

Multiple end-point analysis reveals cisplatin damage tolerance to be a chemoresistance mechanism in a NSCLC model: implications for predictive testing

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Abstract

Systemic chemotherapy is extensively used in cancer therapy, however for many treatments' response rates are limited. Furthermore, certain regimens are frequently associated with significant morbidity and occasional mortality. Consequently, when alternative options exist, it is desirable to reserve a particular chemotherapy for those patients whose tumours will respond. Therefore, attention is turning to the development of techniques that could provide predictive information regarding a tumour's particular chemosensitivity, as a means of enhancing patient selection for that specific treatment. One approach has been to focus on measures of DNA damage formation and repair as being potentially predictive of cancer cell chemosensitivity, the premise being that higher levels of induced DNA damage (resulting from the chemotherapeutic agents) and/or deficiencies in DNA damage repair are indicative of greater sensitivity. In the present study we have investigated the Comet assay response of a panel of NSCLC cell lines towards cisplatin and found an inverse correlation between sensitivity and damage formation resulting from this agent. Moreover, an inverse correlation was found between resistance and extent of damage repair. Further analysis of multiple alternate cellular end-points (including cell cycle analysis, apoptosis and gene expression changes) revealed cisplatin damage tolerance to be a chemoresistance mechanism in this model system. This study highlights damage tolerance mechanisms as potentially confounding factors in attempts to develop predictive tests based on measures of genotoxicity. To address this we would argue that a range of multiple end-points should be analysed in order to ascertain the "complete predictive picture".

Introduction

Systemic chemotherapy is often used during treatment of cancer patients to cure certain disseminated cancers, decrease tumour volume, alleviate symptoms and prolong life. Even though chemotherapy has proven to be highly effective in certain tumour types (*e.g.* testicular cancer) it still suffers from limited response rates in others (*e.g.* lung cancer). Moreover, it is frequently associated with significant side effects and even occasional mortality. Consequently, when equally effective treatment options exist, it is desirable to reserve a specific chemotherapy for patients whose tumours will respond. To facilitate this, attention is turning to the development of techniques that could provide predictive information about a tumour's particular chemosensitivity, as a means of enhancing patient selection for that treatment. To date, much research has focused on measures of DNA damage formation and repair as potential predictors of cancer cell chemosensitivity, the premise being that higher levels of induced DNA damage (resulting from the chemotherapeutic agents) and/or deficiencies in DNA damage repair would be indicative/predictive of greater sensitivity.

The chemotherapeutic drug cisplatin is one of the most frequently used agents in cancer treatment (*e.g.* testicular, ovarian, lung cancer) and its cytotoxic effects are believed to be due to the DNA damage (in the form of crosslinks) it induces in cells. It would therefore be expected that the greater the level of cisplatin adducts present in the tumour tissue of a patient undergoing cisplatin-based chemotherapy, the more likely it is that the tumour cells will die as a result of treatment, with consequent improved patient outcome. In fact, positive correlations between levels of cisplatin-induced DNA crosslinks (in peripheral blood lymphocytes, used as a surrogate tissue) and good clinical outcome have been reported in the literature ¹⁻⁷. However, contradictory results have also been reported ⁸⁻¹⁰. The lack of consensus between these reports indicates that genotoxicity measures may be limited with respect to their predictive potential and may not be suitable in every case. However, a feature common to all of the mentioned studies, and other reports measuring platinum adducts in patients ^{11, 12}, is the strong interindividual variability in platinum adduct formation, even when patients are subjected to the same cisplatin dose. This may be due to variability in DNA repair. Therefore, measuring repair capacity could potentially predict a patients' response to chemotherapy. Predictive tests of how a tumour would react to a particular chemotherapeutic regimen would be extremely useful as this would permit an individual patient to receive the most appropriate/effective drug(s). Furthermore, such predictive tests could help avoid

unnecessary treatments that can have serious side effects, leading to a delay in the effective treatment of the disease.

Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancers (the leading site of incidence and cancer mortality worldwide) ¹³. The panel of NSCLC cell lines used in the present study comprises the three main histological types of this tumour site with three adenocarcinomas (A549, H522 and H23), one squamous cell carcinoma (H520) and one large cell lung carcinoma (H460). A study by Wistuba *et al.* ¹⁴ indicated that NSCLC cell lines retain the properties of their parental tumours for lengthy culture periods, demonstrating they are representative of the original tumour and, therefore, provide suitable model systems for the study of this neoplasm. Cisplatin-based chemotherapy regimens are often used in treating NSCLC patients, however only around 20% of patients respond to the treatment ¹⁵. Therefore, in this study we investigated the effect of cisplatin in a panel of five NSCLC cell lines using a series of different end-points (sensitivity/resistance, crosslink formation and repair, cell cycle analysis, apoptosis and gene expression changes) in an attempt to elucidate their potential use in tumour predictive testing.

Material and methods

Chemicals and reagents

All chemicals and tissue culture reagents were purchased from Sigma (Poole, UK), unless otherwise stated. Cisplatin was from Mayne Pharma (Royal Leamington Spa, UK).

Cell lines and culture conditions

All NSCLC cell lines used in this study (A549, H23, H522, H520 and H460) were purchased from the American Type Culture Collection (ATCC, USA) and grown as a monolayer in RPMI 1640 media containing L-glutamine with 10% foetal calf serum in an incubator at 37 °C with 5% CO₂. Cells were confirmed as mycoplasma contamination-free.

Cell treatments with cisplatin

Cells were plated at an appropriate density and left in a CO₂ incubator at 37 °C for 20 to 24h before treatments. Cells were incubated with freshly prepared solutions of cisplatin in media (0 to 250 µM) for 1h at 37 °C, washed with PBS and then incubated in drug-free media for the stated periods of time.

Cell proliferation and cytotoxicity determination by cell counting

At 0 and 24h after cisplatin incubations, cells were washed with PBS, harvested, counted and viability assessed by the trypan blue exclusion assay with a haemocytometer. Results are the average of two independent cultures and are shown as the percentage of viable cells present at 24h compared to the ones present at 0h.

Clonogenic assay for clonal cell survival

Treated and untreated cells were incubated in drug-free media until colonies with over 50 cells could be clearly seen (2 to 4 weeks, depending on the cell line). The colonies were fixed in 100% methanol, stained with a 0.5% solution of crystal violet and counted.

Alkaline Comet Assay

Crosslink formation and repair were determined by the alkaline comet assay (a sensitive microscopy-based method for the detection of a variety of DNA lesions at the level of individual cells) as previously described¹⁶. Briefly, cisplatin treated and control cells (~1.5×10⁴) were X-irradiated on ice at 10 Gy (250 kV; 1 Gy / min) then suspended in 0.6% low melting point agarose and dispensed onto a clear microscope slide precoated with 1%

normal melting point agarose. Unirradiated controls and cisplatin-treated samples were processed alongside the irradiated samples. Duplicate slides were prepared for each sample. The cells were lysed overnight at 4 °C in lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, pH 10.0) containing freshly added 1% Triton X-100. Slides were then washed twice in ice-cold distilled water for 10 min, incubated in ice-cold alkali buffer (300 mM NaOH, 1 mM NaEDTA, pH > 13) for 20 min followed by electrophoresis in the same buffer at 30 V (0.66 V/cm), 300 mA for a further 20 min. The slides were rinsed with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 20 min followed by washing with ice-cold distilled water for 10 min and left to dry in a 37 °C incubator. All procedures were carried out on ice and under subdued light.

After drying, slides were stained with a freshly made solution of 2.5 µg/ml propidium iodide (PI) for 20 min, and then washed with distilled water. Images were analysed by use of an Olympus fluorescence microscope, fitted with an excitation filter of 515-535 nm and a barrier filter of 590 nm, at × 200 magnification. Images were captured by an on-line charge-coupled device (CCD) camera and analysed by using the Komet Analysis software (version 5.5) from Andor Technology (Belfast, UK).

A total of 100 cells were analysed per sample, 50 per duplicate slide. Olive tail moment (OTM) was calculated for each cell by the Komet Analysis software as the product of the percentage DNA in the comet tail and the distance between the centres of gravity for DNA in the head and tail, based on the definition of Olive *et al.* (1990). OTM was measured from two independent experiments and data from 200 cells pooled together and presented as mean and SEM.

