ERCC1 EXPRESSION AND RAD51B ACTIVITY CORRELATE WITH CELL CYCLE RESPONSE TO PLATINUM DRUG TREATMENT NOT DNA REPAIR

Britta Stordal and Ross Davey*

Bill Walsh Cancer Research Laboratories, Royal North Shore Hospital and The University of Sydney, St Leonards NSW 2065 AUSTRALIA.

*** Correspondence:**

Adjunct Prof Ross Davey
Bill Walsh Cancer Research Laboratories
Royal North Shore Hospital
St Leonards NSW 2065
AUSTRALIA

Tel: +61-2-9926-7456
Fax: +61-2-9926-5253
email: rdavey@med.usyd.edu.au

Abstract

Background: The H69CIS200 and H69OX400 cell lines are novel models of low-level platinum-drug resistance. Resistance was not associated with increased cellular glutathione or decreased accumulation of platinum, rather the resistant cell lines have a cell cycle alteration allowing them to rapidly proliferate post drug treatment.
Results: A decrease in ERCC1 protein expression and an increase in RAD51B foci activity was observed in association with the platinum induced cell cycle arrest but these changes did not correlate with resistance or altered DNA repair capacity. The H69 cells and resistant cell lines have a p53 mutation and consequently decrease expression of p21 in response to platinum drug treatment, promoting progression of the cell cycle instead of increasing p21 to maintain the arrest.
Conclusion: Decreased ERCC1 protein and increased RAD51B foci may in part be mediating the maintenance of the cell cycle arrest in the sensitive cells. Resistance in the H69CIS200 and H69OX400 cells may therefore involve the regulation of ERCC1 and RAD51B independent of their roles in DNA repair. The novel mechanism of platinum resistance in the H69CIS200 and H69OX400 cells demonstrates the multifactorial nature of platinum resistance which can occur independently of alterations in DNA repair capacity and changes in ERCC1.

Keywords: Cisplatin, Oxaliplatin, Resistance, Cell Cycle, DNA Repair, ERCC1, RAD51B, p21, Small Cell Lung Cancer

Introduction

The chemotherapeutic drugs cisplatin and oxaliplatin cause cytotoxicity by covalently binding to DNA forming adducts which hinder both RNA transcription and DNA replication. DNA damage normally causes a coordinated cellular response which involves arrest of the cell cycle to accommodate DNA repair, followed either by resumption of the cell cycle or apoptosis depending on the success or failure of the DNA repair process respectively. This coordinated response protects against mutations and therefore maintains genomic stability.

The H69CIS200 cisplatin-resistant and H69OX400 oxaliplatin-resistant small cell lung cancer cell lines are novel models of low-level platinum resistance [1]. The H69CIS200 and H69OX400 cell lines were developed from parental H69 small cell lung cancer cells with eight 4-day treatments of 200 ng/ml cisplatin or 400 ng/ml oxaliplatin respectively. These cell lines are approximately 2-fold resistant to cisplatin and oxaliplatin and are cross resistant to both drugs. The resistance is not associated with increased cellular glutathione or decreased accumulation of platinum which are common mechanisms of platinum resistance. The H69 platinum sensitive cells enter a lengthy 3 week growth arrest in response to low-level cisplatin and oxaliplatin treatment. This is an example of the coordinated response between the cell cycle and DNA repair. In contrast the H69CIS200 and H69OX400 cells have an alteration in the cell cycle allowing them to rapidly proliferate post drug treatment. The resistant cell lines also have many chromosomal rearrangements most of which are not associated with the resistant phenotype, suggesting an increase in genomic instability in the resistant cell lines [2]. We hypothesised that there was a deregulation between the cell cycle and DNA repair in the resistant cell lines allowing proliferation in the presence of DNA damage which has created an increase in genomic instability. Here we investigate the DNA repair processes involved in this deregulation and their role in permitting cell cycle progression in the platinum-resistant cell lines.

Methods

Cell Culture

The human H69 small cell lung cancer cell line was obtained from the American Type Culture Collection (Virginia, USA). The H69CIS200 and H69OX400 cells were developed over 8 months with eight 4-day treatments of 200 ng/ml cisplatin and 400 ng/ml oxaliplatin respectively [1]. There was no change in growth rate or morphology associated with the resistance. All cells and sublines were maintained in drug and antibiotic-free RPMI (Thermoelectron, Sydney, Australia) with 10% FCS in a humidified atmosphere with 5% CO_2 at 37°C. The cultures were tested regularly and were *mycoplasma* free. Flow cytometry cell cycle analysis and MTT cytotoxicity assays were performed as previously described [1].

Real-Time PCR

Total RNA was extracted and purified for real-time PCR using the Atlas pure total RNA labelling system (BD Biosciences). 2 µg total RNA was converted to cDNA using Bioscript RNase H Minus (Bioline, Sydney, Australia). Primers were designed with Primer 3 [3] with the following parameters, optimum Tm of 60°C, and optimum amplicon length of 120 bases and are presented in Table 1. Primers were Guaranteed Oligos™ synthesised by Sigma-Proligo (Lismore, New South Wales, Australia). The general 25 µl reaction mix for real-time PCR was as follows:- 12.5 µl 2X Immomix (Bioline), 0.75 µl Forward primer 10 µM, 0.75 µl Reverse primer 10 µM, 1.2 µl Sybr-Green 10X stock (Invitrogen, Melbourne, Australia), 1 µl cDNA and 8.8 µl sterile H_2O. The real-time PCR reaction was carried out on a Rotor Gene real-time PCR machine (Corbett Research, Sydney, Australia). FAM-Sybr Green was detected during the 72°C extension step of each cycle and a melt curve was performed at the end of the run to confirm the amplification of a single product. The cycling conditions were as follows:- Step 1 - 95°C 10 minutes, Step 2 - 95°C 20 seconds, 60°C 20 seconds, 72°C 20 seconds (40 cycles). The real-time PCR reactions were analysed with Rotor Gene 6 software (Corbett Research). A β-actin standard curve using H69 control cDNA serially diluted from 1:10 to 1:10000 was performed in each

real-time PCR run. The reaction rate of each primer set was the same as the β-actin primer set. A Ct value was calculated by Rotor Gene 6 for each unknown sample and standard and relative expression of each unknown sample was interpolated from the β-actin standard curve in each run.

Western Blotting

Cells were washed in cold PBS (0.15M NaCl, 0.03M NaH_2PO_4, 0.07M Na_2HPO_4 , pH 7.2), and resuspended in 100µl of lysis buffer (0.01M Tris/HCl, pH 7.4) at 4°C. 10µl complete protease inhibitor (Roche, Sydney, Australia) was added prior to sonnication. 20 µg protein was then electrophoresed and Western blotted as previously described [4], with the following modifications. A 12% acrylamide Tris/glycine gels with a 4% stacking gel was used and Biorad broad range markers were used as indicated (Biorad, Sydney, Australia). The blots were then stained with ponceau-s-red solution (Sigma, Sydney, Australia) to check the protein had transferred properly and to enable quantitation of loading in each lane. The primary antibodies and dilutions used were ERCC1 1:200 (ERCC1 Ab-1(3H11) mAb from Labvision via DKSH, Melbourne, Australia), RAD51B 1:1000 (RAD51B Antibody [1H3/13] from Abcam via Sapphire Biosciences, Sydney, Australia), p21 1:1000 ($p21^{WAF1}$ Ab-11 (Clone CP74) from Labvision via DKSH, Melbourne, Australia) and phospho-H2AX(Ser 139) (Cell Signalling Technology via Genesearch, Brisbane, Australia). The secondary antibody was alkaline phosphatase (Chemicon, Melbourne, Australia) or HRP conjugated (Santa Cruz Biotechnology via Monarch Medical, Brisbane, Australia) mouse immunoglobin diluted 1: 500.

