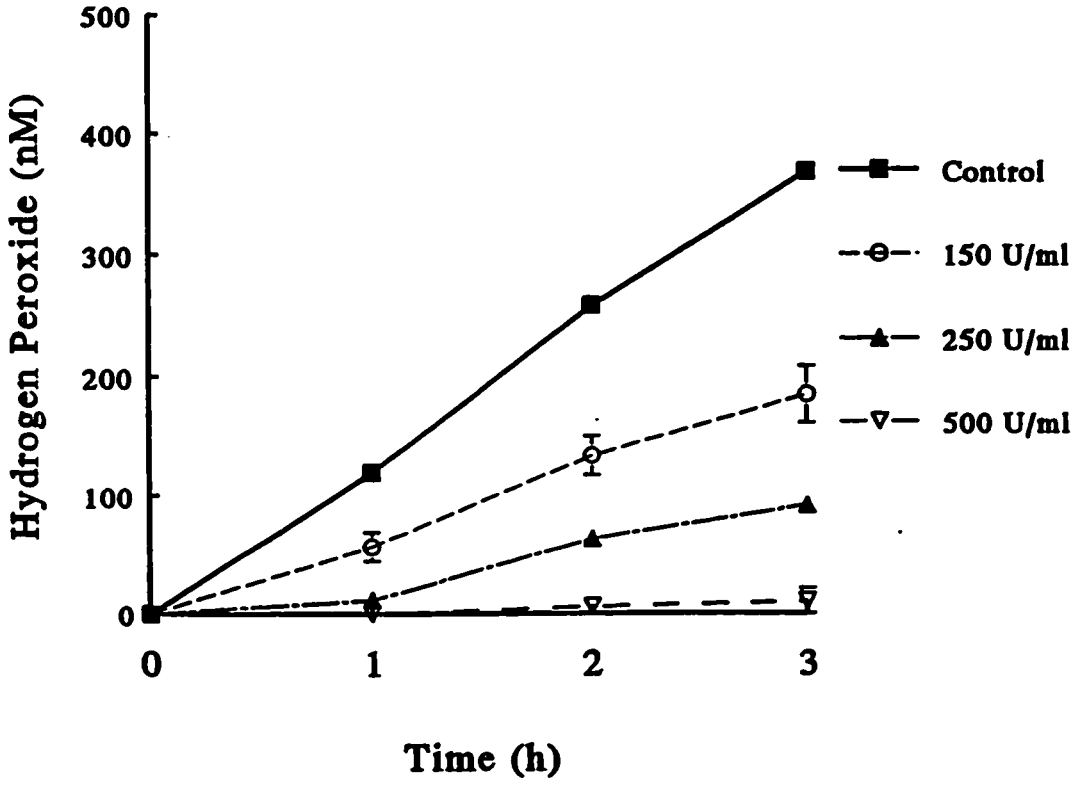


Figure 19: H_2O_2 generation by $5.0x10^4$ adherent C13* cells as measured with the fluorescent indicator A6550. Increasing concentrations of catalase in the assay buffer eliminates fluorescent conversion of A6550 confirming the H_2O_2 specificity of the assay. Data represent mean ± SEM of n=3 independent experiments.

Figure 20: Net Extracellular H_2O_2 Production by HT29 Cells

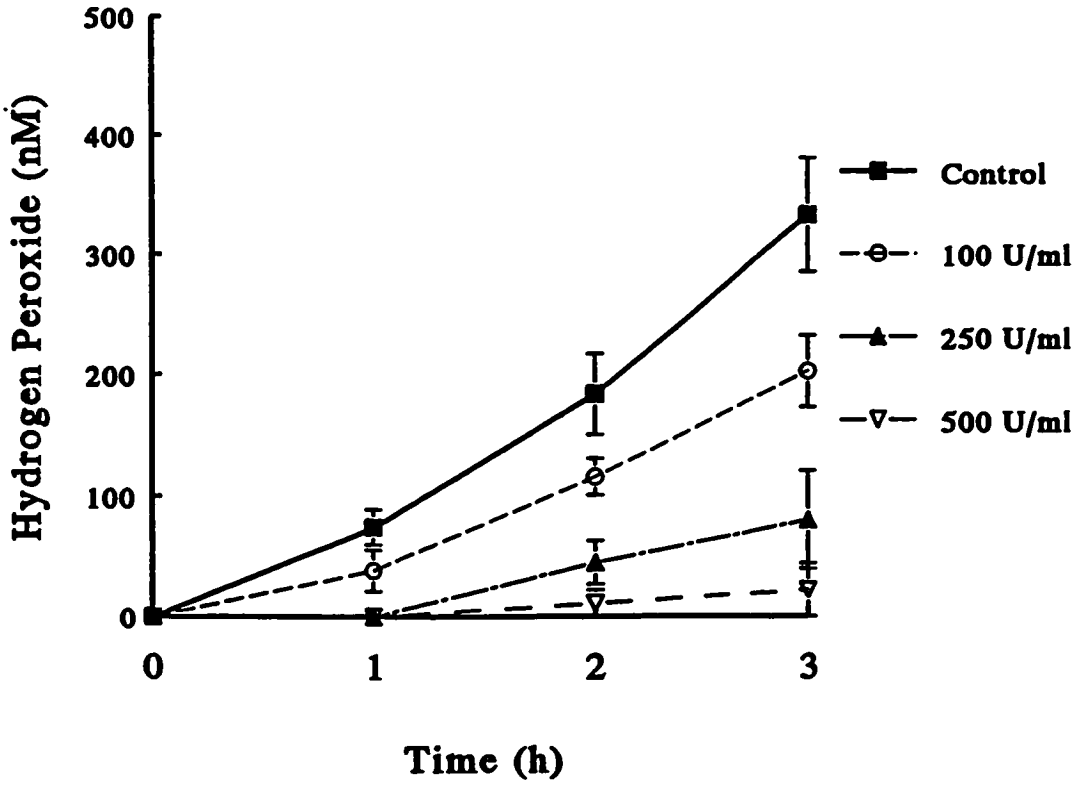


Figure 20: H_2O_2 generation by $5.0x10^4$ adherent HT29 cells as measured with the fluorescent indicator A6550. Increasing concentrations of catalase in the assay buffer eliminates fluorescent conversion of A6550 confirming the H_2O_2 specificity of the assay. Data represent mean ± SEM of n=3 independent experiments.

Figure 21: Net Extracellular H_2O_2 Production by SKOV-3 Cells

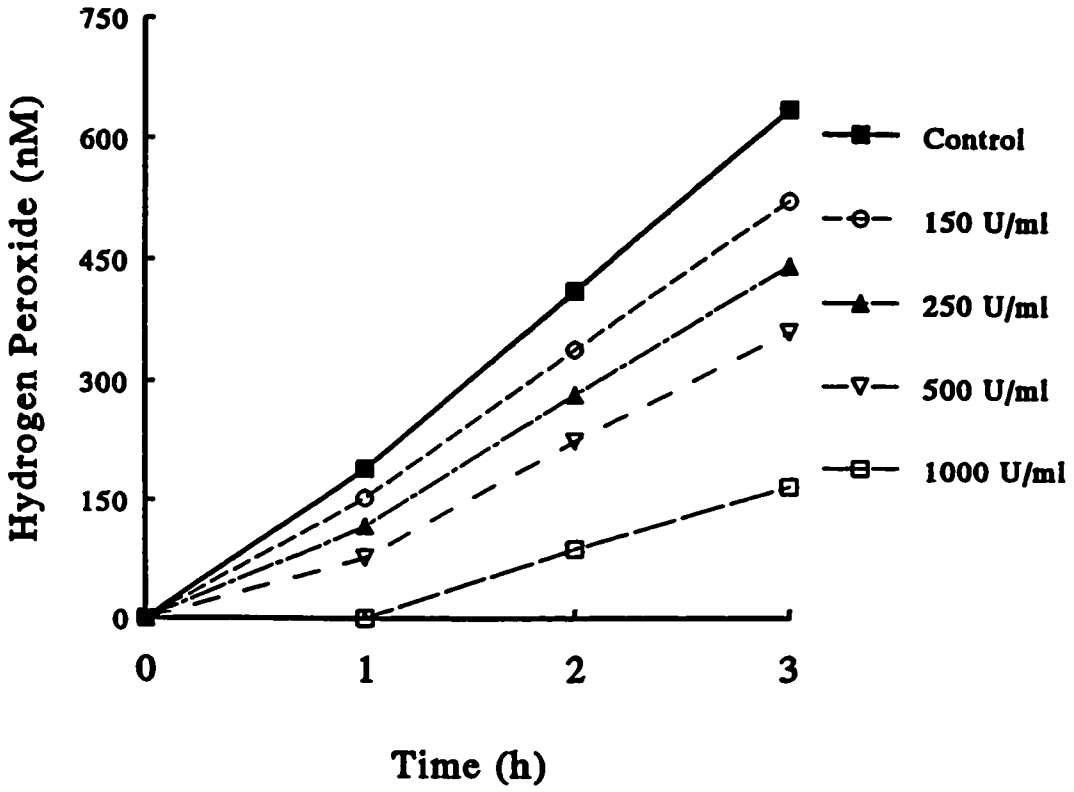


Figure 21: H_2O_2 generation by $2.5x10^4$ adherent SKOV-3 cells as measured with the fluorescent indicator A6550. Increasing concentrations of catalase in the assay buffer eliminates fluorescent conversion of A6550 confirming the H_2O_2 specificity of the assay. Data represent mean of n=2 independent experiments.

Figure 22: H_2O_2 Production Profiles of 2008, C13*, HT29 and SKOV-3 Cells

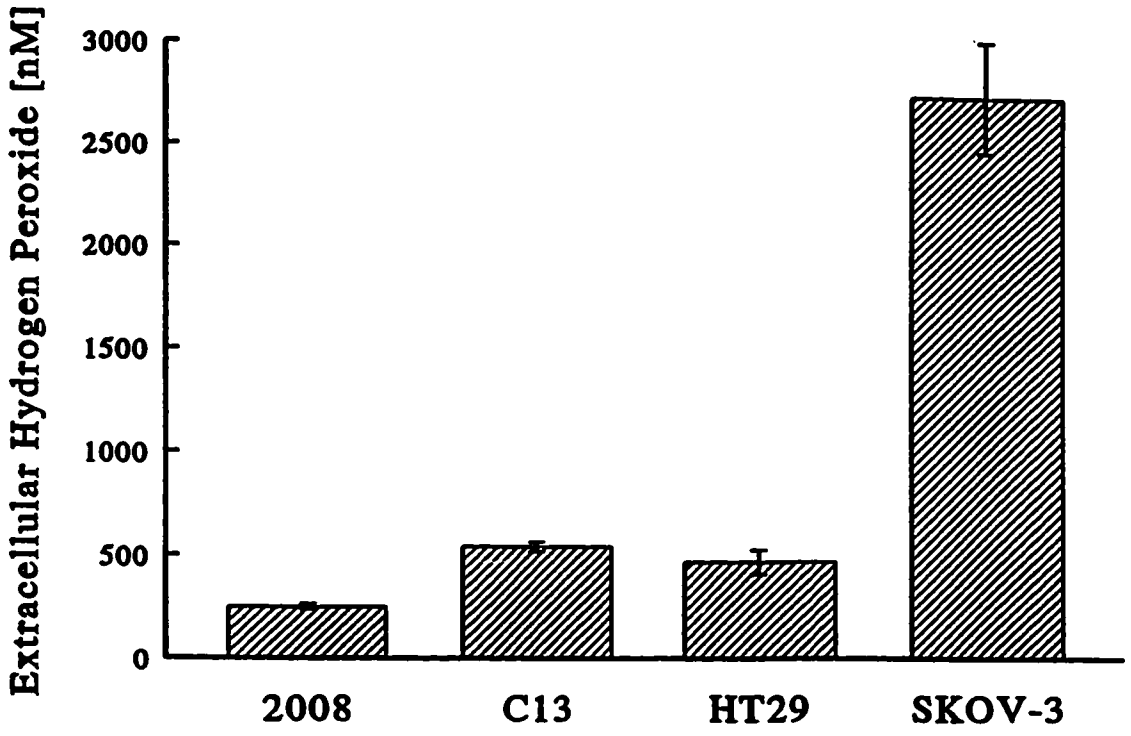


Figure 22: H_2O_2 concentrations generated by $5.0x10^4$ adherent 2008, C13*, HT29 and SKOV-3 cells following a 3h incubation. Data represent mean ± SEM of n>20 experiments for 2008 and C13* cells and n=6 experiments for HT29 and SKOV-3 cells.

Summary of Pharmacological Survey in 2008 and C13 Cells*

Agents that selectively inhibit potential cellular H_2O_2-generating sources were pre-incubated with 2008 and C13* cells to determine their contribution to extracellular H_2O_2 production. Table 8 summarizes the data obtained with agents that target the mitochondrion. The inhibitors of electron transport rotenone and antimycin A, which target complex I and complex III respectively, were very effective at reducing the extracellular H_2O_2 concentration to 56% (rotenone, 50 μM) and 69-75% (Antimycin A, 25μM) of DMSO solvent-treated controls in both 2008 and C13* cells. This indicates that mitochondrial electron transport activity is a major contributor to H_2O_2 concentration in the extracellular compartment.

The inhibitory effect of rotenone at a dose of 5μM was equivalent to that of either 10 μM or 50μM in both 2008 and C13* cells suggesting that maximum inhibition was already achieved at 5μM. The extent of inhibition at 5μM rotenone can then be used to estimate the overall mitochondrial contribution to the extracellular H_2O_2 concentration. The observed 40% reduction in H_2O_2 corresponds to nearly 100nM of the accumulated H_2O_2 for 2008 cells assayed over a 3h period, but equals over 200nM for C13* cells. In the absence of any significant difference in mitochondrial mass, this suggests that C13* mitochondrial contributions to extracellular H_2O_2 are twice that for 2008 cells.

In contrast to the effect of the electron transport inhibitors, the agent oligomycin that inhibits ATP production by the F_oF_1 ATPase (complex V) of mitochondria, had the unique effect of increasing extracellular H_2O_2 production in 2008 cells to >130% of that of DMSO solvent-treated controls (Figure 22). No significant effect was observed in C13* cells even at the highest dose of 5μM oligomycin, representing a novel difference in bioenergetic responses between 2008 and C13* cells (Table 8 and Figure 23). This response distinction was maintained even when oligomycin incubations were reduced to 0.5 or 1h prior to H_2O_2 assay, indicating that any stimulation of H_2O_2 production by C13* cells was not missed during the 2h incubation (data not shown).

Table 8: Impact of Mitochondrial Inhibitors on 2008 and C13* Extracellular H_2O_2 Production

Extracellular H_2O_2 (nM)	Rotenone (μM)			Antimycin A (μM)			Oligomycin (μM)		
	5	10	50	5	10	25	0.5	1.0	5.0
2008 248.9 ± 13.2 (100%)	141.3 ± 12.8 (57%) ✥	154.7 ± 18.6 (62%) ✥	140.1 ± 20.2 (56%) ✥	166.5 ± 9.2 (67%) ✥	169.8 ± 6.5 (68%) ✥	152.2 ± 18.3 (61%) ✥	334.9 ± 21.0 (134%)	341.5 ± 23.4 (137%)	328.7 ± 27.2 (132%)
C13* 542.6 ± 21.3 (100%)	323.8 ± 49.2 (60%) ✥	330.6 ± 41.4 (61%) ✥	310.3 ± 25.6 (57%) ✥	404.7 ± 43.6 (74%) ✥	341.6 ± 34.5 (63%) ✥	360.3 ± 42.0 (66%) ✥	497.6 ± 32.0 (92%)	604.5 ± 48.9 (111%)	530.6 ± 22.8 (98%)

Table 8: The results for native 2008 and C13 cell H_2O_2 production reflect the sum of n>20 experiments, while the absolute values reported for each dose of each agent represent the mean of 3-6 independent determinations. (✥) indicates a significant ($p<0.05$) difference in extracellular H_2O_2 production from untreated cells or solvent treated controls.

