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## Targeting NF- $\kappa$ B-Mediated Inflammatory Pathways in Cisplatin-Resistant NSCLC

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## Highlights

- Altered NF- $\kappa$ B expression was evident in cisplatin-resistant versus cisplatin-sensitive NSCLC cells
- The NF- $\kappa$ B inhibitor, DHMEQ, counteracted a cisplatin-mediated increase in NF- $\kappa$ B expression
- DHMEQ treatment enhanced the sensitivity of cisplatin-resistant NSCLC cells to cisplatin *in vitro*

## Abstract

### Objectives

The majority of patients with non-small cell lung cancer (NSCLC) present with advanced stage disease, at which time chemotherapy is usually the most common treatment option. While somewhat effective, patients treated with platinum-based regimens will eventually develop resistance, with others presenting with intrinsic resistance. Multiple pathways have been implicated in chemo-resistance, however the critical underlying mechanisms have yet to be elucidated. The aim of this project was to determine the role of inflammatory mediators in cisplatin-resistance in NSCLC.

## Materials and Methods

Inflammatory mediator, NF- $\kappa$ B, and its associated pathways were investigated in an isogenic model of cisplatin-resistant NSCLC using age-matched parental (PT) and corresponding cisplatin-resistant (CisR) sublines. Pathways were assessed using mass spectrometry, western blot analysis and qRT-PCR. The cisplatin sensitizing potential of an NF- $\kappa$ B small molecule inhibitor, DHMEQ, was also assessed by means of viability assays and western blot analysis.

## Results

Proteomic analysis identified dysregulated NF- $\kappa$ B responsive targets in CisR cells when compared to PT cells, with increased NF- $\kappa$ B expression identified in four out of the five NSCLC sub-types examined (CisR versus PT). DHMEQ treatment resulted in reduced NF- $\kappa$ B expression in the presence of cisplatin, and re-sensitized CisR cells to the cytotoxic effects of the drug.

## Conclusion

This study identified NF- $\kappa$ B as a potential therapeutic target in cisplatin-resistant NSCLC. Furthermore, inhibition of NF- $\kappa$ B using DHMEQ re-sensitized chemo-resistant cells to cisplatin treatment.

## Keywords

Non-small cell lung cancer, chemotherapy, resistance, cisplatin, DHMEQ, NF- $\kappa$ B.

## 1 Introduction

Lung cancer remains a significant health care burden, accounting for 11.6% (2.094 million) of all cancer cases and 18.4% (1.8 million) of cancer related deaths [1]. With advances in targeted therapeutics and immunotherapy agents [2], five-year survival rates are improving with recent SEER data indicating a rate of 19.4% [3]. However, chemotherapy is still widely used, with more recent trials combining traditional chemotherapy with newer agents [4]. Cisplatin, *cis*-Diamineplatinum (II) dichloride, is a common chemotherapeutic agent used in the treatment of advanced NSCLC [5-9], and is currently one of the most effective chemotherapeutic agents available, with response rates of 40-50% depending on NSCLC sub-type [10, 11]. However, many patients develop refractory disease either through intrinsic or acquired resistance to cisplatin, thus impeding its effectiveness in the management of NSCLC [12, 13].

Mechanisms of cisplatin-resistance are varied and complex [14-27]. Decreased uptake or increased efflux of cisplatin is frequently observed in cisplatin resistant cell lines, the result being reduced intracellular accumulation of the drug [14]. Three copper transporters (CTR1, ATP7A and ATP7B) involved in the uptake and efflux of platinum complexes have been linked with acquired platinum resistance [15-21]. Therefore, any modulation of these mediators may impact on cisplatin concentration in the cell. When DNA integrity is compromised, a complex network of DNA repair pathways recognizes it and initiates destruction of the cell [22]. Tumor cells, however, possess differences in their DNA repair mechanisms compared with

healthy cells [23]. Some of these discrepancies may be acquired, resulting in an increased capacity for repairing cisplatin-induced DNA damage in comparison to cisplatin sensitive cells [14]. Nucleotide excision repair (NER) has been implicated as a central mediator of a tumor's sensitivity to cisplatin [25, 26, 28]. It has also been hypothesized that mismatch repair (MMR) defective tumors infer more resistance to cisplatin than their MMR-proficient counterparts [26].

The inflammatory transcription factor, nuclear factor-kappa B (NF- $\kappa$ B), is linked to chemotherapy response and resistance in lung cancer [29-37]. NF- $\kappa$ B is a critical mediator of cell health and aids in the expression of over 200 genes, thus it is central to many signaling cascades, and is a positive mediator of cell growth and proliferation [30, 31, 38-40]. NF- $\kappa$ B and its target genes are implicated in all of the hallmarks of cancer [41, 42]. Constitutive NF- $\kappa$ B activation has been observed in a variety of tumors, including prostate, breast, cervical, pancreatic and lung cancer [43, 44]. Oncogene activation [34], the presence of an inflammatory environment [45, 46] or exposure to chemotherapeutic agents [47-49] can each result in NF- $\kappa$ B activation. In particular, mutant p53 and RAS are associated with chemo-resistance in lung cancer [50-52], and may promote NF- $\kappa$ B-mediated modulation of chemo-resistance and tumorigenesis via cell-cycle dysregulation and suppression of apoptosis [33]. K-RAS which accounts for 90% of RAS mutations in lung cancer, activates the non-canonical NF- $\kappa$ B pathway [35]. p53 and NF- $\kappa$ B have reciprocal inhibitory activity [36], whereby NF- $\kappa$ B suppresses apoptosis by antagonizing p53 [37]. The collaboration between loss of function p53 and constitutively active K-RAS initiates NF- $\kappa$ B activation in lung cancer cells [34]. NF- $\kappa$ B regulated inflammatory chemokines, CCL2 and CCL5, have also been implicated in chemo-resistance via their influence on IL-6

secretion [53]. Chemotherapeutic agents such as gemcitabine [49], doxorubicin [48] and cisplatin [47], can induce NF- $\kappa$ B activation in cancer cells, potentially promoting resistance to these therapies [29].

Blocking NF- $\kappa$ B has been shown to impede cancer cell survival, metastasis and resistance to therapeutic agents [32, 54-57]. Therefore, targeting NF- $\kappa$ B has the potential to increase the efficacy of anti-cancer therapeutics [32, 36, 58]. Various NF- $\kappa$ B inhibitors exist, including anti-oxidants and proteasome inhibitors, which either directly or indirectly suppress the NF- $\kappa$ B pathway [31, 36, 40, 59-62]. Many of these, however, may exert off-target effects [63-65]. Dehydroxymethylepoxyquinomicin (DHMEQ), a low molecular weight NF- $\kappa$ B specific inhibitor derived from antibiotic epoxyquinomicin C [54, 66], has demonstrated the capacity to impact the expression of NF- $\kappa$ B and that of its downstream effectors. DHMEQ covalently binds to NF- $\kappa$ B in the cytoplasm via the Cys38 residue [54, 66, 67]. This action of DHMEQ impedes NF- $\kappa$ B binding to  $\kappa$ B sites in the nucleus and as such mediation of its downstream targets [54, 67-71]. DHMEQ has been successfully utilized as a drug-sensitizing agent [71-74] and has exerted anti-inflammatory and anti-tumor effects in various neoplastic diseases [75-84]. When used in combination with chemotherapeutic drugs, DHMEQ may result in chemo-sensitization of both intrinsically and acquired drug-resistant tumors [79]. In the clinical setting, this may lead to more effective treatments and improved prognosis in patients with lung cancer [32, 36, 85].

