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Molecular changes in EGFR downstream signalling and intracellular calcium changes associated with the cisplatin-resistant phenotype of lung cancer cells

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Oberschleißheim,22.07.2020
Place, date

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Signature doctoral candidate

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(Venu Pamidiboina)

Einleitung: Lungenkrebs ist weltweit die häufigste Ursache für krebsbedingte Todesfälle. Cisplatin bleibt trotz klinischer Fortschritte in der Medizin die Behandlung der Wahl. Die Resistenz gegen diese Arzneimittel schränkt jedoch ihre klinische Wirksamkeit ein. Verschiedene Tumorzellen haben verschiedene Mechanismen der Arzneimittelresistenz und unterschiedliche Überlebenswege, was es schwierig macht, die zugrunde liegenden Ursachen der Resistenz zu bestimmen.

Zweck: Identifizieren Sie molekulare Veränderungen, die durch Cisplatinresistenz in den EGFR-Signalwegen und im intrazellulären Calcium ausgelöst werden, um potenzielle Ziele für neuartige Kombinationstherapien zu identifizieren, die das Überleben des Patienten verbessern könnten.

Experimentelles Design und Methoden: Ein isogenes klinisches Modell wurde verwendet, um einen Cisplatin-resistenten Phänotyp (CRP) in nicht-kleinzelligen Lungenkrebszellen mit mutiertem EGFR (H838, HCC827, H1975 und H1650) und kleinzelligen Lungenkrebszellen (H1339) zu erzeugen. Die Wirkung von Cisplatin auf das Überleben, die Proliferation, die Koloniebildung und die Apoptose der Zellen wurde in CRP-Zellen und altersangepassten naiven Zellen charakterisiert. Die EGFR-Phosphorylierung und -Signalisierung wurden unter Verwendung eines EGFR-Signalantikörperarrays analysiert. Wir haben die Wirksamkeit von EGFR-Tyrosinkinase-Inhibitoren der dritten Generation (Erlotinib, Gefitinib, Afatinib und Rociletinib) bei klinischen Konzentrationen gemessen.

Ergebnisse: Cisplatin verringerte die Proliferation, erhöhte die Resistenz gegen Zelltod und verbesserte das klonogene Überleben von CRP-Zellen. Die Cisplatinresistenz veränderte die EGFR-Expression, die EGFR-Phosphorylierung und die nachgeschalteten EGFR-Signalmoleküle in CRP-Zellen, dies variierte jedoch zwischen den Zelllinien. Die Wirkung von EGFR-TKIs war in CRP-Zellen und ihren Vorläufern ähnlich. Es gibt keinen signifikanten Unterschied in den Calciumspiegeln zwischen Cisplatin-resistenten und naiven Zellen.

Schlussfolgerung: Die EGFR-Signalübertragung war in CRP-Lungenkrebszellen verändert, und diese Effekte waren zellspezifisch. Darüber hinaus induzierte die Cisplatinresistenz eine Chemosensibilisierung gegen Erlotinib, jedoch nicht gegen andere TKIs in EGFR-Wildtyp-Zellen. Diese Ergebnisse liefern ein tieferes Verständnis der nachgeschalteten zellulären Ereignisse von EGFR, die an der Cisplatinresistenz beteiligt sind. Die Cisplatinresistenz veränderte die intrazellulären Calciumspiegel nicht.

Introduction: Lung cancer is the most common cause of cancer-related death worldwide. Cisplatin remains the treatment of choice, despite clinical advances in medicine. However, resistance to these drugs limits their clinical efficacy. Different tumour cells have various mechanisms of drug resistance and different survival pathways, which makes it difficult to determine the underlying causes of resistance.

Purpose: Identify molecular changes triggered by cisplatin resistance in the EGFR signalling pathways and intracellular calcium to identify potential targets for novel combination therapies that could improve patient survival.

Experimental design and methods: An isogenic clinical model was used to generate a cisplatin-resistant phenotype (CRP) in mutant-EGFR non-small-cell lung cancer cells (H838, HCC827, H1975, and H1650) and small-cell lung cancer cells (H1339). The effect of cisplatin on cell survival, proliferation, colony formation, and apoptosis was characterised in CRP cells and age-matched naïve cells. EGFR phosphorylation and signalling were analysed using an EGFR signalling antibody array. We measured the efficacy of third-generation EGFR tyrosine kinase inhibitors (erlotinib, gefitinib, afatinib, and rociletinib) at clinical concentrations.

Results: Cisplatin decreased proliferation, increased resistance to cell death, and enhanced the clonogenic survival of CRP cells. Cisplatin resistance altered EGFR expression, EGFR phosphorylation, and EGFR downstream signalling molecules in CRP cells, but this varied between cell lines. The effect of EGFR TKIs was similar in CRP cells and their precursors. There no significant difference in calcium levels between cisplatin resistant and naïve cells.

Conclusion: EGFR signalling was altered in CRP lung cancer cells, and these effects were cell-specific. In addition, cisplatin resistance induced chemosensitisation to erlotinib but not to other TKIs in EGFR-wild-type cells. These results provide a deeper understanding of the EGFR downstream cellular events involved in cisplatin resistance. Cisplatin resistance did not change intracellular calcium levels.

1. Introduction

Cancer is caused by accumulated epigenetic changes and mutations that alter normal cell growth and survival [1]. The hallmarks of a malignant phenotype are rapid proliferation, reduced differentiation, and apoptosis [2]. Cancer is accountable for one in three premature deaths worldwide, and cases are expected to rise to over 20 million per year by 2030 [3-11].

1.1. Lung cancer

Lung cancer is the leading cause of cancer-related death worldwide [4, 12-19]. Cigarette smoking is a critical risk factor for developing lung cancer, and other risks include exposure to passive smoke, radon, asbestosis, and radiation, which increase susceptibility to inherited genetic changes [4-6]. Lung cancer is classified according to its histology and the primary forms are non-small-cell lung cancer (NSCLC), small-cell lung cancer (SCLC), and rare tumours. NSCLC can be further divided into squamous cell carcinoma (~40%) and non-squamous cell carcinoma (~50%). Non-squamous cell carcinomas include adenocarcinomas and large-cell carcinomas [5, 6, 20, 21]. Adenocarcinomas arise in distal airways, often have glandular histology, and express specific biomarkers. Squamous cell carcinomas arise in more proximal airways and are strongly associated with smoking and chronic inflammation. Large-cell carcinomas are characterised by exclusion of a glandular or squamous shape or expression of specific biomarkers [7].

1.2. Non-small-cell lung cancer (NSCLC)

NSCLC is the most common type of lung cancer (85% of lung cancers) and has a predicted 5-year survival rate of 15.9% [7]. NSCLC is often diagnosed in advanced stage, and the prognosis is worst and the life expectancy of most patients is very low (median overall survival is 10–12 months) [22]. Until now, chemotherapy has been the treatment of choice for advanced NSCLC

and other advanced solid tumours [6]. Patients often develop resistance to chemotherapy, even in early stages of the disease, which limits therapeutic efficacy and causes relapse and mortality. To complicate matters further, NSCLC tumours are biologically distinct and respond differently to systemic treatments [6, 7, 9, 23]. In NSCLC, genomic aberrations occur in components of various signalling pathways [7, 9, 24]. More than 60% of NSCLC overexpress EGFR and 10% have EGFR mutations. Chemotherapy also modulates the immune response by controlling or enhancing antitumor immune activity, so is a good co-treatment to immunotherapy [25, 26].

1.3. Small-cell lung cancer (SCLC)

SCLC is a neuroendocrine tumour that represents 15–20% of lung cancer cases and characterised by rapid tumour growth and early metastasis [27]. SCLC often originates in the central airways, and 70% of patients have distant vital organ metastasis at the time of diagnosis and if untreated, the median survival is 2–4 months [28]. SCLC is distinguishable from NSCLC by its small cells (only twice the size of lymphocytes), which are round or oval and appear bluish when observed under a light microscope [28]. Alterations in tumour-suppressor genes are common and probably play a vital role in the pathogenesis of SCLC. SCLC tumours are highly sensitive to chemotherapy and radiotherapy, and combined therapy is more effective than sequential therapy [7, 28, 29].

1.4. Diagnosis and treatment

Up to 75% of lung cancer patients have symptoms such as a cough, chest pain, haemoptysis, weight loss, and dyspnoea. Diagnosis involves locating the tumour, taking biopsies, sputum cytology, establishing the metastatic status, and molecular testing to identify targeted therapies [4, 5]. Histology and marker expression are the basis of clinical tumour diagnosis and the tumour genotype can predict the response to therapy. Each tumour is unique, and identifying the specific

histological subtype is critical [4, 24]. There are five ways to manage NSCLC: localised treatments (surgery and radiotherapy) and systemic therapy (chemotherapy, targeted therapy, and immunotherapy).