Figure 23: Oligomycin Stimulates H_2O_2 Production in 2008 cells

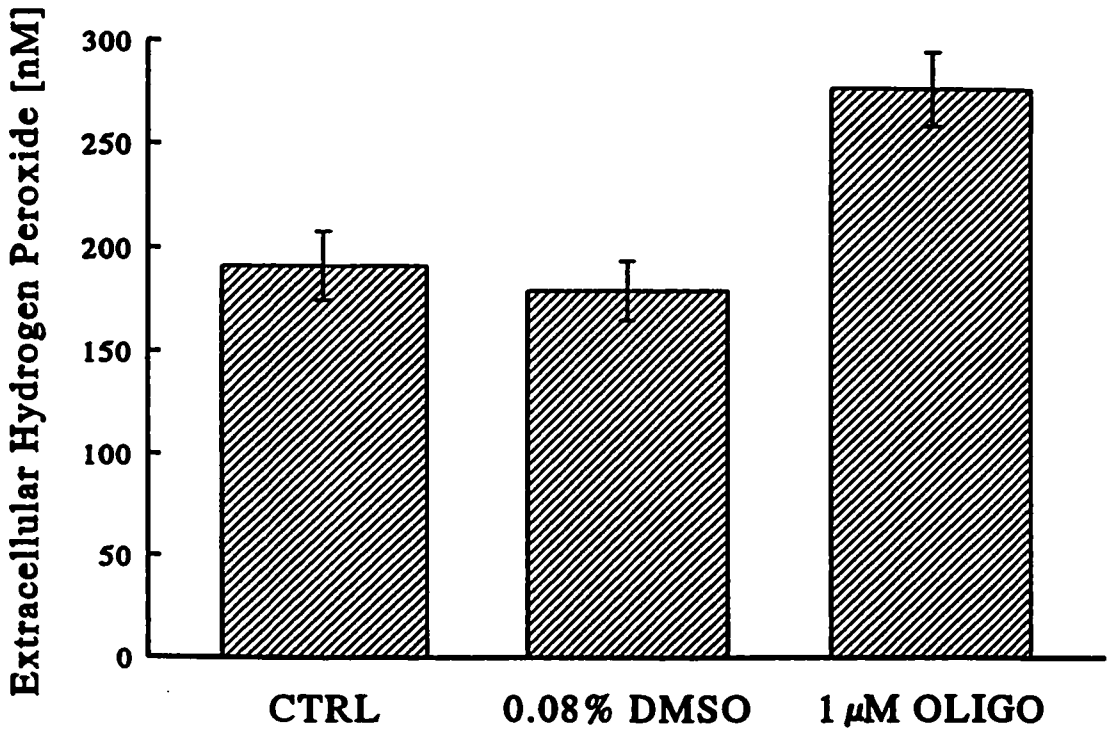


Figure 23: A dose of 1μM oligomycin significantly stimulates extracellular H_2O_2 production in 2008 cells compared to either untreated controls or the equivalent DMSO solvent concentration of 0.08% (p=0.008). Data represent mean ± SEM of n=5 independent determinations.

The influence of the enzyme-selective inhibitors allopurinol, pargyline and capsaicin on H_2O_2 production in 2008 and C13* cells is summarized in Table 9. The xanthine oxidase inhibitor allopurinol incubated at concentrations up to 100μM for a 2h duration had no significant effect on extracellular H_2O_2 concentrations in either cell type; thus indicating that xanthine oxidase generated $O_2^{\cdot}$ is not a contributor to the extracellular H_2O_2 pool. Similarly, the agent pargyline that selectively inhibits monoamine oxidase B, had no significant influence on the amount of extracellular H_2O_2 produced by 2008 or C13* cells. Capsaicin is reported to have inhibitory activity against a plasma membrane-associated NADH oxidase activity found in several tumour cell lines and the serum of cancer patients (Morré, Chueh and Morré 1995; Morré *et al.* 1997). Our investigations showed no significant influence of capsaicin on extracellular H_2O_2 concentrations generated by 2008 or C13* cells with a 2h incubation of 10, 50 or 100 μM.

Table 9: Impact of Enzyme-Specific Inhibitors on 2008 and C13* Extracellular H_2O_2 Production

Extracellular H_2O_2 (nM)	Allopurinol (μM)			Pargyline (μM)			Capsaicin (μM)		
	25	50	100	50	100	200	10	50	100
2008 248.9 ± 13.2 (100%)	209.9 ± 38.0 (84%)	214.0 ± 38.0 (86%)	216.5 ± 41 (87%)	2097 ± 47.0 (84%)	213.8 ± 47.0 (86%)	210.8 ± 54.0 (85%)	222.4 ± 21.0 (90%)	209.6 ± 33.4 (84%)	222.2 ± 25.0 (89%)
C13* 542.6 ± 21.3 (100%)	518.0 ± 92.0 (96%)	472.0 ± 69.0 (87%)	468.0 ± 75.0 (86%)	473.6 ± 37.0 (87.%)	499.4 ± 35.0 (92%)	465.5 ± 24.0 (86%)	542.0 ± 74.0 (100%)	531.6 ± 43.0 (98%)	505.0 ± 50.0 (93%)

Table 9: The results for native 2008 and C13* cell H_2O_2 production reflect the sum of n>20 experiments, while the absolute values reported for each dose of each agent represent the mean of 3-6 independent determinations.

A well-characterized source of extracellular ROS production is the NADPH oxidase assembly of neutrophils and macrophages. Recent discoveries of extracellular $O_2^{\cdot}$ and H_2O_2 generation in several non-phagocytic cell types has led to the biochemical and pharmacological characterization of novel NADH or NADPH-selective oxidase activities (Arbault *et al.*, 1997; Brightman *et al.*, 1992; Gorin *et al.* 1997; Ushio-Fukai *et al.* 1996; Zulueta *et al.* 1995). To investigate whether similar membrane-assemblies contribute to the extracellular H_2O_2 pool of 2008 and C13* cells, we have used two agents that are known to inhibit $O_2^{\cdot}$ and H_2O_2 production in neutrophils - diphenylene iodonium (DPI) and phenylarsine oxide (PAO) – in our model system (Table 10). In both 2008 and C13* cells, an incubation of 10μM DPI was very effective at reducing extracellular H_2O_2 production to 53% and 68% of solvent-treated controls, respectively. As a general flavoprotein inhibitor, the mutual response to DPI suggests a role for flavoprotein activity in H_2O_2 generation in both 2008 and C13* cells, although the lack of target specificity of DPI does not permit conclusions as to the exact source. Interestingly, C13* cells showed enhanced sensitivity to DPI-mediated suppression with a 1.0μM incubation reducing production to 69% of controls, while in 2008 cells, 1.0μM had no significant effect. This difference suggests that H_2O_2 generated via flavoproteins in C13* cells is distinct either in terms of the sensitivity to inhibition by a source that is common to both 2008 and

C13* cells, or represents a novel flavoprotein-dependent activity present only in C13* cells.

Unlike DPI, the trivalent arsenical compound PAO at 0.1 and 0.5μM had no significant effect on either 2008 or C13* H_2O_2 production, although it should be noted that the dose range was strictly limited by the ability of higher concentrations of PAO to induce a loss of cell adherence.

The agent chloroquine is best known for its anti-malarial properties. The medicinal action may be a function of chloroquine's inhibitory effect on electron transfer at the plasma membrane of this parasite and other eukaryotic cells (Toole-Simms, Sun and Morré 1990). In 2008 and C13* cells, incubations of chloroquine at concentrations up to 100μM had no significant impact on extracellular H_2O_2 production (Table 10).

Table 10. Impact of Inhibitors of Plasma Membrane Electron Transfer Reactions on 2008 and C13* Extracellular H_2O_2 Production.

Extracellular H_2O_2 (nM)	Diphenylene Iodonium (DPI) (μM)			Phenylarsine Oxide (PAO) (μM)		Chloroquine (μM)		
	1	5	10	0.1	0.5	25	50	100
2008 248.9 ± 13.2 (100%)	202.1 ± 21.4 (81%)	166.0 ± 26.4 (66.8)	132.2 ± 20.1 (53%) ✦	211.7 ± 44.0 (85%)	236.2 ± 37.0 (95%)	241.3 ± 19.0 (97%)	208.9 ± 21.0 (84%)	211.1 ± 27.0 (85%)
C13* 542.6 ± 21.3 (100%)	376 ± 27.6 (69%) ✦	343.6 ± 21.3 (63%) ✦	369.5 ± 78 (68%) ✦	412.0 ± 67.0 (76%)	464.0 ± 83.0 (86%)	508.0 ± 27.0 (94%)	477.3 ± 30.0 (88%)	535.0 ± 36.0 (98%)

Table 10: The results for native 2008 and C13* cell H_2O_2 production reflect the sum of n>20 experiments, while the absolute values reported for each dose of each agent represent the mean of 3-6 independent determinations. (✦) indicates a significant difference in H_2O_2 production from untreated cells or solvent-treated controls.

Impact of Selected Inhibitors on HT29 and SKOV-3 Extracellular H_2O_2 Production

As epithelium-derived cancer cell lines, both HT29 and SKOV-3 carcinoma cells have been used as a comparison group for 2008 and C13* cells for the effects of oligomycin, rotenone, capsaicin, chloroquine, DPI and PAO on H_2O_2 production. The complete dose-response information is presented in Tables 11 and 12, but the most pertinent data for comparison with 2008 and C13* cells has been summarized in Figure 24 as % control response for only the highest comparable dose of each drug. Similar to 2008 and C13* cells, rotenone (50μM) reduced extracellular H_2O_2 production in HT29 and SKOV-3 cells to approximately 50% of solvent-treated controls. The inhibitory activity of DPI (10μM) was also reproduced in both HT29 and SKOV-3 cells, suggesting the involvement of a flavoprotein in H_2O_2 generation. The stimulatory effect of oligomycin (5μM) in 2008 cells remained unique within the cell panel since oligomycin had no effect on extracellular H_2O_2 concentrations produced by HT29 cells, and had the opposite effect in SKOV-3 cells, inducing a decrease to approximately 65% of controls. Like 2008 and C13* cells, HT29 and SKOV-3 responses to capsaicin (100μM) chloroquine (100μM) and PAO (0.5μM) were not significant.

Table 11: Impact of Rotenone, Oligomycin and Capsaicin on HT29 and SKOV-3 Extracellular H_2O_2 Production

Cell Type & Extracellular H_2O_2 Production (nM)	Rotenone (μM)			Oligomycin (μM)			Capsaicin (μM)		
	5	10	50	0.5	1.0	5.0	10	50	100
HT29 466.8 ± 58.3 (100%)	312.8 ± 66.9 (67%)	329.6 ± 81.6 (70%)	267.7 ± 40.2 (57%) ✥	366.1 ± 50.2 (78%)	408.4 ± 70.4 (87%)	398.2 ± 31.1 (85%)	493.1 ± 77.7 (105%)	472.9 ± 96.6 (101%)	394.6 ± 39.1 (84%)
SKOV-3 2724.0 ± 268.0 (100%)	1945.0 ± 430.0 (71%)	1809.0 ± 296.0 (66%)	1552.0 ± 433.0 (57%)	1827.9 ± 52.6 (67%) ✥	1875.4 ± 92.0 (69%) ✥	1608.0 ± 122.0 (59%) ✥	2700.0 ± 405.0 (99%)	2324.0 ± 103.0 (85%)	2185.0 ± 120.0 (80%)

Table 11: The results for native HT29 and SKOV-3 cell H_2O_2 production reflect the sum of n=6 experiments, while the absolute values reported for each dose of each agent represent the mean of 3 independent determinations. (✥) indicates a significant difference in H_2O_2 production from untreated cells or solvent-treated controls.

Table 12: Impact of DPI, PAO and Chloroquine on HT29 and SKOV-3 Extracellular H_2O_2 Production

Cell Type & Extracellular H_2O_2 Production (nM)	Diphenylene Iodonium (DPI) (μM)		Phenylarsine Oxide (PAO) (μM)		Chloroquine (μM)		
	1	10	0.1	0.5	25	50	100
HT29 466.8 ± 58.3 (100%)	331.6 ± 58.7 (71%)	287.1 ± 31.3 (61.5%)	374.5 ± 60.1 (80%)	372.4 ± 73.8 (79%)	538.3 ± 90.5 (115%)	467.0 ± 103.0 (100%)	455.4 ± 21.0 (97%)
SKOV-3 2724.0 ± 268.0 (100%)	2009.0 ± 147.0 (74%)	1805.8 ± 88.4 (46%) ✥	2310.0 ± 134.0 (85%)	2430.2 ± 184.0 (89%)	2845.0 ± 343.0 (104%)	2731.0 ± 388.0 (100%)	2806.0 ± 385.0 (103%)

Table 12: The results for native HT29 and SKOV-3 cell H_2O_2 production reflect the sum of n=6 experiments, while the absolute values reported for each dose of each agent represent the mean of 3 independent determinations. (✥) indicates a significant difference in H_2O_2 production from untreated cells or solvent-treated controls.

Figure 24: H_2O_2 Production in 2008, C13*, HT29 and SKOV-3 Cells

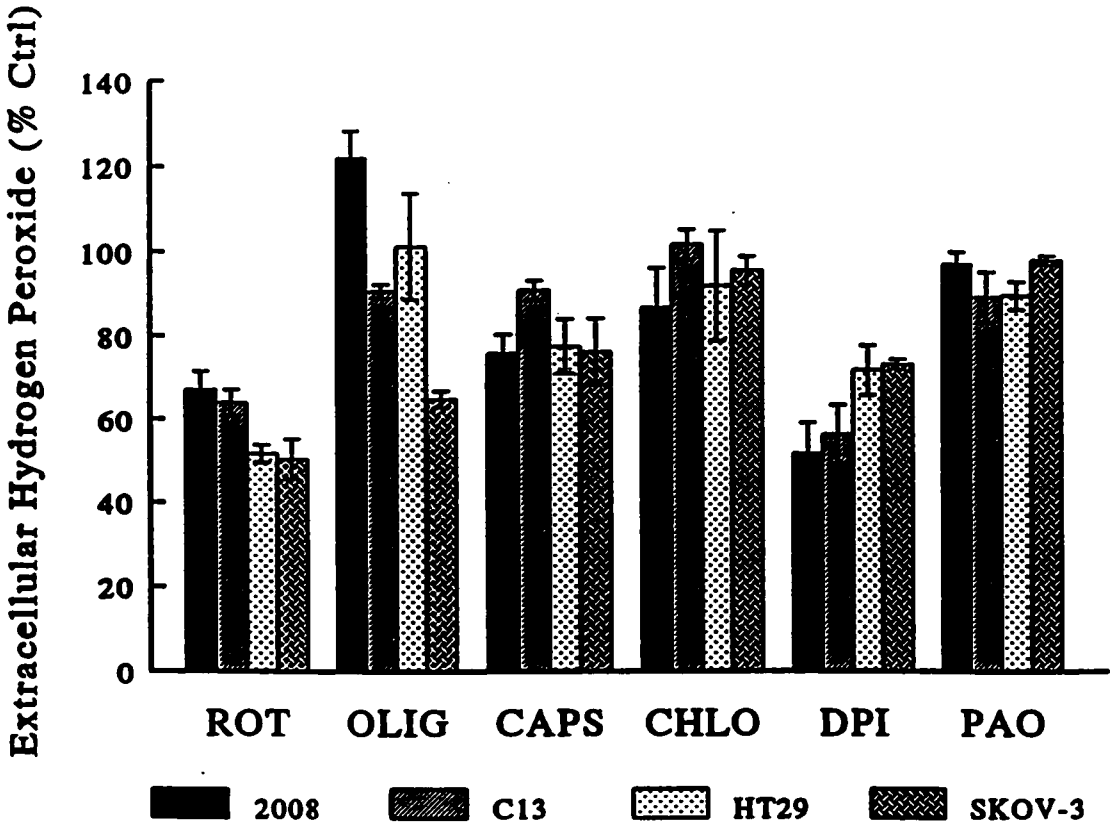


Figure 24: A comparison of the effects of rotenone (ROT, 50μM), oligomycin (OLIGO, 5μM), capsaicin (CAPS, 100μM), chloroquine (CHLO, 100μM), DPI (10μM) and PAO (0.5μM) on extracellular H_2O_2 generation by 2008, C13*, HT29 and SKOV-3 cells, expressed as a percentage of untreated cells. Data represent mean ± SEM of at least n=3 independent experiments.

Cytotoxic Response of 2008 and C13 Cells to Inhibitors of Plasma Membrane Oxidase Activities*

Some of the agents known to inhibit plasma membrane oxidase activities have been used to induce apoptosis in tumour cells (chloroquine), inhibit tumour cell proliferation (capsaicin) and induce selective toxicity in transformed cells (PAO) (Wolvetang *et al.* 1996; Morré *et al.* 1996; Arbault *et al.* 1997). These agents have not yet been tested for their cytotoxic potential in cisplatin-resistant tumour cells, so we performed cytotoxicity assays in 2008 and C13* cells with these agents. Figures 25 through 27 show the toxicity profiles for continuous exposures of these cells to chloroquine, capsaicin and PAO, respectively. No significant survival differences were revealed between the 2008 and C13* cells for any of these agents. These observations match the lack of differential influence of these agents on 2008 and C13* H_2O_2 production, although generally, the concentrations of drug required to induce cytotoxicity were generally lower than those tested in the H_2O_2 assay.