Crosslinking was expressed as the percentage of decrease in mean OTM (MOTM) compared with irradiated controls calculated by the formula: % Decrease MOTM = $[1 - (\text{MOTM}_{\text{di}} - \text{MOTM}_{\text{cu}}) / (\text{MOTM}_{\text{ci}} - \text{MOTM}_{\text{cu}})] \times 100$; where MOTM_{di} is the MOTM of the drug treated irradiated sample, MOTM_{cu} the MOTM of the untreated unirradiated control and MOTM_{ci} the MOTM of the untreated irradiated control¹⁷. Repair capacity was assessed as a percentage calculated by the following formula, adapted from¹⁸: % Repair = $[(\% \text{ decrease MOTM at maximum formation} - \% \text{ decrease MOTM at 72h}) / \% \text{ decrease MOTM at maximum formation}] \times 100$.

Flow cytometric assays

For cell cycle distribution analysis, cisplatin-treated (50 µM) and control cells were harvested at each incubation period of interest, fixed in 70% ethanol and subsequently

resuspended in PBS containing 0.1 mg/ml RNase A and 5 µg/ml propidium iodide (PI), prior to analysis. Cisplatin-induced apoptosis (50 µM) was assayed by the Human Annexin V-FITC Apoptosis Kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The presence of a Sub-G₁ peak was also used to detect cisplatin-induced apoptosis.

Cellular DNA content (for cell cycle distribution analysis and sub-G₁ peak detection) and measurement of phosphatidylserine externalisation were analysed using a Becton Dickinson FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA), plotting 10,000 events per sample. Cell cycle distribution data analysis was subsequently performed using ModFit LT software (Becton Dickinson, Cowley, UK).

Cisplatin-induced gene expression

Cisplatin-induced (50 µM) gene expression changes in A549 cells were analysed by Microarray technology at 10h following cisplatin treatment. Gene expression changes were also analysed, in all cell lines of the NSCLC panel, by real-time quantitative reverse-transcriptase PCR (QRT-PCR) at three different incubation periods post-cisplatin treatment (6, 10 and 24h). For each cell line, control samples were collected alongside the cisplatin-treated samples, all in triplicate. Total RNA was extracted using TRIzol Reagent (Invitrogen, Paisley, UK) and RNA integrity was investigated in an Agilent 2100 Bioanalyzer using the Eukaryote Total RNA Nano assay (Agilent Technologies, Palo Alto, CA, USA), according to the manufacturers' specifications.

DNA microarray analysis

Target preparation (cDNA synthesis, *in vitro* transcription and cRNA fragmentation) and array hybridisation, washing and scanning were performed as described in the Affymetrix GeneChip Expression Analysis Manual 701025 Rev. 3 (Affymetrix, Santa Clara, CA, USA). High-density DNA oligonucleotide expression arrays (HG-Focus, Affymetrix) containing probes representing around 8,500 well-characterised human genes were used in this study. DNA-Chip Analyser (dChip) software¹⁹ was used to calculate model-based expression values from CEL intensity files and to identify genes that are significantly differentially expressed between control and cisplatin-treated samples ($P < 0.05$), each in triplicate. Genes exhibiting fold changes below – 1.5 and above 1.5 were filtered for the analysis. The data discussed in this publication were deposited in NCBI's Gene Expression Omnibus (GEO,

<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE6410.

Real-time QRT-PCR

The primer sequences for *p21*, *Gadd45a*, *XPC*, *ERCC1*, *DDB2*, *BRCA1*, *CSA*, *MLH1*, *Bax*, *Bcl2*, *Fas* and *Hprt1* were designed using Primer 3 software²⁰. The forward and reverse oligonucleotide sequences, along with the expected product length, are listed as supplementary Table I.

Possible genomic DNA contamination was removed from total RNA using the DNA-free Turbo Kit (Ambion, Huntingdon, UK) and the first strand cDNA was prepared using the StrataScript First-Strand Synthesis System (Stratagene, Amsterdam, Netherlands) according to the manufacturers' instructions. Real-time QRT-PCR was performed on a MX4000 spectrofluorometric thermal cycler (Stratagene) using the SYBR Green JumpStart Taq ReadyMix (Sigma), with 3.5 mM MgCl₂, according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 95 °C for 4min, then 40 cycles at 95 °C for 30s, 57 °C for 1min and 72 °C for 30s. The amplification of a single product was verified by the existence of a single peak in the dissociation curve and by the presence of a single product band in a 1% agarose gel electrophoresis.

The relative expression level of each gene was determined by the Standard Curve method²¹ using the housekeeping gene *Hprt1* to normalise expression results between samples. Control samples were analysed at 10 and 24h and no difference in expression levels was detected between time points (data not shown). Therefore, and for ease of presentation, the information from the six control samples was pooled together and considered to be representative of the control values for all time points. Results were analysed using the statistical software package Minitab 13 (Minitab Ltd., Coventry, UK). The non-parametric Mann-Whitney test was used to estimate the statistical significance of the differences in expression levels between cisplatin-treated and control samples. Results were considered to be statistically significant when $P < 0.05$.

Western blotting and antibodies

Cisplatin-induced (50 µM) protein expression changes in A549 and H460 cells were analysed by western blotting at 24h following cisplatin treatment. Treated and control cell lysates were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were blocked using Odyssey blocking buffer (LI-Cor Biosciences, Cambridge, UK) for 1h at

room temperature and incubated with primary antibodies overnight at 4 °C. Membranes were then incubated with the appropriate secondary antibodies for 1h at room temperature and detection (simultaneously at 700 nm and 800 nm) and quantification performed with the Odyssey Infrared Imaging System (LI-Cor Biosciences). Normalisation of gel bands was accomplished by ratiometric analysis of two wavelengths, where β -Actin or α -Tubulin was used as loading control. Results are the average of at least three independent experiments with representative blots being shown.

Bax was detected with a mouse monoclonal antibody (sc-20067) (Santa Cruz Biotechnology, Inc., Santa Cruz, USA), XPC (X1129) with a rabbit monoclonal antibody (Sigma), Bcl2 (M0887) and P21 (M7202) with mouse monoclonal antibodies (Dako, Glostrup, Denmark), β -Actin (sc-1616-R) with a rabbit polyclonal antibody (Santa Cruz) and α -Tubulin (CP06) with a mouse monoclonal antibody (Calbiochem, Darmstadt, Germany). Secondary antibodies used were IRDye 800CW Goat (polyclonal) Anti-Mouse and IRDye 680 Goat (polyclonal) Anti-Rabbit (LI-Cor Biosciences).

Results

Cisplatin sensitivity

Sensitivity to the chemotherapeutic agent cisplatin was investigated in the five cell lines that constitute the NSCLC panel via clonogenic assay. Different susceptibilities to cisplatin were observed (Fig. 1), the most resistant cell line being A549 followed by H460, then H520 and finally H23. The cell line H522 was not capable of forming colonies under the conditions studied and therefore another method, cell counting, was used to assess the sensitivity of this cell line to cisplatin. Briefly, cisplatin-treated and control cells were counted by the trypan blue exclusion assay at 0 and 24h following the 1h incubation with the agent. The number of viable cells present at 24h was compared with the number present at 0h, and the rank order of resistance determined by taking into account cisplatin-induced cell proliferation, inhibition and cytotoxicity (*e.g.* the more resistant cell lines being those with less cisplatin-induced inhibition of cell proliferation, and the most sensitive cell lines being those with higher cisplatin-induced cytotoxicity). For A549, H460, H522 and H23, results obtained by cell counting were in accordance to those obtained by the clonogenic assay and inclusion of H520 placed this cell line as being intermediate between H460 and H520 (Supplementary Fig. 1). The rank order of cisplatin resistance obtained in the NSCLC panel was therefore A549 >> H460 > H522 > H520 > H23.