RAD51B Immunocytochemistry

1.25×10^5 cells in 100 µl PBS were cytospun onto Superfrost® Plus slides (Menzel-Glasier via Lomb Scientific, Sydney, Australia.) using reusable Shandon cytospin funnels and disposable filter cards (Thermoscientific, Melbourne, Australia). The slides were air dried and cells were then fixed by incubating the slides in 100% ice cold methanol for 5 minutes. The slides were air dried and stored at –20°C prior to analysis. The slides were incubated with a serum free protein block (Dako, Sydney, Australia.) for 10 minutes at room temperature in a humidified atmosphere. All

further incubations were also at room temperature in a humidified atmosphere. The blocking solution was tapped off and a 1: 100 dilution of RAD51B primary antibody (Abcam clone 1H3/13) was added in antibody diluent (Dako) and incubated for 2 hours. The slides were then washed in D-PBS for 5 minutes. A 1: 300 dilution of FITC conjugated anti-mouse secondary antibody in antibody diluent was then added and incubated for 1 hour in the dark. The slides were then washed in D-PBS for 5 minutes. Slides were incubated with a DAPI counterstain (1: 50 of 5 mg/ml stock solution in D-PBS) for 5 minutes in the dark and then again washed in D-PBS for 5 minutes. Slides were air dried and then coverslipped using PermaFlour^{TM} Aqueous Mounting Medium (Thermoelectron). Slides were photographed at x 60 magnification using a Nikon Eclipse 80i Microscope. Two photographs were taken of each region of interest, one FITC image for the stained primary antibody of interest and one DAPI image for the nuclei.

Platinated Plasmid DNA Repair Assay

DNA repair was examined using a platinated β-galactosidase reporter plasmid transfected into cells and then β-galactosidase activity was detected in cell lysates using an enzymatic assay. Similar assays have been used in the literature using platinated luciferase [5] and Xgal [6] reporter plasmids. The pEF-Bos-β-galactosidase plasmid (a gift from Cancer Genetics, Kolling Institute, Royal North Shore Hospital) was purified from JM109 cells grown in Luria broth in the presence of 0.1 mg/ml ampicillin, using a Qiagen (Melbourne, Australia) Endofree Plasmid Maxi Kit according to the manufacturers instructions. The purified plasmids (0.1 mg/ml in TE buffer) were incubated in the dark at 37°C with different concentrations of cisplatin. The reaction was stopped by adding NaCl to a final concentration of 0.5M. Plasmid DNA was precipitated with 2 volumes of 100% ethanol at –70°C in the presence of 2.5M ammonium acetate, washed in 70% ethanol, dried and dissolved in TE buffer.

3.25 x 10^5 H69, H69CIS200 or H69OX400 cells were resuspended in 400 µl RPMI with serum in a 24 well plate. All transfections were performed in triplicate. 0.2µg plasmid DNA (Control, 1% or 2% platinated) was diluted in 25 µl Qiagen Buffer EC then 1.6 µl Qiagen Enhancer was added and vortexed for 1 second and incubated for 5

minutes at room temperature. 10 µl Qiagen Effectene transfection reagent was then added and incubated for 10 minutes at room temperature. 225 µl RPMI with serum and antibiotics was then added to the transfection mix which was then added dropwise to the cells. Cells were harvested for the β-galactosidase assay at 24 hours after transfection.

Transfected H69 cells were sonnicated for 20 pulses in 100 µl 1X Reporter Assay Lysis Buffer (Promega, Sydney Australia). 40µl of lysate was added in duplicate to a 96 well plate, 150µl of Assay Buffer (8.8 ml Phosphate Buffer (0.618g Na_2HPO_4 anhydrous salt, 0.623g $NaH_2PO_4.2H_2O$ adjusted to pH 7.3 made up to 100ml with deionised H_2O and frozen in aliquots at –20°C), 900 µl 100 mM $MgCl_2$ and 70 µl Mercaptoethanol made fresh for each assay.) and 50µl of CPRG (1.5 mg/ml, Roche Applied Sciences, Sydney, Australia) was added to each well. The plate was then incubated at 37°C for 6 hours until an orange coloured product was seen. Absorbencies were then read at 595 nm.

Analysis and Statistics

Changes in mRNA and protein expression were determined relative to the untreated H69 control. Means and standard deviations are presented in the figures. Significant differences from the H69 control were determined on the raw data using a two tailed student's t-test assuming the samples were of equal variance.

Results

Growth and Cell Cycle Characteristics of the Platinum Resistant Cell Lines

There was no change in growth rate of the H69CIS200 and H69OX400 resistant cell lines compared to the parental H69 cell line (Fig. 1). Fig. 1 also shows the cell viability of the H69, H69CIS200 and H69OX400 cells after a 4-day 200 ng/ml cisplatin or 400 ng/ml oxaliplatin drug treatment, the same doses of drug and length of treatment used in development of the resistant cell lines. All cell lines show decreased growth with treatment. However, the two resistant cell lines have significant growth advantage over the parental cells in response to both agents ($p < 0.01$ t-test).

The cell cycle profile of each cell line at the end of the 4-day platinum drug treatments better demonstrates the difference between the sensitive and resistant cells. Fig. 2A shows the cell cycle profiles of the untreated sensitive and resistant cells were all the same and typical of cells in log-phase growth. After 4 days of treatment with cisplatin or oxaliplatin, the H69 sensitive cells were in a G_2M arrest with increased numbers in sub-G_0 and G_2M, decreased numbers in G_1 and no change in S phase (Fig. 2B). The cisplatin treated H69CIS200 cells were also arrested showing similar but not as extensive cell cycle changes as the H69 cells (Fig. 2B) while in contrast, the H69OX400 cells did not arrest following oxaliplatin treatment but showed a profile similar to log-phase growth (Fig. 2C). The major difference between the resistant and sensitive cell lines is their rate of growth recovery post platinum drug treatment. Fig. 2D shows the effect of treatment on the time to double cell numbers. The dotted line at 4 days indicates the timepoint where drug was removed. For the treated H69 cells the arrest lasted for 3 weeks compared to the oxaliplatin-treated H69OX400 cells that doubled in 5 days ($p < 0.001$ t-test) and the cisplatin-treated H69CIS200 cells showed an intermediate recovery time of 10 days ($p < 0.05$ t-test).

The ability of cells to enter a protective cell cycle arrest and then proliferate later is known as regrowth resistance [1,7]. This kind of resistance is likely to be underestimated by short term growth curves and toxicity assays. The resistant cells have a greater potential for growth recovery after the toxic agent is removed as can be

seen from the cell cycle profile of the H69OX400 cells in Fig. 2C and the time to doubling data in Fig. 2D.

p21

$p21^{WAF1/CIP1}$ inhibits cell cycle progression at the G_1/S checkpoint by binding to and inhibiting the S-phase promoting Cdk2-CyclinE and Cdk4-CyclinD complexes [8]. We examined the protein expression of p21 by Western blot at the end of the four day platinum drug treatment (Fig. 3A and B). p21 appeared as two distinct bands the upper is the phosphorylated form of the protein [9]. The expression of the upper band significantly decreases in response to platinum drug treatment in the H69 cells relative to untreated cells (Fig. 3A). The upper band was also decreased in both untreated resistant cell lines. The expression of the lower p21 band shows less variability, however it was significantly decreased in the untreated H69OX400 cells compared to the H69 control (Fig. 3B). This decrease in the lower p21 band in the H69OX400 cells was partially reversed by platinum drug treatment but not fully to the level of untreated H69 cells.