The enhanced sensitivity of C13* cells to the inhibitory effect of DPI on extracellular H_2O_2 generation was also explored further in a proliferation assay (Figure 28). C13* cells were also more sensitive to the cytotoxic effects of a continuous 96h exposure to DPI with an approximate 5-fold IC_{50} resistance ratio for 2008 (IC_{50} = 574nM) versus C13* (IC_{50} = 98nM) cells. This association requires further exploration to determine whether a reduction in H_2O_2 production from a DPI-sensitive source is important to the mechanism of cytotoxicty.

Figure 25: Cytotoxic Effect of Chloroquine in 2008 and C13* Cells

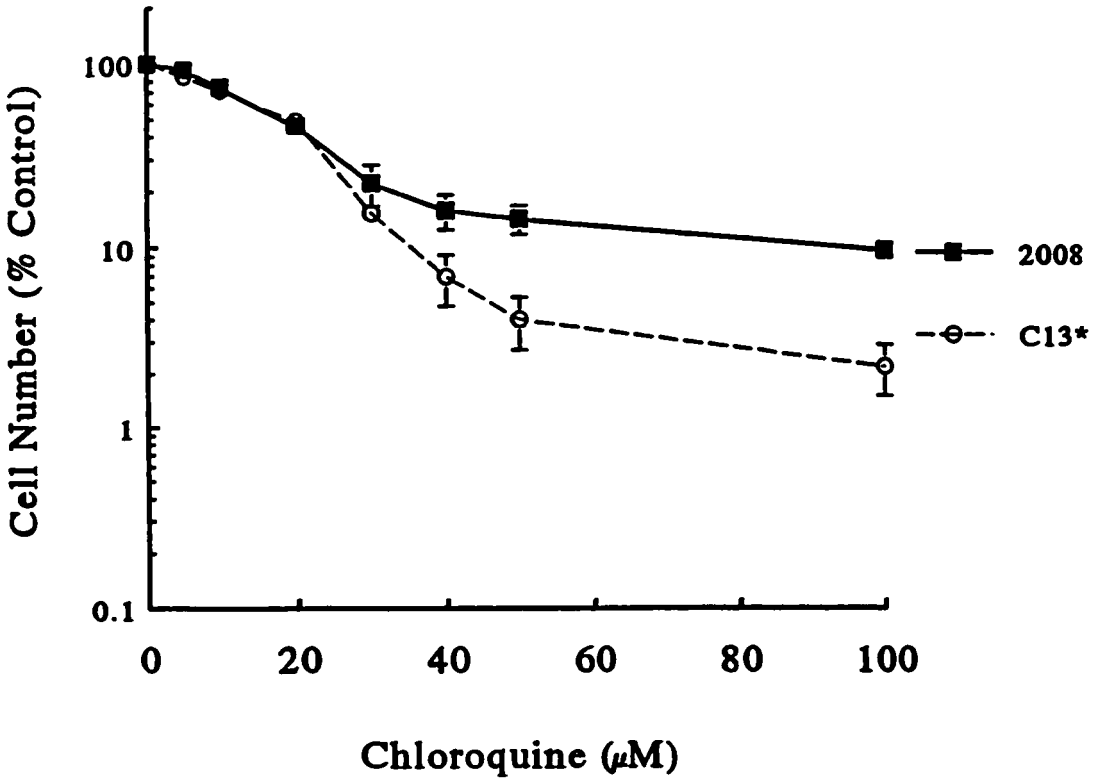


Figure 25: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

Figure 26: Cytotoxic Effect of Capsaicin on 2008 and C13* Cells

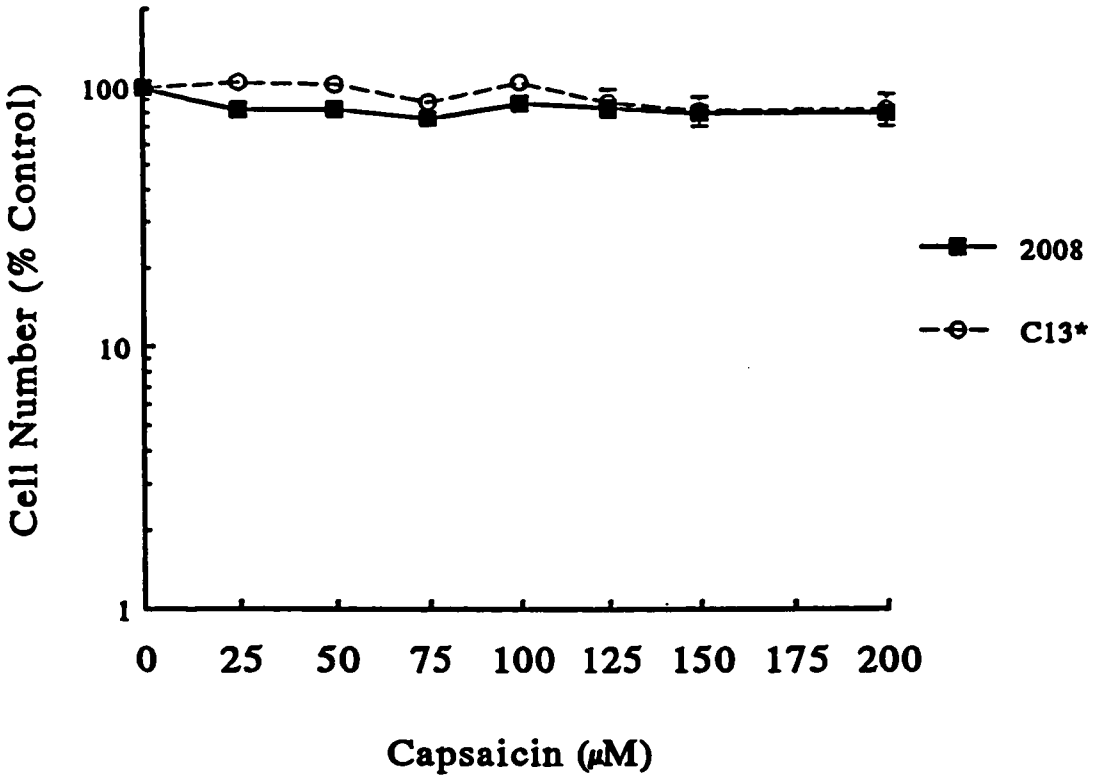


Figure 26: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

Figure 27: Cytotoxic Effect of Phenylarsine Oxide on 2008 and C13* Cells

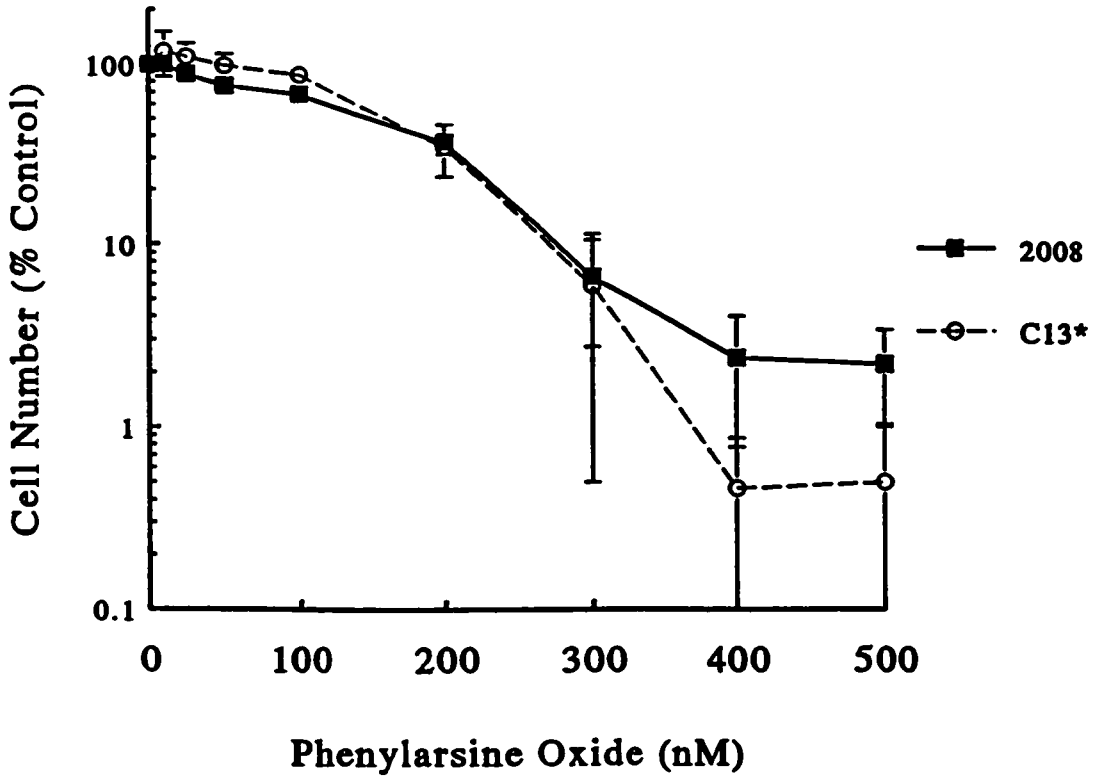


Figure 27: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

Figure 28: Cytotoxic Effect of Diphenylene iodonium on 2008 and C13* Cells

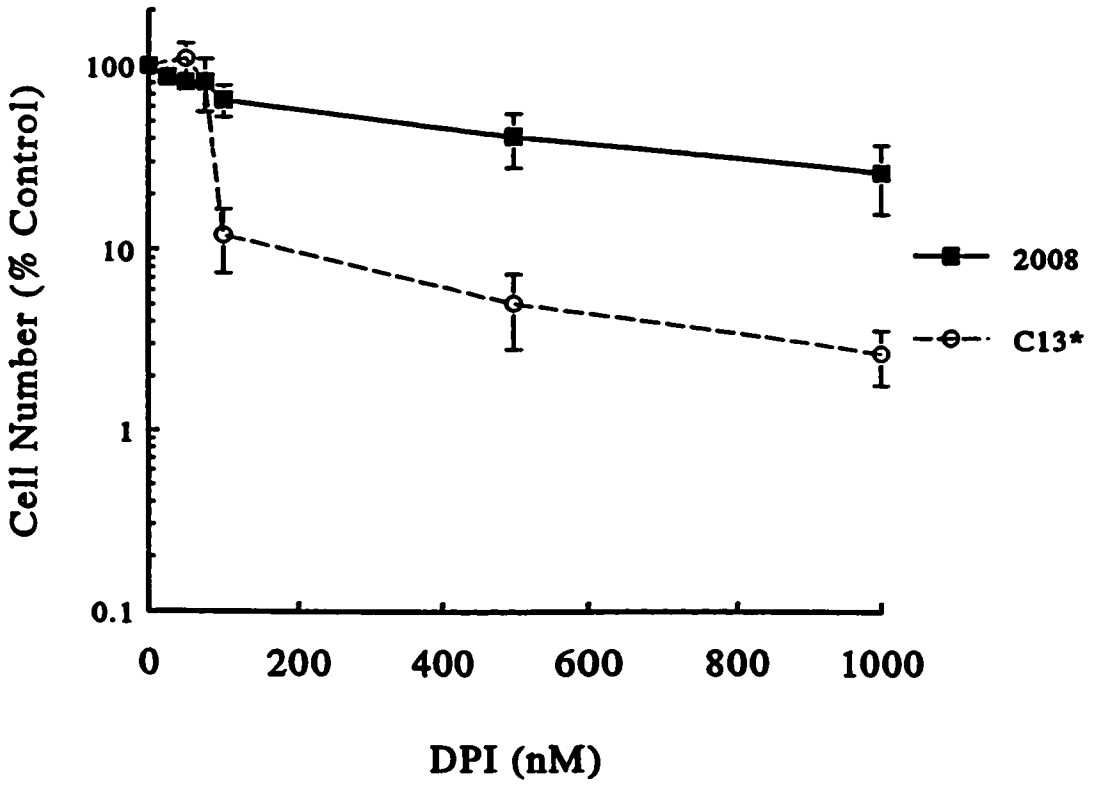


Figure 28: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

3.4 HYDROGEN PEROXIDE IS REQUIRED FOR PROLIFERATION

Extracellular Catalase has an Anti-Proliferative Effect

It is a novel observation that C13* cells with acquired resistance to cisplatin constitutively generate extracellular H_2O_2 at a significantly greater rate than that of the 2008 parental line. Of interest is the fact that C13* cells seem to have an overall shift in their redox set point since they have both an increased content of the antioxidant peptide GSH in addition to increased an "oxidative" stress load in the form of H_2O_2 generation. In the history of investigations pertaining to cisplatin resistance mechanisms, the frequently encountered characteristic of elevated GSH was suspected of playing a direct role in resistance through the promotion drug-detoxification reactions. In the 2008, C13* and RH4 model of resistance, elevated GSH is not expected to play such a mechanistic role since elevated GSH is an attribute of both cisplatin-sensitive RH4 and cisplatin-resistant C13* cells. We hypothesize that the alternate characteristic of increased H_2O_2 generation could make a significant contribution to cisplatin resistance in C13* cells, and that elevated GSH is a required survival response to protect C13* cells from oxidative stress. Modeling our investigation on the previous strategy that was used with elevated GSH in ovarian cancer, we wanted to reduce H_2O_2 concentrations to see if C13* cells could be sensitized to cisplatin. The enzyme catalase was added to the culture medium as a means of selectively reducing extracellular H_2O_2 concentrations, since this enzyme is

substrate specific for H_2O_2 conversion to H_2O. The experimental plan was to combine continuous catalase treatment with a 1h cisplatin pulse to evaluate potential synergy in terms of the overall cytotoxic response. The preliminary data for the effects of adding a sterilized preparation of catalase alone to the culture medium were surprising however, in the dramatic reduction of colony numbers for 2008 and C13* cells treated with catalase alone (Figure 29). This was dependent on catalase activity since the effect was eliminated if the preparation was denatured by heating to 100°C for 5 minutes. Repetition of this experimental strategy with 2008 cells in the DNA adherence assay revealed a dose-response curve between increasing catalase concentrations (Units of activity/ml) and reduced 2008 cell number (Figure 30). The appearance of the dose-response effect also inferred that the enzymatic activity of catalase to remove extracellular H_2O_2 was the influential factor leading to inhibition of cell growth.

Figure 29: Catalase in the Culture Medium Eliminates Colony Formation in 2008 and C13* Cells

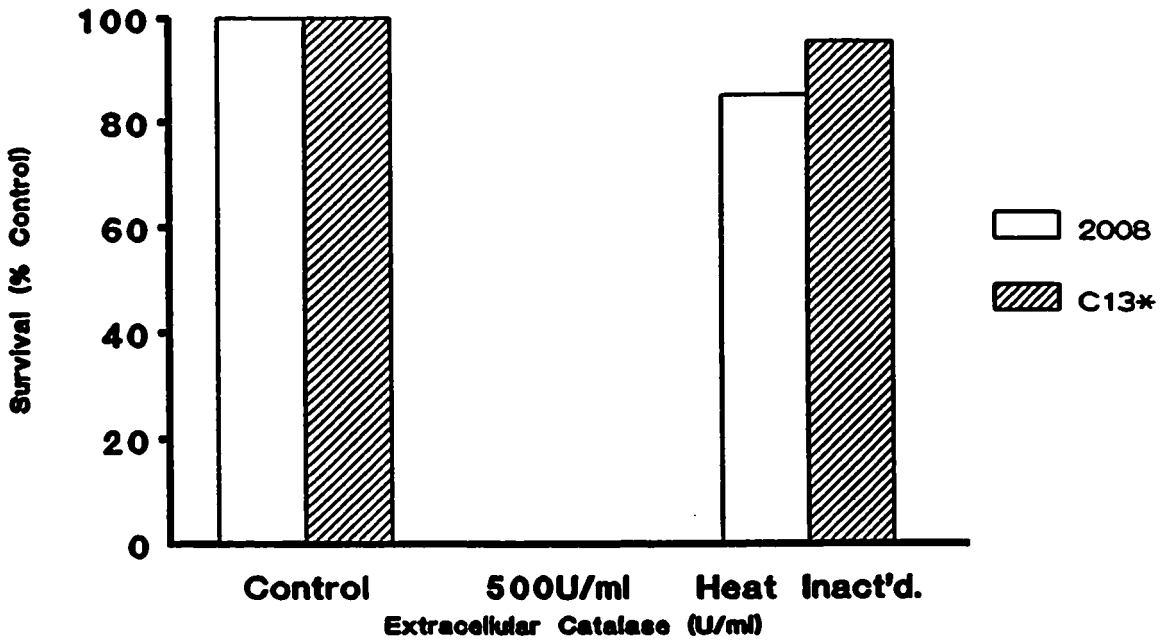


Figure 29: % Inhibition of colony formation when 500U/ml of catalase activity is present in the culture medium. The enzymatic activity of catalase is required since heat denaturation (I-CAT) of the preparation eliminates the effect. (Data represent the mean results of 2 independent experiments).