DNA crosslink formation and repair

Results obtained with the alkaline Comet assay show that the incubation time at which the cell lines reached the maximum of crosslink formation (expressed as % decrease in MOTM) varied between cell lines but generally occurred between 6 and 10h (Fig. 2). The amount of crosslinks formed was also variable between the cell lines and cisplatin concentration studied. Results show that the most resistant cell lines, A549, H460 and H522, formed a greater amount of crosslinks at all cisplatin concentrations when compared to the sensitive cell lines, H520 and H23 (Fig. 2 and Table I). Crosslink repair became noticeable in all cell lines following maximum of crosslink formation, especially between the 10-, 24- and 72-hour incubation periods. Repair efficiency, estimated as the percentage of crosslinks repaired, was determined for all cell lines and cisplatin concentrations. The extent of repair varied between cell lines. For instance, only the sensitive H23 cell line was capable of repairing the majority of the crosslinks formed at all the cisplatin concentrations studied by

72h, while the resistant A549 cells still had a substantial amount of crosslinks remaining at the same time point (Fig. 2 and Table I).

Cisplatin-induced cell cycle distribution changes and apoptosis

Analysis of the A549 and H460 cell cycle distribution in response to 50 μ M cisplatin revealed that there was an abrupt accumulation of cells in the S-phase of the cell cycle by 10h followed by a rapid increase in the number of cells in the G₂ and/or M phases by 24h, which then began to decrease by 48h (Fig. 3). By 72h the percentage of cells in each phase of the cell cycle is clearly approaching the levels present in the control, indicating the cells were re-establishing the normal cell cycle distribution (Fig. 3). A different effect was observed in H23, H522 and H520 cell lines, where there was a more gradual accumulation of cells in the S-phase over 24h - 48h followed by an accumulation of cells in the G₂ and/or M phases that persisted at least until 72h (Fig. 3). Generally, the cell cycle distribution of control samples did not differ throughout the duration of the experiments, therefore (and for ease of presentation) only values for control at 10h are shown. The only exceptions were in the A549 and H460 cell lines, in which an increase of cells in the G₀/G₁ phase of the cell cycle was observed at 48 and 72h (data not shown). This occurred because these cell lines have shorter doubling times and therefore control cells reached confluence at the longer incubation periods, which causes cell cycle arrest at G₀/G₁²².

Cisplatin-induced apoptosis results obtained by both flow cytometric assays (Annexin V-FITC/PI assay and Sub-G₁ peak detection) were in general accordance with each other and showed that only three of the five cell lines in the panel were undergoing apoptosis, namely H460, H23 and H522 (Fig. 4). As expected, the most resistant cell line (A549) did not exhibit any cisplatin-induced apoptosis, whereas two of the three most sensitive cell lines had an elevated apoptotic index (H23 and H522). The high percentage of apoptotic cells observed in H460 cells was, however, somewhat unexpected considering that this is one of the most cisplatin-resistant cell lines.

Cisplatin-induced gene expression changes

Gene expression changes induced at 10h after cisplatin treatment were studied by Microarray technology in the most cisplatin-resistant cell line (A549) in order to identify genes of interest for a more detailed gene-expression analysis of the NSCLC panel by real-time QRT-PCR. Results show that cisplatin up-regulated the expression of genes involved in cell cycle arrest, apoptosis and DNA repair, and down-regulated genes involved in different

functions (Table II). The Microarray data were validated by analysing the expression levels of nine genes by real-time QRT-PCR (supplementary Fig. 2).

Cisplatin-induced gene expression changes were subsequently studied for eleven genes, in all cell lines that constitute the NSCLC panel, by real-time QRT-PCR. Six genes (*p21*, *Gadd45a*, *XPC*, *DDB2*, *Fas* and *Bax*) were selected because they were up-regulated in the A549 cell line in response to cisplatin as shown in the Microarray experiment, and another five genes because they are believed to be involved in cisplatin resistance (*ERCC1*, *BRCA1*, *CSA*, *MLH1* and *Bcl2*).

The results obtained by real-time QRT-PCR (Table III) showed that two of the most resistant cell lines from the NSCLC panel, A549 and H460, were able to effectively up-regulate several of the studied genes in response to cisplatin. These two cell lines showed similar transcriptional responses to cisplatin, although the H460 cells presented higher fold changes in all genes (especially at 24h) when compared to A549. Genes that were shown to be significantly up-regulated, in a time-dependent manner, in both cell lines were *p21*, *Gadd45a*, *Fas*, *Bax*, *DDB2* and *XPC* (all p53-regulated genes). Also statistically significant was the up-regulation of the DNA repair gene *ERCC1* in H460 cells at 24h and the down-regulation of the anti-apoptotic *Bcl2* gene in A549 at all incubation periods but only slightly down-regulated at 6h in H460 cells (Table III). Additionally, the up-regulation of P21, Bax, XPC and the down-regulation of Bcl2 were confirmed at the protein level in both cell lines by Western blotting analysis (supplementary Fig. 3).

The H522 cell line, of intermediate sensitivity, showed statistically significant up-regulations in the transcription of *p21* (at all incubation periods studied), *DDB2* and *BRCA1* (at 24h) and also a down-regulation in *Bcl2* at 6 and 10h (Table III). In the most sensitive cell line, H23, we only observed a 2-fold down-regulation in *Bcl2* at 6h after cisplatin treatment, with the transcription of this gene appearing to return to control levels by 24h. The transcription of the DNA repair gene *BRCA1* and of the pro-apoptotic gene *Fas* was only slightly up-regulated at 24h following cisplatin and those changes are probably not biologically significant. Of the cell lines tested, the H520 is the only one that did not seem to exhibit biologically significant changes in the expression of any of the genes studied (Table III).

The NSCLC cell lines could therefore be divided into two groups according to their transcriptional response to cisplatin, with the first group (A549 and H460) showing earlier and increased transcriptional response than the second (H23, H522 and H520).

Discussion

Correlation between cisplatin-induced crosslink formation and/ or repair and cell survival

There was an inverse correlation between sensitivity to cisplatin and crosslink formation *in vitro*, in which the most sensitive cells were the ones with the lowest levels of crosslinks being formed, and an inverse correlation between cisplatin resistance and repair efficiency, in which the most resistant cell lines were the ones repairing crosslinks least efficiently. These results were somewhat unexpected considering that cisplatin cytotoxicity is believed to be due to DNA crosslinking²³. The possibility that the most cisplatin-resistant cells from the NSCLC panel are able to tolerate/bypass the induced DNA lesions and continue performing their cellular functions (*e.g.* replication) provides an explanation to the observed results. A second possibility is that the more cisplatin-sensitive cells are indeed repairing the damage, as seen by the ‘excision’ of DNA crosslinks, but that they might not be repairing the DNA damage with high fidelity. Consequently, this ‘mis-repair’ could prove to be lethal at later times. In support of our findings, this lack of correlation between the level of DNA platination and sensitivity to cisplatin (at equimolar cisplatin doses) has been observed before²⁴⁻²⁶, indicating that sensitivity to cisplatin may not always be directly associated with DNA damage.

Cisplatin-induced cell cycle changes and damage tolerance

In terms of cisplatin-induced cell cycle variations, the panel of NSCLC cell lines could be clearly divided into two groups: A549 and H460 cell lines arrested their cell cycle at earlier time-points and recovered from it by 72h, whereas the other cell lines (H23, H522 and H520) showed cell cycle arrest at later time-points and remained arrested by 72h (Fig. 3). Taking into account the results presented in Fig. 2, it is possible that the most resistant cell lines tolerate higher levels of cisplatin-induced crosslinks because they are able to re-enter the cell cycle despite the high level of DNA damage still present in their DNA at 72h. In contrast, the cisplatin-sensitive cell lines continue in their effort to repair the damage via prolonged cell cycle arrest. In fact, the analysis of cisplatin-induced cell cycle changes seems to be a good indicator of drug-resistance, as the more cisplatin-resistant cell lines are able to recover from cell cycle arrest and the more sensitive cells are not. Our results support the view that

increased tolerance to cisplatin adducts constitutes one of the possible mechanisms of resistance to cisplatin²⁷.