DNA Repair

ERCC1

ERCC1 is involved in the nucleotide excision repair removal of platinum adducts cutting on the 5' side of the damaged DNA to be excised and replaced. ERCC1 is one of many rate limiting proteins involved in the damage recognition and excision process [10]. Increases in the expression of ERCC1 have been previously shown in response to cisplatin treatment and in cisplatin resistant cell models [10]. In contrast, we observed decreases in ERCC1 mRNA and protein expression (Fig. 4A and B). The decreases in ERCC1 protein expression were associated with the formation of a lower molecular weight band of approximately 26 kDa (marked with arrow Fig. 4B). We believe this to be the alternative spliced variant of ERCC1 which is missing exon 8 and has been associated with decreased repair activity [11]. When the changes in ERCC1 mRNA and protein expression were analysed in reference to the cell cycle (Fig. 4C and D), the samples in cell cycle arrest (grey background) had a significant

decrease in mRNA and protein expression compared to the untreated control cells. This suggests that ERCC1 expression is more related to the cell cycle state of all cell lines than the resistant phenotype. The samples in cell cycle recovery were not significantly different from the untreated control cells but had lower levels of mRNA and protein suggesting that part of restoring normal cell cycle activity was associated with restoring normal ERCC1 levels.

RAD51B

Homologous recombination repair is in part mediated by the RAD51 proteins [8]. A downregulation of RAD51-mediated homologous recombination repair in knockout chicken B lymphocyte DT40 cells resulted in sensitivity to cisplatin treatment [12,13,14]. Therefore an increase in homologous recombination could mediate platinum resistance by increasing the repair of platinum induced double-strand DNA breaks. We chose to examine RAD51B as it has been linked to cell cycle control as well as DNA repair [15]. We observed some increases in RAD51B mRNA (Fig. 5A) but no change in protein expression (data not shown). In response to DNA damage RAD51 becomes concentrated in multiple discrete foci. These are thought to represent nuclear domains for homologous recombination repair [16]. The morphology of RAD51 can therefore indicate if it is actively repairing DNA. RAD51B foci were examined by immunocytochemistry in the H69, H69CIS200 and H69OX400 cell lines (Fig. 5B). The number of cells positive for RAD51B foci was counted for 6 fields of view under the microscope, analysing in total around 400 cells per slide. Cells were deemed positive for RAD51B foci if they had greater than 5 foci in their nuclei, this criteria has been used in other RAD51 studies [17]. The parental H69 cells had higher levels of RAD51B foci in response to oxaliplatin drug treatment than the H69OX400 cells (Fig. 5C). This is the opposite of what would be expected, since the resistant cells would be expected to have a higher level of repair than the sensitive parental cells.

When the changes in RAD51B mRNA and foci were analysed in reference to the cell cycle state of the sample a pattern emerges (Fig. 5D and E). Both the RAD51B mRNA and activity were increased significantly in the arrested cells compared to the non-arrested controls, suggesting that its expression and activity are related more to

the cell cycle than to platinum resistance. The samples in cell cycle recovery had no change in RAD51B foci from the untreated cells suggesting that part of restoring normal cell cycle activity was restoring normal RAD51B foci activity.

DNA Repair Activity

The analysis of in vitro DNA repair activity is a compromise at best; some studies determine activity from whole cell extracts or nuclear extracts which may not accurately reflect repair in intact live cells. We have chosen two methods of determining DNA repair activity in intact cells, the phosphorylation of γH2AX and the repair of a transfected platinated plasmid. The expression of phospho-γH2AX is a marker of the early steps of DNA repair, particularly that of homologous recombination of double strand breaks. Phospho-γH2AX is a marker of the detection of these double strand breaks by the cell [18] and not necessarily successful DNA repair. However, cell lines with repair defects have been found to be deficient in phospho-γH2AX [19]. The repair of platinated reporter plasmids best corresponds to nucleotide excision repair, however the transfected plasmid is likely to be in the cytoplasm of the cell rather than the nucleus. Resistant cells with increases in DNA repair [5] or defects in DNA repair [6] have been detected by this method. By using these two methods we have examined the DNA repair pathways in which ERCC1 and RAD51B participate.

Phospho-γH2AX was examined by Western blot in H69, H69CIS200 and H69OX400 cells which had been drug treated for 4 days with either 200 ng/ml cisplatin or 400 ng/ml oxaliplatin. However, phospho-γH2AX was undetectable at this time point (data not shown). The phosphorylation of γH2AX is an early event in DNA damage detection and repair [18], therefore H69, H69CIS200 and H69OX400 cells were treated with 200 ng/ml or 5 μg/ml cisplatin for 24 hours and examined for γH2AX phosphorylation by Western blot (Fig. 6A and B). Cisplatin treatment at 200 ng/ml induced the same amount of γH2AX phosphorylation in all cells. Cisplatin treatment at 5 μg/ml induced a higher amount of γH2AX phosphorylation in the resistant cell lines but this result was more variable and was not statistically significant. The higher dose of 5 μg/ml cisplatin is also above the clinically relevant doses used in the rest of

this study. These results suggest that there was no difference in the detection of DNA damage between the sensitive and resistant cell lines as measured by phospho-γH2AX.

β-galactosidase plasmids were platinated with cisplatin at two different doses, designed to place adducts on 1% and 2% of bases within the plasmid. These were then transfected into the H69, H69CIS200 and H69OX400 cells and β-galactosidase activity compared to an unplatinated control plasmid. β-galactosidase activity was measured at 24 hours post transfection, this time point allowed sufficient time for β-galactosidase to be expressed in all samples. Fig. 6C shows that there was no increase in DNA repair of the platinated plasmids in the resistant cell lines compared to the parental sensitive cells. There was a small decrease in DNA repair in the H69OX400 cells, however due to slight differences in transfection efficiency between cell lines this was not statistically significant. These results suggest that there is no difference in DNA repair between the sensitive and resistant cell lines as measured by the repair of platinated plasmids.

The H69, H69CIS200 and H69OX400 cells were examined for their response to ionising radiation as cells with increased DNA repair capacity are often radiation resistant. The H69CIS200 cells are not radiation resistant as measured by this 5-day MTT assay (Fig. 6D). The H69OX400 cells were 2.68 fold resistant to radiation compared to the parental cells ($p < 0.05$ t-test).

Discussion

The H69 parental cells enter a lengthy three week growth arrest in response to cisplatin or oxaliplatin drug treatment (Fig. 2D). Resistance in the H69CIS200 and H69OX400 cells is associated with a more rapid recovery from this growth arrest. Increased expression of p21 causes the cell to arrest at the G_1S checkpoint of the cell cycle [8], and has also been found to enhance the cytotoxic effect of cisplatin [20,21]. Therefore a decrease in the expression of p21 as observed in response to platinum treatment in both the sensitive H69 cells and in the resistant cell lines (Fig. 3) could promote platinum resistance by reducing the cytotoxic effect of the drug and enabling the cell to progress through the cell cycle. The H69 cells have a mutation in p53 [22].