Figure 30: Catalase in the Culture Medium Inhibits 2008 Cell Growth

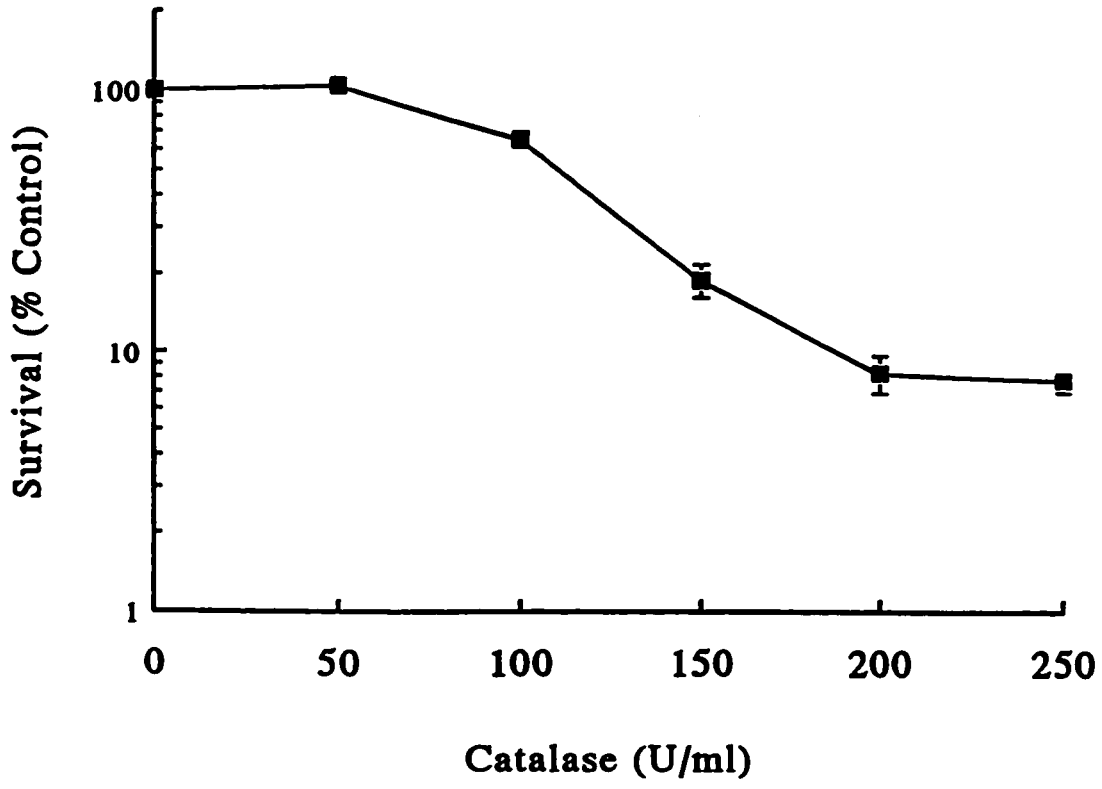


Figure 30: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

Since the 2008 and C13* cells are of a common cellular origin, it was necessary to verify the effect of extracellular catalase is several other cell lines of different tissue and species origin that have measurable extracellular H_2O_2 production. Table 13 shows the H_2O_2 production rate in pmol/h for $5x10^5$ normal human LLO ovarian epithelial cells, human HT29 colon carcinoma cells, human SKOV-3 ovarian carcinoma cells, human Hf172 immortalized fibroblasts and murine 3T6 fibroblasts compared to the human 2008 cells. Utilizing the DNA fluorescence assay for cell number, the catalase experiment was performed for each cell line grown in their respective FBS-supplemented medium. The addition of catalase to the extracellular medium of all the cell lines studied had an inhibitory effect on cell growth measured over a 96h period irrespective of their normal, immortalized or transformed phenotype (Table 13). It was uncertain whether the reduction in cell number was the result of a cytotoxic effect or an overall anti-proliferative effect in the population. The absence of a dose-response curve for a range of catalase concentrations in the preliminary colony formation assay with 2008 and C13* cells suggested that catalase had an anti-proliferative effect, since survival endpoints in this assay procedure are dependent on cell proliferation to form colonies. The anti-proliferative effect of extracellular catalase was confirmed in 2008, HT29 and SKOV-3 cells using DNA fluorescence staining and flow cytometry to assess cell cycle profiles and DNA synthesis activity 24h after the addition of catalase. A double fluorescence-

labelling experiment was used to identify cell cycle stage according to their DNA content and active DNA synthesis activity was assessed by the extent of incorporation of the nucleotide analogue bromodeoxyuridine (BrDU). Figure 31 shows a representative profile of HT29 control cells with active DNA synthesis versus cells that are growth-inhibited by catalase. The cell cycle stage is evident by PI fluorescence on the X-axis, while S phase activity is estimated by the appearance of FITC signal on the Y-axis, a measure of BrDU incorporation. Table 14 summarizes the cell cycle profile analysis and, more importantly, the reduction in DNA synthesis activity that is evident when 2008, HT29 or SKOV-3 cells are treated for 24h with catalase using hydroxyurea as a positive control for growth arrest.

Table 13: Catalase Inhibits the Growth of Various H_2O_2-Generating Cell Lines

Cell Type	IC_{50} for Extracellular Catalase (Units/ml)	Extracellular H_2O_2 Generation Rate (pmol/h)
SKOV-3	205 ± 34	181.6 ± 17.0
LLO	105 ± 8	46.7 ± 8.2
HT29	116 ± 6	31.1 ± 3.7
2008	117 ± 3	16.6 ± 1.5
Hf172	43 ± 5	12.9 ± 1.1
3T6	48 ± 6	3.8 ± 1.2

Table 13: Extracellular H_2O_2 generation rate for $5.0x10^4$ adherent cells in comparison to the calculated IC_{50} for a 96h continuous exposure to extracellular catalase in the culture medium. Data represent mean ± SEM of a minimum of n=3 independent experiments.

Figure 31: Catalase Inhibits DNA Synthesis Activity in HT29 Cells

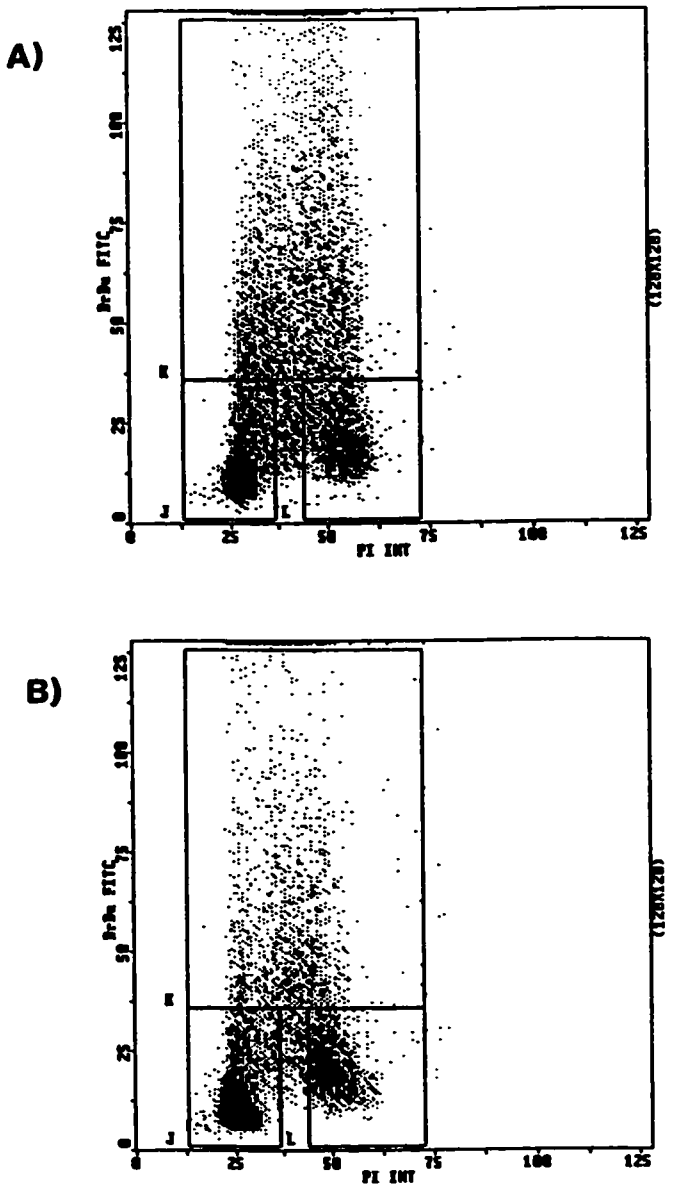


Figure 31: A representative plot of cell cycle stage (PI fluorescence) vs BrDU incorporation (FITC fluorescence) for a) control HT29 cells and b) cells treated for 24h with 500U/ml of catalase in the culture medium.

Table 14: Cell Cycle Profile Analysis of 2008, HT29 and SKOV-3 Cells Following Catalase or Hydroxyurea (HU) Incubation

Cell Type	Label	Untreated Control			500 U/ml Catalase			1.5 mM Hydroxyurea		
		%G_1	%S	%G_2	%G_1	%S	%G_2	%G_1	%S	%G_2
2008	PI	55.0 ± 2.4	35.3 ±2.1	9.6 ±0.4	44.9 ± 4.4	53.2 ±4.7	1.8 ±0.8	70.3 ± 2.2	28.4 ±1.6	1.3 ±0.7
	BrDu/ FITC	-	21.6 ±2.3	-	-	8.3 ± 1.3	-	-	3.2	-
HT29	PI	38.1 ± 2.1	43.5 ± 1.3	18.3 ± 2.4	60.3 ± 0.7	20.0 ± 1.7	19.5 ± 1.2	59.3 ± 3.7	38.8 ± 4.4	1.8 ± 0.9
	BrDu/ FITC	-	27.2 ± 4.1	-	-	2.3 ±4.0	-	-	8.8 ±2.2	-
SKOV-3	PI	56.8 ± 3.4	28.9 ± 4.8	14.2 ± 1.9	64.5 ± 4.0	17.0 ± 3.5	18.4 ± 2.3	77.5 ± 1.1	17.8 ± 0.4	8.9 ± 1.8
	BrDu/ FITC	-	22.3 ± 0.8	-	-	11.0 ±4.0	-	-	13.6 ±3.2	-

Table 14: Alterations of cell cycle distribution in 2008, HT29 and SKOV-3 cells following 24h treatment with catalase or a 16h incubations with 1.5mM hydroxyurea. Cell cycle profiles generated by MCYCLE software analysis of PI stained populations. Population fractions undergoing active DNA synthesis (S-fraction) were estimated by antibody detection of BrDU incorporation. Data represent mean ± SEM of 3 experiments unless n=1 values reported.

Proliferation Rescue of Catalase-mediated Cell Growth Inhibition

In general, the amount of catalase required to inhibit growth in each cell line examined was positively correlated to their individual H_2O_2 production rate. This suggests that the enzymatic activity of catalase to decompose H_2O_2 is associated with its growth inhibitory effect and that H_2O_2 is a required factor for growth. To verify this hypothesis, we designed a competition experiment for the co-incubation of catalase and the H_2O_2-generating enzyme glucose oxidase in the extracellular medium. If H_2O_2 is the critical growth regulator, a measurable rescue of cell number would be expected when H_2O_2 supplied by glucose oxidase exceeds the consumption of catalase.

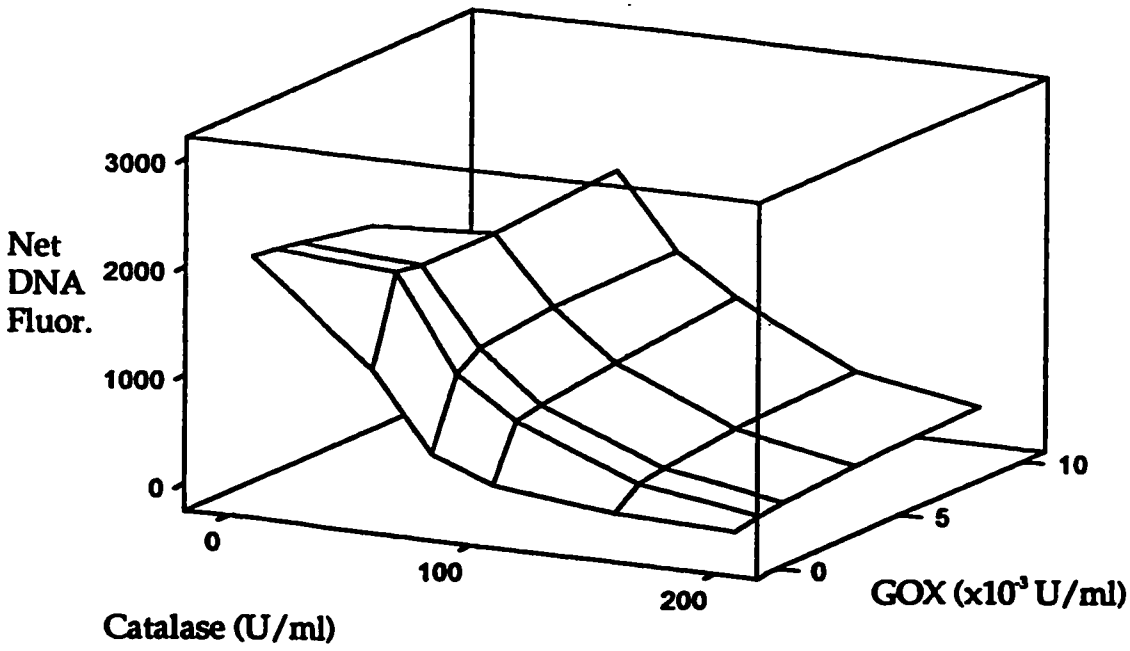
For the competition rescue experiments we chose 3T6, 2008 and SKOV-3 cells to represent a range of low, moderate and high H_2O_2 production rates. The three-dimensional plots shown in Figures 32, 33 and 34 graphically represent the accompanying tabulated cell survival data for 3T6, 2008 and SKOV-3 cells receiving catalase alone versus catalase in combination with 4 different concentrations of glucose oxidase. The most important observation from these experiments with 2008, 3T6 and SKOV-3 cells is that catalase-mediated growth inhibition can be significantly reversed with a co-incubation of glucose oxidase, suggesting that H_2O_2 is a key regulator of growth. Furthermore, we have shown that this rescue of cell number is not the result of D-gluconolactone generation, the alternate product of glucose oxidase activity. 2008 cells dosed with an IC_{90}

equivalent of 200 U/ml of catalase were co-incubated D-gluconolactone at concentrations up to 5mM, the maximum amount of D-gluconolactone that would be generated if all the D-glucose present in the culture medium was converted to this product. Figure 35 shows that D-gluconolactone was not toxic to 2008 cells, and in comparison to glucose oxidase, concentrations up to 5mM could not rescue growth (Figure 36).

The capacity of glucose oxidase to rescue cell growth in 3T6, 2008, and SKOV-3 cells dosed with catalase is not an infinite response, but represents a balance of several factors. For instance, glucose oxidase alone was toxic to each cell line, a response likely contributed to by the consumption of essential D-glucose from the medium and the generation of excess H_2O_2 which triggers a cytotoxic response depending on each cell's individual level of tolerance to oxidative stress. In accordance with this idea 3T6 cells seemed more resistant to the addition of glucose oxidase alone and also required less H_2O_2 to restore their normal complement of extracellular H_2O_2. This combination of factors could explain why cell number rescue was observed at many concentrations of glucose oxidase beyond the minimum required to reverse growth inhibition at a dose of 50U/ml of catalase. In contrast, a significant rescue of cell number was more difficult to achieve in SKOV-3 cells due to their higher requirements for H_2O_2 replacement at catalase concentrations of 150 U/ml. Aside from the conclusion that H_2O_2 is a required factor for growth in culture, these data suggest that each

cell population requires a balance of H_2O_2 production and consumption to satisfy proliferation requirements while maintaining viability.