Cisplatin-induced apoptosis and sensitivity to cisplatin

From the cell survival results, it would be expected for the most cisplatin-resistant cells of the NSCLC panel to exhibit less cisplatin-induced apoptosis than the more cisplatin-sensitive cell lines. This was indeed the case for the most resistant cell line (A549), in which no cisplatin-induced apoptosis was detected, and for two of the most sensitive cell lines (H23 and H522), which undergo prominent apoptosis as a result of the cisplatin treatment. However, it is difficult to understand why the H460 cell line, which is the second most cisplatin-resistant, after A549, has a higher percentage of cells undergoing apoptosis than all of the more cisplatin-sensitive cell lines (H522, H520 and H23). One hypothesis is that even though this cell line has increased cisplatin-induced apoptosis, the fewer cells that manage to survive proliferate more than the surviving cells of the most sensitive cell lines. This is supported by the fact that H460 cells still exhibit some cell growth at a cisplatin concentration of 50 μ M, even at 72h after treatment (data not shown). It is thus possible that, at later incubation times, the H460 cells are able to recover from that initial high percentage of apoptosis, which could explain the fact this is the second most resistant cell line of the panel in the clonogenic assay. Indeed, the accelerated repopulation of tumours by surviving cells during chemotherapy is an important cause of treatment failure²⁸. This accelerated repopulation in H460 cells could be due to the presence of a higher percentage of cancer stem cells in this particular cell line.

Cisplatin-induced gene expression in A549 as determined by Microarray technology

The apoptosis-related genes that were significantly up-regulated include the p53-regulated pro-apoptotic genes *Fas*²⁹, *TRAF4*³⁰, *Bax*³¹, *TP53I3*,³² and *PMAIP1*, which is a mediator of DNA damage-induced apoptosis³³. Interestingly, the *Pim-2* gene, an inhibitor of apoptosis³⁴, was also up-regulated indicating that there are conflicting apoptotic signals being up-regulated in response to this agent.

Genes involved in cell cycle and/or proliferation processes that were up-regulated include *p21* and *Gadd45a* (p53-regulated genes) which are responsible for the up- or down-regulation of several genes involved in cell cycle arrest and DNA repair³⁵. Other p53-regulated genes whose expression was up-regulated in response to cisplatin include the *cyclins G1* and *G2* (involved in cell cycle arrest), the anti-proliferative *BTG2* gene, which is

also important in DNA damage response ³⁶, and the *PPMID* gene ³⁷. Interestingly, the latter gene also possesses negative feedback effects on p53 activity thus functioning as a positive regulator of cell proliferation and being able to rescue cells from apoptosis ³⁸. This could possibly explain why these cells do not undergo cisplatin-induced apoptosis even though several pro-apoptotic genes are being up-regulated.

Cell cycle regulation genes that were down-regulated in response to cisplatin include *CDC20*, whose protein is required for nuclear movement prior to anaphase and chromosome separation ³⁹, and *PLK1*, a regulator of cell cycle progression that is essential for mitotic entry following recovery from DNA damage ⁴⁰. In addition, PLK1 functions as a p53 inhibitor, being capable of influencing not only cell cycle progression but also apoptosis in response to DNA damage ⁴¹. These results give some indication of the mechanisms that may be causing the observed cisplatin-induced cell cycle arrest in this cell line.

Two DNA repair genes were up-regulated in response to cisplatin, *XPC* and *DDB2*. *XPC* is a DNA damage-inducible NER gene whose protein has been shown to have an important role in the cisplatin-mediated cell cycle arrest and apoptosis ⁴². *DDB2* is also involved in the NER pathway and the transcription of both these genes is known to be regulated by p53 ^{43, 44}. These two observations suggest that these cells may be starting to up-regulate cisplatin lesion repair by NER, which was in fact observed in the comet assay data.

Transcriptional response to cisplatin, correlation with cell cycle arrest, apoptosis and DNA damage formation and repair

Cisplatin treatment resulted in transcriptional activation of several apoptotic, DNA repair and cell cycle regulator genes. Again, the panel of NSCLC cell lines could be divided into two groups according to their transcriptional response (A549 and H460 with earlier and increased transcriptional response when compared to the other cell lines), which probably results from the differences in terms of p53 status. In fact, A549 and H460 cells are p53 wild type ⁴⁵, which explains the elevated induction of p53-responsive genes in these cell lines but not in the p53 mutated cells (H23, H522 and H520) ^{45, 46}. The latter cell lines do however show elevated expression of certain p53-regulated genes (*e.g.* *p21*, *DDB2*), which is most likely happening via a p53-independent pathway, *e.g.* ^{47, 48}.

Cell cycle arrest genes (*p21* and *Gadd45α*) were up-regulated in the two most cisplatin-resistant cell lines (A549 and H460), which agrees with the observed induction of cell cycle arrest by cisplatin in these cell lines. The fact that the other three cell lines only showed mild or no up-regulation of these genes can be a direct consequence of them being

p53 negative. However, it is important to bear in mind that whilst the transcriptional response was only studied up to 24h, the majority of the cell cycle arrest in the most sensitive cell lines (H23, H522 and H520) takes place at later time-points; so it is possible that the up-regulation of certain cell cycle arrest genes would be further pronounced at later incubation periods.

In terms of apoptosis induction, it is interesting to observe that the most cisplatin-resistant cell line (A549) does not undergo apoptosis at any of the time points studied even though this agent causes the up-regulation of the pro-apoptotic genes *Fas* and *Bax*, and the down-regulation of the anti-apoptotic *Bcl2* gene. In addition, cisplatin has been shown to up-regulate protein expression of Bax and down-regulation of Bcl2 in this cell line which seems to indicate that there is a good correlation between transcriptional and translational response, at least for these two proteins. These observations seem therefore to indicate that this cell line has a defect in the apoptosis pathway downstream of these genes/proteins. Additionally, the translational response might be counteracted by other factors that inhibit the induction of apoptosis and that were not measured in this particular study. Conversely, the H460 cell line, the second most resistant, shows high cisplatin-induced up-regulation of pro-apoptotic genes (and proteins) and subsequent extensive cisplatin-induced apoptosis.

Genes involved in DNA damage recognition and repair that were shown to be up-regulated in response to cisplatin include *XPC*, *DDB2* and *ERCC1*. This only occurs in the most cisplatin resistant cells (A549 and H460), indicating that they are attempting to respond to the cisplatin insult by increasing the repair of the DNA lesions, which is in fact verified by the comet assay (Fig. 2). However, the comet assay data also showed that H23, H522 and H520 were more efficient at repairing the cisplatin-induced crosslinks than A549 and H460 cells, which seems to be independent of the transcriptional up-regulation of the *XPC*, *DDB2* and *ERCC1* genes. Nevertheless, it is necessary to bear in mind that only the mRNA levels were studied and these may not always correlate with the levels of the respective functional proteins.

Our results show that the most cisplatin-resistant cell lines, A549 and H460, were the ones exhibiting higher crosslink levels and lower repair efficiency, a more transient cell cycle arrest and greater cisplatin-induced variations in the expression of the genes under study. However, it could be that the more sensitive cell lines take longer to react to the cisplatin insult and this is the reason why delayed and/or fewer responses were detected.

Conclusions of the study and implications for predictive testing

Overall, the results discussed above suggest that lesion tolerance (possibly via replicative bypass or defects in the apoptosis pathway) is an important mechanism for cisplatin resistance in this NSCLC panel of cell lines. A possible mechanism might be the preferential induction of DNA polymerases that effectively bypass cisplatin lesions such as Polymerase β or η ^{49, 50}. Lesion tolerance might be a major confounding factor in the implementation and use of predictive tests of outcome measuring the formation and repair of cisplatin-induced crosslinks in patient samples. Although the NSCLC panel used here comprises the three main histological types of this tumour site, it is worth noting that the present study has only included five cell lines. Also, further studies are required in the clinical setting since the *in vitro* studies only provide indications of what might occur in the clinic.

Additionally, the fact that different assays generated different ranks of sensitivity/resistance to the chemotherapeutic agent studied here (Supplementary Table II) indicates the pitfalls of attempting to predict outcome based on the measure of a single endpoint. In our model, only the cell cycle data seemed to be in agreement with the actual sensitivity to cisplatin. Overall, this study highlights the importance of analysing multiple cell features and responses to generate a valid understanding of cell sensitivity, since reliance on a single measurement can be misleading and/or deliver an incomplete picture of what is occurring at the cellular and molecular level. Finally, even though these and other tumour predictive tests are of potential use it is important to bear in mind that strategies to circumvent the limited tumour sample availability will be pivotal to ensure their successful implementation in the clinic for patients where tumour samples are restricted.