I. Chemotherapy: Cytotoxic chemotherapy is the most common class of antineoplastic drugs for treating malignant diseases. Agents in current clinical use include alkylating agents (cisplatin and carboplatin), antimetabolites (pemetrexed and gemcitabine), anti-microtubule agents (vinorelbine and taxanes), topoisomerase inhibitors (camptothecin, topotecan, and etoposide), and cytotoxic antibiotics (bleomycin and doxorubicin) [4, 7, 30].

II. Targeted therapy: Genomically targeted therapies are used to treat advanced-stage disease, when surgery and radiation therapy are no longer indicated. These drugs have shown promising results in cancers with specific genetic mutations. Current clinically approved treatment targets include EGFR, ALK, ROS1, B-Raf, and MET [28].

III. Immunotherapy: NSCLC can be treated by targeting immune checkpoint proteins, which are expressed on the surface of immune cells and regulate immune function. Cancer cells use immune checkpoint proteins to suppress tumour-specific T-cells and blocking these proteins with immune checkpoint inhibitors can overcome immune evasion by cancer cells. Inhibitors have been developed against cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), programmed death-1 (PD-1), and programmed death-ligand 1 (PD-L1) [5, 31, 32].

1.5. Cisplatin and resistance

Cisplatin, the most active and widely used cytotoxic anti-cancer drug [33]. In clinical settings, cisplatin is the primary treatment for advanced-stage NSCLC and stage II–IIIA NSCLC following or preceding surgical resection. Combination treatments with platinum agents have overall response rates of 25–35%, a median progression-free survival of 4–6 months, and a median overall survival of approximately 8–10 months [34].

Mechanism of action: Cisplatin is activated by the substitution of one or both cis -chloro groups for water molecules in the cytoplasm and generates highly reactive mono- and bi-aquated cisplatin forms. These molecules interact with cytoplasmic nucleophiles and removes reducing agents to alter the redox balance and induce oxidative stress in the cell, causing DNA damage response and mitochondrial apoptosis [30, 35]. Although effective at first, cancer cells develop resistance to cisplatin, which limits the therapeutic efficacy [36, 37]. Surviving cancer cells can also divide to rejuvenate tumours between chemotherapy cycles, which is one of the main reasons for treatment failure [38].

Resistance mechanisms: Various mechanisms underpin the drug resistance in tumour cells and leads to treatment failure. Furthermore, augmented drug metabolism, inadequate drug exposure, secondary mutations in the drug target, or activation of alternative or parallel cell survival pathways all promote resistance to cancer treatment [5, 39]. The four known mechanisms of cisplatin resistance are:

I. **Pre-target resistance (cisplatin binding to DNA):** Increased or decreased expression of copper transporter 1, ATP7A/ATP7B, multidrug resistance protein 2 (ATP-dependent cellular efflux of cisplatin), ATP-binding cassette family, GSH/g-GCS/GST (cisplatin extrusion), and metallothionein (detoxification of metal ions) is involved in this process [30].

II. **On-target resistance (DNA–cisplatin adducts formation):** Cisplatin-mediated inter- and intra-strand DNA adducts induce apoptosis. However, cisplatin-resistant cancer cells either repair these adducts or tolerate unrepaired DNA lesions. The factors influencing this process are BRCA1/BRCA2 (nucleotide excision repair), MLH1 and MSH2 genes (DNA mismatch repair), POLH (DNA polymerase eta), and REV3/REV7 genes (cisplatin sensitivity in tumour cells), and cisplatin-binding proteins [30, 40].

III. **Post-target resistance (signalling activated by cisplatin-mediated DNA damage):** Cisplatin disrupts apoptosis in response to DNA damage. Non-repairable cisplatin-induced DNA damage

activates a pro-apoptotic signalling cascade. The genetic and epigenetic alterations to signalling components are associated with resistance to cisplatin. The most effective mechanisms of post-target resistance involve TP53 inactivation (DNp63 alpha expression) and pro-apoptotic signal transducers such as mitogen-activated protein kinase (MAPK) may also contribute to cisplatin resistance [30, 40].

IV. Off-target resistance (affects molecular circuitries): Cisplatin resistance can also be caused by alterations in signalling pathways that are not directly linked to cisplatin-elicited signals but compensate for cisplatin-induced lethal signals. These include autophagy, dual-specificity Y-phosphorylation-regulated kinase 1B, HER2 (HER-2), heat-shock proteins, and transmembrane protein 205 [30, 40, 41].

Strategies to overcome the epigenetic forms of therapeutic resistance have not yet shown a clinical success. By understanding resistance mechanisms to cisplatin, therapies can be developed to overcome resistance and treatment failure.

1.6. Epidermal growth factor receptor (EGFR) and clinical significance

More than 60% of NSCLC tumours overexpress EGFR and 10% express EGFR-activating mutations. Mutations in the EGFR kinase domain enhance sensitivity to tyrosine kinase inhibitors (TKI). Initial response rates to TKIs are over 75% in patients with mutant-EGFR NSCLC tumours, and these drugs have been validated [25].

EGFR protein, ligands, adaptor proteins, and phosphorylation: The EGFR protein has four extracellular domains, one transmembrane domain, and three intracellular domains. Ligands bind to the third extracellular domain, inducing receptor dimerisation and tyrosine autophosphorylation, which activates cell proliferation. The EGFR family of RTKs and their ligands are essential regulators of tumour cell proliferation, angiogenesis, and metastasis. The

EGFR family has four receptors, and ten different ligands bind selectively to these receptors [42, 43]. This information is described in the table below.

Table 1: EGFR receptor family, ligands, their phosphorylation sites

Receptor	Ligand	Phosphorylation	Adaptor protein	Physiology	Disease	Inhibitors
EGFR	EGF TGF AR HB-EGF BTC EPR EPG	Tyr845, Tyr891 Tyr920, Tyr974 Tyr992, Tyr1045 Tyr1068, Tyr1086 Tyr1101, Tyr1114 Tyr1148, Tyr1173 Tyr654, Tyr669 Ser1046, Ser1047 Ser1070	Grb2, SOS Shc Shp1 cSrc Gab1 PLCγ PKC cCbl	Control of cell growth & differentiation	Overexpress in cancer of breast, lung, prostate, pancreas, head & neck, colon, ovary, and bladder	Afatinib Erlotinib Gefitinib
Her2	Not known ligands	Tyr882, Tyr 899 Tyr958, Tyr1023 Tyr1028, Tyr1139 Tyr1143, Tyr1196 Tyr1221/22, Tyr1226, Tyr1227 Tyr1249, Tyr1253	Grb2SOS Shc	Essential for neuregulin receptor complex	Like EGFR1	Trastuzumab Lapatinib
Her3 **It cannot auto-phosphorylate due to the impaired kinase.**	Neuregulin, Ebp1, SH2 domain of p85	Tyr1035, Tyr1178 Tyr1180, Tyr1203/5 Tyr1241, Tyr1243 Tyr1257, Tyr1270 Tyr1309	Grb2/ 7SOS Shc PI3K	Development of variety of tissues	Like EGFR1	Patritumab
Her4	Neuregulin1 βcellulin	Tyr1066, Tyr1162 Tyr1066, Tyr1188 Tyr1189, Tyr1242 Tyr1258, Tyr1284	Shc Grb2SOS PI3K	Interacts with neuregulin (NRG2NRG3) & HB-EGF like growth factor	Like EGFR1	AST-1306

EGFR mutations: The most common activating mutations are in-frame deletions in exon 19: in-frame deletions of amino acids 747–750 account for 45% of mutations. L858R substitutions in exon 21 account for 40–45% of mutations, and the remaining 10% involve exons 18 and 20. Partially activated mutant EGFRs can become constitutively active without ligand binding in the presence of other site substitutions, such as the T790M mutation in exon 20. These mutants are resistant to first- and second-generation EGFR TKIs [43].

EGFR downstream signalling cascade: Under normal physiological conditions, ligand binding to the EGFR causes the receptor to dimerise, thereby activating the receptor complex. This activates signalling pathways that promote cell growth, proliferation, and survival. In cancer, EGFR downstream signalling is constitutively activated, which means cancer cell proliferation and survival are no longer controlled [43]. The EGFR signalling network is highly complex and

interacts with downstream (MAPK, phosphoinositide-3-kinase/protein kinase B [PI3K/AKT], and Janus kinase /signal transducers and activators of transcription [JAK/STAT]) cascade and other signalling pathways, such as phospholipase C gamma (PLCγ) and hepatocyte growth factor receptor (HGF/MET) pathways.

I. MAPK pathway: The MAPKs are a highly conserved family of serine/threonine protein kinases that coordinate extracellular signalling pathways involved in cell growth and survival [44]. The MAPK family cascade members are extracellular signal-related kinases (ERK1/2), c-JUN N-terminal kinase (JNK1/2/3), p38-MAPK, and ERK5 [45]. MEK1 or ERK2 knockout is lethal during embryonic development in mice, whereas MEK2 or ERK1 knockout mice are viable, fertile, and healthy [46].