Novel strategies are needed to overcome cisplatin-resistance and enhance response to chemotherapy. The identification of novel markers to effectively stratify patients for

such cytotoxic therapy is warranted. The aim of this study was to further improve our understanding of the inflammatory-related molecular mechanisms fundamental to chemo-resistance in NSCLC, through the utilization of an isogenic cell line model of cisplatin-resistance [14]. . We identified significant dysregulation of NF- $\kappa$ B signaling in cisplatin-resistant lung cancer cells. Furthermore, we have demonstrated the potential of DHMEQ as a cisplatin-sensitizing agent for the first time in this cancer type.

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## 2 Materials and Methods

### 2.1 Cell lines

A panel of isogenic cisplatin resistant NSCLC cell lines were used in this study. The panel consisted of age-matched A549 (adenocarcinoma), H460 (large cell carcinoma), MOR (adenocarcinoma), H1299 (carcinoma) and SKMES-1 (squamous cell carcinoma) cells, from which cisplatin resistant sublines were generated from corresponding parental (wild-type) counterparts [14]. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in RPMI 1640 medium; with the exception of A549 cells which were maintained in Ham's F12 nutrient mix supplemented with 1% L-Glutamine, and H1299 and SKMES-1 cells which were maintained in MEME supplemented with 1% L-Glutamine and 1% non-essential amino acids (NEAA). Cell culture media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). All cell culture reagents were supplied by Thermo Fisher Scientific (Waltham, MAUSA). Cisplatin resistant cell lines were maintained in the presence of cisplatin (IC<sub>25</sub>) (Merck KGaA, Darmstadt, Germany).

### 2.2 Reagents

*cis*-Diamineplatinum (II) dichloride (cisplatin) (Merck, Germany) was dissolved in 0.15 M NaCl to a final concentration of 3.3 μM, and sterile filtered before use. Dehydroxymethylepoxyquinomicin (DHMEQ) was dissolved in DMSO and was supplied by Professor Kazou Umezawa [71].

### **2.3 CellTiter-Glo luminescent cell viability assay**

Cell viability was assessed using the CellTiter Glo assay (Promega Corporation, Madison, WI, USA). Briefly, cultured cells were treated with CellTiter-Glo (1:1), placed on an orbital shaker for 2 min to induce cell lysis and incubated at RT for 10 min to stabilize the luminescent signal, in accordance with the manufacturer's instructions. Luminescence was recorded using a microplate reader (FLUOstar Omega, BMG Labtech, Germany). Background values were subtracted from test wells and the data normalized to untreated controls.

### **2.4 Cell viability using High content analysis**

Cellular viability was assessed using the Cytell™ Cell Viability Kit (GE Healthcare, Chicago, IL, USA) as per manufacturer's instructions. Cells were imaged using the Cytell Imaging System (GE Healthcare), and the data analyzed using the IN Cell Analyzer software (GE Healthcare).

### **2.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was prepared from cell cultures using the ISOLATE II RNA Mini Kit (Bioline, London, UK). cDNA was synthesized from 1 µg total RNA using Oligo (dT) primers and Superscript™ III reverse transcriptase (200 U/µL) as per manufacturer's instructions (Thermo Fisher Scientific). Prior to qRT-PCR, cDNA was either dispensed into RT<sup>2</sup> Profiler™ PCR arrays (QIAGEN, Hilden, Germany), or into standard qRT-PCR plates. qRT-PCR cycling conditions consisted of 95°C for 10

min, followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C and 15 sec at 95°C, with a final extension at 72°C for 10 min using an Applied Biosystems ViiA7 (Thermo Fisher Scientific) real-time thermal cycler. *NF-κB* (RT<sup>2</sup> qPCR Primer Assay, QIAGEN) and *CCL2* (forward primer 5'-ATATCAGTGCAGAGGCTCGC-3'; reverse primer 5'-TCGGATTTGGGTTTGCTTG-3') (Integrated DNA Technologies, Coralville, IA, USA) transcript levels were normalized to *7SL* (forward primer ATCGGGTGTCCGCACTAAGTT; reverse primer CAGCACGGGAGTTTTGACCT) (Merck) transcript levels. Array transcript levels were normalized to the appropriate house-keeping gene. Fold change levels were calculated using the  $\Delta\Delta C_t$  method.

## 2.6 Western blotting

Protein lysates were extracted from cell cultures using ice cold lysis buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 5% glycerol, 10 mM Mg<sub>2</sub>Cl<sub>2</sub>, 0.5 mM EDTA, 0.05% IGEPAL) supplemented with protease inhibitor cocktail (Merck) and phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA). Lysates were separated by SDS/PAGE, transferred onto nitrocellulose membranes and blocked for 1 h with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NA, USA) prepared in PBS-T (1:1). Membranes were incubated overnight at 4°C with NF-κB p65 monoclonal antibody (1:1000) prepared in 1% fish gelatin, before incubation for 2 h at RT with the control antibody GAPDH (1:10000), and 1 h at RT in the appropriate secondary antibodies (1:10000). Blots were developed using an Odyssey Imaging System (LI-COR Biosciences) at the appropriate wavelengths. Densitometry analysis was carried out using Image J software [86].

## 2.7 Enzyme Linked Immunosorbent Assay (ELISA)

Supernatants were collected from cell cultures and analysed for the presence of secreted CCL2 using the Human MCP-1 (CCL2) ELISA kit (Jomar Life Research, Australia), according to the manufacturer's instructions. The optical density (OD) of the samples were determined using a microplate reader set at 450 nm. Data were analysed using SoftMax Pro 7 (Molecular Devices, San Jose, MA, USA). CCL2 concentrations were determined by interpolating from a standard curve (4PL model) of known concentrations.

## 2.8 Nano liquid chromatography tandem mass spectrometry

Whole cell lysates (20  $\mu$ g) were prepared in SDS-Tris lysis buffer and subjected to filter-aided sample preparation (FASP) as previously described [87]. Peptide concentrations were determined using the Pierce™ Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific). Peptide spectral data from approximately 400 ng – 1  $\mu$ g of injected tryptic peptides per sample were generated using nano liquid chromatography tandem mass spectrometry (nanoLC-nanoESI-MS/MS) on a TripleTOF® 5600+ instrument (SCIEX). Following chromatography, peptides were injected into the instrument and analyzed by data-dependent acquisition (DDA) using a 95 min method. For quantitation, eluted peptides were subjected to a cyclic data-independent acquisition (DIA) using variable isolation windows SWATH-MS™ acquisition for 95 min. DDA data was searched against a human library (Proteome UP000005640 containing 20,373 proteins, downloaded from UniProt.org) using ProteinPilot 5.0 (SCIEX). A False Discovery Rate threshold (FDR) cut-off of 1% was

applied before results were loaded into PeakView 2.0 software (SCIEX). A spectral library was exported as text from PeakView. The spectral library and DIA (SWATH) raw data were loaded into Skyline for quantitative analysis [88]. Briefly, Skyline employs an mProphet peak-picking model to identify and quantify transition peaks for each peptide [89]. Summary statistics and pairwise comparisons were generated in Skyline using the MSstats package [90]. Signaling network analysis was conducted using Ingenuity® Pathway Analysis (IPA) (QIAGEN).