Decreasing the expression of p21 or no induction is a known response of mutant p53 cells to cisplatin [23] or oxaliplatin treatment [24]. Therefore it is unlikely that p21 is causing the platinum induced cell cycle arrest in the H69 cells. The H69 cells and resistant cell lines also have an amplification of the c-myc gene on chromosome 8 [2] which would increase their ability to cycle after DNA damage [25] However, there was no increase in this amplification in the resistant cell lines compared to the sensitive parental cell line [2].

A similar pattern of growth arrest and recovery was observed in the development of cisplatin-resistant IGROV1 ovarian carcinoma cells [26] as in the H69CIS200 and H69OX400 cell lines. Development of resistance to cisplatin in IGROV1 cells was also associated with the ability of the treated cells to progress through the cell cycle beyond the G1/S checkpoint [26]. We have characterised this type of resistance as "regrowth resistance" where the cells arrest and then rapidly proliferate in response to a previously cytotoxic dose of drug [1]. This type of resistance is not related to the p53 status of the cell as the IGROV1 cells are wild-type p53 and increase p21 expression in response to cisplatin drug treatment. The similarity of phenotype is rather linked to the low, clinically relevant dose of drug used in development and the pulsed selection strategy where the cells are allowed to recover in drug free media between treatments.

No change in DNA repair capacity associated with platinum resistance

Although increased nucleotide excision repair can cause cisplatin resistance [10] it appears not to be responsible for the platinum resistance of the H69CIS200 and H69OX400 cell lines as these cells showed no increases in ERCC1 mRNA or protein expression (Fig. 4A and B) and no increase in repair in the platinated repair assay (Fig. 6C). An increase in homologous recombination is also not responsible for the platinum resistance as there was no increase in RAD51B expression or nuclear foci formation (Fig. 5) nor was the level of phospho-γH2AX increased in the resistant relative to the sensitive H69 cell line (Fig. 6A).

A decrease in mismatch repair has also been previously associated with cisplatin resistance, as the binding of the mismatch repair complex to Pt–DNA adducts appears

to increase the cytotoxicity of the adducts, either by activating apoptosis or by causing "futile cycling" during trans-lesion synthesis past Pt–DNA adducts [27]. Mismatch repair protein MSH2 was examined by Western blot and real time PCR and found not to have changed in the resistant cell lines (data not shown). The activity of oxaliplatin in some cisplatin-resistant cell lines is thought to be due to repair or damage recognition processes that discriminate between cisplatin and oxaliplatin adducts. This has been best established for mismatch repair, defects in mismatch repair increase resistance to cisplatin adducts, but have no effect on oxaliplatin adducts [27]. The H69CIS200 and H69OX400 cells are cross resistant to both cisplatin and oxaliplatin, this combined with no decrease of MSH2 suggests that there is no loss of mismatch repair mediating resistance to platinum.

The resistance of the H69CIS200 and H69OX400 cells is therefore unlikely to be the result of changes in the DNA repair pathways which have been previously associated with platinum resistance. This highlights the multifactorial nature of platinum resistance and therefore the difficulty in using DNA repair proteins as markers of platinum resistance in the clinic. Some trials have found an association between ERCC1 and response to cisplatin combination therapy [28-30], but many more have found no association [31-35]. High ERCC1 may correlate with platinum resistance, but low or absent ERCC1 may not always indicate sensitivity as the H69CIS200 and H69OX400 when actively dividing show no change in ERCC1 despite being platinum resistant.

Changes in ERCC1 and RAD51B are associated with cell cycle arrest

The changes in the DNA repair pathways are associated with the platinum induced cell cycle arrest rather than the resistant phenotype. There was a significant decrease in ERCC1 mRNA and protein expression (Fig. 4C and D) and increased RAD51B foci formation (Fig. 5E) associated with the samples in cell cycle arrest. The samples in cell cycle recovery have the same levels of expression of ERCC1 and RAD51B foci as untreated cells. This suggests that these DNA repair proteins are being modulated for reasons other than DNA repair and are potentially participating in the regrowth resistance mechanism of cell cycle arrest and recovery.

There is some evidence to suggest that ERCC1 and RAD51B could mediate a cell cycle arrest. Hepatocytes from ERCC1 knockout mice are arrested in the G_2 phase of the cell cycle [36]. The H69 cells enter a G_2 arrest in response to platinum drug treatment (Fig. 2) associated with a decrease in ERCC1 expression (Fig. 4). The expression of full length ERCC1 decreases in association with the cell cycle arrest, however this is associated with the formation of an ERCC1 splice variant which has been previously reported to have reduced DNA repair activity [11]. It is possible that this splice variant may have an increased role in the process of cell cycle arrest. Fibroblasts from ERCC1 knockout mice also show a decreased rate of cell growth and disruptions in cell cycle [37] suggesting that the decrease in ERCC1 may contribute to the lengthy growth arrest in the sensitive cells.

Transfection of RAD51B into CHO cells induces a cell cycle G_1 delay similar to what was observed in the H69 cells in response to platinum treatment [38]. The cell cycle arrest in both cases appears as a flattening of the G_1 peak. Transfection of RAD51 into human and rat fibroblasts also induces a G_1 arrest [16].

Confirmation of Mechanism of Cell Cycle Arrest by Transfection or RNAi ?

The next logical step in many research studies of this kind would be to increase the expression of RAD51B by transfection and/or decrease the expression of ERCC1 by RNAi or other methods. However, we believe that these experiments would not conclusively prove the mechanism of regrowth resistance which we have proposed.

The changes we have found are transient and associated with cell cycle arrest after platinum treatment, not permanent changes in the resistant cell lines. The resistance produced in this model is low level, and as such is likely to be comprised of many small changes of which we have only identified two in this study. Replicating this mechanism by altering the expression of two genes is very unlikely.

Increasing the expression of RAD51B would most likely lead to an increase in platinum resistance due to an increase in homologous recombination based DNA repair. This model shows no increase in DNA repair as the increase in RAD51B is transient during the cell cycle arrest. Decreasing the expression of ERCC1 would most likely lead to platinum sensitivity due to a downregulation of nucleotide excision repair as it did in response to ERCC1 siRNA in HeLa S3, MCF-7 and HCT116 cells in a recent study [39]. Again this is not a change observed in this model, and the alteration of ERCC1 may be post-translational modification of the protein rather than a decrease in expression.

Platinum Resistance and Checkpoint Adaptation

The H69CIS200 and H69OX400 cells do not use any of the well characterised mechanisms of platinum resistance such as increased intracellular glutathione, decreased cellular accumulation of drug [1] or increased DNA repair (Fig. 6). Rather, their resistance is dependent on a rapid cell cycle progression after drug treatment. The regrowth resistance arrest is the same in all cells, the resistant cell lines quickly exit this cell cycle arrest despite the presence of DNA damage and continue to cycle. Therefore the resistant cells have a decrease in DNA repair in response to platinum drug treatment, not because of a downregulation of a DNA repair pathway but because of the reduced time in cell cycle arrest where the repair occurs.