II. PI3K/AKT pathway: Promotes tumour growth by regulating proliferation, migration, metastasis, and chemotherapy resistance [44, 47-49]. The cascade is activated by RTKs such as EGFR, HER2, IGF-1, VEGFR, and PDGFR [50]. PI3K signalling supports cancer development by promoting angiogenesis, genomic instability, and inflammatory cell recruitment [51].

III. JAK/STAT pathway: The pathway regulates haematopoiesis, and disruption of signalling promotes cell growth and prevents apoptosis and are involves in many cellular events [52]. Aberrant JAK/STAT signalling has been reported in various human malignancies, and signalling is constitutively activated in cancer cell lines and tumour tissue. Activated signalling is associated with resistance to radiotherapy and genotoxic chemotherapy in human cancers [16]. STATs can be activated by many cytokines and growth factors, as well as G-protein-coupled receptor agonists, and this activation is cell type- and ligand specific [53].

IV. Phospholipase C (PLC) pathway: PLC consists of six isoforms (β, γ, δ, ε, ζ, η), and distinct mechanisms regulate each isoform. PLCγ is activated by RTKs (EGF, PDGF, FGF, NGF, and HGF), which induces PIP2 production. PLCγ consists of two isozymes, PLCγ1 and PLCγ2. PLCγ1 is ubiquitously expressed and regulates cell growth, migration, and differentiation [37,

54-56]. Pharmacological (U73122) or molecular (RNA interference) inhibition of PLCγ signalling reduces cell invasiveness, but not tumour cell proliferation and apoptosis [37, 55].

V. MET/HGF signalling: Upon ligand (HGF) binding, c-MET dimerises and autophosphorylates, activating MAPK, Pl3K/AKT, SRC, and STAT signalling. This triggers cell survival, proliferation, migration, scattering, motility, invasion, angiogenesis, tumorigenesis, and tumour progression [57]. Combinational treatment with c-met inhibitors and cytotoxic agents can be used to treat c-MET-positive tumours. c-MET inhibitors (Onartuzumab) have shown good clinical efficacy with a manageable toxicity profile in NSCLC patients [58].

1.7. Calcium signalling

Calcium is a critical cofactor for cellular signalling, and multiple proteins regulate the concentration of cytosolic Ca^{2+}. This regulation is essential for physiological functions, including cell cycle control, survival, apoptosis, migration, and gene expression [2, 59, 60]. Components of the calcium machinery include: Ca^{2+} channels, inositol-1,4,5-trisphosphate receptors, ryanodine receptors, transducers, RTKs, G-protein-coupled receptors, Na^{+}/Ca^{2+} exchangers, mitochondrial channels, Ca^{2+}-ATPases, Golgi pumps, calcium buffers, Ca^{2+}-binding proteins, and calcium-sensitive enzymes [61, 62].

Intracellular Ca^{2+} concentration: Cytoplasmic calcium is maintained at low levels (~100 nM) by active mechanisms. Intracellular Ca^{2+} is stored in the mitochondria, Golgi apparatus, nucleus, and endoplasmic reticulum (ER), with most being stored in the ER [2, 59]. An increase in intracellular calcium concentration initiates multiple signalling pathways, depending on the pattern and subcellular localisation [63]. Upregulation of proliferation- and apoptosis-related pathways alters cell growth and cell death and eventually leads to cancer [60].

2. Hypothesis

Cisplatin evokes different cellular responses, and cisplatin resistance is associated with the activation of cell survival signals [34, 64-67]. We hypothesised that cisplatin resistance alters EGFR phosphorylation, downstream EGFR signalling, EGFR TKI sensitisation, and intracellular calcium levels. To test this hypothesis, we quantified total and phosphorylated levels of HER family proteins and their downstream signalling components in cisplatin-resistant EGFR-mutant lung cancer cells. We also examined the effect of EGFR TKIs on the acquired CRP, as this is currently disputed in lung cancer cells.

3. Translational Relevance

Resistance to oncotherapy is an inevitable scenario that leads to tumour relapse and patient mortality. Cisplatin is one of the treatments of choice for advanced lung cancer, and EGFR-targeted therapy has benefited a proportion of NSCLC patients. The reasons for cisplatin resistance are numerous and complicated, and translational approaches are needed to overcome cisplatin resistance in lung cancer. Cisplatin resistance alters EGFR, and calcium signalling – a better understanding of these changes may aid the design of novel and less toxic combination therapies. Specific screening may lead to the development of personalised treatments that enhance the patient quality of life and overall survival.

4. Materials and Methods

4.1. Instruments and laboratory consumables

4.1.1. Instruments

Instruments	Model	Source
ELISA-Reader	Mithras LB 940 Multimode Microplate Reader	Berthold Technologies GmbH & Co. KG, 75323 Bad Wildbad, DE
Fluorescence microscope	Axiovert 200M, SIP 79800	Carl Zeiss AG, Jena, DE
Flow cytometry (FACS)	BD FACSCANTO II	BD Biosciences, D 69126 Heidelberg
Luminescent Image Analyzer	LAS-4000	Fuji Film Europe GmbH, D-40549, Düsseldorf
ChemiDoc	Touch Imaging System	Bio-Rad Laboratories GmbH, Muenchen, DE

4.1.2. Laboratory equipment

Instruments	Model	Source
Cell Incubator	HERA Cell 240	Thermo Scientific, Heraeus, DE
Laminar airflow	HERA Safe	Thermo Scientific, Heraeus, DE
Tube/Plate-centrifuge	Mutifuge X3R	Thermo Scientific, Heraeus, DE
Eppendorf-centrifuge	Kalte mittel R1349	Eppendorf AG, 22331 Hamburg
Precision Balance	KB240-3N	Kern & Sohn GmbH
Analytic Balance	SBC21	Scaltec Instruments, Heiligenstadt
Invert Microscope	Zeiss Primo Vert	Zeiss GmbH, 81241, DE
Nano drop	ND-2000C Spectrophotometer	Thermofisher Life Technologies GmbH, 64293
Water bath	1083	Gesellschaft fur Labortechnik GmbH, D 3006
Thermomixer	Compact	Eppendorf-Netheler-Hinz GmbH, Hamburg, DE
Plate Shaker	KS260 Basic	IkaMag RH, Janke & Kunkel IKA-Labortechnik, DE
Roller	CAT, RM5.40	CAT, M. Zipperer GmbH, D79219
Shaker/ Vortex	G560E VortexGene-2	Scientific Industries, USA
Single-channel pipettes **Multi-channel pipettes**	10, 100, 200 and 1000 µl 0.1-10 & 10-100 µl 30-300 µl	Eppendorf AG, 22331 Hamburg 4661030N, Thermo scientific 64293 Darmstadt, DE

4.1.3. Laboratory consumables

Material	Type	Source
Culture flasks	25, 75 & 175 cm2 and 2 µm vent cap	Corning Incorporated, NY, US Greiner Bio-One GmbH, DE
Multi-well dishes	6-well and 96-well	Falcon, BD Biosciences Labware, US
Cell culture dishes	35 Å~ 10 mm and 100 Å~ 20 mm	Falcon, BD Biosciences Labware, US and Greiner Bio-One GmbH, DE
Centrifuge tubes	15 ml and 50 ml	Sarstedt AG & Co., Nümbrecht, DE
Cryotubes	Cryo Vials	Greiner Bio-One GmbH, DE
Cover slides	24 Å~ 32 mm and 18 Å~ 18 mm	Menzel-Gläser, Mezel GmbH Braunschweig, DE
Counting chamber	0.1 Å~ 0.0025 mm2	Neubauer, Brand, DE
Cell culture pipettes	5, 10 and 25 ml	Corning Incorporated, US.33
Pipette tips	10, 200 and 1000 µl,	Sarstedt AG, Nümbrecht, DE
PCR 96-well plate	Light Cycler 480 Multi-well Plate 96, white	Cat.no:04729692001, Roche Diagnostische GmbH

4.2. Drugs and Chemicals

4.2.1. Drugs

Compound	Source	Mechanism of action (MOA)	Properties
Cisplatin	Apotheke LMU Klinikum, München, DE	Formation of DNA injuries followed by activation of the DNA damage response and the induction of mitochondrial apoptosis. Conventional cytotoxic, approved for the treatment of many malignancies.	Mol.Wt.300 gm/mol (Soluble in saline)
Erlotinib HCl (OSI-776)	Cat.no: E-4007, LC Laboratories, USA	Competing with ATP and reversibly binds to EGFR TK at the intracellular catalytic domain through inhibiting EGFR phosphorylation and blocking the signal transduction. First & second-line therapy for NSCLCs patients; Active against the del E746-A750 deletions in exon 19.	Mol.Wt.429.9 gm/mol (Soluble in DMSO)
Gefitinib (ZD-1839/ Iressa)	Cat.no: G440, LC laboratories, USA	Similar to the erlotinib MOA. First & second-line therapy for NSCLCs patients; Active against the L858R point mutation in exon 21.	Mol.Wt.446.9 gm/mol (Soluble in DMSO)
Afatinib (BIBW2992/ Giotrif)	Cat.no: A-8644, LC Laboratories, USA	Irreversible ATP-competitive drugs make covalent bonds with a cysteine residue at position 797 in EGFR and also affects HER2 & HER4. First-line therapy for subjects bearing metastatic NSCLC tumours has EGFR exon 19 deletions or exon 21 (L858R) substitution mutations.	Mol.Wt.485.9 gm/mol (Soluble in DMSO)
Rociletinib (CO-1686/ AVL-301)	Cat.no: R-3692, LC laboratories, USA	Blocks the kinase activity of EGFR carrying the T790M mutation, and do not inhibit wild-type EGFR significantly. Specific to the patients have the EGFR T790M mutation.	Mol.Wt.555.5 gm/mol (Soluble in DMSO)