## 2.9 Bioinformatics and statistical analysis

Overall survival of chemotherapy treated NSCLC patients were stratified into high versus low according to median NF- $\kappa$ B (p65; probe 201783\_s\_at) expression using Kaplan-Meier Plotter [91]. Transcript expression was assessed in microarray datasets from the Cancer Biomedical Informatics Grid, the Gene Expression Omnibus and The Cancer Genome Atlas. Kaplan-Meier plots were generated using GraphPad Prism 7 where the hazard ratio (HR) with 95% confidence intervals and log-rank  $P$  values were calculated. All data from *in vitro* experiments are expressed as mean  $\pm$  SEM from three independent experiments ( $n=3$ ), unless stated otherwise. Significance was determined via one-way analysis of variance (ANOVA) where the number of groups in the experiment was three or more, or an appropriate student's  $t$  test with a group of two. Tukey's multiple comparisons test or Welch's correction *post-hoc* analyses were used, where appropriate. Differences were considered significant when  $p < 0.05$ . Statistical analysis was performed using GraphPad Prism 7 (San Diego, CA, USA).

### 3 Results

#### 3.1 NF- $\kappa$ B is dysregulated in cisplatin-resistant NSCLC cells

In order to investigate a potential role for NF- $\kappa$ B in treatment resistance, this study utilized a panel of cisplatin-resistant isogenic cell lines. This panel consisted of cisplatin-resistant (CisR) cell lines, which were derived from original, age-matched parent (PT) cells (drug sensitive) [14]. Analysis of basal protein expression in H460 CisR versus PT cells (n=3) was carried out by SWATH quantitative mass spectrometry. The number of proteins exhibiting increased and decreased expression was quantified and expressed as a percentage of the total protein count. In comparing proteomes from CisR versus PT cell lines, 25.2% of all proteins assessed exhibited increased expression, while 16.3% of proteins showed decreased expression (1078 proteins represented; expression denoted by  $\pm 0.25$  fold change).

H460 PT and CisR cells treated with their respective IC<sub>50</sub> cisplatin concentrations (PT: 7.5  $\mu$ M; CisR: 16.6  $\mu$ M) (Supplementary Figure 1) for a period of 72 h (n=3) also underwent SWATH quantitative mass spectrometry analysis. Comparing the proteomes of H460 PT cisplatin (IC<sub>50</sub>) treated versus untreated, 16.6% of all proteins exhibited increased expression while 18.2% of proteins exhibited decreased expression (1091 proteins represented; expression denoted by  $\pm 0.25$  fold change). In H460 CisR cells (IC<sub>50</sub> cisplatin treated versus untreated), 40.2% of all proteins

exhibited increased expression and 9.4% of proteins exhibited decreased expression (1060 proteins represented; expression denoted by  $\pm 0.25$  fold change).

The data generated by mass spectrometry was subjected to Ingenuity Pathway Analysis® (IPA) (QIAGEN Bioinformatics) in order to identify any prominent and potentially modified signalling pathways in H460 CisR versus PT, H460 PT (IC<sub>50</sub> cisplatin treated versus untreated) and H460 CisR (IC<sub>50</sub> cisplatin treated versus untreated) data sets. Appropriate parameters were applied to the data sets: log<sub>2</sub> fold change cut-offs =  $\pm 0.5$ ;  $p < 0.05$ ; activated z-score threshold = 2; and the data was compared to the user defined original data set. IPA analysis revealed a number of NF- $\kappa$ B targets exhibiting increased and decreased expression (CisR versus PT) (Figure 1 (A)). In addition, cisplatin treatment resulted in altered protein expression in both H460 PT and CisR cells (IC<sub>50</sub> cisplatin versus untreated), as demonstrated in Figure 1 (B) and (C), respectively. NF- $\kappa$ B signaling was prominent as the second highest ranked pathway in each of these proteomic data sets. Pathways in H460 CisR versus PT were identified as cell cycle, cardiovascular system development and function, and organ morphology (Figure 1 (A)); with cellular development, cellular growth and proliferation, and embryonic development identified in H460 PT (IC<sub>50</sub> cisplatin versus untreated) (Figure 1 (B)); and in H460 CisR (IC<sub>50</sub> cisplatin versus untreated) pathways were identified as cancer, hematological disease and immunological disease (Figure 1 (C)).

### 3.2 NF- $\kappa$ B expression is altered in cisplatin-resistant NSCLC

As NF- $\kappa$ B was identified as prominently increased in cisplatin-resistance *in vitro*, a bioinformatics approach was undertaken to examine the association between NF- $\kappa$ B expression and patient outcome, in a cohort of NSCLC patients using the KMplot tool [91]. Adenocarcinoma (AC) cases (n=36), identified via exploration of the Cancer Biomedical Informatics Grid (caBIG), the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) [91], were stratified on the basis of median *NF- $\kappa$ B p65* transcript level whereby *NF- $\kappa$ B<sup>low</sup>* and *NF- $\kappa$ B<sup>high</sup>* tumors were considered as below and above the median transcript level, respectively. Univariate Kaplan-Meier analysis indicated that patients receiving a chemotherapy regimen with *NF- $\kappa$ B<sup>high</sup>* tumors had a poorer outcome than those with *NF- $\kappa$ B<sup>low</sup>* tumors (HR = 4.765, CI (95%): 2.011-11.29, log rank  $p < 0.0001$ ) (Figure 2 (A, B)).

Given these data, we next sought to examine the expression of NF- $\kappa$ B at the transcript (qRT-PCR) and protein (western blot) levels in an expanded panel of matched parental (PT) and corresponding cisplatin-resistant (CisR) A549, H460, MOR, H1299 and SK-MES-1 cell lines [14]. Basal *NF- $\kappa$ B* mRNA levels, normalized to housekeeping gene *7SL*, indicated dysregulated *NF- $\kappa$ B* expression between matched PT and CisR cell lines (Figure 2 (C-G)). Four out of the 5 subtypes exhibited increased *NF- $\kappa$ B* expression in the CisR relative to the PT cells: A549 ( $p < 0.0001$ ) (Figure 2 (C)), H460 ( $p < 0.01$ ) (Figure 2 (D)), H1299 (Figure 2 (F)), and SK-MES-1 (Figure 2 (G)). Decreased *NF- $\kappa$ B* expression was evident in the MOR model (CisR versus PT) ( $p < 0.0001$ ) (Figure 2 (E)).

In order to determine if the observed alteration in *NF-κB* gene transcript expression (CisR versus PT) in the NSCLC panel was similar at the protein level, basal NF-κB expression was also determined by means of western blot (Figure 2 (H)). NF-κB was normalized to the housekeeping control GAPDH (37 kDa). These data indicated a significant increase in NF-κB expression between CisR and PT cell lines for A549 ( $p < 0.05$ ) (Figure 2 (I)), H460 ( $p < 0.05$ ) (Figure 2 (J)), H1299 (Figure 2 (L)) and SK-MES-1 ( $p < 0.05$ ) (Figure 2 (M)). A decrease in NF-κB expression in the MOR cell line (Figure 2 (K)), was not statistically significant. A similar trend in protein expression was found relative to that observed at the mRNA level.

### 3.3 Downstream targets of NF- $\kappa$ B signaling are dysregulated in H460 cisplatin-resistant cells

A qRT-PCR screen was conducted comparing H460 CisR versus PT cells to identify specific NF- $\kappa$ B responsive genes, which might play a role in cisplatin-resistance. RT<sup>2</sup> Profiler™ PCR arrays (QIAGEN) were used to screen for the expression of genes involved in NF- $\kappa$ B signaling pathways, in addition to NF- $\kappa$ B signaling targets within these pathways. Data were normalized to the array house-keeping genes (ACTB; B2M; GAPDH; HPRT1; RPLP0) before being expressed as a fold-change using the  $\Delta\Delta$ Ct method (CisR versus PT). Data from the NF- $\kappa$ B signaling pathway arrays showed a greater than 2-fold increased expression in 52% of genes examined between CisR and PT cells, while 24% of genes were downregulated (Supplementary Figure 2). A similar trend was evident within the NF- $\kappa$ B signaling targets array, with 38% of genes demonstrating greater than 2 fold increased expression, and 33% exhibiting downregulation.