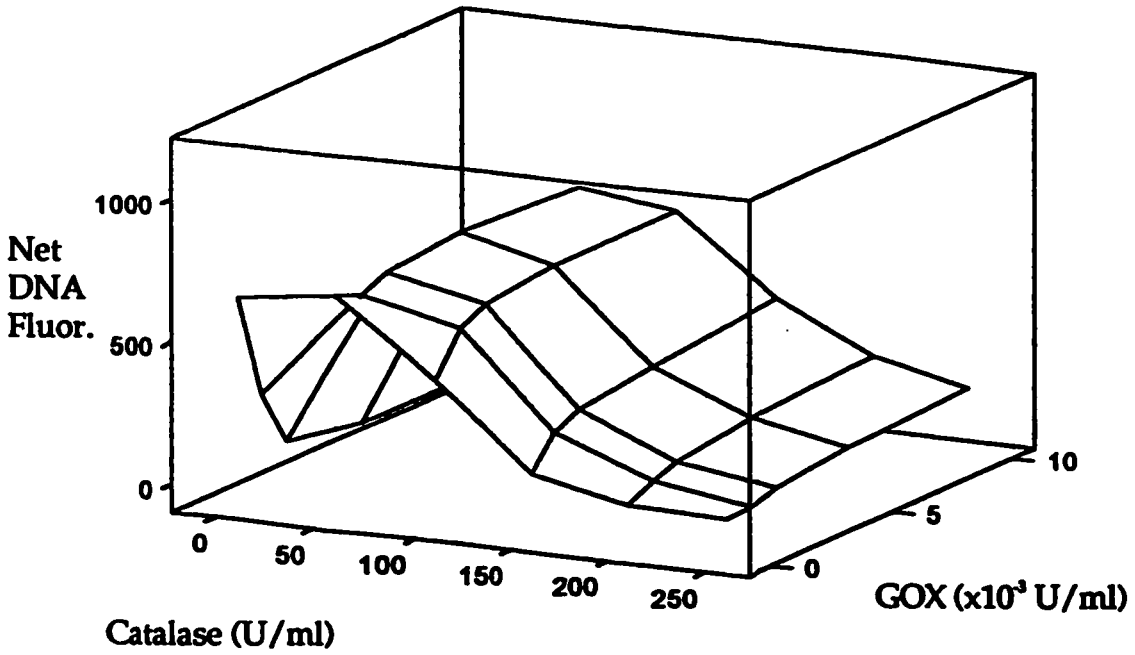
Figure 32: Catalase-Mediated Growth Inhibition is Reversed by the Addition of Glucose Oxidase to 3T6 Cells



Glucose Oxidase (x10³ Units/ml)	Catalase (Units/ml)					
	0	50	75	100	150	200
0	2425.2 ± 242.1	1203.2 ± 123.9	447.2 ± 74.8	231.8 ± 40.8	74.6 ± 19.0	49.3 ± 15.2
1	2282.7 ± 72.0	2185.3 ± 296.6	1157.4 ± 44.1	736.8 ± 21.3	285.7 ± 60.3	89.2 ± 19.6
2	2340.3 ± 332.8	2151.4 ± 206.3	1308.7 ± 126.2	798.6 ± 41.7	309.7 ± 114.0	113.1 ± 41.5
5	2263.0 413.7±	2126.7 ± 254.4	1388.8 ± 118.7	894.5 ± 90.9	386.3 ± 164.7	153.2 ± 45.7
10	11.6 ± 3.0	2259.7 ± 275.7	1430.4 ± 53.3	1004.1 ± 89.4	414.7 ± 150.8	211.3 ± 76.6

Figure 32: Data represent mean ± standard deviation of net DNA fluorescence units for 3 independent experiments. Shaded cells represent combinations of glucose oxidase and catalase that permitted significantly increased cell growth versus catalase alone ($p < 0.05$)

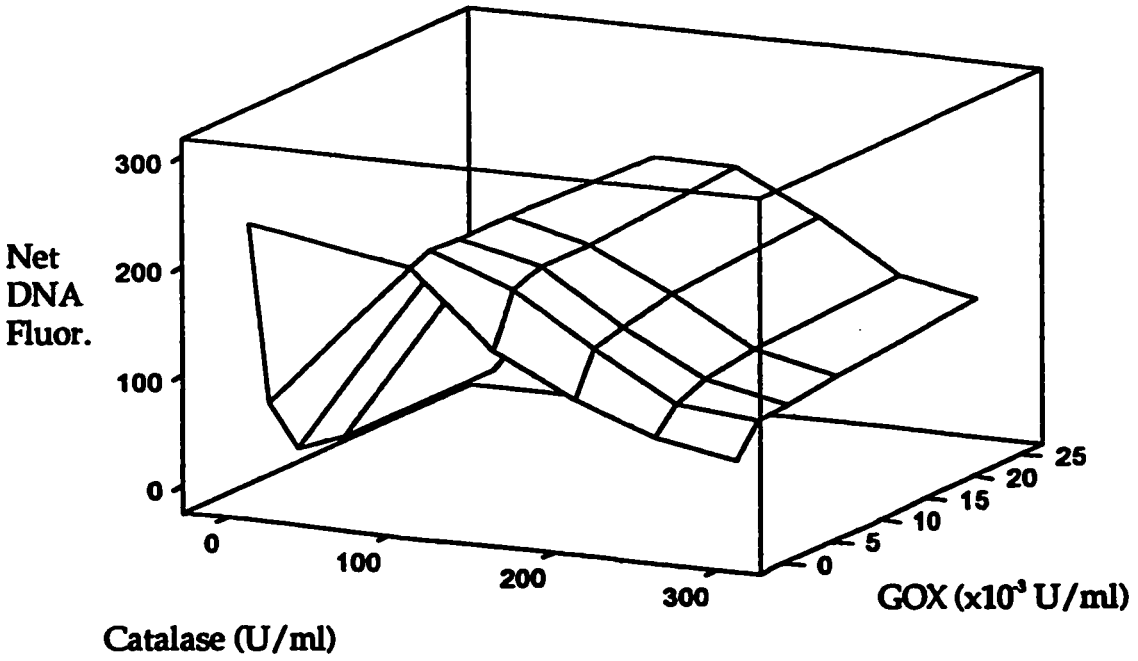
Figure 33: Catalase-Mediated Growth Inhibition is Reversed by the Addition of Glucose Oxidase to 2008 Cells



Glucose Oxidase (x10³ Units/ml)	Catalase (Units/ml)					
	0	50	100	150	200	250
0	538.2 ± 128.2	562.8 ± 134.9	335.9 ± 65.4	83.4 ± 30.5	36.0 ± 14.2	36.8 ± 10.1
1	83.2 ± 128.0	520.4 ± 144.8	496.7 ± 120	194.1 ± 49.4	76.8 ± 25.1	36.8 ± 9.7
2	13.8 ± 6.2	562.0 ± 149.6	527.2 ± 127.7	237.3 ± 37.4	111.1 ± 22.7	68.2 ± 16.5
5	12.8 ± 6.8	573.7 ± 168.9	539.5 ± 152.6	272.8 ± 54.0	143.3 ± 37.1	85.7 ± 22.9
10	8.2 ± 4.6	564.4 ± 143.8	522.1 ± 145.2	304.2 ± 66.2	155.2 ± 39.3	99.4 ± 24.8

Figure 33: Data represent mean ± standard deviation of net DNA fluorescence units for 3 independent experiments. Shaded cells represent combinations of glucose oxidase and catalase that permitted significantly increased cell growth versus catalase alone ($p < 0.05$).

Figure 34: Catalase-Mediated Growth Inhibition is Reversed by the Addition of Glucose Oxidase to SKOV-3 Cells



Glucose Oxidase (x10³ Units/ml)	Catalase (Units/ml)						
		0	100	150	200	250	300
	0	227.4 ± 10.4	211.0 ± 3.6	142.1 ± 26.0	105.2 ± 32.4	78.3 ± 43.0	64.1 ± 29.7
	2	68.2 ± 87.2	218.6 ± 18.1	190.6 ± 25.7	143.8 ± 37.0	100.7 ± 32.6	93.2 ± 22.9
	5	0.0 ± 0.0	216.2 ± 4.8	198.8 ± 28.9	150.8 ± 38.9	110.7 ± 34.6	95.2 ± 25.6
	10	0.0 ± 0.0	216.6 ± 14.8	197.9 ± 29.5	161.8 ± 40.1	118.6 ± 39.9	101.9 ± 24.0
	25	0.0 ± 0.0	210.0 ± 9.1	208.0 ± 22.2	170.8 ± 32.0	124.3 ± 38.2	89.2 ± 54.0

Figure 34: Data represent mean ± standard deviation of net DNA fluorescence units for 4 independent experiments. Shaded cells represent combinations of glucose oxidase and catalase that permitted significantly increased cell growth versus catalase alone ($p < 0.05$).

Figure 35: D-Gluconolactone is not Cytotoxic to 2008 Cells

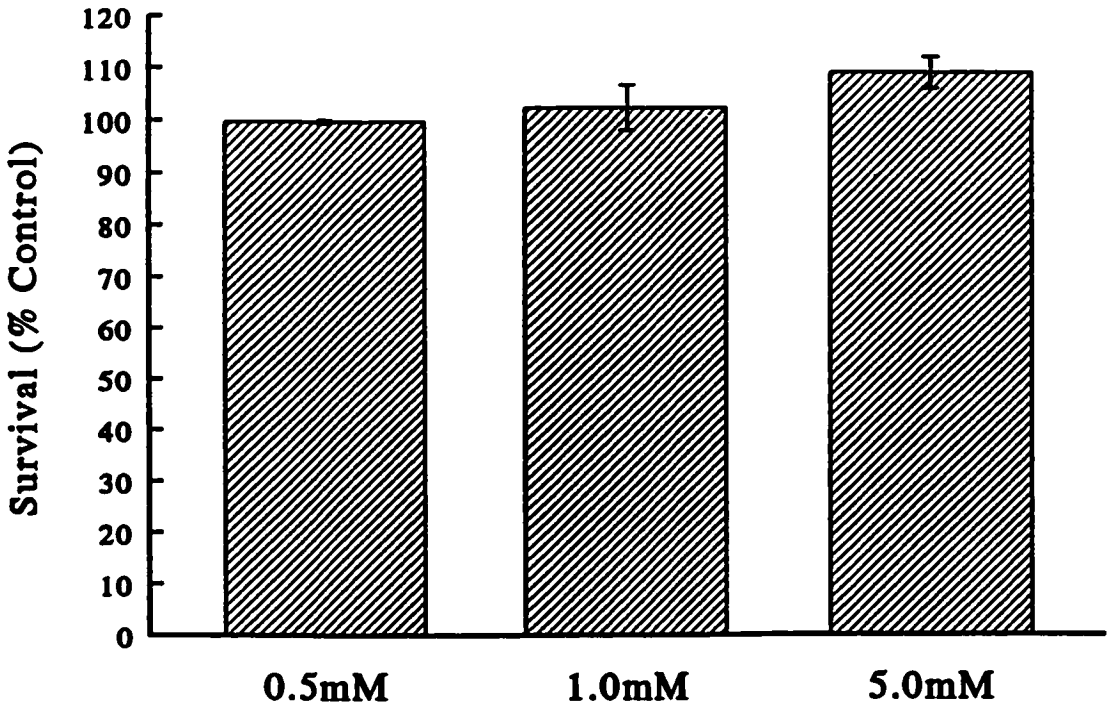


Figure 35: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments.

Figure 36: D-Gluconolactone Does Not Rescue Catalase-Mediated Growth Inhibition in 2008 Cells

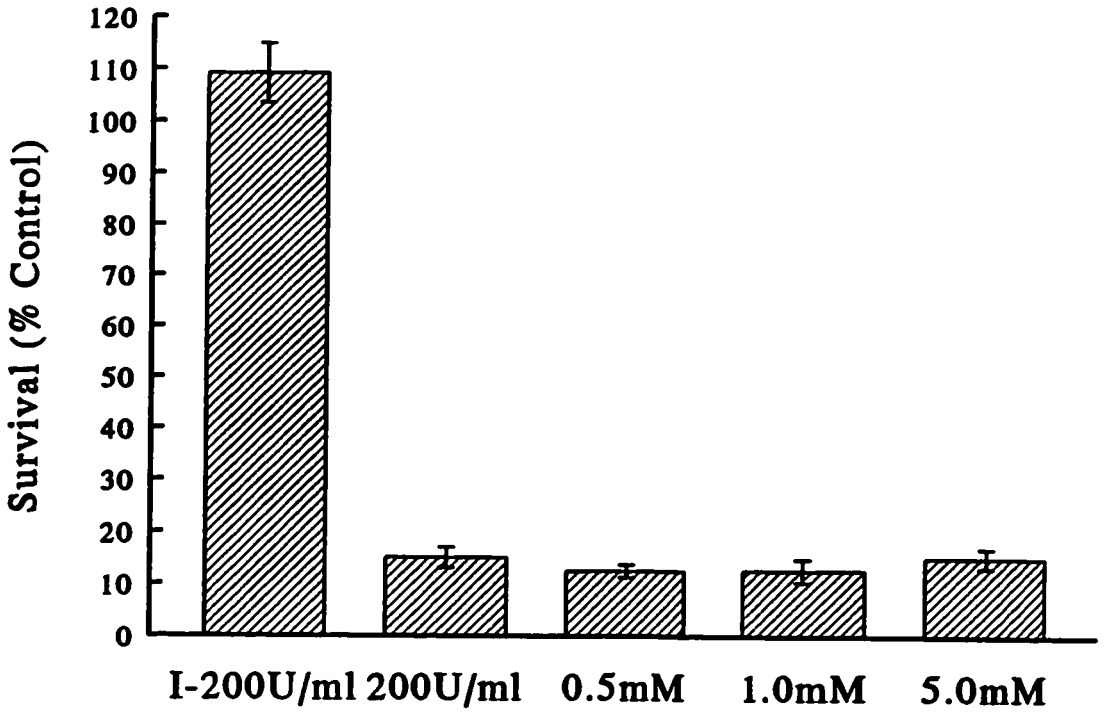


Figure 36: Cell number measured following a 96h continuous exposure. These data represent mean ± SEM of n=3 independent experiments. Cell number was not rescued by co-incubations of D-gluconolactone with 200 U/ml of active catalase. Heat inactivated catalase (I-200) is represents a negative control.

4.0 DISCUSSION

4.1 ENERGETIC CHANGES IN CISPLATIN-RESISTANT C13* CELLS

The bioenergetic properites of cancer cells have been a subject of interest since Warburg's early observations that Ehlich ascites cancer cells expressed a significant shift in their major source of energy production from mitochondrial respiration to glycolysis as a result of a heritable deficiency in respiratory capacity (Warburg 1956). This process has been termed aerobic glycolysis and represents increased glycolytic activity as a common property of cancer cells that operates even when oxygen concentrations are not limiting for respiration (Baggetto 1992). The alterations of energetic and associated metabolic pathways in tumour cells have been investigated for their potential as selective therapeutic targets, their contribution to the process of cancer development and their role in therapeutic resistance (Chen 1988; Lyon *et al.* 1988; Arora and Pedersen 1988; Baggetto 1992; Jones 1992; Sharp *et al.* 1992; Toussaint, Houbion and Remacle 1994). Based on this history, the reported involvement of mitochondrial alterations in our model culture system of cisplatin-resistant ovarian cancer cells stimulated an investigation of the energetic properties of these cells. A comparison of the glycolytic and mitochondrial respiration capacity of the 2008 parental line, the C13* cisplatin-resistant line and the cisplatin-sensitive RH4 revertant revealed no significant changes in glycolytic capacity, but a

significantly reduced capacity for respiration was evident in both C13* and RH4 cells. The existence of a similar defect in both cisplatin-resistant C13* and cisplatin-sensitive RH4 cells suggested that this defect alone was not associated with resistance, but may act in combination with an elevated mitochondrial membrane potential present only in C13* cells (Andrews and Albright 1992). The absence of any induction of glycolytic capacity in either C13* or RH4 cells suggests that the defect of mitochondrial respiration was not a limiting factor for ATP production. Confirmation of this has been provided by Berghmans *et al.* (1992) who used ^{31}P NMR to show that C13* cells have an equivalent baseline content of ATP in comparison to the parental 2008 cells. From these data we concluded that energy-production by mitochondria may not be the relevant factor involved in mitochondrially-associated cisplatin resistance, and subsequently widened our investigation to consider other mitochondrial functions in 2008 and C13* cells.