4.2.2. Chemicals and reagents

Compound	Source	Category	Concentration
Human Epithelial Growth Factor (hEGF)	Cat. No. 8916SE, Cell Signaling Technology or Cat. No. AF-100-15-100UG Peprotech, London	EGFR and PLC gamma stimulator	Stock: 100 µg/ml Final: 100ng/ml In saline
Crystal violet [C.I. 42555]	Cat.no: T123.1, Carl Roth GmbH	Staining agent	0.5%W/V in PBS
Trypan blue	Cat.no. T8154, Sigma-Aldrich	Staining agent	0.4%
paraformaldehyde	Cat.no: A2156,0100 AppliChem GmbH	Fixing agent	4%

4.2.3. Assay kits

Compound	Source	Purpose
Cell Titer Blue Reagent	Cat.no #G8081, Promega GmbH, D-68199 Mannheim	Cell Viability Assay
Annexin V-FITC apoptosis assay	Cat. No. 556547, BD Biosciences, San Diego, CA	Cellular apoptosis
Human EGFR Phosphorylation Antibody Array	Cat.no: AAH-PER-1-8 Ray Biotech, Inc. GA 30092	EGFR phosphorylation
PathScan EGFR Signaling Antibody Array	Cat.no: #12622, Cell Signaling Technology, MA	EGFR downstream
DC protein assay kits Bio-Rad Laboratories	Protein Assay Reagent A #5000113 Protein Assay Reagent B #5000114 Protein Assay Reagent S #5000115	Protein estimation
Fura-2 AM	Cat. F14185, Molecular Probes, Invitrogen, US (50 µg Å~ 20)	The Intracellular calcium

4.3. Cells lines and cell culture

4.3.1. Tumour cell lines and characteristics

Cell lines	Source	Histology	Characteristics	Mutations
NCI-H838	Cat. No: ATCC-CRL5844	AD & NSCLC	The 3B staged metastasis lymph node tissue of 59 years, Caucasian male, and a smoker.	Wild-type EGFR KRAS, and p53
HCC827	Cat. No: ATCC-CRL2868	AD (BAC features) and NSCLC	Lung epithelium tissue of 38-years Caucasian female, long smoker.	EGFR activating mutation, deletion in exon 19 (del E746-A750). c-Met mutated wt. KRAS & p53
NCI-H1975	Cat. No: ATCC-CRL5908	AD and NSCLC	Lung epithelium tissue of female & Non-Smoker.	Missense mutations in exon 21 (L858R) and exon 20 (T790M-20 /gatekeeper). KRAS & wt p53
NCI-H1650	Cat. No: ATCC-CRL5883	NSCLC, Bronchi alveolar carcinoma	The 3B stage metastatic, pleural effusion of 27 Caucasian male Non-Smoker.	Exon19(delE746A750) & 20 (T790M) on EGFR gene. del PTEN, activated Akt/PI3K signalling . KRAS & p53 wt.
NCI-H1339	Cat. No: ATCC-CRL5979	Small Cell Lung Carcinoma (SCLC)	From the metastatic site: pleural effusion of female.	Wild-type EGFR

4.3.2. Cell culture medium and supplements

Reagent	Catalogue	Source	Strength
RPMI 1640	F1415 BE12-167F	Biochrom AG, Berlin Lonza, B-4800 Verviers, Belgium	Very low Endotoxin
L-glutamine	M11-004	PAA Laboratories GmbH, Austria	200 mM
Pen strep	DE17-602	Lonza, B-4800 Verviers, Belgium	Penicillin & Streptomycin (10,000 U)
Fungi zone	P11-001	PAA Laboratories GmbH, Austria	Amphotericin-B, 250 µg/ml
Fetal Bovine Serum (FBS)	10270	Gibco, Life technologies, DE	Sterile
Trypsin-EDTA	L2143	Biochrom GmbH, D-12247, Berlin	0.05%/0.02% W/V in D-PBS without Ca^{2+} Mg^{2+}
Phosphate Buffered Saline	BE17-516F	Lonza, B-4800 Verviers, Belgium	without Ca^{2+} & Mg^{2+}
Phosphate Buffered Saline	BE17-513F	Lonza, B-4800 Verviers, Belgium	With Ca^{2+} & Mg^{2+}
Ethanol & Methanol		Apotheke, Klinikum Groß Hadern, Münch	100%
Dimethyl sulfoxide (DMSO)	D2650	Sigma-Aldrich Chemie GmbH, DE	100%

4.3.3. Cell culture medium composition

A. 10% FBS medium: Heat-inactivated FBS (10% [v/v]), L-glutamine (200 mM), penicillin and streptomycin (50 mg/ml), and amphotericin B (Fungi zone, 2.5 µg/ml) in RPMI 1640 (90%) medium for H838, HCC827, H1650, and H1975 cells.

B. 20% FBS medium: Heat-inactivated FBS (20% [v/v]), L-glutamine (200 mM), penicillin and streptomycin (50 mg/ml), and amphotericin B (Fungi zone, 2.5 µg/ml) in RPMI 1640 (80%) for H1339 cells.

4.3.4. Cell culture method: H838, HCC827, H1650, H1975, and H1339 cells were cultured in 175 cm^2 culture flasks with 10–12 ml medium at 37°C and 5% CO^2. Culture medium was changed every 2–3 days. Cells were passaged when they reached 60–70% confluency. Cells were detached by incubating in Trypsin-EDTA solution for 3–10 minutes at 37°C and 5% CO^2. Cells

were frozen in cell culture medium containing 20% FBS and 10% DMSO. The effect of EGFR TKIs was compared in cisplatin-resistant cells and naïve cells. For cisplatin treatment, cells were exposed to 1 µg/ml cisplatin for 3 hours, based on previous findings that plasma cisplatin concentrations persist for 3 hours in humans [68, 69].

Table 2: Pharmacokinetic and pharmacodynamics of the cisplatin and the EGFR TKIs

Compound	Category	Human Dose (mg)	Human Plasma (C_{max})	Concentrations used
Cisplatin	Cytotoxic	60-100 mg/m^2, i.v/21days *	3.3 µM or 1 µg/ml	Stock (1mg/ml) Final (1µg/ml)
Erlotinib	Reversible EGFR inhibitor	150-O. D/oral	4.7 µM or 2 µg/ml (1.26 – 2.93 µg/ml)	Stock (2mg/ml) Final (2µg/ml)
Gefitinib	Reversible EGFR inhibitor	250-O. D/oral	0.4 µM or 200 ng/ml (0.16 – 0.24 µg/ml)	Stock (0.2 mg/ml) Final (2µg/ml)
Afatinib	Irreversible EGFR inhibitor	40-O. D/oral	62 nM or 30 ng/ml	Stock (30 µg/ml) Final (30ng/ml)
Rociletinib	Specific inhibitor (T790M mutation)	625-BID/oral	360 nM or 200 ng/ml	Stock (0.2 mg/ml) Final (2 µg/ml)

* Cisplatin can also be given every 28 days depending on the treatment scheme and the combination therapy.

In our experiments, cells were treated with established clinical concentrations of cisplatin (3.3 µM) [68, 70], erlotinib (4.7 µM) [71, 72], gefitinib (0.4 µM) [71, 72], afatinib (62 nM) [72-75], and rociletinib (360 nM) [76], except in drug sensitivity and cell proliferation assays.

4.4. Experimental methods and procedures

4.4.1. Generation of cisplatin-resistant phenotype (CRP) cell lines

Cisplatin resistance was induced in EGFR-mutated NSCLC cells (H838, HCC827, H1975, and H1650) and in SCLC cells (H1339). Cisplatin resistance was induced by exposing the cells to 1 µg/ml cisplatin for 3 hours at 37°C, after which the medium was changed. Cells were exposed to cisplatin once a week for four weeks to induce the CRP [68, 70].

4.4.2. Cell growth assay/ Cell viability assay

To check whether cells were resistant to cisplatin and whether inhibiting EGFR signalling affected this resistance, cell growth and cell viability assays were performed.