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Table I. (a) Crosslinks at time of maximum formation per cell line and cisplatin concentration, determined as % decrease in MOTM \pm SEM. **(b)** Percentage of crosslinks repaired per cell line and cisplatin concentration 72 hours after treatment.

	A549	H460	H522	H520	H23
(a)					
50 μM	42 \pm 1.4	37 \pm 1.8	36 \pm 1.7	33 \pm 1.3	26 \pm 1.8
150 μM	70 \pm 1.1	71 \pm 1.3	67 \pm 1.3	54 \pm 1.2	54 \pm 1.6
250 μM	83 \pm 0.8	79 \pm 1.3	80 \pm 0.9	65 \pm 1.1	65 \pm 1.5
(b)					
50 μM	30	14	91	58	95
150 μM	46	68	88	59	97
250 μM	54	56	67	65	100

Table II. Genes with altered expression in response to 50 μ M cisplatin (1h treatment and 10h incubation in drug free media) in A549 cells.

<i>Gene ID</i>	<i>Gene Name</i> (official symbol)	<i>Fold Change</i>
Apoptosis		
581	Bcl2 associated X protein (BAX) Ψ	2.6
5366	phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) Ψ	1.7
11040	pim-2 oncogene (PIM2)	1.7
970	TNF ligand, member 7 (TNFSF7)	1.7
8744	TNF ligand, member 9 (TNFSF9)	2.1
355	TNF receptor superfamily, member 6 (FAS) Ψ	4.2
8795	TNF receptor, member 10b (TNFRSF10B)	1.6
9618	TNF receptor-associated factor 4 (TRAF4) Ψ	1.8
9540	Tumour protein p53 inducible protein 3 (TP53I3) Ψ	1.6
Cell Cycle and/or proliferation		
8465	aurora kinase A (AURKA) Ψ	- 1.5
7832	BTG family member 2 (BTG2) Ψ	2.9
991	CDC20 cell division cycle 20 homolog (<i>S. cerevisiae</i>) (CDC20)	- 1.7
1058	centromere protein A (CENPA)	- 1.6
9133	cyclin B2 (CCNB2)	- 1.6
899	cyclin F (CCNF)	- 1.8
900	cyclin G1 (CCNG1) Ψ	1.7
901	cyclin G2 (CCNG2) Ψ	1.6
1026	cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A) Ψ	6.1
2012	epithelial membrane protein 1 (EMP1)	1.7
1647	growth arrest and DNA-damage-inducible, alpha (GADD45 α) Ψ	2.6
4674	nucleosome assembly protein 1-like 2 (NAP1L2)	1.7
5347	polo like kinase 1 (<i>Drosophila</i>) (PLK1) Ψ	- 1.9
10957	proline-rich nuclear receptor coactivator 1 (PNRC1)	1.5
8493	protein phosphatase 1D magnesium-dependent, delta	3.1

	isoform (PPM1D) Ψ	
6790	serine/threonine kinase 6 (STK6)	- 1.7
27244	Sestrin 1 (SESN1) Ψ	2.5
DNA repair		
7508	<i>xeroderma pigmentosum</i> complement, group C (XPC) Ψ	2.1
1643	damage-specific DNA binding protein 2, 48kDa (DDB2) Ψ	2.1
5890	RAD51-like 1 (<i>S. cerevisiae</i>) (RAD51L1)	- 1.9
Transcription		
467	activating transcription factor 3 (ATF3) Ψ	1.8
57103	chromosome 12 open reading frame 5 (C12orf5)	1.9
10432	RNA binding motif protein 14 (RNM14)	- 1.7
4609	v-myc myelocytomatosis viral oncogene homolog (avian) (c-Myc)	- 1.5
Others		
136	Adenosine A2b receptor (ADORA2B)	1.6
220	aldehyde dehydrogenase 1 family, member A3 (ALDH1A3)	1.7
6310	ataxin 1 (ATXN1)	- 2.2
1793	dedicator of cytokinesis 1 (DOCK1)	- 1.8
1730	diaphanous homolog 2 (<i>Drosophila</i>) (DIAPH2)	- 1.8
1806	dihydropyrimidine dehydrogenase (DPYD)	- 3.4
2232	ferredoxin reductase (FDXR) Ψ	4.3
10243	Gephyrin (GPHN)	- 1.6
2762	GDP-mannose 4,6-dehydratase (GMDS)	- 2.0
2539	glucose-6-phosphate dehydrogenase (G6PD)	- 1.6
3008	histone 1, H1e (HIST1H1E)	- 1.6
4277	MHC class I polypeptide-related sequence B (MICB)	1.6
3778	potassium large conductance calcium-activated channel, subfamily M, alpha member (KCNMA1)	- 2.2
6586	slit homolog 3 (<i>Drosophila</i>) (SLIT3)	- 2.6

Ψ, genes previously reported as p53-regulated; Gene ID, name and official symbol according to EntrezGene.

Table III. Gene expression data from NSCLC cells incubated with 50 μ M of cisplatin for 1h and further incubated in drug-free media for up to 24h.