The presence of defective mitochondrial respiration in cisplatin-resistant C13* cells seems consistent with the available literature for cisplatin-mediated toxicity in proximal tubule cells proceeding via disruption of mitochondrial respiration and ROS generation (Gordon and Gattone, 1986; Brady *et al.* 1990; Tsutsumishita *et al.* 1998). There are fewer comparable investigations for the effect of cisplatin on tumour cell mitochondria, although mitochondrial alterations have been speculated to support resistance in other tumour cell lines

besides our 2008 and C13* model (Shinomiya *et al.*1992; Ara *et al.* 1994). Furthermore, ROS production has been implicated as a component of the acute cytotoxic response to cisplatin in KU7 bladder cancer cell lines (Miyajima *et al.* 1997). We have subsequently investigated 2008 and C13* cells for alterations in cellular and mitochondrial ROS production.

4.2 SHIFT OF REDOX BALANCE IN CISPLATIN-RESISTANT CELLS:

An increasing number of non-phagocytic cell types have been shown to release ROS such as $O_2^{\cdot}$ and H_2O_2 into the extracellular compartment (Table 5). In each cell type there is evidence that ROS have a functional role as signaling mediators, although the downstream impact of these signals seems to be cell type specific (Table 4). Tumour cells cultured *in vitro* have also been shown to constitutively generate low levels of H_2O_2 that can be measured extracellularly, although it has not yet been determined what impact this might have on intracellular signaling pathways.

We have used a sensitive and specific fluorescent detection assay to measure extracellular H_2O_2 production in 2008 and SKOV-3 ovarian carcinoma cells, the cisplatin resistant C13* subline of 2008 cells, and HT29 colon carcinoma cells cultured under standard conditions of 95% air (21% O_2) and 5% CO_2 ($pO_2 \approx$ 150mmHg). Extracellular H_2O_2 production by HT29 colon and SKOV-3 ovarian

carcinoma cells has been previously reported by Szatrowski and Nathan using a fluorescence assay with the indicator scopoletin (1991). In order to record detectable levels of H_2O_2, HT29 and SKOV-3 cells were plated at a high density of $2x10^5$ cells per well of 96-well plates and assayed for a 4h period, although the rate of production is reported for $1x10^4$ cells/h. Our attempts to use the scopoletin indicator to measure H_2O_2 were not successful for cell types with low-level H_2O_2 production like the 2008 line. The A6550 indicator, however, proved to be more sensitive such that a direct assay of $5x10^4$ cells over a 3h period gave consistent results for each cell type, with confirmation that fluorescence development was cell number dependent, and specific for H_2O_2 based on catalase competition. A direct comparison of the accumulated concentration of H_2O_2 for $5.0x10^4$ plated cell revealed that SKOV-3 cell generate approximately 5-fold more H_2O_2 than either C13* or HT29 cells, and nearly 10-fold more than 2008 cells. The magnitude of difference between SKOV-3 and HT29 is half of the 10-fold difference reported by Szatrowski and Nathan for $2.0x10^5$ plated SKOV-3 versus HT29 cells, however, these values cannot be directly compared due to significant differences in assay technique. For example, Szatrowski and Nathan inlcude 1mM sodium azide in the assay buffer to eliminate the intracellular activity of catalase, thus overestimating the net production of H_2O_2 by these cells.

Our observation that C13* cells have significantly increased (2-fold) constitutive extracellular H_2O_2 production compared to 2008 cells is a novel

finding for a cell line with acquired resistance to cisplatin. The shift towards increased, net production of H_2O_2 in C13* cells may reflect either a reduction in the cell's antioxidant capacity to neutralise H_2O_2 or an increase in the generation of this product. There are two reasons to suspect the latter case is true in C13* cells. Firstly, it has been shown in kidney proximal tubule cells that mitochondria are a target for the cytotoxic action of cisplatin, a mechanism that involves enhanced mitochondrial production of ROS (Brady *et al.*1990; Zhang and Lindup 1993; Kruidering *et al.* 1997; Tsutsumishita *et al.* 1998). This information in combination with the observed defect in C13* mitochondrial respiration suggests that the mitochondria of C13* cells have been similarly targeted, with the potential consequence of increased ROS production (Shinomiya *et al.* 1992; Suzuki *et al.* 1998). Secondly, there is evidence that antioxidant defenses in C13* cells are up-regulated compared to their 2008 counter-parts, as exemplified by a 2-fold increase in cellular GSH content (Zinkewich-Péotti and Andrews 1992). For these reasons we have begun our investigation of increased extracellular H_2O_2 production in C13* cells with the expectation that the net increase stems from an increase in H_2O_2 generation.

Endogenous Sources of H_2O_2 Production in Carcinoma Cells: A Pharmacological Investigation

The major sources of intracellular O_2^- and H_2O_2 production are summarized in Figure 9. In our pharmacological survey, we have tried to evaluate the targets that may operate in ovarian carcinoma cells, with particular emphasis on mitochondrial contributions. In addition, the increasing number of reports for novel NADH and NADPH plasma membrane oxidase assemblies in non-phagocytic cells has led us to investigate whether agents that target these activities are effective inhibitors of constitutive H_2O_2 production in our panel of carcinoma cells (Meier *et al.* 1993; Ushio-Fukai *et al.* 1996; Souren, Aken and Van Wijk 1996; Cool *et al.* 1998).

Mitochondrial Contributions

Mitochondria are generally considered a prominent source of intracellular H_2O_2 production since these organelles consume the majority of cellular oxygen (80-90%) and an estimated 1-2% of this consumption results in superoxide (O_2^-) production (Boveris and Chance, 1973). Superoxide is generated by the non-enzymatic transfer of electrons from reduced electron carriers to O_2 primarily at complexes I (NADH dehydrogenase), and III (ubiquinone), although the prominent contributor may be cell type specific (Kwong and Sohal 1998) (Figure 8). H_2O_2 is subsequently generated from O_2^- by spontaneous dismutation or enzymatic conversion by manganese superoxide dismutase (MnSOD) localized

in the mitochondrial matrix (Weisiger and Fridovich 1973). The membrane permeability of H_2O_2 is roughly equivalent to H_2O and diffusion of H_2O_2 across mitochodrial, peroxisomal and plasma membranes has been observed (Freeman and Crapo 1982).

We have examined the effect of two mitochondrial electron transport inhibitors, rotenone and Antimycin A, on the extracellular concentration of H_2O_2 produced by 2008 and C13* cells. In each cell type, these inhibitors had a dramatic effect on the amount of extracellular H_2O_2 detected, reducing the measured concentration to approximately 60% of solvent-treated controls following a 2h incubation with either agent. Rotenone acts as complex I of the transport chain, irreversibly inhibiting the transfer of electrons from NADH dehydrogenase to subsequent Fe-S protein, while Antimycin A acts at complex III, preventing electron transfer from cytochrome b to cytochrome c_1 (Figure 8). Historical experiments performed by Boveris and Chance (1973) showed that these inhibitors can immediately increase H_2O_2 production in isolated pigeon mitochondria, as would be predicted by the law of mass action if the upstream electron carriers became highly reduced. A reasonable explanation for the depressive effect of these agents on 2008 and C13* H_2O_2 production is the loss of mitochondrial membrane potential that occurs after long term blockade of electron transport (Simbula *et al.* 1997). Other research groups have also

reported that rotenone and Antimycin A can depress cellular production of ROS. In sheep and bovine pulmonary endothelial cells Sanders *et al.* (1993) and Zulueta *et al.* (1995) used 20 and 100 μM rotenone, respectively, to reduce basal H_2O_2 production to 40% and 70% of untreated cells. Similarly, in rat thymocytes that generate H_2O_2 during dexamethasone-induced apoptosis, both rotenone (100μM) and Antimycin A (20μg/ml) significantly reduced cellular production to 26% and 18% of untreated thymocytes undergoing apoptosis (Wang, Jerrells and Sptizer 1996). In our study, a similar depressive effect of rotenone was observed in HT29 colon carcinoma and SKOV-3 ovarian carcinoma cells, reducing the measurable H_2O_2 concentration to nearly 57% of controls. This suggests that mitochondrial electron transport activity is a major contributor to endogenous H_2O_2 generation in the extracellular compartment in 2008, C13*, SKOV-3 and HT29 cultured lines irrespective of their H_2O_2 production phenotype.

In both 2008 and C13* cells, the extent of H_2O_2 reduction was similar with rotenone incubations from 5 to 50μM, suggesting that the maximum mitochondrial contribution to extracellular H_2O_2 concentration can be estimated. With an incubation of 50μM rotenone, the mitochondrial contribution (44%) in 2008 cells is equivalent to a concentration of 100nM, and in C13* cells the contribution is 211nM (43%). Given the absence of any significant difference in

mitochondrial mass, this suggests that C13* mitochondria produce a net 2-fold more H_2O_2 than the mitochondria of 2008 cells. There are two general means by which mitochondrial H_2O_2 production is increased overall, one is a loss of antioxidant defenses to eliminate H_2O_2, and the other is a genuine increase in production that is not matched by an increase in antioxidant defense. In most mitochondria, glutathione peroxidase (GPX) is the dominant enzyme involved in neutralization of H_2O_2, since catalase is found only in heart mitochondria (Reed 1990; Radi *et al* 1991; Phung, Ezieme and Turrens 1994). Furthermore, efficient GPX activity is dependent on the concentration of mitochondrial GSH, a critical determinant of how much H_2O_2 is free to diffuse to the intracellular space (Reed 1990; García-Ruiz *et al.* 1995) (Figure 9). As yet, we have no evidence for reduced mitochondrial antioxidant capacity in C13* versus 2008 cells, but we do have a measured deficiency in oxygen consumption. The consumption of oxygen by mitochondrial cytochrome c oxidase is recognized to serve two purposes: one is the coupling of oxygen consumption to the generation of a proton gradient that is used for ATP production. The other purpose is oxygen consumption as a contribution to antioxidant defense (Skulachev 1996; Papa, Guerrieri and Capitanio 1997). This role for cytochrome c oxidase was concluded from evidence that the complex can consume oxygen in the absence of coupled proton pumping, and these so-called "slips" are responsible for the enzyme's role in respiratory protection to keep oxygen concentrations low (Papa,

Guerrieri and Capitanio 1997). The defect of oxygen consumption in C13* cells would increase the localized concentration of O_2 in C13* mitochondria, and in the presence of reduced electron carriers this would be expected to increase $O_2^{\cdot}$ and H_2O_2 production overall. These data further the evidence that mitochondria of cisplatin-resistant C13* cells have sustained permanent alterations following cisplatin selection *in vitro* and we propose that enhanced H_2O_2 production may be the mechanistic link between drug resistance and the mitochondrial changes previously described in this system.

The other mitochondrial inhibitor oligomycin used in this investigation does not target mitochondrial electron transport directly, but ATP synthesis at the F_0F_1 ATPase (complex V), creating an up-stream arrest of proton pumping similar to State IV respiration. The cellular responses to oligomycin were the most disparate of any agent: 2008 cells responded to a 5.0μM incubation with a significant increase (28%) in extracellular H_2O_2 generation, C13* cells and HT29 cells showed no significant effect, and SKOV-3 cells showed a significant (40%) decrease in extracellular H_2O_2 generation. Overall, these data are not readily explained, however, oligomycin has been shown previously to increase the mitochondrial membrane potential of hepatocytes and the ROS production of thymocytes (Simbula *et al.* 1997; Wang, Jerrells and Spitzer 1996). It remains our objective to measure the effect of oligomycin on the mitochondrial membrane potential in these four cell lines to explain these unique responses, with

particular interest in the difference between 2008 and C13* cells to further define the altered bioenergetic properties of C13* cells.

Enzymatic Contributions

In neurons, the enzyme monoamine oxidase A (MAO-A) generates H_2O_2 as direct product in the conversion of dopamine to 3,4-dihydroxyphenylacetaldehyde (Cohen, Farooqui and Kesler 1997). The B isoenzyme of MAO is involved in the extra-neuronal metabolism of monoamines, so we have examined the potential contribution of MAO-B to cellular H_2O_2 production with the specific inhibitor pargyline (Berry, Juorio and Paterson 1994). In 2008 and C13* cells incubations of pargyline at concentrations up to 200μM had no significant effect on extracellular H_2O_2 generation, suggesting that MAO-B is not a significant contributor. Szatrowski and Nathan also reported that the agent pargyline had no effect on H_2O_2 production in their panel of tumour cell lines.

Xanthine oxidase (XO) is an intracellular source $O_2^{\cdot}$ with H_2O_2 as a spontaneously or enzymatically converted dismutation product. Xanthine oxidase is not usually present in cells, but can be converted from xanthine dehydrogenase under conditions that stimulate proteolytic conversion (Sanders, Eisenthal and Harrison 1997). To examine whether xanthine oxidase contributes to H_2O_2 production in 2008 and C13* cells, the cells were incubated with

allopurinol at a maximum concentration of 100μM for 2h prior to assay. We observed that allopurinol had no significant effect on extracellular H_2O_2 production in either cell type suggesting that xanthine oxidase is not a significant contributor. Similarly, Szatrowski and Nathan reported no effect of allopurinol on H_2O_2 production by a variety of tumour cell lines (1991).

Potential Contributions by Novel Plasma Membrane Oxidase Assemblies

The agent DPI is an effective inhibitor of stimulated ROS production by activated neutrophils at concentrations of 10μM (Cross and Jones 1986; Cross 1987; Hancock and Jones 1987; O'Donnell *et al.* 1993). DPI is a general flavoprotein inhibitor that can influence other flavoprotein-containing enzymes such as nitric oxide synthase, xanthine oxidase, NADPH cytochrome p450 reductase, and the NADH ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport chain (Majander, Finel and Wilstöm 1994; Sanders, Eisenthal and Harrison 1997). DPI has been commonly used to confirm the flavoprotein dependence of ROS-generating plasma membrane assemblies present in non-phagocytic cells. In spermatozoa, ras-transformed fibroblasts, normal fibroblasts, pulmonary neuroepithelial bodies, pulmonary endothelial cells and neuroblastoma cells, DPI is effective at reducing extracellular ROS detection in the concentration range of 1-20μM (Aitken *et al.* 1997; Irani *et al.* 1997; Meier *et al.* 1993; Wang *et al.* 1996; Zulueta *et al.* 1995; Szatrowski and Nathan 1991).

DPI was very effective at reducing extracellular H_2O_2 generation in 2008, C13*, HT29 and SKOV-3 cells to approximately 50% of control production. Although it is not possible to conclude whether DPI targets the generation of H_2O_2 from mitochondria or other flavoprotein sources, incubations with DPI revealed that H_2O_2 production in C13* cells was more sensitive to inhibition by DPI than in 2008 cells. This sensitivity was also maintained in a cytotoxicity assay with DPI where the IC_{50} ratio for 2008 versus C13* cells was approximately 5-fold. This suggests that C13* cells have increased dependence on DPI-inhibitable flavoprotein activity which may be a reflection of H_2O_2 dependence.

Another compound that is a potent inhibitor of ROS generation by the phagocytic NADPH oxidase is the agent phenylarsine oxide (PAO), with an IC_{50} in macrophages and neutrophils of approximately $1x10^{-7}M$ (Roussin *et al.* 1997). PAO is a hydrophobic trivalent arsenical compound that can form covalent adducts with two closely spaced sulfhydryl groups. The mechanism of action of PAO is distinct from DPI, since it does not target the flavoprotein but inhibits a critical phosphotyrosine phosphatase activity that is required for activation of the NADPH oxidase (Kutsumi *et al.* 1995). PAO has also been used to reduce H_2O_2 production in plasma membrane preparations of pig thryoid and intact SV40-transformed fibroblasts, at concentrations of 10μM and 100μM, respectively (Gorin *et al.* 1997; Arbault *et al.* 1997). In our study the administration of PAO was limited to very low concentrations given the ability

of this agent to induce a significant loss of cell adherence. Incubations of low doses (0.1 and 0.5μM) that did not induce cell lifting had no dramatic influence on H_2O_2 generation by 2008, C13*, HT29 or SKOV-3 cells. Further investigations with PAO will require an adherence-independent assay for H_2O_2 to increase the testable dose-range and improve the reliability of the results.