The cell cycle associated changes in DNA repair proteins may also be contributing to the genomic instability of the cells which will increase the mutagenic potential of the cells in response to further drug treatment. Decreases in ERCC1 [40,41] and increases in RAD51 [42] have also been associated with increased genomic instability which correlate with the large amount of chromosomal aberrations found in the resistant cell lines [2]

The normal exist from the cell cycle arrest after the successful completion of DNA repair is termed checkpoint recovery. Normal checkpoint recovery in the H69 parental cells is the 3 week growth arrest (Fig. 2D). Checkpoint adaptation is related to checkpoint recovery and promotes cell cycle re-entry even when unrepairable DNA damage is present [43]. Checkpoint adaptation has been well characterised in yeast cells but more recently has been shown to also occur in human cells in response to ionising radiation [44]. The H69CIS200 and H69OX400 cells appear to have the checkpoint adaptation phenotype, the cell cycle continuing despite the presence of DNA damage. The H69OX400 cells exit the cell cycle arrest faster than the H69CIS200 cells and this correlates with the greater amount of chromosomal aberrations in the H69OX400 cell line [2]. This suggests that resistance is largely dependent on the speed at which the cell cycle arrest can be overcome. The H69OX400 have the more 'aggressive' phenotype and this correlates with their cross resistance to ionising radiation (Fig. 6D). The MTT toxicity assay is a 5-day assay, it is likely that the H69CIS200 cells are more radiation resistant than the parental H69 cells, but at this time point they are both in cell cycle arrest and appear the same.

Conclusions

Resistance in the H69CIS200 and H69OX400 cells is not associated with an increase in DNA repair, rather it is associated with the speed of the recovery from the cell cycle arrest which may involve modulation of ERCC1 and RAD51B. These cell models highlight the multifactorial nature of platinum resistance and that clinical markers such as ERCC1 will not identify all types of platinum resistance.

Acknowledgements

The authors thank Nghia Le, Laura Castelnoble and David Campbell from Sutton Laboratories at Royal North Shore Hospital for advice on real-time PCR. Thanks to Amy Au and Inge Stewart from Cancer Genetics, Kolling Insitute at Royal North Shore for providing the β-galactosidase plasmid and advice on the β-galactosidase

assay. Thanks to Regina Bromley from Radiation Oncology at Royal North Shore Hospital for irradiating the cell lines.

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Table 1 – Real Time PCR primers

		Forward Primer				Reverse Primer				
Gene Name	**Accession**	**Pos**	**Tm**	**%GC**	**Sequence**	**Pos**	**Tm**	**%GC**	**Sequence**	**Amplicon**
ERCC1	NM001983	841	62.86	55	TCTCCCGGGTGACTGAATGT	970	60.93	55	GGGCATAAGGCCAGATCTTC	129
MSH2	NM000251	2271	60.66	50	ATCCTCAGGTCTGCAACCAA	2409	60.68	40	CAAACATGCAAAAAGCACCA	138
RAD51B	NM002875	1021	57.84	45	TCGCTGATGAGTTTGGTGTA	1143	60.15	40	ATGCATGGGCGATGATATTT	122
β Actin	NM001101	1642	59.8	45	TTGAATGATGAGCCTTCGTG	1771	58.93	52.2	CTGGTCTCAAGTCAGTGTACAGG	129

Pos – Position

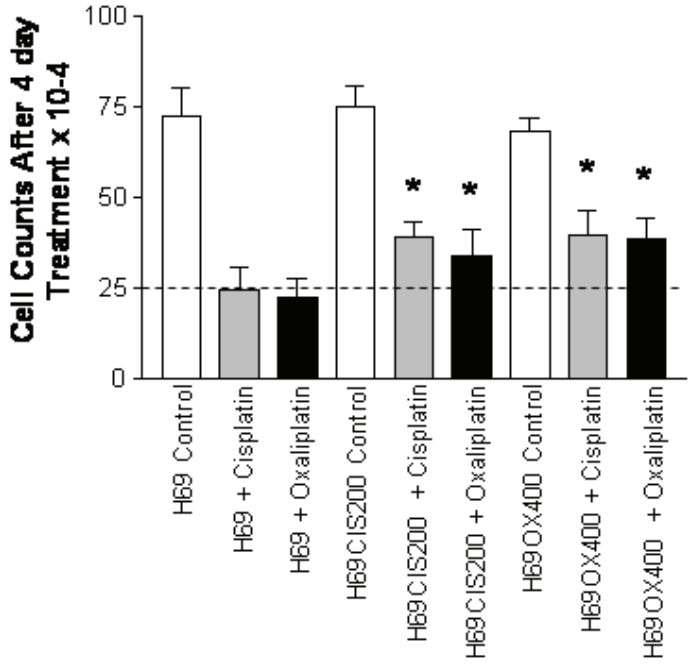


Fig. 1 – Effect of platinum drug treatment on cell growth. H69, H69CIS200 and H69OX400 cells were treated with either 200 ng/ml cisplatin, 400 ng/ml oxaliplatin or an untreated control for 4 days and viable cells were counted using trypan blue exclusion. The line at 25 indicates the starting density of the 4-day culture, 2.5 x 105 cells/ml. * indicates a significant increase in growth of the resistant cells from the sensitive cells.