Inflammatory chemokines *CCL5* and *CCL2* were among the top genes exhibiting the greatest fold change (CisR versus PT) in both the NF- $\kappa$ B signaling pathway (*CCL5*: fold increase = 808.27; *CCL2*: fold increase = 62.97) (Figure 3 (A)) and NF- $\kappa$ B targets (*CCL5*: fold increase = 417.58; *CCL2*: fold increase = 119.85) (Figure 3 (B)) arrays (n=1). *CCL2* has previously been implicated in chemo-resistance [92-95]. This data reinforced the outcome of the SWATH quantitative mass spectrometry analysis and confirmed dysregulated NF- $\kappa$ B activity in cisplatin-resistant NSCLC.

### 3.4 DHMEQ inhibits NF- $\kappa$ B signaling and CCL2 expression

As we identified dysregulated NF- $\kappa$ B pathways and NF- $\kappa$ B responsive genes in cisplatin-treated and CisR NSCLC cell lines, using proteomics and qRT-PCR, we next sought to determine the impact of NF- $\kappa$ B blockade using a potent NF- $\kappa$ B inhibitor, DHMEQ [71]. Western blot analysis was first performed to assess total and phosphorylated NF- $\kappa$ B levels in H460 PT and CisR cells in the presence or absence of cisplatin ( $IC_{50}$ ) or DHMEQ (20  $\mu$ g/mL) [96] for 72 h (Figure 3 (C)). NF- $\kappa$ B and phosphorylated NF- $\kappa$ B protein levels were normalized to GAPDH and expressed relative to the untreated sample. The data obtained indicated elevated NF- $\kappa$ B (Figure 3 (C, D)) and phosphorylated NF- $\kappa$ B (Figure 3 (C, E)) expression in H460 PT cells in the presence of cisplatin. Conversely, cisplatin treatment resulted in reduced expression of NF- $\kappa$ B ( $p < 0.01$ ) (Figure 3 (C, F)) and phosphorylated NF- $\kappa$ B (Figure 3 (C, G)) in the CisR cell line. In contrast with cisplatin, DHMEQ treatment resulted in a reduction in NF- $\kappa$ B expression in the PT cells (Figure 3 (C, H)). Levels of phosphorylated NF- $\kappa$ B, however, were increased (Figure 3 (C, I)). DHMEQ significantly decreased levels of both NF- $\kappa$ B ( $p < 0.01$ ) (Figure 3 (C, J)) and phosphorylated NF- $\kappa$ B ( $p < 0.0001$ ) (Figure 3 (C, K)) in the CisR cell line.

The augmented expression of NF- $\kappa$ B target gene, *CCL2*, as observed in the gene arrays (CisR versus PT), was validated with qRT-PCR using primers specific for *CCL2* (Figure 3 (A, B)). *7SL* was utilized as a house keeping control. qRT-PCR analysis verified the result obtained by the arrays with *CCL2* expression significantly increased in the CisR versus the PT cells ( $p < 0.05$ ) (Figure 3 (L)). *CCL2* was utilized to represent a surrogate marker of NF- $\kappa$ B responsive mediators in order to assess the effect of cisplatin ( $IC_{50}$ ) and DHMEQ on NF- $\kappa$ B downstream targets. H460 PT

cells were treated with IC<sub>50</sub> cisplatin (7.5  $\mu$ M) and DHMEQ (20  $\mu$ g/mL) for 72 h, after which time the supernatant was collected for analysis using ELISA. Cisplatin treatment significantly increased secreted CCL2 expression ( $p < 0.05$ ) (Figure 3 (M)) in H460 PT cells compared to the untreated control. DHMEQ treatment significantly reduced secreted CCL2 expression in the PT cell line, compared to the untreated control ( $p < 0.05$ ) (Figure 3 (N)).

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### 3.5 Cisplatin in combination with DHMEQ inhibits NF- $\kappa$ B protein expression and re-sensitizes lung cancer cells to the cytotoxic effects of cisplatin

NF- $\kappa$ B protein expression in the presence of both cisplatin and DHMEQ was assessed in order to determine the ability of DHMEQ to overcome cisplatin-induced NF- $\kappa$ B expression. H460 PT and CisR cells were treated with cisplatin at their respective IC<sub>50</sub> concentrations (PT = 7.5  $\mu$ M; CisR = 16.6  $\mu$ M) in combination with 20  $\mu$ g/mL DHMEQ for 72 h. Controls included cisplatin (IC<sub>50</sub>), DHMEQ (20  $\mu$ g/mL) and untreated cells (Figure 4 (A)). NF- $\kappa$ B levels were normalized to GAPDH and expressed relative to untreated controls. NF- $\kappa$ B expression was significantly reduced in H460 PT cells treated with DHMEQ alone and the cisplatin/DHMEQ combination compared to cisplatin alone ( $p < 0.05$ ) (Figure 4 (A, B)). Conversely, in H460 CisR cells, DHMEQ alone and in combination with cisplatin induced a marked reduction in NF- $\kappa$ B expression relative to control cells ( $p < 0.0001$ ) and to cisplatin alone ( $p < 0.001$ ) (Figure 4 (A, C)).

To evaluate whether DHMEQ re-sensitized CisR cells to cisplatin, cell viability was next assessed in H460 PT and CisR cells treated with their respective IC<sub>50</sub> cisplatin concentrations or DHMEQ (20  $\mu$ g/mL) alone and in combination. Cellular viability was assessed using the CellTiter-Glo luminescent cell viability assay with samples normalized to their respective untreated controls and viability expressed as a percentage. All treatments significantly reduced viability in both H460 PT ( $p < 0.0001$ ) (Figure 4 (D)), and CisR ( $p < 0.0001$ ) (Figure 4 (E)) cell lines, compared to their untreated controls. Treatment with DHMEQ alone resulted in reduced viability compared to cisplatin alone in both PT ( $p < 0.01$ ) (Figure 4 (D)) and CisR cells

( $p < 0.0001$ ) (Figure 4 (E)). DHMEQ also had a greater detrimental effect on the health of H460 CisR cells than on PT, with percentage viabilities of 26.8% and 42% after 72 h, respectively (Figure 4 (D, E)). However, it was the cells treated with both cisplatin and DHMEQ that displayed the lowest viability. The combination of cisplatin and DHMEQ significantly reduced H460 PT viability compared to cisplatin alone ( $p < 0.0001$ ) and DHMEQ alone ( $p < 0.0001$ ) (Figure 4 (D)). While in H460 CisR cells, DHMEQ alone and in combination with cisplatin resulted in reduced H460 CisR viability compared to cisplatin alone ( $p < 0.0001$ ) (Figure 4 (E)). These results suggest that NF- $\kappa$ B inhibition re-sensitizes cisplatin-resistant cells to the cytotoxic effects of cisplatin.