Naïve and CRP H838, HCC827, H1650, H1975, and H1339 cells were seeded separately into 25 cm^2 culture flasks and left overnight to settle. Naïve and CRP cells were treated with 0.1% DMSO as an assay control. CRP cells were treated with 1 μg/ml cisplatin for 3 hours. Naïve and CRP cells were treated with erlotinib, gefitinib, afatinib, and rociletinib (see Table 2 for concentrations). Briefly, after 24 hours of drug exposure, cells were trypsinised and centrifuged at 10,000 rpm for 5 minutes to obtain a cell pellet. The pellet was resuspended in 1 ml fresh culture media and diluted 1:10 in 0.4% trypan blue solution. The cell suspension was loaded into a cell counting chamber (0.1 Å~ 0.0025 mm^2) and viable cells (i.e., unstained cells) were counted under a microscope. Viable cells were quantified every 24 hours for four days [68].

4.4.3. Cell proliferation assay/ drug sensitivity assay

To see whether cisplatin resistance alters the growth inhibitory concentration (IC_{50}), and what effect EGFR TKIs have on this, we performed cell proliferation and drug sensitivity assays [77]. Naïve and CRP H838, HCC827, H1650, H1975, and H1339 cells were plated in 96-well tissue culture plates in a complete culture medium. H838 and H1975 cells were plated at a concentration of 3,000 cells per well, and HCC827, H1650, and H1339 cells were plated at a concentration of 5,000 cells per well. Twenty-four hours after plating, cells were exposed to cisplatin (0.01 μM, 0.1 μM, 1 μM, 10 μM, and 100 μM) for 3 hours, after which the medium was replaced with fresh medium containing 10% FBS. Cells were exposed to EGFR inhibitors (0.01 μM, 0.1 μM, 1 μM, 10 μM, and 100 μM) for three days in medium containing 10% FBS. Cell viability was measured after 72 hours of drug incubation using the CellTiter Blue cell viability assay. In this assay, living cells produce a fluorescent end product, which can be quantified, and the amount of fluorescence

is directly proportional to the number of viable cells. Fluorescence was measured on a Mithras LB 940 multimode microplate reader. The IC_{50} was calculated using Graph Pad Prism software (CA, USA) according to the formula below:

$$\text{Fold Resistance} = \frac{IC_{50}\text{ of Resistant Cell Line}}{IC_{50}\text{ of Parental Cell Line}}$$

4.4.4. Clonogenic assay/Colony formation assay

To measure the effect of cisplatin and EGFR inhibitors on colony formation in CRP and naïve cells, we performed colony formation assays. Naïve and CRP H838, HCC827, H1650, H1975, and H1339 cells were plated into six-well culture plates at a concentration of 100 cells per well in a complete culture medium. Twenty-four hours after plating, cells were exposed to 1 μg/ml cisplatin for 3 hours, after which the culture medium was replaced with fresh medium containing 10% FBS. After 2–3 weeks, cell colonies formed, and plates were washed with cold isotonic PBS, then fixed and stained for 30 minutes at room temperature in a 4% paraformaldehyde and 0.5% w/v crystal violet solution in PBS. The plates were then washed gently with water to remove excess stain and air-dried in the dark [78]. Colonies were counted manually under the microscope, and the plating efficiency and surviving fraction (SF) was calculated as follows:

$$PE = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} \times 100\% \quad SF = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded} \times PE}$$

4.4.5. Apoptosis assay by FACS

To quantify the effect of cisplatin resistance and EGFR inhibitors on cisplatin-mediated apoptosis, we performed apoptosis assays. We used the annexin V-FITC apoptosis kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Naïve and CRP H838,

HCC827, H1650, H1975, and H1339 cells were seeded into 25 cm^2 culture flasks and cultured for 24 hours. Cells were then exposed to 1 μg/ml cisplatin for 3 hours. Cells were also treated with erlotinib, gefitinib, afatinib, and rociletinib (Table 2). After 24 hours of drug exposure, we quantified the number of apoptotic cells. First, cells were trypsinised, pelleted by centrifugation, and resuspended in annexin V binding buffer. Next, cells were incubated in a solution of 1 μg/ml FITC-conjugated annexin V and 50 μg/ml propidium iodide in the dark for 30 minutes at room temperature. Stained cells were analysed on a FACS CANTO II flow cytometer (Becton Dickinson, Mountain View, CA), and data were analysed by Flowjo 8.7 and Graph Pad Prism software.

4.4.6. Human EGFR Phosphorylation antibody array

To measure EGFR phosphorylation in cisplatin-resistant and naïve lung cancer cells, we performed EGFR phosphorylation arrays.

Assay procedure: Cells were plated in small dishes and cultured until 70–80% confluent. Naïve and cisplatin-resistant cells were treated with 1 ug/ml cisplatin for 3 hours. Naïve cells were stimulated with hEGF (100 ng/ml for 20 minutes) as a positive control. Cells were washed in ice-cold PBS, and 1× cell lysis buffer was added. Cells were incubated in 0.5 ml lysis buffer on ice for 2 minutes; then cell lysates were collected by scraping. Lysates were left to stand on ice for 3 minutes before centrifuging at 10,000 *g* for 10 minutes. The supernatant was immediately stored at -80°C, and cell lysates were diluted to 0.2–1.0 mg/ml in diluent buffer. Protein in the cell lysates was quantified using the DC protein assay kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Membranes were blocked with 1× blocking buffer for 1 hour and then incubated in 1.2 ml of cell lysate supernatant (test sample) (500 ug/ml) overnight at 4°C. Membranes were washed three times in 1× wash buffer I and then 1× wash buffer II at room temperature with shaking for 5 minutes per wash. Membranes were incubated in 1 ml diluted

biotin-conjugated anti-EGFR antibody overnight at 4°C and washed as described above. Membranes were incubated in 1.5 ml 1× HRP-conjugated streptavidin overnight at 4°C. After incubation, the membranes were washed as mentioned above. Equal portions of detection buffer C and detection buffer D were added to the membranes, and the membranes were imaged using ChemiDoc. Spot intensity was measured by ImageJ (Dot Blot Analyzer) software and data were analysed using Prism software.

Normalisation of signals: One array was defined as the "reference" to which the signal intensities of the other arrays should be compared. Array signals were normalised based on the positive controls:

Pos (1) = Reference average signal intensity of positive controls

Pos (2) = Array 2 average signal intensity of positive controls

X (2) = Intensity of a specific spot on array 2

X(N2) = Normalised value for a specific spot on array 2

X(N2) = X (2) * Pos (1)/Pos (2)

4.4.7. PathScan EGFR signalling antibody array

EGFR downstream signalling was investigated in CRP and naïve EGFR-mutant and EGFR- wild type lung cancer cells using the PathScan EGFR signalling antibody array. In this array, nitrocellulose-coated glass slides spotted with antibodies against phosphorylated EGFR, HER2, c-met, and other EGFR downstream signalling components are used to detect target proteins in the cell lysates [79, 80].

Lysates were prepared from CRP, naïve, and positive control (naïve cells stimulated with 100 ng/ml hEGF for 5 minutes) H838, HCC827, H1650, H1975, and H1339 cells as described in section 4.3.1. The array was performed as described in the manufacturer's instructions. Briefly, a multi-well gasket was fixed to the glass slides, and non-specific binding sites were blocked by

adding blocking buffer to each well. After blocking, 75 µl diluted cell lysate was added to each well and left to incubate for 2 hours at room temperature. Cell lysates were removed, and the wells were washed for 5 minutes in 1× array wash buffer before incubating in detection antibody cocktail for 1 hour at room temperature. After four 5-minute washes, 75 µl 1× HRP-linked streptavidin was added to each well and incubated for 30 minutes. After washing, the glass slide was exposed to a LumiGLO/peroxide reagent, and chemiluminescent signals were detected. Images were captured and analysed using a LAS4000 imaging system (Fuji Photo Film). Array spot intensity was measured using ImageJ (Dot Blot Analyzer) software. Data were analysed using Graph Pad Prism software.

4.4.8. Calcium quantification assay

We measured differences in cytoplasmic calcium concentration in CRP and naïve lung cancer cells. We also examined the impact of EGFR inhibitors at clinical concentrations on cytoplasmic Ca^{2+} concentrations in naïve and CRP cells.