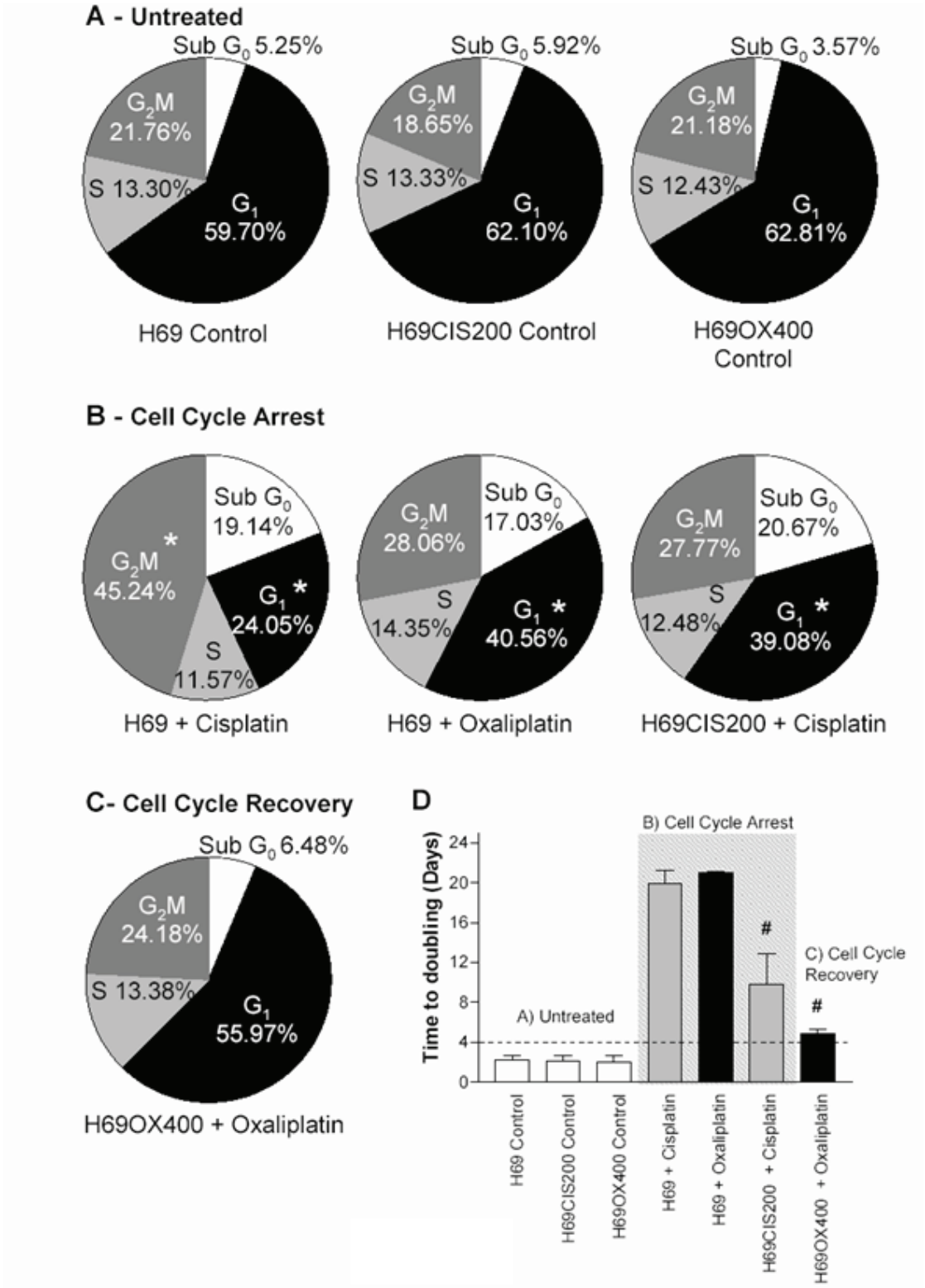


Fig. 2 – Effect of platinum drug treatment on cell cycle. H69, H69CIS200 and H69OX400 cells were treated with either 200 ng/ml cisplatin, 400 ng/ml oxaliplatin or an untreated control for 4 days. A) Untreated cells, B) samples in cell cycle arrest, C) samples in cell cycle recovery. * indicates a significant difference from the untreated control. D) time to doubling after a 4-day exposure to cisplatin or oxaliplatin determined by counting cells twice a week using trypan blue exclusion. The line at 4 days indicates the time point analysed in parts A, B and C. Samples in cell cycle arrest at 4-days are indicated with a grey background. # indicates a significant difference in recovery of the resistant cells from the sensitive cells.

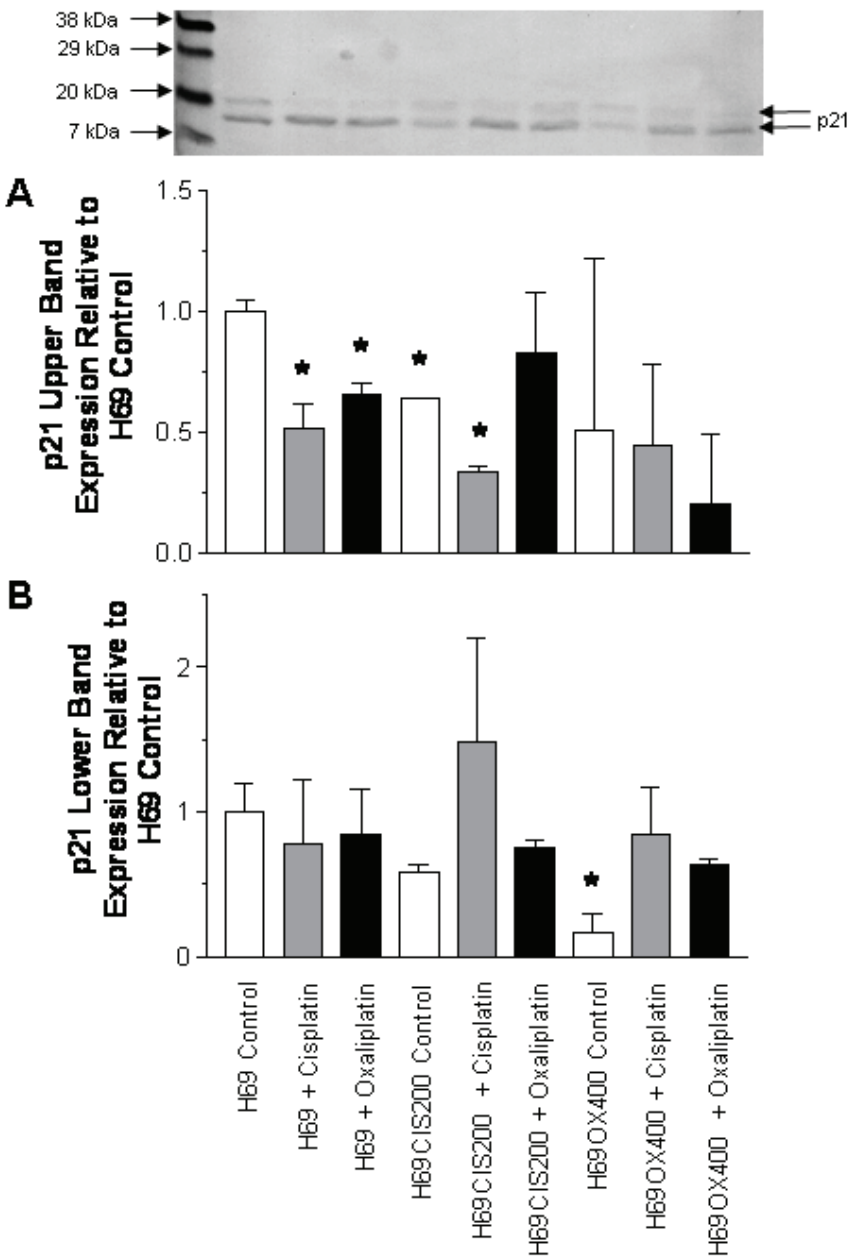


Fig. 3 – Analysis of p21 protein expression in H69, H69CIS200 and H69OX400 cells after a 4-day exposure either 200 ng/ml cisplatin or 400 ng/ml oxaliplatin. A) Expression of upper p21 band, B) Expression of lower p21 band. * indicates a significant difference in expression from the H69 control.