In addition to being an effective inhibitor of H_2O_2 production, Arbault *et al.* (1997) reported that PAO induced selective toxicity in SV-40 transformed fibroblasts compared to untransformed cells. We have also used PAO as a cytotoxic agent against 2008 and C13* cells with a maximum dose of 500nM. No dramatic differences were observed in the cytotoxic response of 2008 and C13* cells to continuous PAO exposure.

Chloroquine is a well-known anti-malarial agent that is an identified inhibitor of plasma membrane electron transport activities in protozoa, yeast and mammalian cells (Toole-Simms, Morré and Crane 1990; Barr *et al.* 1991; Santos-Ocaña *et al.* 1995). Chloroquine has been used to initiate apoptosis in human and murine tumour cell lines based on its ability to inhibit PMOR activity, although the exact mechanism of toxicity is not well understood (Wolvetang *et al.* 1996). It has been speculated that an inhibition of electron transfer to recognized PMOR substrates like ascorbate radical could lead to a situation similar to electron transport block in the mitochondrion, where highly reduced electrons carriers in

the presence of oxygen could lead to the generation of ROS like H_2O_2. Chloroquine incubated with cells at concentrations capable of inhibiting the diferric transferrin reductase activity of PMOR (100μM) had no significant impact on H_2O_2 production in 2008, C13*, HT29 or SKOV-3 cells. This suggests either that this PMOR system is not present in these cells, or that oxygen is not an alternate substrate for this system. Chloroquine was also toxic to 2008 and C13* cells, although there was no dramatic difference in sensitivity to this agent between these cell types. It is yet to be identified whether chloroquine induces toxicity in these cells via inhibition of the PMOR as measured by an inhibition of ascorbate reduction or ferricyanide reduction.

Capsaicin is recognized therapeutically for its efficacy as a local analgesic in conditions of neuropathic pain (Fusco and Giacovazzo 1997). Capsaicin has also been identified as a specific inhibitor of an NADH oxidase activity found in tumour cell plasma membranes, in the conditioned medium of tumour cells, and in the sera of patients with cancer (Morré, Chueh and Morré 1995; Morré *et al.* 1996). This enzymatic activity is a potential target for cancer therapy since inhibition of this activity with capsaicin is associatated with growth arrest and apoptosis induction in tumour cell lines (Morré, Chueh and Morré 1995; Morré *et al.* 1996; Wolvetang *et al.* 1996). The physiologically-relevant electron acceptor of this activity has not been determined, but we hypothesized that oxygen could be

a potential substrate and incomplete reduction of oxygen may contribute to the cellular production of ROS like H_2O_2. Pre-incubation of a maximum dose of 100μM capsaicin prior to the extracellular H_2O_2 assay had no significant impact on production by 2008, C13*, HT29 or SKOV-3 cells. In the absence of a cell line to serve as a positive control for this particular NADH oxidase activity it cannot be concluded whether oxygen serves as a potential substrate for this oxidase or if this activity contributes to cellular ROS production. Similarly, it is not known if this activity is present in our panel of cells, but unlike the reports of Morré *et al.*, we observed no growth inhibitory effect of capsaicin in 2008 and C13* cells, suggesting that this activity may not be present in these cells (1996). A follow-up assay for NADH oxidase activity in isolated plasma membranes of 2008 and C13* cells based on NADH consumption will be necessary to confirm this suspicion.

4.3 INCREASED H_2O_2 PRODUCTION IN CISPLATIN-RESISTANT CELLS: IMPLICATIONS FOR DRUG RESISTANCE

Resistance to Cisplatin and H_2O_2 is Associated with Common Stress Responses

Our evidence for increased constitutive H_2O_2 production in C13* cells is a novel feature of cell population with acquired resistance to cisplatin. Based on the extensive literature for H_2O_2 as a participant in intracellular signaling pathways and as a modulator of gene expression, it is feasible that increased H_2O_2 production could be a significant factor involved in resistance. *In vitro*

culture models of cells have been examined for their responses to excess oxidative stress through the administration of exogenous H_2O_2 treatments, administered either as acute or continuous doses, or through conditions of increased endogenous ROS production, usually derived from mitochondria. It is frequently observed in surviving cells that various stress-response and antioxidant genes are up-regulated including members of the heat shock protein family, the growth arrest and DNA damage (*gadd*) genes and antioxidant enzymes like catalase, MnSOD and GPX (Fornace *et al.* 1992)(Appendix 5). Interestingly, these same stress response factors – GSH, heat shock proteins and *gadd* genes - have been investigated for their associations with cisplatin resistance development in C13* cells (Zinkewich-Péotti and Andrews 1992; Kimura *et al.* 1993a; Delmastro *et al.* 1997). The association between oxidative stress resistance and cisplatin resistance is also strengthened by the fact that HA-1 fibroblasts made resistant to H_2O_2-mediated oxidative stress are cross-resistant to cisplatin, suggesting that common pathways are involved (Spitz *et al.* 1993; Sood and Buller 1998).

Increased endogenous H_2O_2 production in C13* cells seems to be associated with an oxidative-stress resistant phenotype since C13* cells are 2.4-fold resistant to exogenously applied H_2O_2 compared to the parental 2008 line. The elevated GSH content of C13* cells is expected to be an important component of their antioxidant response since 1) GSH is a selectively up-regulated element in acute

responses to H_2O_2 in several model systems 2) the supply of reducing equivalents such as GSH are a critical component of antioxidant defense system (Kehrer and Lund 1994), and 3) C13* cells undergo a cytotoxic response when treated with the GSH-depleting agent BSO. This suggests that elevated GSH is an essential component of redox balance in C13* cells, where increased endogenous H_2O_2 production is matched by increased antioxidant defenses (GSH) to ensure survival.

In accordance with this concept of oxidative stress resistance in C13* cells, the up-regulation of hsp60 protein in C13* versus 2008 cells may reflect a localized protective response for the mitochondria of C13* cells which we now know contribute significantly to increased H_2O_2 production in C13* cells. This hypothesis stems from the evidence that heat shock proteins are a recognized component of the oxidative stress response and that mitochondria are a focal point for protection from oxidative stress to deter permeability transition and cell death via apoptosis (Reed 1999) (Appendix 5). In accordance with this idea, Polla *et al.* have shown that induction of heat shock proteins in human pre-monocytic U937 cells is important for resistance to subsequent H_2O_2 exposure (1996).

Increased expression of the growth-arrest and DNA damage (*gadd*) genes is also a common feature of the cellular response to oxidative stress (Appendix 5). The expression of *gadd 45* and *gadd 153* has already been investigated by

Delmastro *et al.* (1997) in C13* cells, but in the context of cisplatin-resistance and not resistance to oxidative stress. This study provided evidence for altered regulation of these stress genes between 2008 and C13* cells. Following an IC_{30} dose of cisplatin, the expression of *gadd 45* mRNA was greater in C13* cells than 2008 cells, but the expression of *gadd 153* was reduced in C13* versus 2008 cells (Delmastro *et al.* 1997). The authors concluded that the regulatory pathways for these genes were distinct between the two cell types. Our evidence for distinct H_2O_2 production profiles may contribute to the altered regulation of stress-responsive *gadd* genes in 2008 and C13* cells.

The transcription factor NFκB is also known to increase its DNA binding activity in response to oxidative stress, either from exogenous or endogenous sources (Appendix 5). NFκB regulates the expression of multiple genes involved in stress resistance and may play a role in cancer cell development (Gilmore *et al.* 1996; Luque and Gélinas 1997). Of particular interest is the recent finding that NFκB plays a critical role in resistance to TNFα-initiated apoptosis in human lymphoma cells by inducing the expression of anti-apoptotic proteins (Van Antwerp *et al.* 1996; Wang, Mayo and Baldwin 1996; Beg and Baltimore 1996; Wang *et al.* 1998; Giri and Aggarwal 1998). Considering the distinct H_2O_2 production phenotypes of 2008 and C13* cells, it would be interesting to measure NFκB DNA binding activity to elucidate whether NFκB activity is a downstream effector of increased H_2O_2 production in C13* cells.

Potential Impact of Increased Endogenous H_2O_2 Production to Cisplatin Resistance

The increased expression of oxidative stress-responsive factors like GSH, hsp60 and altered regulation of *gadd* genes in cisplatin-resistant C13* cells suggests that increased endogenous H_2O_2 production in C13* cells could be the relevant stimulus for these changes. Constitutive H_2O_2 production may represent a constant signal for up-regulation of these pathways that ultimately confer a cross-resistance advantage when cisplatin is re-introduced. We also suspect that endogenous H_2O_2 may serve a pro-survival function based on our observation that endogenous H_2O_2 is a required factor for the growth of multiple cell types cultured *in vitro*. Resistance to cisplatin may be conferred by the ability of the H_2O_2 proliferative signal to override growth arrest responses that would normally occur following cisplatin exposure and DNA damage. The premise for this hypothesis comes from evidence that the *myc* transcription factor, a strong proliferation signal associated with aberrant growth in cancer cells, can actively down-regulated *gadd* gene expression (Amundson *et al.* 1998) H_2O_2 has also been regarded as a "life signal" in U937 lymphoma cells that contributes to apoptosis resistance (Del Bello *et al.* 1999). Our hypothesis also has particular relevance to cisplatin resistance since defective growth arrest responses have been associated with acquired resistance in the IGROV1 ovarian cancer model (Poulain *et al.* 1998).

4.4 H_2O_2 IS AN AUTOCRINE PROLIFERATION SIGNAL *IN VITRO*

The biological significance of secreted H_2O_2 is likely to be cell-type specific, but there is abundant evidence for exogenous H_2O_2 as an effector of mitogenic signal transduction pathways in multiple cell lines *in vitro* (Table 4). We have investigated the role of endogenous, extracellular H_2O_2 in the regulation of cell growth by adding extracellular catalase to the serum-supplemented medium of a panel of cells that included normal human ovarian epithelial cells (LLO), immortalized murine and human fibroblasts (3T6 and Hf172) and human carcinoma cells (2008, HT29 and SKOV-3). Each of these cells generated a measurable amount of extracellular H_2O_2 as measured by the A6550 fluorescence assay. In each cell line the addition of catalase to the respective, serum-supplemented culture medium led to a dose-responsive reduction in cell number in a 96h growth assay, irrespective of the cell's normal, immortalized or transformed phenotype. The anti-proliferative effect of extracellular catalase could also be measured within 24h of dosing as a reduction in DNA synthesis activity of 2008, HT29 and SKOV-3 cells. Furthermore, the IC_{50} for catalase was positively correlated with the H_2O_2 production phenotype of each cell, suggesting that the activity of catalase to neutralise H_2O_2 was the important factor involved in growth inhibition. In order to provide conclusive evidence for H_2O_2 as the key mediator of this phenomenon, we designed a growth-rescue experiment by adding the H_2O_2 generating enzyme glucose

oxidase (GOX) to catalase-treated cultures. Using 3T6, 2008 and SKOV-3 cells as representative of a range of H_2O_2 production phenotypes, we have shown that combinations of GOX with catalase can significantly increase cell growth relative to catalase treatment alone. In addition, we have used 2008 cells to show that growth rescue depends on H_2O_2 generation, and is not a function of the alternate product of GOX activity, D-gluconolactone. Thus we conclude that cell-derived H_2O_2 is not only a participant in mitogenesis, but is an absolute requirement for the growth of both cancer cell and normal cells in culture even in the presence of serum-derived growth factors. H_2O_2 has been shown to regulate proliferation in fibroblasts, prostate carcinoma cells, leukemic cells and pancreas cells based on the evidence for low doses of exogenously added H_2O_2 to stimulate growth (Appendix 6). In studies more similar to our design, some investigators have observed cell growth dependence on endogenous H_2O_2 production derived from novel plasma membrane oxidases in transformed fibroblasts and primary vascular smooth muscle cells (Irani *et al.*1997; Zafari *et al.* 1998).

There are few studies available that have investigated the significance of endogenous H_2O_2 production to proliferation control in tumour cells, and the potential contribution of mitochondrially-derived H_2O_2 has not been examined at all in this field. Our evidence for mitchondria as a major contributor to extracellular H_2O_2 generation in 2008, C13*, HT29 and SKOV-3 carcinoma cells

implies that this organelle may be a relevant source of H_2O_2 that is capable of regulating proliferation.

5.0 FUTURE DIRECTIONS

Our investigation of energetic changes in C13* ovarian carcinoma cells with acquired resistance to cisplatin revealed increased endogenous production of H_2O_2 compared to the 2008 parental line. This finding was followed up by a pharmacological survey to begin identification of contributing sources. Also, as part of a study to examine the potential significance of increased H_2O_2production to cisplatin resistance, the finding that H_2O_2 is a required autocrine factor for the growth of several cell types in culture was revealed. These initial results can be expanded into several research projects:

1) Identification of the relevant molecular targets of H_2O_2 that are involved in growth control in cultured cells.
2) Investigation of endogenous H_2O_2 production as a contributor to apoptosis resistance concurrent with its "pro-proliferation" role.
3) Further biochemical characterization and description of the cellular compartmentalization of H_2O_2-generating sources.
4) Further evaluation of the antioxidant defense capacity of 2008 and C13* cells to determine their potential contribution to the net increase of H_2O_2 production by C13* cells.

5) Evaluation of other tumour cell culture pairs with intrinsic or acquired resistance to cisplatin to record their H_2O_2 production phenotype.

Endogenous H_2O_2 Production and Tumour Cell Metastatic Potential:

There is evidence in the literature that suggests the onset of cisplatin resistance in tumour cells is concurrent with an increased metastatic potential (Mitsumoto *et al.* 1998). It is a reasonable hypothesis that cell-derived extracellular H_2O_2 could contribute to this phenomenon based on the fact that exogenous H_2O_2 treatment of fibroblasts and endothelial cells stimulates the production and activation of matrix metalloproteinases, key players in the metastatic cascade (Belkhiri *et al.* 1997; Brenneisen *et al.* 1997). Exogenous H_2O_2 has also been shown to stimulate lymphocytes and angiogenesis (Monte, Davel and Sacerdote de Lustig 1997). It would be interesting to co-incubate 2008 or C13* cells with these cell types to observe whether cell-derived, extracellular H_2O_2 can support these responses, and whether C13* cells have an increased potential to promote these processes.

Endogenous H_2O_2 Production and Proliferation Control: Relevance of In Vitro Findings In Vivo

The majority of investigations concerning the intracellular messenger activity of H_2O_2 have been performed on cultured cell lines exposed to standard culture conditions and 21% oxygen ($pO_2 \approx 150$mmHg). A fundamental question

arising from our results is whether or not ROS like H_2O_2 regulate gene expression and/or proliferation *in vivo*. One area of research that provides positive evidence for H_2O_2 as a regulator of proliferation *in vivo* concerns the hepatic carcinogens and peroxisomal proliferators fenofibrate and ciprofibrate. The transforming potential of these agents originates from increased intracellular H_2O_2 production via increased expression of peroxisomal H_2O_2-generating enzymes like fatty acyl-Coa oxiase and urate oxidase (Arnaiz *et al.* 1995; Chu *et al.* 1995; Chu *et al.* 1996). In this model increased intracellular H_2O_2 generation is directly involved in NFκB activation and growth control, suggesting that H_2O_2 has the potential to regulate proliferation *in vivo* (Nilakantan, Spear and Glauert 1998).

The data gathered in this investigation suggests that mitochondria are a major contributor to H_2O_2 production in cells cultured *in vitro* under standard conditions (21% O_2). Hyperoxic conditions have a stimulatory impact on mitochondrial $O_2^{\cdot}$ and H_2O_2 production due to the non-enzymatic mechanism of $O_2^{\cdot}$ generation at this site (Boveris and Chance 1973; Gnaiger *et al.* 1995). If one considers the mean physiological oxygen concentration to be approximately 40 mmHg (5% O_2), then estimates of mitochondrially-derived ROS production may be substantially inflated in cell lines cultured *in vitro* at 150 mmHg (Vaupel, Kallinowski and Okunieff 1989; Lawrence, Colinas and Walsh 1996).). Nohl and Hegner proposed that mitochondrial generation of $O_2^{\cdot}$ and H_2O_2 is likely to occur

under normoxic conditions *in vivo* based on the existence of mitochondrial MnSOD and the presence of lipid peroxidation products localized to the mitochondria (1978). Whether or not this is true in solid tumours is uncertain since "normoxic" pO_2 estimates are substantially higher than the pO_2 in "hypoxic" regions of solid tumours (pO_2 range 2-30 mmHg) that are irregularly perfused by disorganized vasculature (Vaupel, Kallinowski and Okunieff 1989; Kuppusamy *et al.* 1998; Movsas *et al.* 1999). An additional complication in solid tumours with irregular blood flow is the impact of ischemia/reperfusion injury as a stimulus for ROS generation (Parkins *et al.* 1997). It seems that the question of H_2O_2 as a regulator of proliferation *in vivo* can be addressed two ways: one is to assess ROS production *in vivo* in solid tumour models with the use electron paramagnetic resonance spectroscopy and the appropriate trapping agent, and the other more flexible route is to repeat *in vitro* experiments under more physiological pO_2 concentrations and/or conditions of hypoxia/reoxygenation.