Measurement of cytoplasmic Ca^{2+}

HCC827 and H1975 naïve and CRP cells were seeded in cell culture dishes. After the cells adhered, Fura-2 AM was added at a final concentration of 10 µM and the cells were incubated for 90 minutes at 37°C to stain cytoplasmic calcium. Cells were then incubated another 30 minutes in PBS (with Ca^{2+} and Mg^{2+}) to allow complete dye de-esterification before examining the cells under a fluorescent microscope. To capture the images, the following were used: microscope (Axiovert 200M, SIP 79800, Carl Zeiss AG), HBO lamp (103W/2, short Arc mercury lamp, Osram GmbH), objective fluor (20 × 0.75, transmission wavelength from 340 nm, Zeiss AG), excitation filter (wavelength 340 nm and 380 nm), emission filter (wavelength 510 nm) (both filters: Cat. 340 AF 15 and 380 AF 15, Laser Components GmbH), and a CCD digital camera (AxioCam MRm, Carl Zeiss Vision). Images of the same field were taken at excitation

wavelengths 340 nm and 380 nm with the image-processing program Axio Vision 4.1 (Carl Zeiss). ROIs were defined in the cytoplasm of each cell, and the average fluorescence of ROIs was measured with the image-processing program Scion Image 4.0 (Scion). $[Ca^{2+}]$ c was calculated as follows:

$$[Ca^{2+}]_{free} = K_d \times \left[\frac{R-R_{min}}{R_{max}-R}\right] \times \left[\frac{F^{380}{}_{max}}{F^{380}{}_{min}}\right]$$

The ratio (R) of emission intensities is calculated as the emission intensity from 340 nm excitation at 510 nm, divided by the emission intensity at 510 nm from 380 nm excitation (R = F340/F380). According to the equation above, the $[Ca^{2+}]$c was obtained from the fluorescence emission intensity ratio [81].

4.5. Statistical analysis

Data are presented as mean values ± SEM and mean values ± SD. Nonlinear fit curves were used to determine the inhibition concentration (IC_{50}) using Graph Pad Prism. Paired Student's t-test was used to compare groups. A p-value < 0.05 indicated statistical significance.

5. Results

5.1. Generation and characterisation of cisplatin-resistant phenotype (CRP) cells

To explore how EGFR signalling is affected by cisplatin resistance, we first generated cells with a cisplatin-resistant phenotype (CRP). We used a panel of EGFR-mutant lung cancer cells (summarised in Table 3). The peak plasma levels of cisplatin are between 0.2 and 11 μM in patients receiving 60–100 mg/m^2 of the drug. Therefore, using higher concentrations and prolonged exposure in cells lines is not clinically relevant [34, 67, 82-93]. We induced cisplatin resistance in EGFR-mutant lung cancer cells using cisplatin concentrations of the patient plasma C_{max} (3.3 μM [1 μg/ml] for 3 hrs every week for four weeks) to mimic the clinical condition [70].

Table 3: Histology, mutations, and EGFR TKI sensitivity of the cell lines

Cell lines	Histology	EGFR	KRAS	PTEN	EGFR TKI sensitivity
H838	AD, NSCLC	Wild type	Wild type	-	Not sensitive
HCC827	AD, NSCLC	Exon 19 deletion (del E746-A750)	Wild type	-	Very sensitive
H1975	AD, NSCLC	Exon 21 & 20 deletion (L858R, T790M)	Wild type	-	Sensitive to 2nd & 3rd generation EGFR TKIs
H1650	AD, NSCLC	Exon 19 deletion (delE746A750)	Wild type	+	Not sensitive
H1339	SCLC	Wild type	Wild type	-	Not sensitive

The EGFR-mutant human lung cancer lines (H838, HCC827, H1650, H1975, and H1339) were subjected to repeated cisplatin treatment in a clinical isogenic cell model to induce cisplatin resistance. Derived CRP cells were characterised by measuring viability, proliferation, colony formation, and apoptosis following treatment with 1μg/ml cisplatin. CRP cells showed weak sensitivity to cisplatin in viability assays (Figure 1), with higher IC_{50} values compared with naïve cells (Figure 2). Colony formation assays showed larger colony sizes for CRP cells compared with naïve cells following cisplatin treatment (Figure 3). Furthermore, cisplatin-induced apoptosis was reduced in CRP cells compared with their corresponding naïve cells (Figure 4).

5.1.1. CRP cells show enhanced survival after cisplatin treatment

Cell survival inhibition curves of H838, HCC827, H1650, H1975, and H1339 cells were assessed by trypan blue dye exclusion/phase-contrast microscopy and are presented in Figure 1. Viable cell numbers were not different in CRP cells and naïve cells treated with cisplatin (1µg/ml) on day one, but decreased significantly in naïve cells compared with CRP cells from the 2nd day to the 4th day in a time-dependent manner.

Figure 1

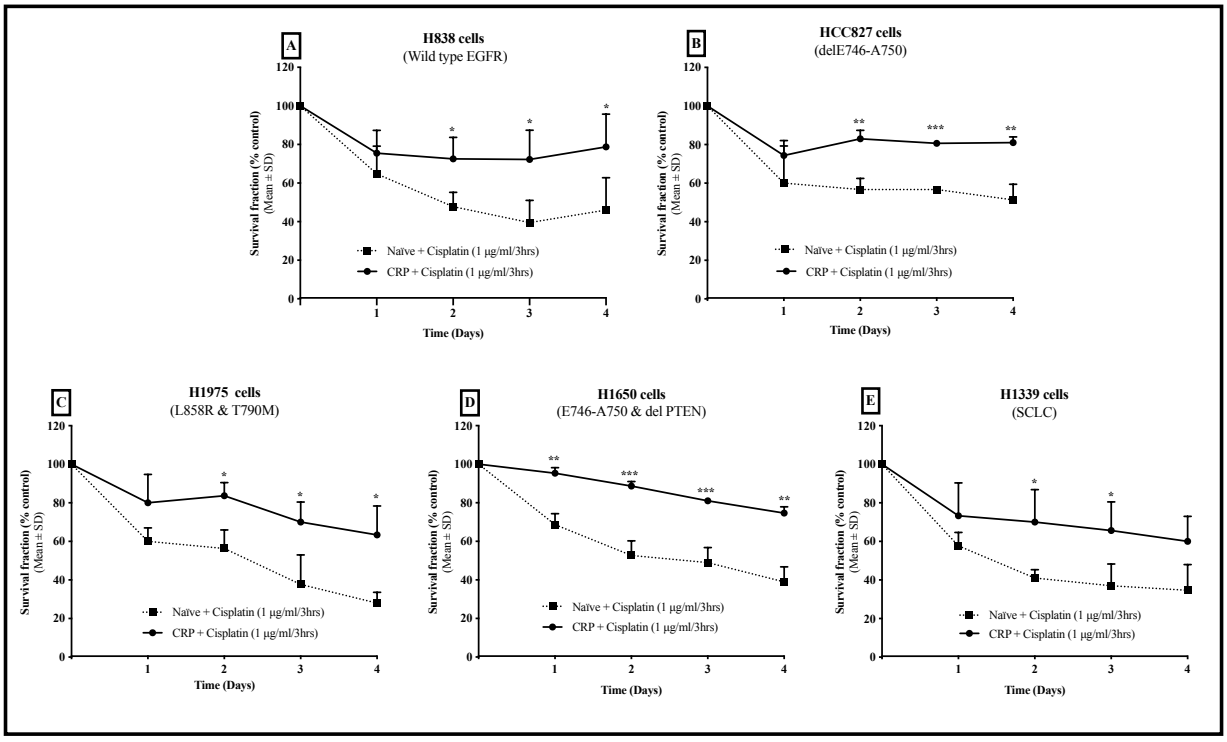

Figure 1A–E: Cell viability assay in EGFR-mutant lung cancer cell lines in response to cisplatin. Viable cells were visualised by trypan blue dye exclusion in EGFR-wild-type H838 cells; in EGFR-mutant HCC827, H1650, and H1975 cells; and in the SCLC H1339 cells. CRP cells were incubated with cisplatin (1 µg/ml) for three hours and survival was measured in a time-dependent manner for four days. CRP cells showed significantly higher survival compared with naïve cells. Data are expressed as mean ± SD from three independent experiments (n = 3, *P < 0.05, **P < 0.01, and ***P < 0.001).

5.1.2. Determination of cisplatin IC_{50} in CRP cells

To determine the IC_{50} values of CRP cells and their corresponding naïve cell lines (H838, HCC827, H1650, H1975, and H1339), cells were treated with log concentrations of cisplatin ranging from 0.01 to 100 µM for 3 hours, after which the medium was replaced with fresh medium. Cell proliferation was measured after 72 hours of drug incubation using the CellTiter-Blue cell viability assay. Dose-response curves were generated and IC_{50} concentrations (Figure 2) and fold resistance were calculated (Table 4) for all cell lines. Cisplatin concentrations (IC_{50}) varied between the tested cell lines.