#### 4. Discussion

Chronic inflammation is known to support cancer progression [97-100], and increased expression of the inflammatory transcription factor, NF- $\kappa$ B, has been associated with the promotion of a pre-cancerous inflammatory environment [101]. Furthermore, high levels of tumor cell NF- $\kappa$ B have been linked with chemo-resistance, disease progression and poor prognosis in patients with lung cancer [31, 44, 102, 103]. This study set out to profile proteomic differences between cisplatin resistant and cisplatin sensitive NSCLC and investigate the therapeutic potential of targeting NF- $\kappa$ B to overcome cisplatin-resistance. This study identified noteworthy patterns in protein expression in CisR versus PT cell lines, and has demonstrated the ability of an NF- $\kappa$ B inhibitor, DHMEQ, to increase cisplatin sensitivity in this model of cisplatin-resistant NSCLC.

Our bioinformatics analysis of a cohort of AC patients suggested that high *NF- $\kappa$ B* expression is prognostic of poor outcome for patients that have received chemotherapy. SWATH quantitative mass spectrometry revealed dysregulated protein expression in H460 CisR versus PT cells at a basal level, as well as in both H460 PT and CisR cells treated with their respective  $IC_{50}$  cisplatin doses. Subsequent pathway analysis revealed prominent NF- $\kappa$ B signaling in H460 CisR versus PT, as well as in H460 PT and CisR ( $IC_{50}$  cisplatin versus untreated) data sets, and identified NF- $\kappa$ B targets implicated in chemo-resistance. Decreased expression of CDK6 (cell division protein kinase 6) was identified in the H460 CisR versus PT data set. CDK6 has been implicated in chemo-resistance in ovarian cancer, and furthermore has been proposed as an actionable target that can be

exploited to improve the efficiency of platinum-based chemotherapeutic agents [104, 105]. ALDH7A1, a member of the aldehyde dehydrogenase family [106], expression was increased in H460 PT (IC<sub>50</sub> cisplatin versus untreated) cells. Recent research has revealed the cisplatin-induced emergence or expansion of ALDH1-positive cellular subpopulations in cisplatin-sensitive and resistant NSCLC cell lines, respectively, which further enhanced cisplatin resistance [13]. Targeting ALDH1 significantly re-sensitized resistant NSCLC cells to the cytotoxic effects of cisplatin [13]. Decreased expression of GSR (Glutathione reductase) was identified in the H460 CisR (IC<sub>50</sub> cisplatin versus untreated) data. GSR is responsible for maintaining the supply of reduced glutathione (GSH) [107]. Increased levels of both GSR and GSH in cancer cells have been linked with chemo-resistance [108, 109]. As NF-κB signaling ranked highly it was decided to examine its behaviour in cisplatin sensitive and resistant NSCLC cell lines in greater detail.

Further, *in vitro* analysis of a panel of 5 isogenic models of cisplatin-resistance in NSCLC identified elevated NF-κB expression in all but one of the paired CisR versus PT cell lines at the transcript and protein level. In addition, gene array data revealed dysregulated expression of NF-κB targets in H460 CisR versus PT cells, pointing to the impact of drug resistance on the activity of nuclear localized and transcriptionally active NF-κB. Further investigation is required to define the molecular regulation of NF-κB during the evolution of platinum resistance. Nonetheless, these data point to a role for dysregulated NF-κB pathways in our cell line model of chemo-resistance [110, 111].

Next, we assessed if a potent small molecule NF- $\kappa$ B inhibitor, DHMEQ, was capable of re-sensitizing cisplatin-resistant NSCLC cells to therapy. . In our study, DHMEQ (20 $\mu$ g/mL) reduced NF- $\kappa$ B protein expression in H460 PT and CisR cell lines. Furthermore, the inhibitory capability of DHMEQ was sufficient to counteract the cisplatin-mediated induction of NF- $\kappa$ B in both cell lines. DHMEQ-mediated NF- $\kappa$ B inhibition was greater in the CisR compared to the PT cells, both alone and in the presence of cisplatin (IC<sub>50</sub>). As basal levels of NF- $\kappa$ B were elevated in H460 CisR versus PT cells, the superior inhibitory effect of DHMEQ on NF- $\kappa$ B in the CisR cell line may suggest that DHMEQ is more potent in those cells with increased NF- $\kappa$ B. Viability data demonstrated the ability of DHMEQ to enhance cisplatin-mediated toxicity in both the CisR and cisplatin-sensitive PT cells. The DHMEQ-mediated reduction of NF- $\kappa$ B expression, in both the CisR and PT cells, appears to have increased the efficacy of cisplatin in these cells. The impact of DHMEQ on cellular viability is greatest in the CisR cell line, which corresponds with the greater reduction in NF- $\kappa$ B expression in these cells in response to DHMEQ treatment. These findings further implicate NF- $\kappa$ B in cisplatin-resistance in NSCLC and confirm DHMEQ as a cisplatin-sensitizing agent.

While inhibition of NF- $\kappa$ B has proved effective *in vivo* [112-115], we must be mindful of its systemic impact [29, 116, 117]. This study therefore proposes further scrutiny of the NF- $\kappa$ B gene array and proteomic data, which identified de-regulated expression in cisplatin-resistant versus cisplatin-sensitive NSCLC cells. While the proteomic data was conclusive (n=3), validation of other mediators identified in both the proteomic and gene array screens is necessary. These investigations would encompass not only validation of expression levels, but also ascertain the impact of

modified expression levels on cellular function as well as cisplatin sensitivity.

Reduced tumor size has previously been observed in response to NF- $\kappa$ B inhibition in animal cancer models [113, 114, 118]. As such, further investigation utilizing murine models of cisplatin-resistance in NSCLC may yield novel data as regards the efficacy of DHMEQ in an *in vivo* setting. This would encompass initial dosing studies followed by a pilot study investigating the ability of DHMEQ to re-sensitize cisplatin-resistant NSCLC cells to therapy, signified by enhanced tumor inhibition in response to the combination treatment of cisplatin and DHMEQ, compared to cisplatin alone.

Determining the appropriate dose of cisplatin to combine with DHMEQ, however, would require much optimization in order to balance the associated detrimental effects of cisplatin with an effective concentration [119]. Currently, tissue biopsy is conducted for the majority of NSCLC patients in order to obtain molecular information about their tumors [120]. Future work examining tissue samples may reveal a correlation between protein expression and treatment response and/or outcome in a clinical setting.

## 5. Conclusions

Gene array and proteomic data generated in this study will provide significant data sets for further interrogation of novel NF- $\kappa$ B-regulated targets implicated in the cisplatin-resistant phenotype in NSCLC. Furthermore, the identification of novel therapeutic targets and biological markers may be utilised in the clinical setting to stratify patients for therapy. In summary, this study identified NF- $\kappa$ B as a potential therapeutic target in overcoming cisplatin-resistance in NSCLC and demonstrated the potential of DHMEQ as a cisplatin-sensitizing agent in cisplatin-resistant NSCLC.

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**Conflict of Interest**

The authors declare no conflict of interest.