		Cell cycle		Apoptosis			DNA Repair					
		<i>p21</i>	<i>Gadd45a</i>	<i>Fas</i>	<i>BAX</i>	<i>Bcl2</i>	<i>DDB2</i>	<i>XPC</i>	<i>BRCA1</i>	<i>ERCC1</i>	<i>CSA</i>	<i>MLH1</i>
A549	Control	1.0 \pm 0.3	1.0 \pm 0.2	1.0 \pm 0.2	1.0 \pm 0.3	1.0 \pm 0.1	1.0 \pm 0.3	1.0 \pm 0.2	1.0 \pm 0.1	1.0 \pm 0.2	1.0 \pm 0.2	1.0 \pm 0.2
	6h	<u>6.2 \pm 2.1</u>	<u>2.4 \pm 0.6</u>	<u>3.1 \pm 1.6</u>	1.7 \pm 1.0	<u>0.6 \pm 0.2</u>	<u>2.2 \pm 0.9</u>	<u>2.0 \pm 0.6</u>	0.7 \pm 0.3	1.2 \pm 0.6	0.9 \pm 0.1	0.9 \pm 0.2
	10h	<u>9.1 \pm 1.0</u>	<u>3.4 \pm 0.1</u>	<u>5.5 \pm 0.9</u>	<u>1.8 \pm 0.1</u>	<u>0.6 \pm 0.1</u>	<u>2.7 \pm 0.3</u>	<u>2.3 \pm 0.2</u>	1.0 \pm 0.0	1.1 \pm 0.2	1.1 \pm 0.1	1.1 \pm 0.1
	24h	<u>14.8 \pm 1.6</u>	<u>4.3 \pm 0.3</u>	<u>8.6 \pm 0.7</u>	<u>2.7 \pm 0.4</u>	<u>0.7 \pm 0.2</u>	<u>4.2 \pm 0.6</u>	<u>4.2 \pm 0.2</u>	1.3 \pm 0.3	1.3 \pm 0.2	1.3 \pm 0.2	1.4 \pm 0.2
H460	Control	1.0 \pm 0.2	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.2	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.2	1.0 \pm 0.1	1.0 \pm 0.1
	6h	<u>9.0 \pm 1.3</u>	<u>4.3 \pm 0.7</u>	<u>2.8 \pm 1.3</u>	1.3 \pm 0.2	<u>0.7 \pm 0.1</u>	<u>2.0 \pm 0.4</u>	1.6 \pm 0.2	0.7 \pm 0.3	1.1 \pm 0.1	1.0 \pm 0.2	1.0 \pm 0.2
	10h	<u>16.9 \pm 3.0</u>	<u>9.7 \pm 2.3</u>	<u>4.0 \pm 1.5</u>	<u>1.6 \pm 0.4</u>	0.8 \pm 0.0	<u>3.5 \pm 0.5</u>	<u>2.6 \pm 0.4</u>	0.8 \pm 0.2	1.1 \pm 0.1	1.0 \pm 0.1	1.1 \pm 0.1
	24h	<u>52.3 \pm 2.4</u>	<u>52.2 \pm 5.9</u>	<u>13.3 \pm 2.2</u>	<u>3.7 \pm 0.3</u>	1.1 \pm 0.1	<u>11.7 \pm 0.1</u>	<u>6.9 \pm 0.7</u>	1.3 \pm 0.3	<u>1.8 \pm 0.1</u>	1.4 \pm 0.3	<u>1.6 \pm 0.2</u>
H522	Control	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.2	1.0 \pm 0.2	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.3	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.1
	6h	<u>2.0 \pm 0.1</u>	<u>1.3 \pm 0.2</u>	0.6 \pm 0.2	0.9 \pm 0.1	<u>0.5 \pm 0.0</u>	1.1 \pm 0.2	1.1 \pm 0.2	0.6 \pm 0.1	1.2 \pm 0.1	1.0 \pm 0.1	<u>0.8 \pm 0.1</u>
	10h	<u>1.7 \pm 0.1</u>	1.3 \pm 0.1	1.2 \pm 0.0	<u>0.8 \pm 0.0</u>	<u>0.3 \pm 0.1</u>	1.0 \pm 0.0	1.5 \pm 0.1	1.0 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.0	0.9 \pm 0.0
	24h	<u>2.2 \pm 0.3</u>	1.0 \pm 0.1	1.3 \pm 0.1	1.3 \pm 0.1	0.7 \pm 0.1	<u>1.7 \pm 0.2</u>	1.1 \pm 0.1	<u>2.0 \pm 0.2</u>	0.9 \pm 0.1	0.9 \pm 0.1	1.0 \pm 0.1
H23	Control	1.0 \pm 0.3	1.0 \pm 0.3	1.0 \pm 0.2	1.0 \pm 0.3	1.0 \pm 0.2	1.0 \pm 0.3	1.0 \pm 0.2	1.0 \pm 0.2	1.0 \pm 0.2	1.0 \pm 0.1	1.0 \pm 0.1
	6h	1.1 \pm 0.1	1.1 \pm 0.2	0.7 \pm 0.1	0.9 \pm 0.1	<u>0.5 \pm 0.1</u>	1.1 \pm 0.1	0.9 \pm 0.1	0.8 \pm 0.1	0.8 \pm 0.1	0.9 \pm 0.1	<u>0.8 \pm 0.1</u>
	10h	1.4 \pm 0.3	1.4 \pm 0.3	1.2 \pm 0.0	1.1 \pm 0.2	0.7 \pm 0.1	1.3 \pm 0.3	1.1 \pm 0.2	1.0 \pm 0.1	0.9 \pm 0.2	1.0 \pm 0.1	<u>0.9 \pm 0.1</u>
	24h	1.3 \pm 0.3	0.9 \pm 0.1	<u>1.6 \pm 0.1</u>	1.3 \pm 0.3	0.9 \pm 0.1	1.2 \pm 0.2	1.0 \pm 0.2	<u>1.5 \pm 0.2</u>	1.0 \pm 0.2	1.1 \pm 0.1	1.1 \pm 0.2
H520	Control	1.0 \pm 0.4	1.0 \pm 0.1	1.0 \pm 0.3	1.0 \pm 0.3	1.0 \pm 0.1	1.0 \pm 0.3	1.0 \pm 0.1	1.0 \pm 0.3	1.0 \pm 0.4	1.0 \pm 0.1	1.0 \pm 0.1
	6h	1.1 \pm 0.1	<u>1.3 \pm 0.1</u>	0.9 \pm 0.2	0.9 \pm 0.1	0.9 \pm 0.0	0.9 \pm 0.0	1.0 \pm 0.1	0.9 \pm 0.2	0.9 \pm 0.1	0.9 \pm 0.1	<u>0.9 \pm 0.1</u>
	10h	1.0 \pm 0.1	1.3 \pm 0.2	1.4 \pm 0.3	0.8 \pm 0.1	0.9 \pm 0.1	0.8 \pm 0.1	0.9 \pm 0.1	1.1 \pm 0.2	0.7 \pm 0.1	0.9 \pm 0.1	<u>0.9 \pm 0.0</u>
	24h	1.5 \pm 0.5	1.1 \pm 0.2	1.7 \pm 1.1	1.3 \pm 0.4	<u>0.7 \pm 0.1</u>	1.0 \pm 0.3	1.0 \pm 0.2	1.1 \pm 0.6	1.0 \pm 0.3	0.9 \pm 0.1	0.9 \pm 0.1

Underlined values are significantly different from control ($P < 0.05$) as determined by the Mann-Whitney test.

Figure 1. Percentage of cell survival in response to cisplatin (0 to 50 μ M) in the panel of NSCLC cell lines determined by the clonogenic assay. Results are expressed as mean and SEM of two to four independent experiments, each in triplicate.

Figure 2. Cisplatin-induced crosslink formation and repair in the panel of NSCLC cell lines at several incubation times (hours) in drug-free medium, following a 1 hour incubation with 50 μ M (■), 150 μ M (□) or 250 μ M (▣) of the agent. Results are expressed as the percentage of decrease in mean Olive tail moment and SEM.

Figure 3. Cell cycle distribution in NSCLC cell lines at several incubation times in drug-free medium, following a 1 hour incubation with 50 μ M cisplatin. Phases of the cell cycle: G₀/G₁ (■); S (□) and G₂/M (▣). Results are the mean and SD of four to six independent experiments. For ease of presentation only values for control at 10h are shown.

Figure 4. Percentage of early apoptotic cells (A) and cells with sub-G₁ peak (B) in cultures incubated with 50 μ M cisplatin for 1 hour and left to recover in drug-free medium up to 72 hours. Results are the mean \pm SD of two to four independent experiments.

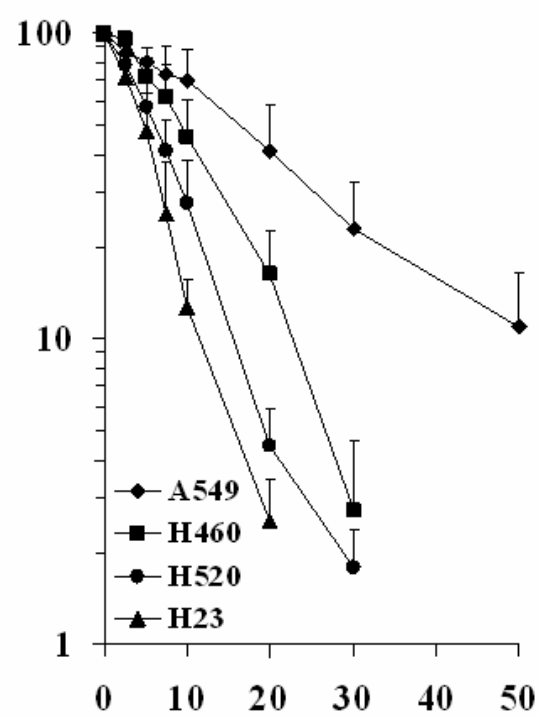


Figure 1.