Figure 2

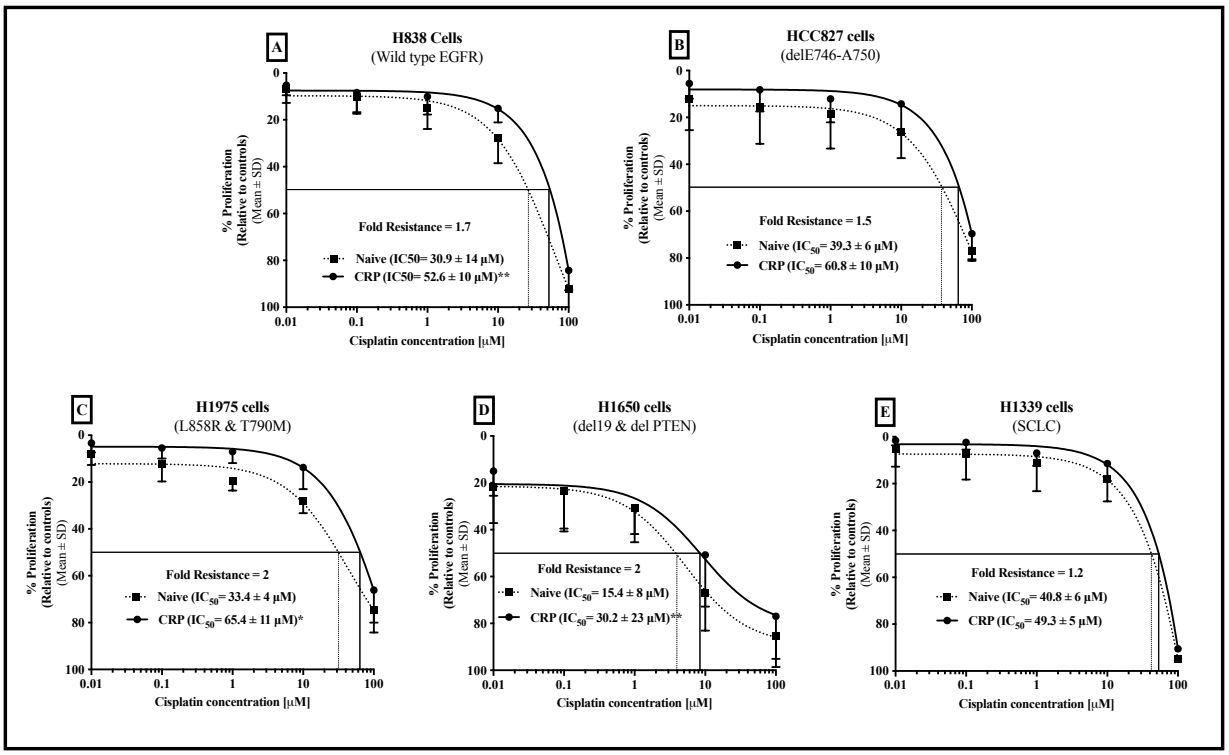

Figure 2A–E: The inhibitory effects of cisplatin on the proliferative capacity of cisplatin -resistant EGFR-mutant lung cancer cells. Naïve and CRP cell lines were treated with log concentrations of cisplatin for 3 h incubation. Proliferation was measured after 72 h of cisplatin application using the CellTiter-Blue cell viability assay. While cisplatin inhibited the growth of both naïve and CRP cells, the inhibitory effect of cisplatin was greatly reduced in CRP cells relative to naïve cells. Data are expressed as mean ± SD from three independent experiments (n = 3, *P < 0.05, **P < 0.01, and ***P < 0.001).

Table 4: Increased cisplatin IC_{50} in CRP lung cancer cells

Cell line	Naïve cells IC_{50} (μM)	CRP cells IC_{50} (μM)	Fold resistance
H838	30.9 ± 6	52.6 ± 9**	1.7
HCC827	39.3 ± 6	60.8 ± 10	1.5
H1975	33.2 ± 4	65.4± 11*	2.0
H1650	3.2 ± 1	5.9 ± 1**	1.8
H1339	40.8 ± 6	49.3± 5	1.2

Table 4: IC_{50} values of cisplatin in naïve and CRP EGFR-mutant lung cancer cells. The IC_{50} increased from 1.2-fold to 2-fold in CRP cells compared with naïve cells 72 hours after cisplatin treatment. Data are expressed as mean ± SD from three independent experiments (n = 3, *P < 0.05, **P < 0.01, and ***P < 0.001). Three CRP cell lines (H838, H1975, and H1650) showed significantly higher IC_{50} values compared with naïve cells.

5.1.3. CRP lung cancer cells demonstrate enhanced clonogenic survival

Survival of naïve and CRP EGFR-mutant lung cancer cells following treatment with cisplatin (1 μg/ml) was assessed using the clonogenic survival assay. These assays showed that more colonies were formed by CRP cells than by naïve cells (Figure 3) in all cell lines. H838, HCC827, H1975, H1650, and H1339 naïve cells showed decreased colony formation potential after cisplatin treatment, but colony formation by CRP cells was not altered after cisplatin treatment.

Figure 3

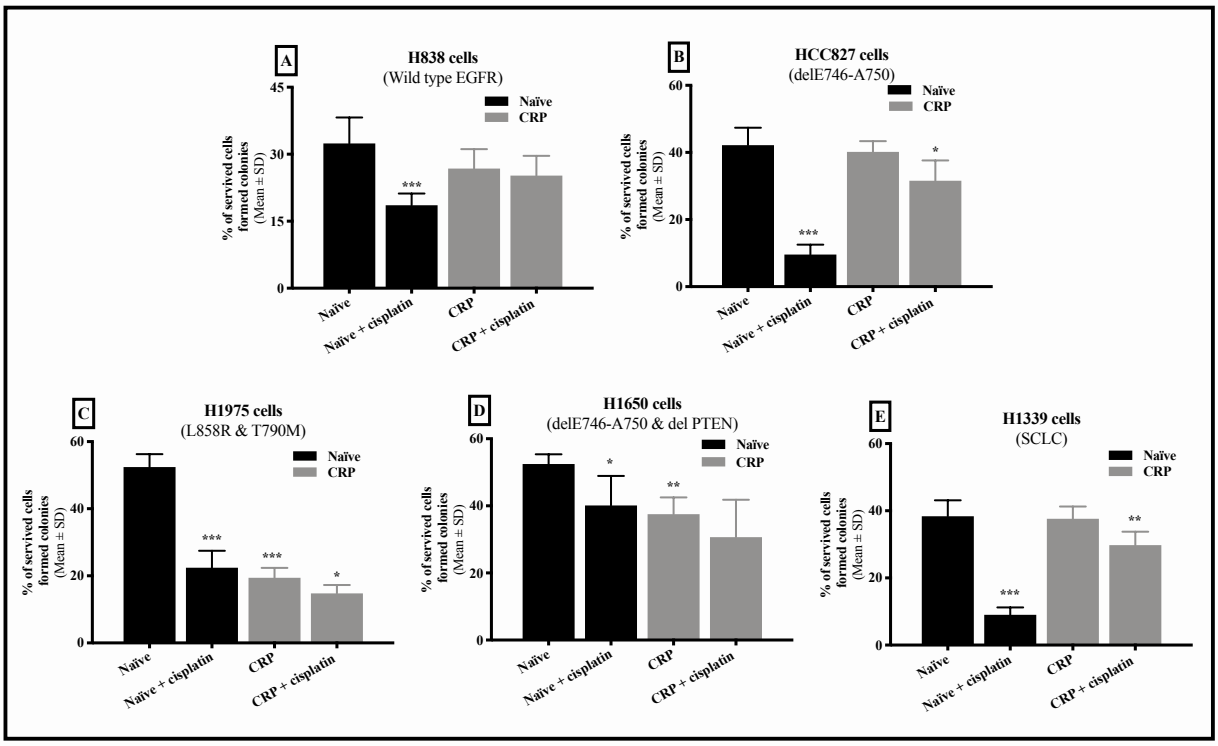

Figure 3A–E: Clonogenic survival is higher in CRP cells than naïve cells after cisplatin treatment. Naïve and CRP EGFR-mutant lung cancer cells were seeded in 6-well plates at optimised seeding densities. Following treatment with cisplatin (1 µg/ml) for 3 hours, media was replaced, and cells were allowed to recover for 14 days. After this, surviving colonies were stained with crystal violet and counted under the microscope. H838, HCC827, H1975, H1650, and H1339 CRP cells formed significantly fewer colonies than naïve cells (A–E). CRP cells treated with cisplatin formed a similar number of colonies as CRP cells not treated with cisplatin. Cisplatin-treated naïve cells formed fewer colonies than naïve cells without treatment. Data are expressed as mean ± SD from six independent experiments (n = 6; *P < 0.05, **P < 0.01, and ***P < 0.001).

5.1.4. Reduced cisplatin-induced apoptosis in CRP cells

Apoptosis was quantified in EGFR-mutant lung cancer CRP cells by flow cytometry. Cells were incubated with 1 µg/ml cisplatin for 3 hours; after 24 hours of drug exposure, we quantified the number of apoptotic cells by flow cytometry using annexin V-FITC and PI (Figure 4). There were fewer apoptotic CRP cells than apoptotic naïve cells after cisplatin treatment (for H838, HCC827, H1650, H1975, and H1339 cell lines), indicating that CRP cells are more resistant to cisplatin than naïve cells are.