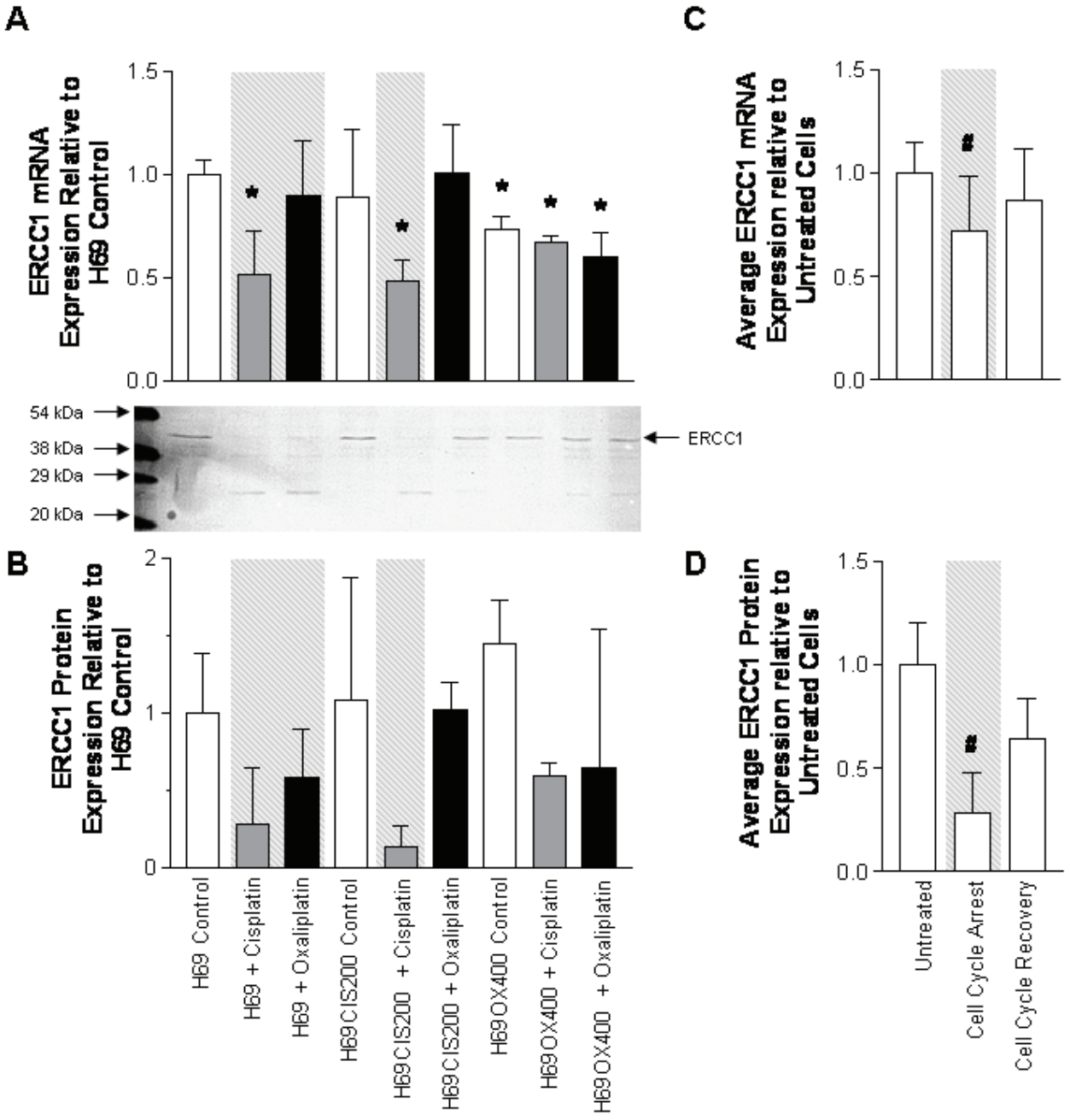


Fig. 4 – Analysis of ERCC1 mRNA and protein expression in H69, H69CIS200 and H69OX400 cells after a 4-day exposure either 200 ng/ml cisplatin or 400 ng/ml oxaliplatin. A) ERCC1 mRNA expression determined by real-time PCR, B) ERCC1 protein expression determined by Western Blot. Samples in cell cycle arrest are indicated with a grey background. * indicates a significant difference in expression from the H69 control. Analysis of ERCC1 C) mRNA and D) protein in reference to the cell cycle, means and standard deviations are presented from pooled data from parts A and B. Untreated is the control cells, cell cycle arrest is the drug treated samples in cell arrest indicated with grey background shading, cell cycle recovery is the drug treated cells not in cell cycle arrest. # indicates a significant difference in compared to the untreated samples.

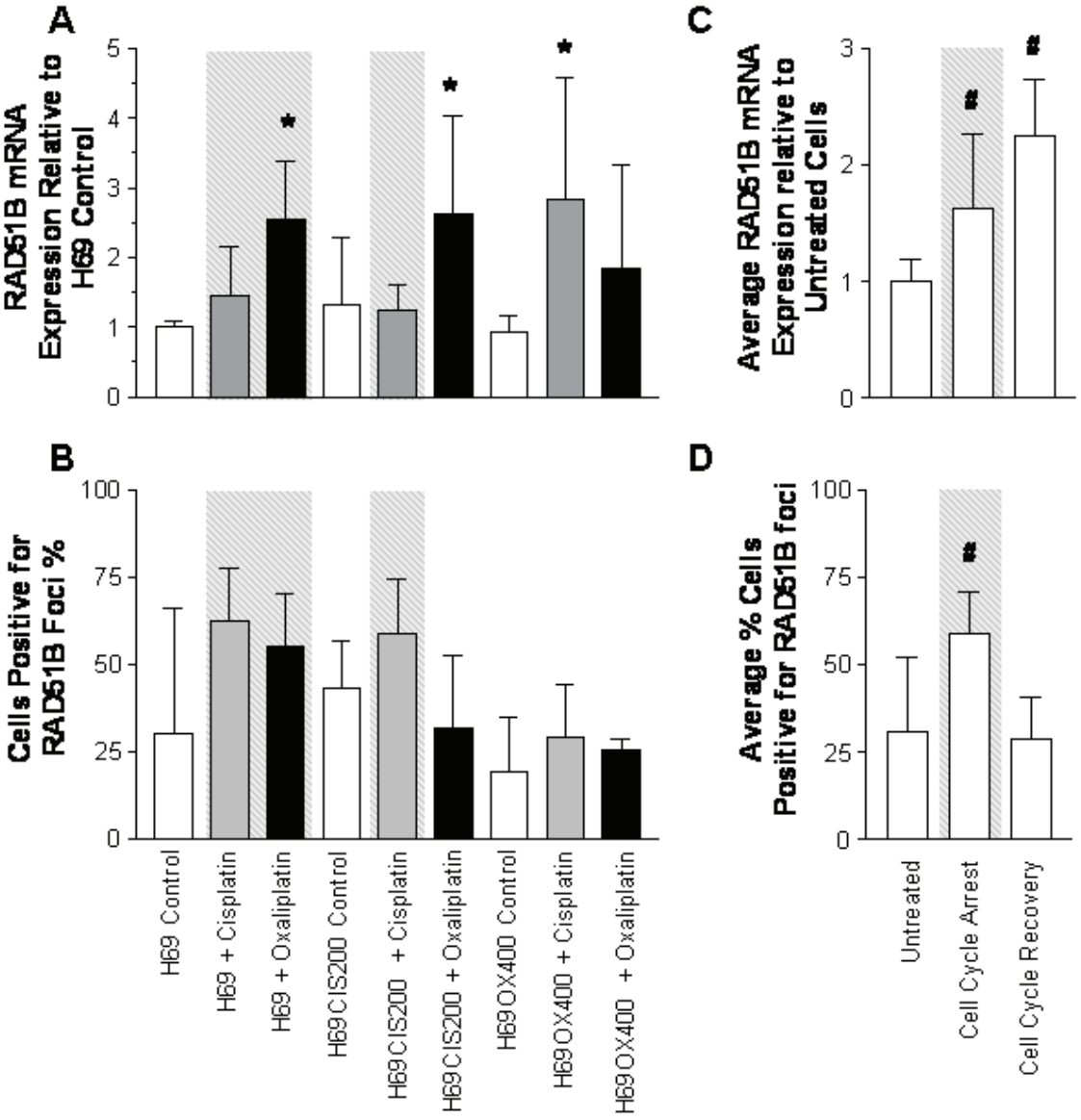
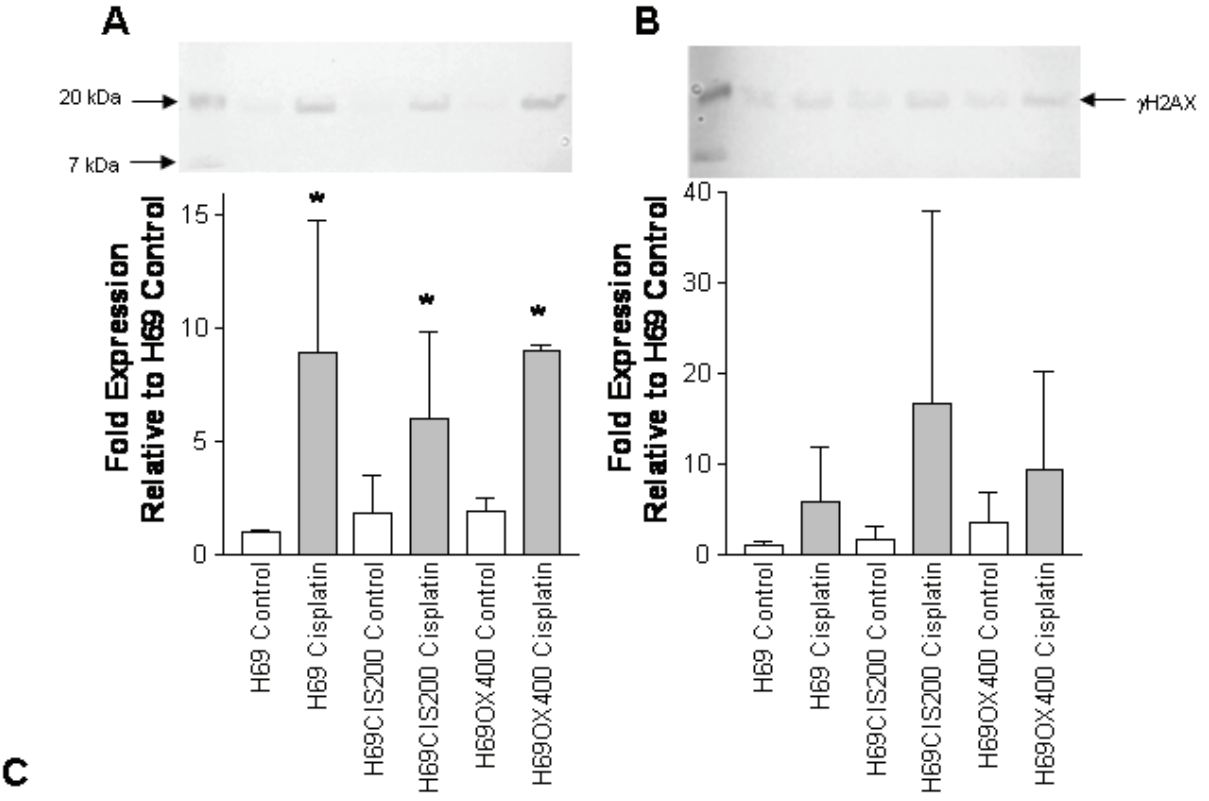
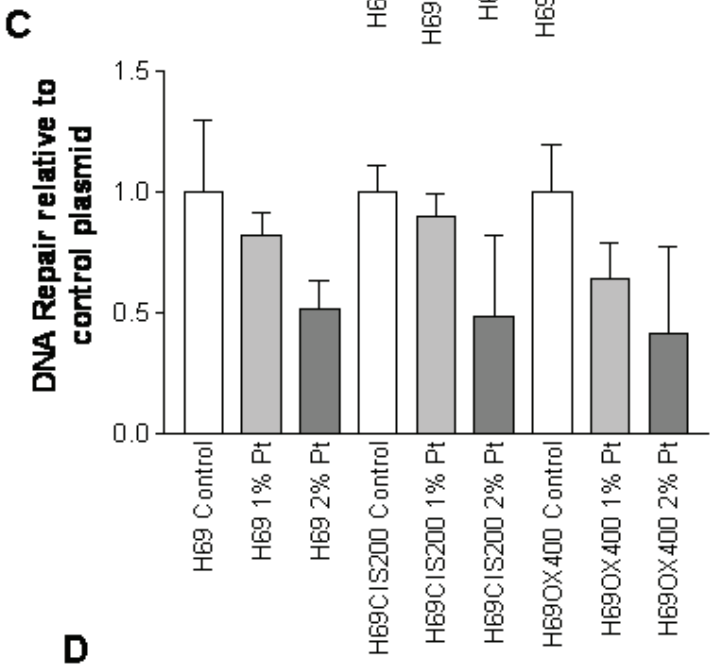