ACCEPTED MANUSCRIPT

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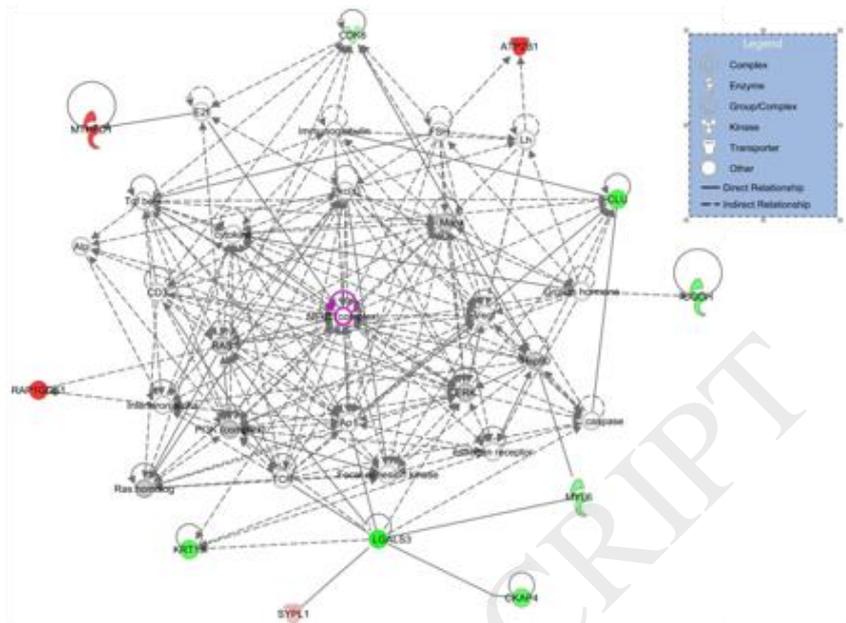
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A

## Signalling networks

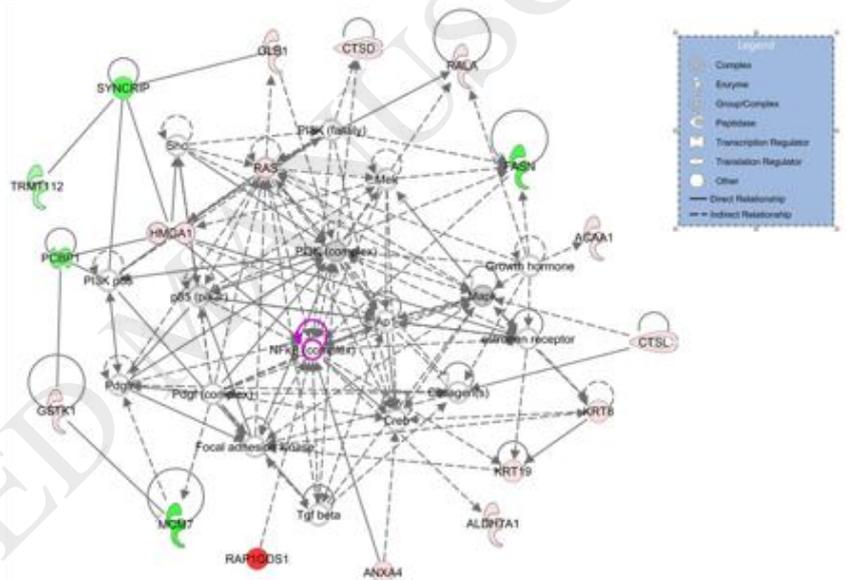
- 1 Cell Morphology, Cellular Movement, Cellular Assembly and Organization
- 2 Cell Cycle, Cardiovascular System Development and Function, Organ Morphology
- 3 Cell Morphology, Tissue Morphology



B

## Signalling networks

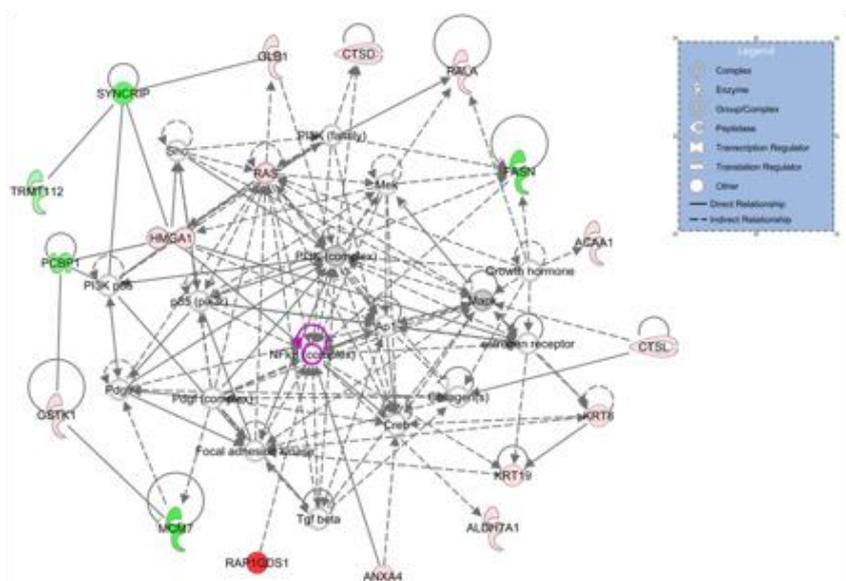
- 1 Cellular Assembly and Organization, Cancer
- 2 Cellular Development, Cellular Growth and Proliferation, Embryonic Development
- 3 Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance



C

## Signalling networks

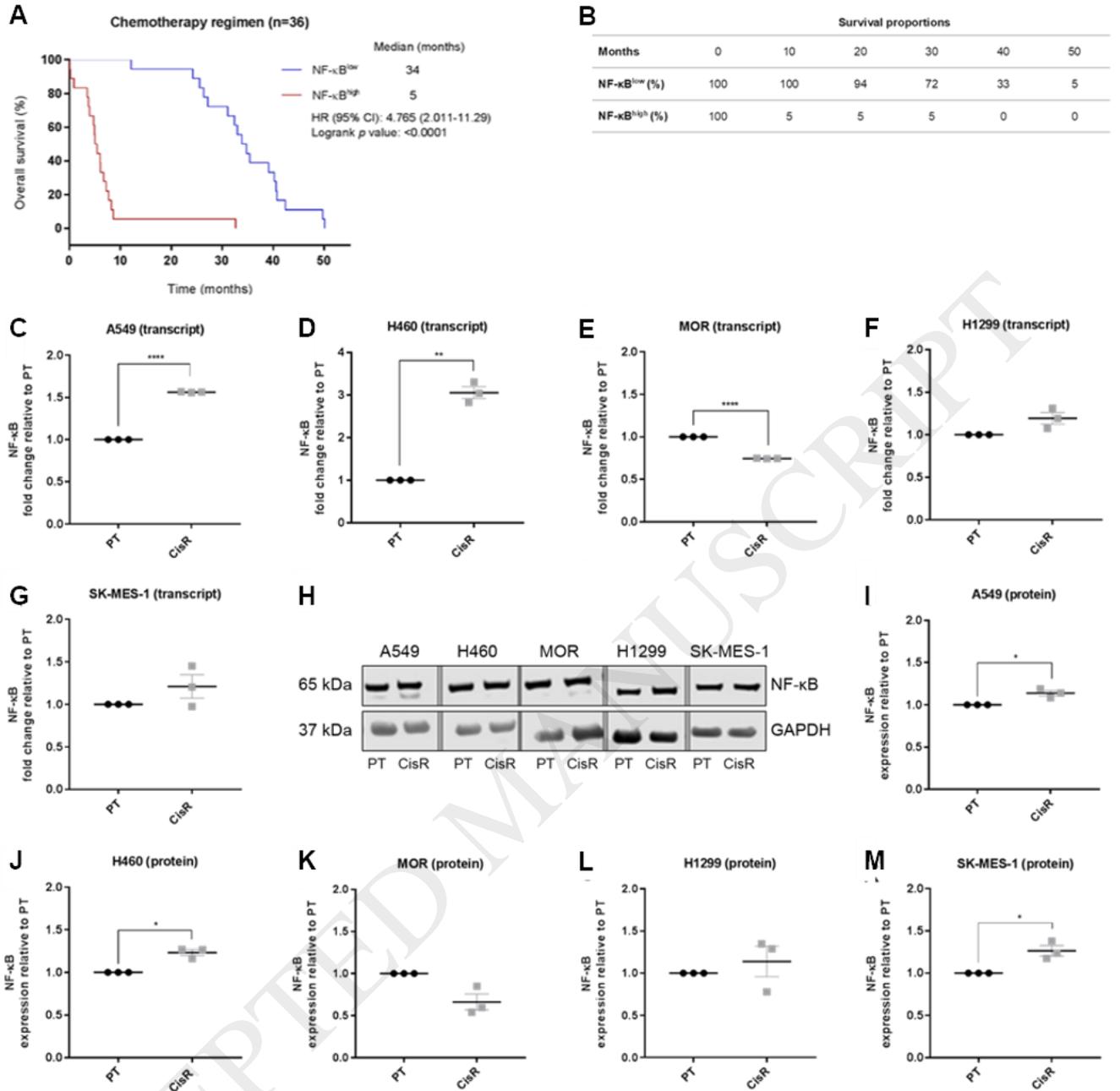
- 1 Cell-To-Cell Signalling and Interaction, Cellular Compromise, Inflammatory Response
- 2 Cancer, Haematological Disease, Immunological Disease
- 3 Cellular Compromise, Cellular Assembly and Organization, Cellular Function and Maintenance