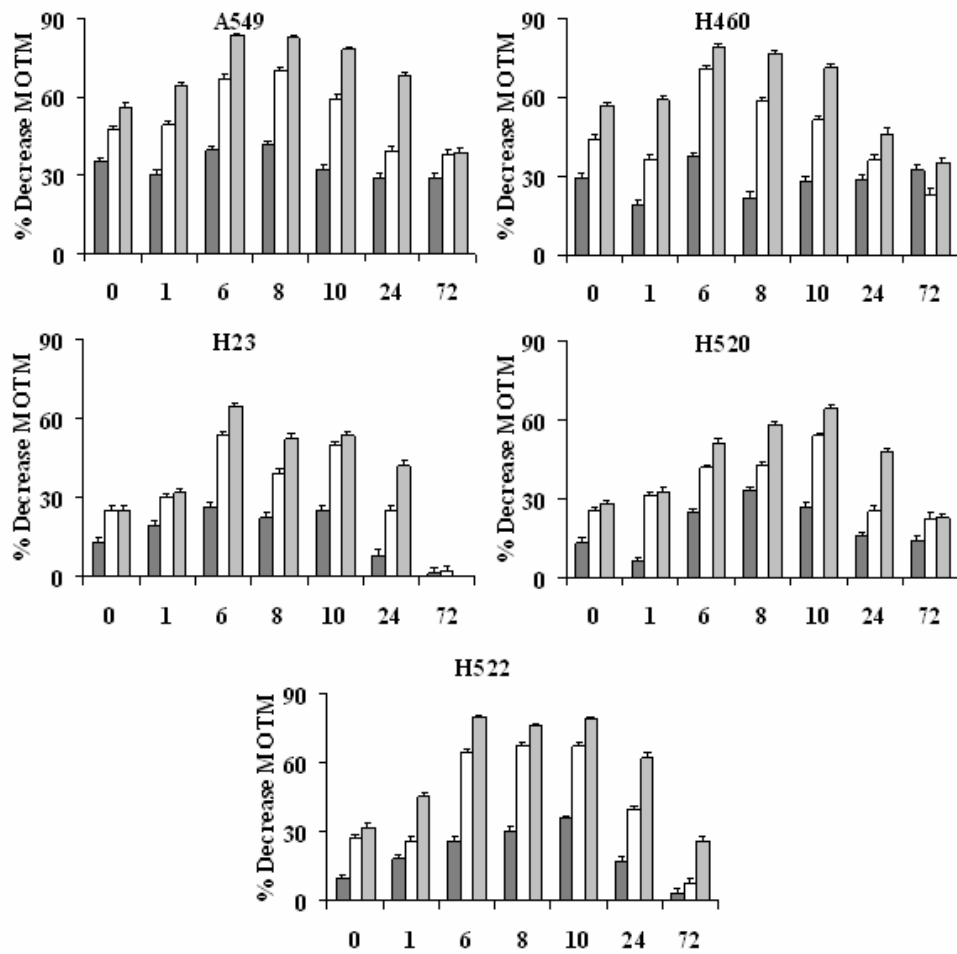


Figure 2.

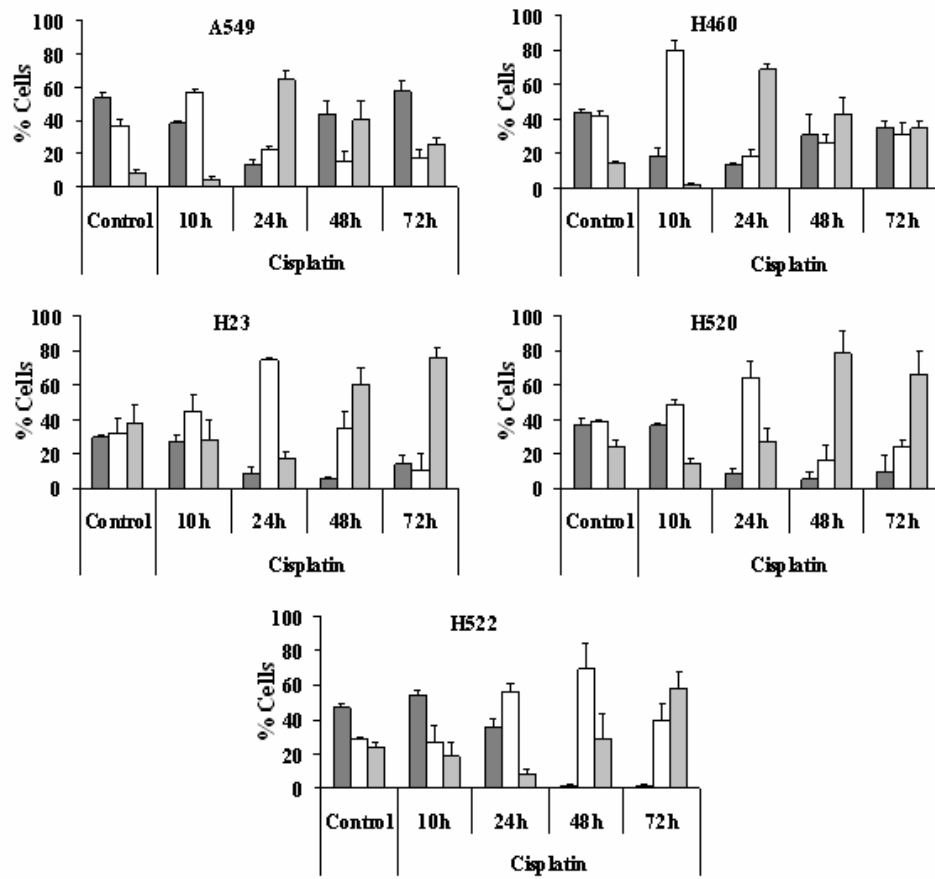


Figure 3.

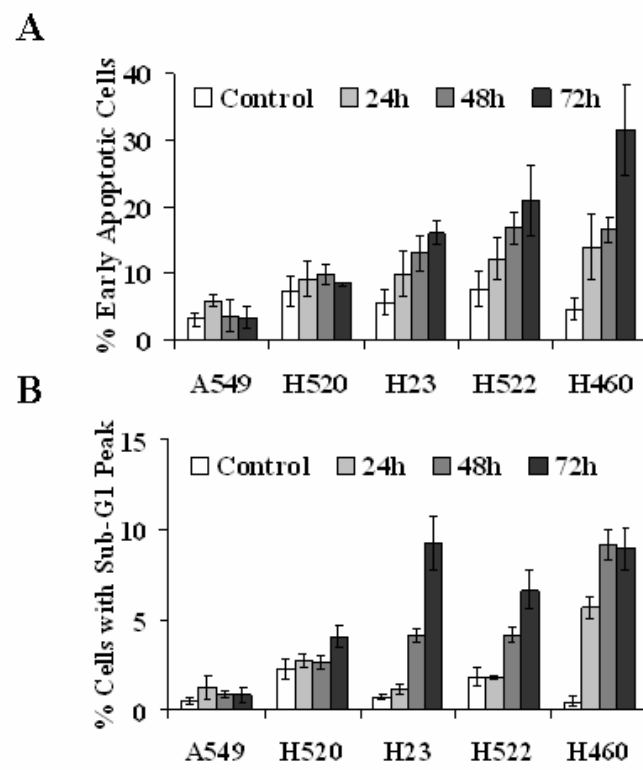


Figure 4.

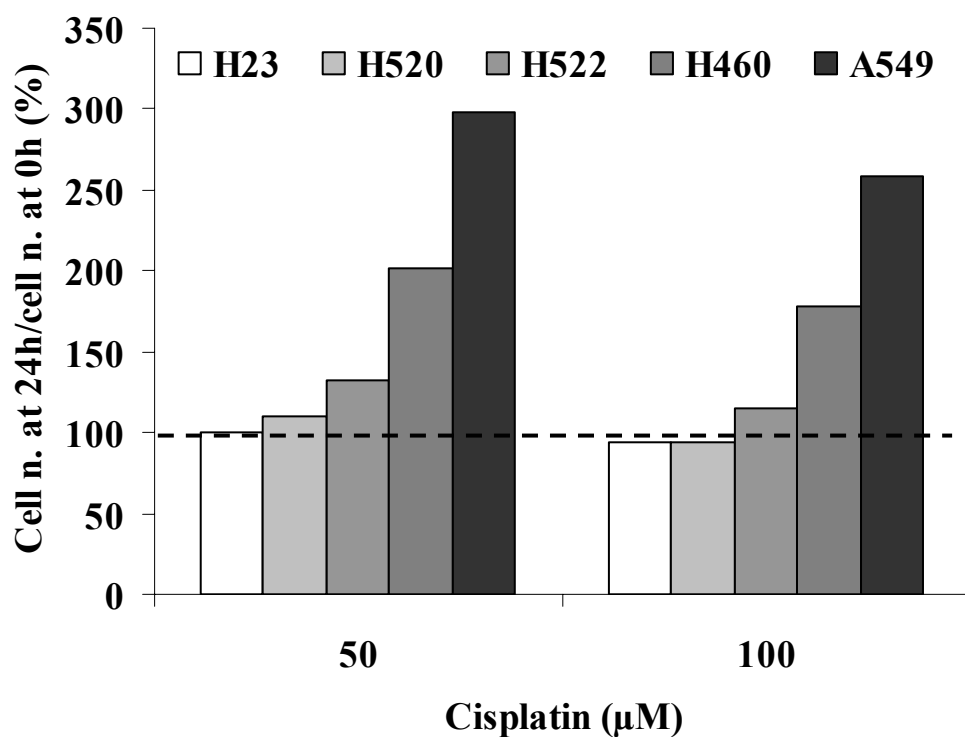
SUPPLEMENTARY MATERIAL:

Supplementary Table I. Primer sequences and respective expected product length.