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APPENDICES

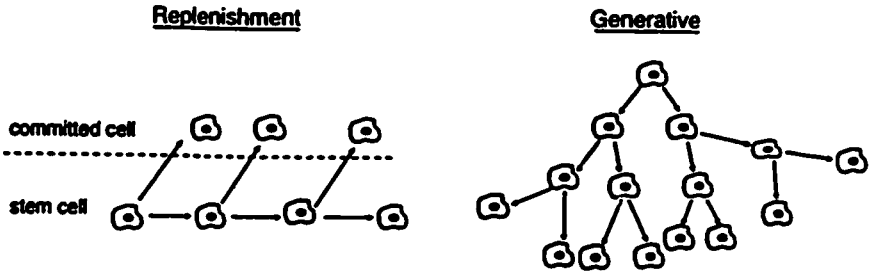
APPENDIX -1

Ovarian Carcinoma Staging Criteria of the International Federation of Gynecologists and Obstetricians (FIGO)

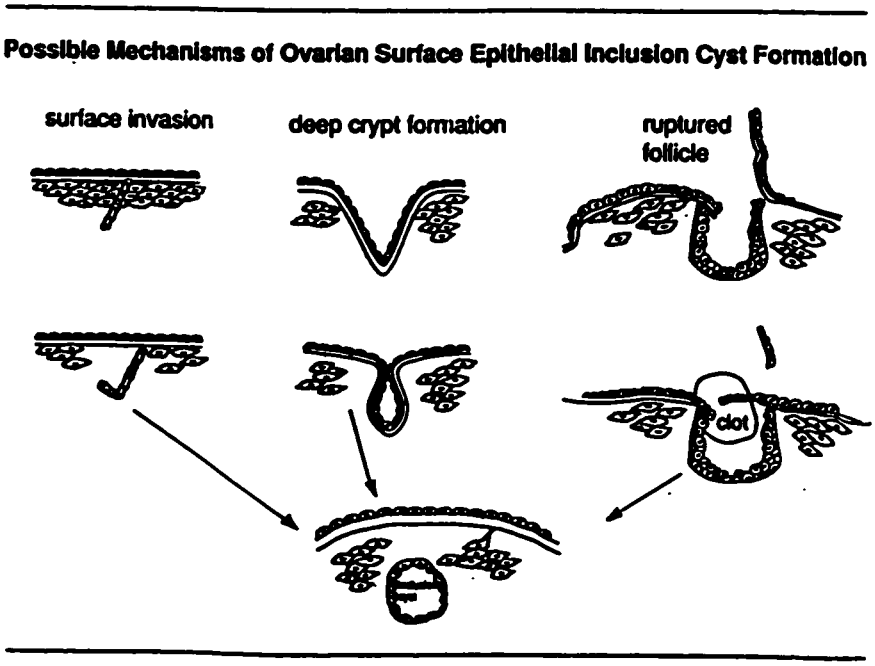
Stage I	Growth limited to the ovaries.
Stage Ia	Growth limited to one ovary; no ascites. No tumor on the external surface; capsule intact.
Stage Ib	Growth limited to both ovaries, no ascites. No tumor on the external surface; capsule intact.
Stage Ic	Tumor either stage Ia or Ib, but with tumor on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage II	Growth involving one or both ovaries with pelvic extension.
Stage IIa	Extension and/or metastases to the uterus and/or tubes.
Stage IIb	Extension to other pelvic tissues.
Stage IIc	Tumor either stage IIa or IIb, but with tumor on surface of one or both ovaries; or with capsule(s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage III	Tumor involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals stage III. Tumor is limited to the true pelvis but with histologically proven malignant extension to small bowel or omentum.
Stage IIIa	Tumor grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces.
Stage IIIb	Tumor involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes are negative.
Stage IIIc	Abdominal implants greater than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present there must be positive cytology to allow a case to stage IV. Parenchymal liver metastasis equals stage IV.

APPENDIX - 2

Ovarian Epithelial Cells Behave Like a Generative Stem Cell Population (Reprinted from Hamilton 1992)



Epithelial Cells of Inclusion Cysts (Reprinted from Hamilton 1992)



APPENDIX - 3

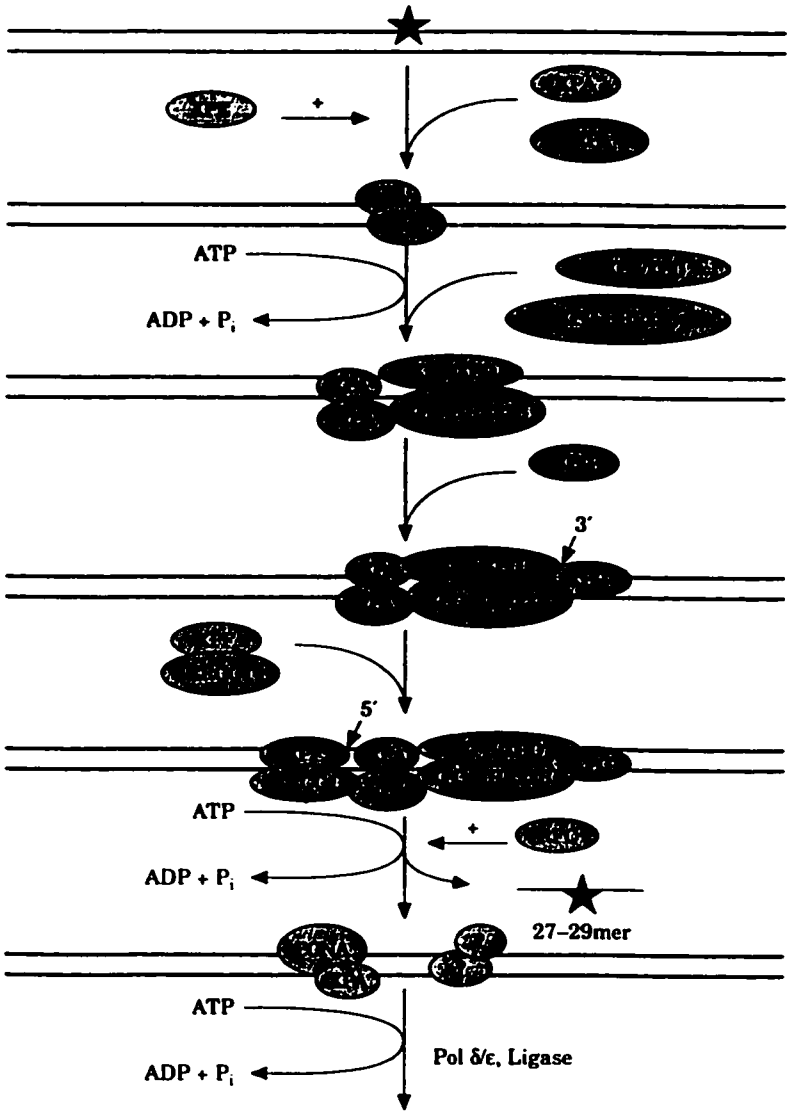
Five Year Survival Rates in Ovarian Cancer Patients Classified by Histologic Subtype, Tumour Grade and FIGO Stage

Tumour Characteristic		Frequency of Cases (%)	5-Year Survival Rate (%)
Histology	Serous	30-70	20-35
	Mucinous	5-20	40-60
	Endometrioid	10-20	40-60
	Clear Cell	3-10	35-50
	Unclassified	1	15-20
Grade	Well Differentiated	10	70-80
	Moderately Differentiated	25	30-45
	Poorly Differentiated	65	5-25
FIGO Stage	I	-	60-90
	II	-	37-66
	III	-	5-50
	IV	-	0-17

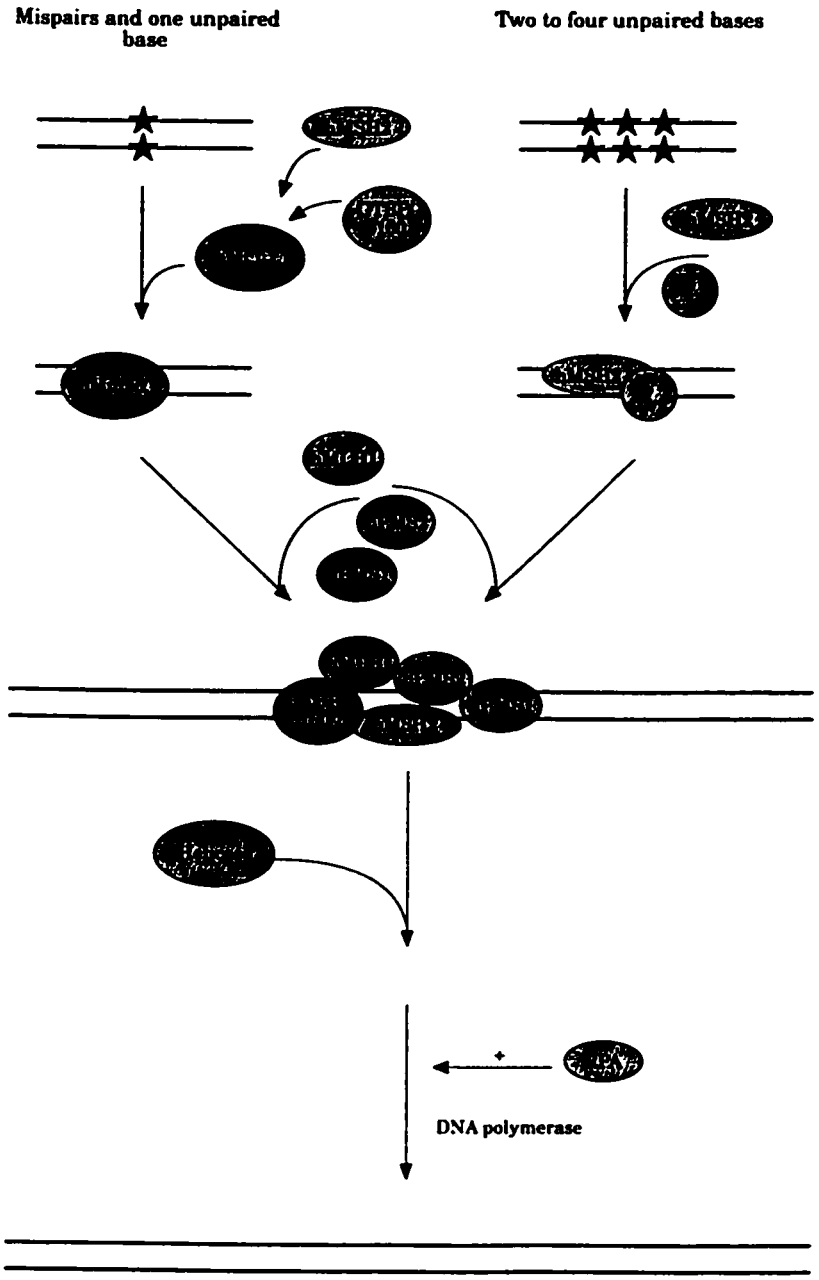
Data Summarized from (Friedlander 1998).

APPENDIX – 4

Steps Involved in the Nucleotide Excision Repair Pathway (Reprinted from Crul *et al.* 1997)



Steps Involved in DNA Mismatch Repair (Reprinted from Crul *et al.* 1997)



APPENDIX – 5

H_2O_2 Stimulates the Expression of Antioxidant and Stress-Response Genes

Cell Type	Endogneous/ Exogenous Oxidative Stress	Cell Response/ Gene Expression	Reference
Drosophila cells	Acute H_2O_2 Exposure	↑ heat shock protein expression (hsp 70, hsp 23)	Courgeon, *et al.* 1988
Hepatocytes (primary)	↑ Endogenous H_2O_2 production from mitochondria	↑ transcriptional activity of NFκB	García-Ruiz *et al.* 1995
HeLa cells Embryonic kidney (293) cells	Acute H_2O_2 Exposure	↑ transcriptional activity of NFκB	Wesselborg *et al.* 1997
HeLa cervical carcinoma cells	Acute H_2O_2 Exposure	↑ transcriptional activity of NFκB	Wang *et al.* 1998
U937 pre-monocytic cells	Acute H_2O_2 Exposure	Nuclear translocation of the heat-shock factor-1 transcription factor	Jacquier-Sarlin and Polla 1996
H441-4 pulmonary adenocarcinoma Cells	Acute H_2O_2 Exposure	↑ *MnSOD* mRNA	Warner, *et al.* 1996
AG8 H_2O_2-resistant fibroblasts (CHO-derived)	Continuous H_2O_2 Treatment (resistance generation)	↑ catalase and GSH reductase activity	Cantoni *et al.* 1996
HA-1 fibroblasts (CHO-derived)	Acute H_2O_2 Exposure	↑ *adapt15* mRNA ↑ *gadd45, gadd 153*mRNA	Crawford, Schools and Davies 1996
OC14 H_2O_2-resistant fibroblasts (HA-1derived)	Continuous H_2O_2 Treatment (resistance generation) + acute exposure	↑ catalase, GST, GPX, MnSOD and Cu/ZnSOD activity, ↑GSH ↑*gadd153, c-jun,HO-1* mRNA	Spitz *et al.* 1993; Guyton, Spitz and Holbrook 1996b
HeLa carcinoma SV40-transformed lung fibroblasts	Acute H_2O_2 Exposure	↑*gadd153*mRNA	Guyton, Xu and Holbrook 1996c
Macrophages (NR8383, alveolar)	Acute H_2O_2 Exposure	↑ *gadd45* and *gadd 153*mRNA	Patton, Paciga and Shelley 1997
U937 pre-monocytic cells	↑ Endogenous H_2O_2 production from mitochondria	↑ *GPX and HO-1* mRNA ↑ GPX activity	Brambilla *et al.* 1997
Hep3B liver cells	↑ Endogenous H_2O_2 production from mitochondria	↑ transcriptional activity of hypoxia-inducible factor-1 (HIF-1)	Chandel *et al.* 1998

APPENDIX - 6

H_2O_2 is Implicated as a Regulator of Proliferation in Numerous Cell Types

Cell Type	Exogenous/ Endogenous H_2O_2	Result	Reference
Human skin fibroblasts	Endogenous O_2^- and H_2O_2	SOD or Catalase Inhibit Thymidine Incorporation	Murrell, Francis and Bromley 1990
Hamster embryo fibroblasts	Exogenous (paraquat stimulated O_2^-)	Increased colony-formation following low level stimulation	Nicotera *et al.* 1994
Pancreas cells (RINm5F)	Exogenous (quinone- recycling stimulated)	Stimulation of thymidine incorporation	Dypbukt *et al.* 1994
Rat vascular smooth muscle cells	Endogenous H_2O_2 (PDGF stimulated)	Increased intracellular catalase inhibits DNA synthesis	Sundaresan *et al.* 1995
Hamster kidney fibroblasts (BHK-21)	Exogenous H_2O_2	Increased cell number	Burdon, Gill and Alliangana 1996
Human lymphoblastic leukemia cells (CCRF-CEM)	Exogenous H_2O_2 (direct or adriamycin stimulated)	Increased cell number	Yang *et al.*1996
Human prostate cancer cell spheroids (DU-145)	Exogenous H_2O_2	Increased cell number	Sauer *et al.* 1997
NIH 3T3 fibroblasts (Ras transfected)	Endogenous O_2^- and/or H_2O_2 (constitutive)	Increased intracellular catalase inhibits DNA synthesis	Irani *et al.*1997
Rat vascular smooth muscle cells	Endogenous O_2^- and H_2O_2 (Angiotensin II stimulated)	Increased intracellular catalase inhibits proliferation	Zafari *et al.* 1998