**Figure 1: De-regulated NF- $\kappa$ B signaling was identified in H460 CisR versus PT cells (basal level), and in H460 PT and CisR cells in the presence of IC<sub>50</sub> cisplatin.**

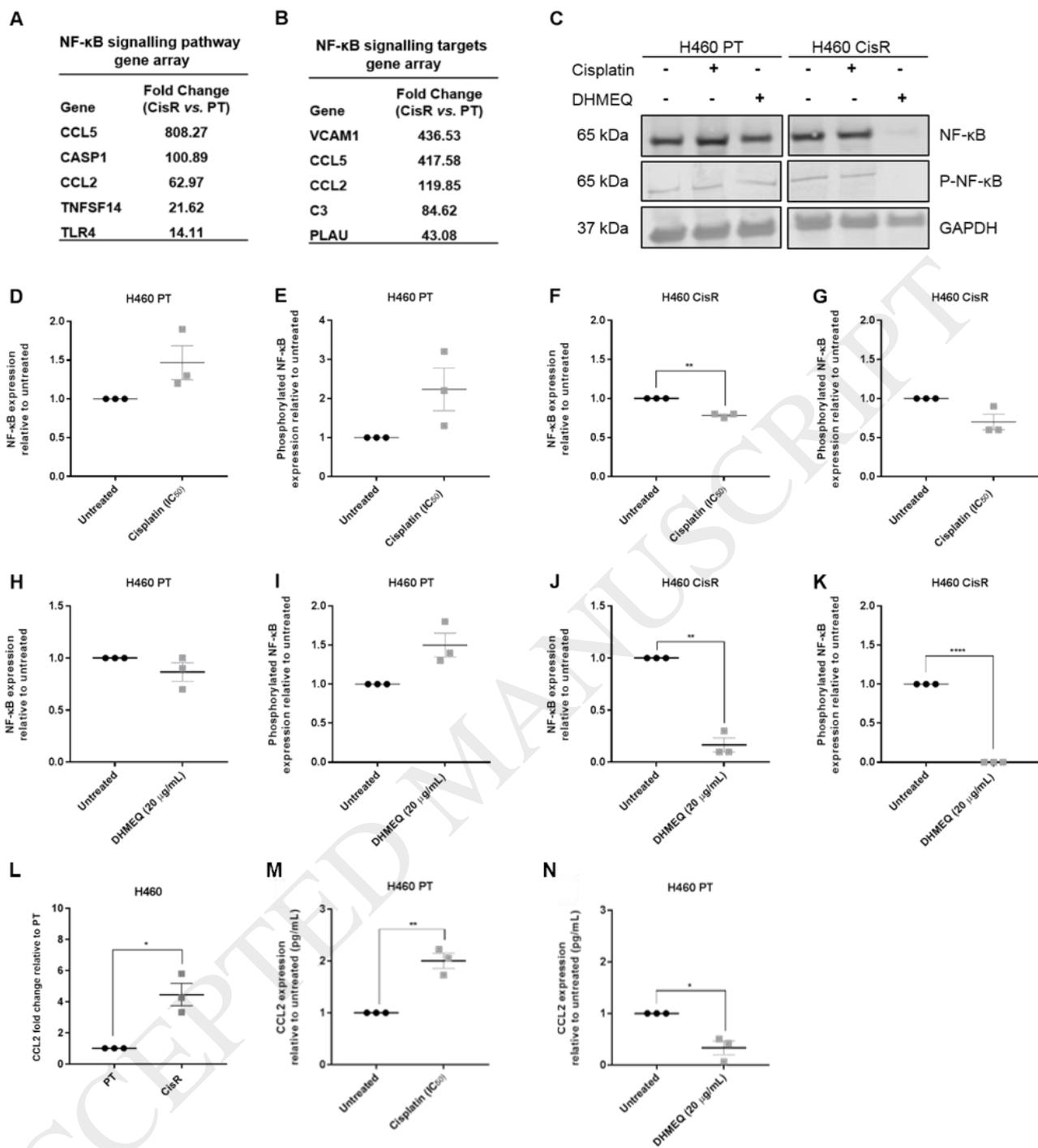
Increased (red) and decreased (green) expression of NF- $\kappa$ B target proteins in H460 CisR versus PT (untreated) (A), H460 PT (IC<sub>50</sub> cisplatin versus untreated) (B), and H460 CisR (IC<sub>50</sub> cisplatin versus untreated) (C). The NF- $\kappa$ B complex is highlighted in pink. The prominent signaling networks identified in each data set are listed in the left hand panels, with NF- $\kappa$ B signaling as the second most prominent network in each case (highlighted).

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**Figure 2: NF- $\kappa$ B expression is altered in patient samples and *in vitro* cell line models**

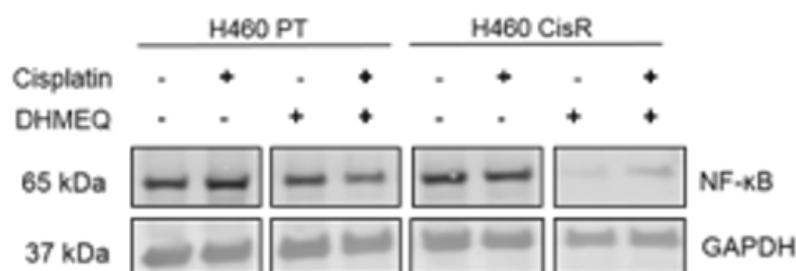
Kaplan-Meier analysis of AC patients treated with a chemotherapy regimen (Affymetrix ID 201783\_s\_at; n=36) comparing high versus low *NF- $\kappa$ B* (*p65*) transcript levels with respect to survival (A, B) [81]. HR = Hazard Ratio; CI = Confidence Interval. Dysregulated *NF- $\kappa$ B* mRNA expression in the NSCLC panel (CisR versus PT) as determined by qRT-PCR analysis using primers specific for *NF- $\kappa$ B* normalized to house-keeping gene *7SL* (C-G). Representative composite western blot image of NF- $\kappa$ B (65 kDa) and GAPDH (37 kDa) in the NSCLC panel (H). Corresponding densitometry analysis of NF- $\kappa$ B expression, normalized to GAPDH loading control, in the NSCLC panel (CisR versus PT) (L-M). Data graphed as mean  $\pm$  SEM (n=3). Statistical analysis was performed using an unpaired two-tailed Welch's t test (\*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001).