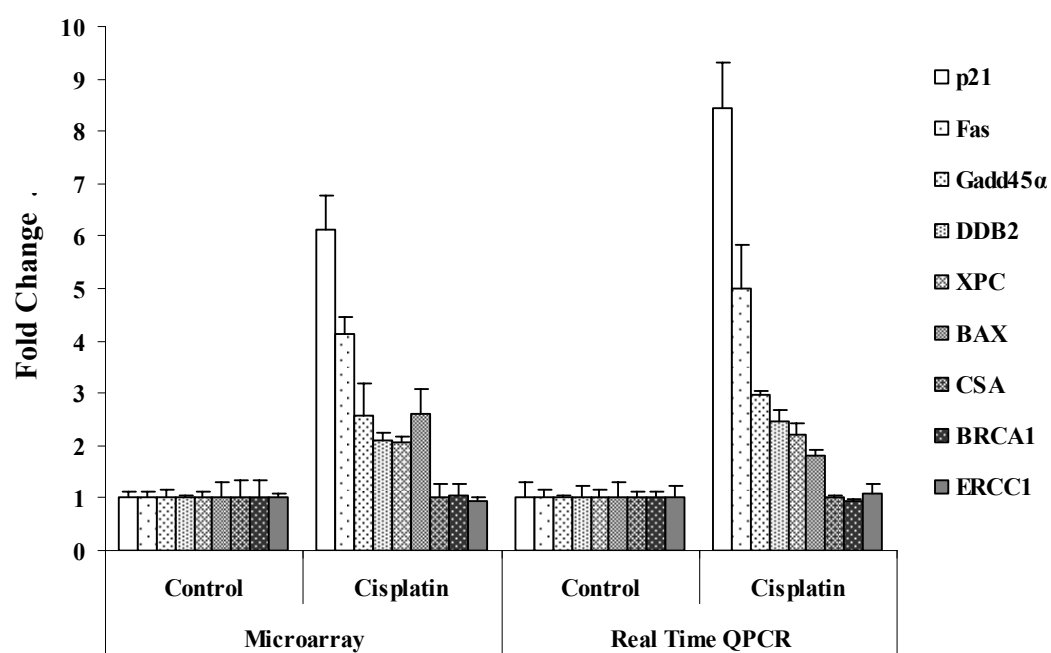
Gene	Length (bp)	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
<i>p21</i>	91	CCCAAACACCTTCCAGCTCCTGT	GTCTAGGTGGAGAAACGGGAACCAG
<i>Gadd45a</i>	85	AGGATTACAGAACTGATGCCAAGGG	TCCATCTGCAAAGTCATCTATCTCCG
<i>XPC</i>	93	GGAGATTCTGGATGGTGGTGCATC	AACCGAGGCGAGTGAACCTGTCTG
<i>ERCC1</i>	103	TGGAGAAGCTAGAGCAGGACTTCGTC	CCAAATGTGGTCAGGAGGGTCTGA
<i>DDB2</i>	85	CCACACATGGGATCAAGTCCTGC	CCCCTGCTCCAACCCCTAACCTTG
<i>BRCA1</i>	100	AAGGTCCAAAGCGAGCAAGAGAATC	AGTTGATCTGTGGGCATGTTGGTG
<i>CSA</i>	107	CATTCTGGCTTGGGTTCATCCTT	CACTGCTGCTCCAGGCATCTTCA
<i>MLH1</i>	89	CAGCTTGCTAACCTGCCTGATCTATACA	GAGAAAGAAGAACACATCCCACAGTGC
<i>Bax</i>	104	CATCTACCCTGCTGACCTCCCAGT	GAGCAATTCCAGAGGCAGTGGAG
<i>Bcl2</i>	95	CAAGCAAACATCCTATCAACAACAAGG	ATTGAGCGAGCCTTTCCATCCTC
<i>Fas</i>	106	TCCACAGGCTAACCCCACTCTATGA	TCAGAGGTAATTTAGAGGCAAAGTGGC
<i>Hprt1</i>	103	GCAGACTTTGCTTTCCTTGGTCAG	GTCTGGCTTATATCCAACACTTCGTG

Supplementary Table II. Summary of the results obtained.

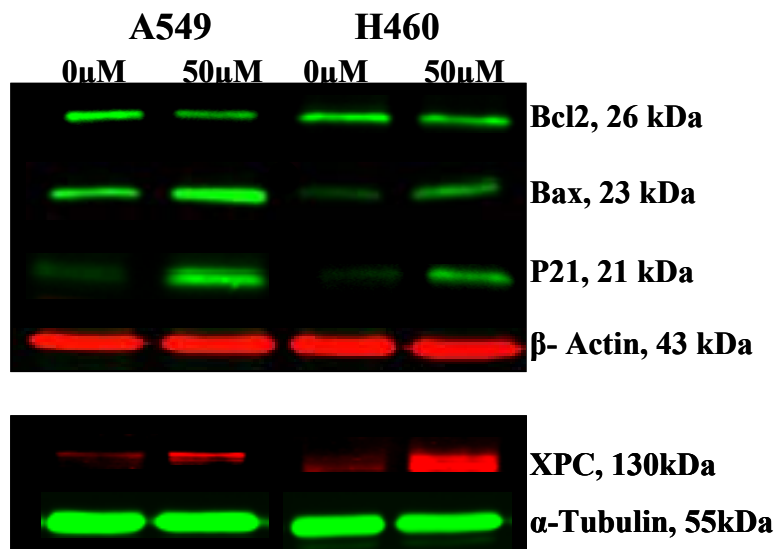
Measured End-Point	Rank Order or Grouping	
Cellular Sensitivity (Clonogenics & Cell Counting)	Resistant	Sensitive A549 >> H460 > H522 > H520 > H23
Adduct Formation (Comet Assay)	Low	High H23 < H520 < H522 < H460 < A549
Adduct Repair (Comet Assay)	Efficient	Deficient H23 > H522 > H520 > H460 > A549
Cell Cycle Arrest	Abrupt/Transient A549, H460	Prolonged/Sustained H522, H520, H23
Induced Apoptosis (Sub G1 & Annexin V)	Low	High A549 < H520 < H23 = H522 < H460
Transcriptional Changes (RT-QRT PCR)	Prominent H460, A549	Minimal H522, H23, H520



Supplementary Figure 1. Cell proliferation/cytotoxicity at 50 and 100 μM of cisplatin in the panel of NSCLC cell lines. Cell proliferation/cytotoxicity is expressed as the percentage of viable cell count at 24 hours compared to cell count at zero hours for control sample. Results are the average of two independent cultures. When values are > 100 , cell proliferation has occurred; values equal 100 when there is total growth inhibition; and values are < 100 when cytotoxicity has occurred.



Supplementary Figure 2. Validation of microarray results (A549 cell line) by real-time QRT-PCR. Results are shown as the mean and SD of three independent experiments.



Supplementary Figure 3. Western blotting of cisplatin-induced proteins in A549 and H460 cells. In A549, cisplatin up-regulated the translation of P21 (4.7 FC), Bax (2.7 FC), XPC (2.4 FC) and down-regulated the translation of Bcl2 (-1.6 FC). In H460, cisplatin up-regulated the translation of P21 (3.2 FC), Bax (2.0 FC), XPC (5.2 FC) and down-regulated the translation of Bcl2 (-1.3 FC). Normalisation of gel bands was accomplished by ratiometric analysis of two wavelengths, where β -Actin (for Bcl2, Bax and P21) or α -Tubulin (for XPC) was used as loading control. At least three independent experiments were performed and representative blots of the obtained results are shown. Fold changes (FC) are given in brackets.