Fig. 5 – Analysis of RAD51B mRNA and foci expression in H69, H69CIS200 and H69OX400 cells after a 4-day exposure either 200 ng/ml cisplatin or 400 ng/ml oxaliplatin. A) RAD51B mRNA expression determined by real-time PCR, B) Percentage of RAD51B foci determined by immunocytochemistry. Samples in cell cycle arrest are indicated with a grey background. * indicates a significant difference in expression from the H69 control. C) Example of RAD51B foci in H69 and H69OX400 cells treated with oxaliplatin, left FITC panels RAD51B, right DAPI panels cell nuceli. Analysis of RAD51B D) mRNA and E) foci expression in reference to the cell cycle, means and standard deviations are presented from pooled data from parts A and B. Untreated is the control cells, cell cycle arrest is the drug treated samples in cell arrest indicated with grey background shading, cell cycle recovery is the drug treated cells not in cell cycle arrest. # indicates a significant difference in compared to the untreated samples.





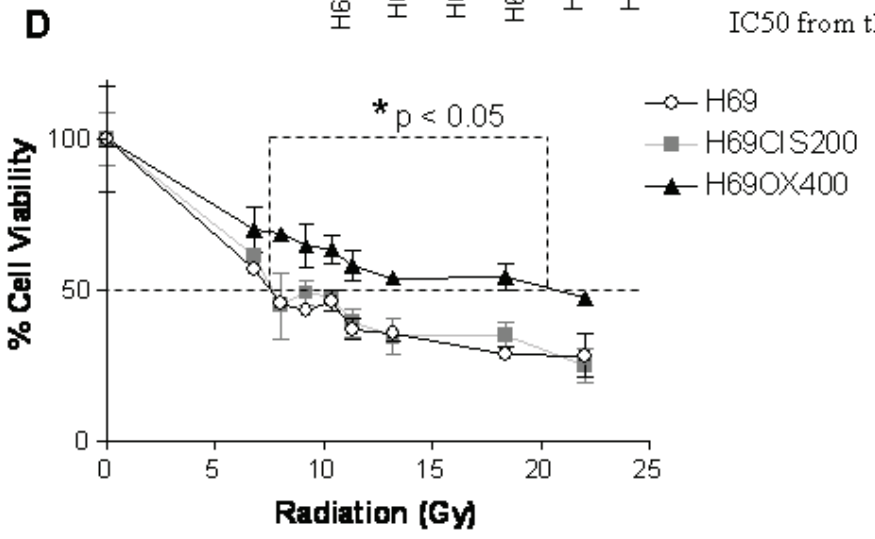


Fig. 6 – Analysis of DNA repair in H69, H69CIS200 and H69OX400 cells. Analysis of the expression of phospho-γH2AX in response to a 24 hour drug treatment of A) 200 ng/ml or B) 5 μg/ml cisplatin. C) Analysis of the repair of a platinated plasmid over 24 hours. * indicates a significant difference in expression from the untreated control for each cell line. D) Radiation resistance in H69, H69CIS200 and H69OX400 cells. Cells were irradiated with the dose indicated, grown for 5 days and cell viability measured using the MTT assay. * indicates a significant difference in IC50 from the H69 control.