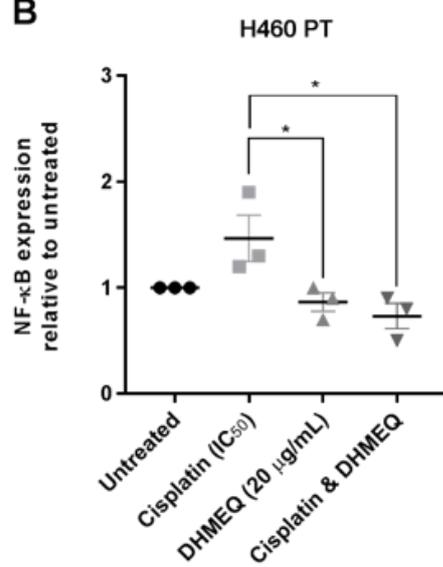
**Figure 3: Cisplatin and DHMEQ-mediated NF- $\kappa$ B expression in H460 PT and CisR cells**

Top altered genes by fold change (H460 CisR versus PT) in NF- $\kappa$ B signaling pathway (A), and NF- $\kappa$ B signaling targets (B), as determined by gene arrays (n=1). NF- $\kappa$ B and phosphorylated NF- $\kappa$ B expression in H460 PT and CisR cells in response to cisplatin (IC<sub>50</sub>) and DHMEQ (20  $\mu$ g/mL) treatment (composite image representative of n = 3), as determined by western blot (C). NF- $\kappa$ B and phosphorylated NF- $\kappa$ B expression levels were normalized to GAPDH, which acted as a loading control, and all treatment data was expressed relative to the untreated controls (D-K). Densitometry analysis indicated enhanced NF- $\kappa$ B (D) and phosphorylated NF- $\kappa$ B (E) expression in H460 PT cells in response to cisplatin treatment. Conversely, cisplatin reduced expression levels of NF- $\kappa$ B (F) and phosphorylated NF- $\kappa$ B (G) in H460 CisR cells. Densitometry analysis indicated reduced NF- $\kappa$ B (H) and increased phosphorylated NF- $\kappa$ B (I) expression in DHMEQ-treated H460 PT cells. DHMEQ reduced both NF- $\kappa$ B (J) and phosphorylated NF- $\kappa$ B (K) expression in H460 CisR cells. qRT-PCR analysis verified the results witnessed in (A) and (B) and indicated increased expression of CCL2 (CisR versus PT) (L). Increased (M) and decreased (N) CCL2 expression in H460 PT cells in response to cisplatin and DHMEQ respectively, as determined by ELISA. Data graphed as mean  $\pm$  SEM (n=3). Statistical analysis was performed using a paired student's *t* test (\*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001).

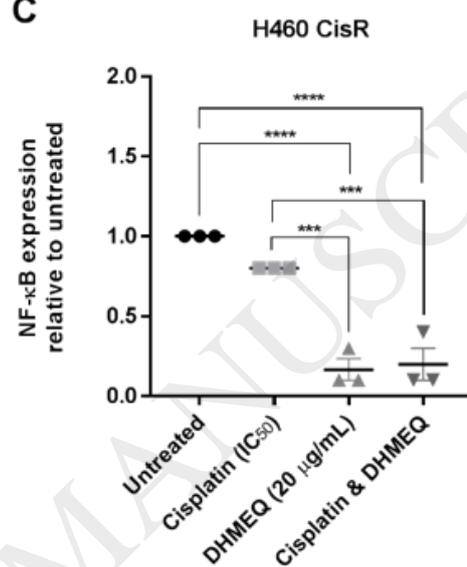
A



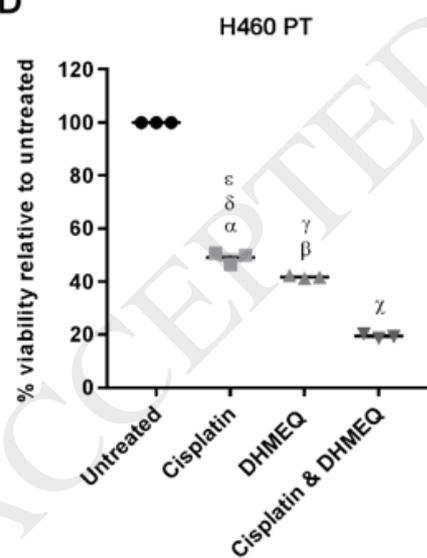
B



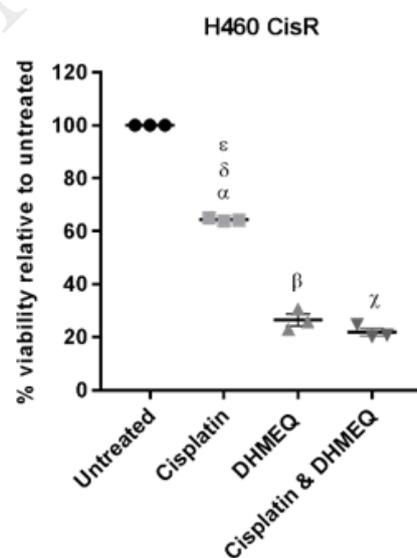
C



D



E



**Figure 4: DHMEQ (20 µg/mL) counteracted cisplatin-mediated NF-κB protein expression and enhanced cisplatin sensitivity in H460 PT and CisR cells.**

NF-κB expression in H460 PT and CisR cells in response to cisplatin ( $IC_{50}$ ), DHMEQ (20 µg/mL) and cisplatin/DHMEQ treatment (composite image representative of  $n=3$ ) (A). NF-κB was normalized to GAPDH loading control. Corresponding densitometry analysis demonstrated reduced NF-κB protein expression in DHMEQ-treated and cisplatin/DHMEQ treated H460 PT (B) and CisR (C) cells relative to the untreated control and to cisplatin alone. Data graphed as mean  $\pm$  SEM ( $n=3$ ). Statistical analysis was performed using one-way ANOVA (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ ). The combination of cisplatin and DHMEQ resulted in a greater reduction in H460 PT (D) and CisR (E) viability compared to cisplatin or DHMEQ alone. Data graphed as mean  $\pm$  SEM ( $n=3$ ). Statistical analysis was performed using one-way ANOVA. Symbols: A ( $\alpha$   $p<0.0001$  Cisplatin versus Untreated;  $\delta$   $p<0.01$  Cisplatin versus DHMEQ;  $\epsilon$   $p<0.0001$  Cisplatin versus Cisplatin & DHMEQ;  $\beta$   $p<0.0001$  DHMEQ versus Untreated;  $\gamma$   $p<0.0001$  DHMEQ versus Cisplatin & DHMEQ;  $\chi$   $p<0.0001$  Cisplatin & DHMEQ versus Untreated). B ( $\alpha$   $p<0.0001$  Cisplatin versus Untreated;  $\delta$   $p<0.0001$  Cisplatin versus DHMEQ;  $\epsilon$   $p<0.0001$  Cisplatin versus Cisplatin & DHMEQ;  $\beta$   $p<0.0001$  DHMEQ versus Untreated;  $\chi$   $p<0.0001$  Cisplatin & DHMEQ versus Untreated).