Figure 4

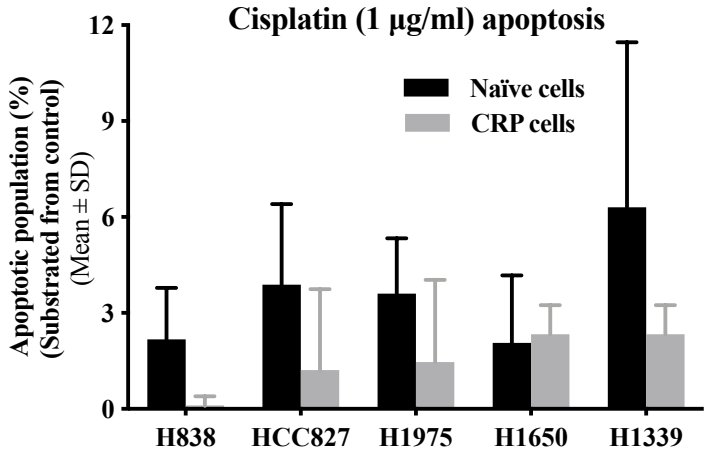

Figure 4: Impact of cisplatin treatment on apoptosis in EGFR-mutant lung cancer cells. H838, HCC827, H1650, H1975, and H1339 cells were treated with 1 µg/ml cisplatin for 3 hours, and apoptosis was quantified after 24 hours of drug exposure. To measure apoptosis, the cells were treated with annexin V-FITC and PI and subjected to flow cytometry. The number of apoptotic CRP cells was lower than apoptotic naïve cells after cisplatin treatment, but there was no significant difference in apoptosis cell proportions between naïve and CRP cells without cisplatin treatment. Data are expressed as mean ± SD from three independent experiments (*P < 0.05, **P < 0.01, and ***P < 0.001).

5.2. EGFR family protein expression and phosphorylation is altered in CRP cells

Alterations in EGFR expression can transform healthy cells into cancer cells with a metastatic phenotype [89]. Levels of EGFR expression and phosphorylation convey essential information about downstream signalling in cancer [88]. An objective of this study was to investigate expression of EGFR family proteins and reveal phosphorylation sites that may regulate cellular responses to cisplatin resistance.

To elucidate how expression of EGFR is affected by cisplatin resistance, we measured EGFR expression in EGFR-wild-type cell line (H838), EGFR-mutant cell lines (HCC827 and H1975), and a SCLC cell line (H1339). We measured the expression of EGFR1, Her2, Her3, and Her4 and quantified total EGFR and phosphorylated EGFR (pEGFR) in cell lysates of naïve and CRP cells (Figure 5). Total EGFR expression (Figure 5 and Table 5) and EGFR phosphorylation

(Figure 6 and Table 6) were both higher in CRP cells than naïve cells, and this difference was cell specific.

5.2.1. EGFR receptor family expression in CRP lung cancer cells

EGFR1 and Her2 are often overexpressed in cancers [94]. Cisplatin resistance did not induce EGFR1 expression in EGFR-mutant lung cancer cells. Her2 was overexpressed in wild-type EGFR cells (H838) and SCLC (H1339) cells (Figure 5A and 5D). In contrast, Her3 and Her4 were only overexpressed in wild-type EGFR cells (H838).

Figure 5

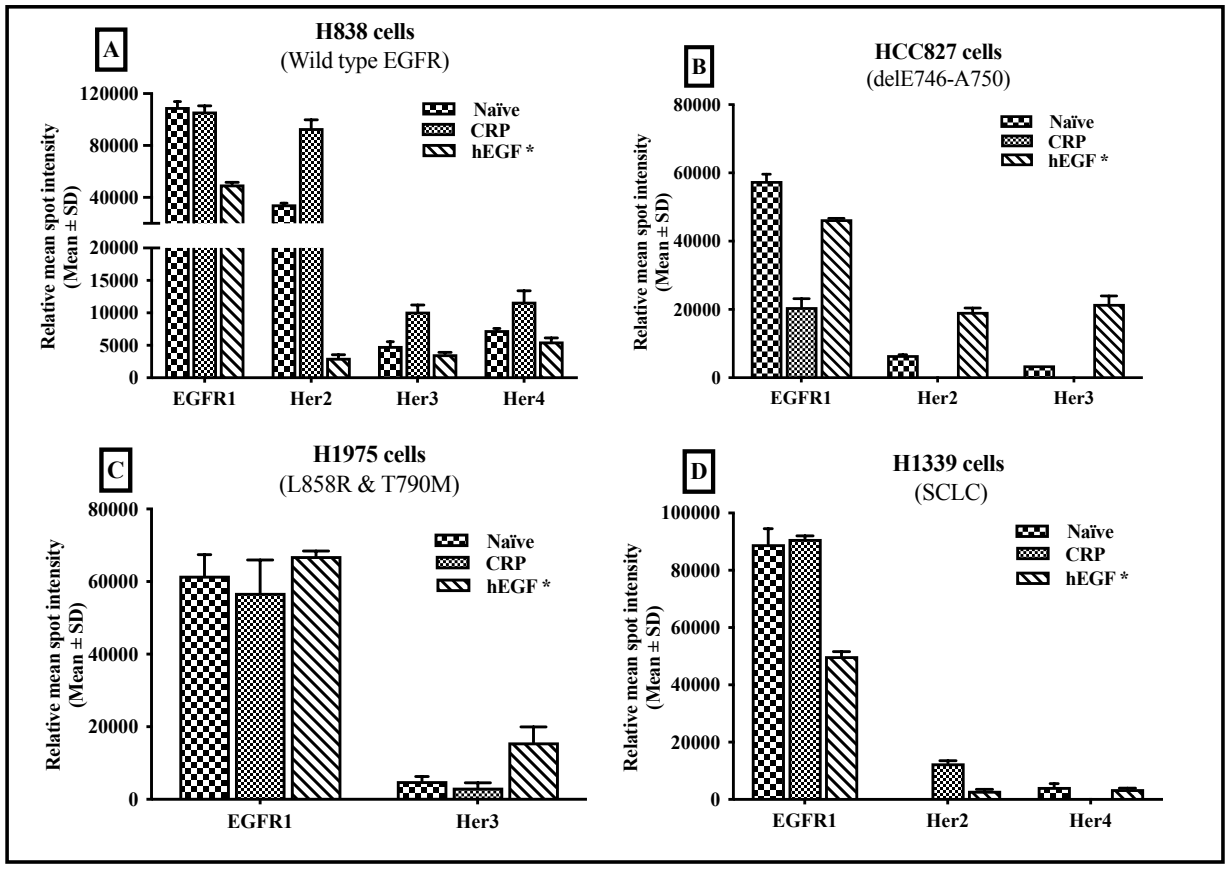

Figure 5 A–D: Cisplatin resistance induces EGFR family receptor expression. In CRP EGFR-mutant lung cancer cells (H838, HCC827, H1975, and H1339), total expression of EGFR family proteins was assessed using a human EGFR family phosphorylation array. Dot intensities were quantified and represented as bar graphs (A–D). Naïve cells were stimulated with hEGF (100 ng/ml for 20 minutes) as a positive control. Data are expressed as mean ± SD. EGFR family receptor expression was altered in CRP lung cancer cells compared with naïve cells (A–D). (A) H838 (wild type-EGFR) CRP cells overexpressed Her2, Her3, and Her4 receptors. (B) HCC827 (EGFR-activating

mutation) CRP cells had reduced EGFR1 expression and did not express Her2 and Her3 receptors. (D) H1339 (wild type-EGFR) SCLC cells showed elevated levels of Her2. Data are expressed as mean ± SD from technical duplicates of two independent experiments (*P < 0.05, **P < 0.01, and ***P < 0.001).

Table 5: EGFR receptor family expression in CRP of lung cancer cells

Receptor	Functional consequences	H838 (wild type)	HCC827 (delE746-A750)	H1975 (L858R &T790M)	H1339 (SCLC)
EGFR1	Ligand binding results in receptor dimerization, autophosphorylation, activation and lysosomal degradation. GRB2, through SHC-RAS–RAF–MEK–ERK: GAB1-PI3K–AKT–mTOR; c-SRC- JAK-STAT; PLCγ–PKC; VAV- Rho family GTPase pathway; Cbl, leading to its ubiquitination and degradation.	**0**	↓	**0**	**0**
ErbB2	ErbB2 possesses an extracellular domain that does not bind any known ligand.. ErbB2 overexpression associated with cisplatin resistance in NSCLC patients. ErbB2 conveys pro-survival signals via PI3K and MAPK.ErbB2 upregulation leads to cell migration and PLCγ activation.	↑	-	-	↑
ErbB3	The EGFR family member with no kinase activity, and can only function in heterodimers, with ERBB2 being its preferred heterodimerization partner. ERBB3 conveys pro-survival signals via PI3K and MAPK.	↑	-	**0**	-
Erb4	ERBB2 and ERBB4, ligand-stimulated ERBB4 can either homodimerize or form heterodimers with ERBB2 resulting in trans-auto-phosphorylation. ERBB4 conveys pro-survival signals via PI3K and MAPK.	↑	-	-	-

Note:([↑] =Increase, [↓] =Decrease, [-] = Not expressed and [0] =similar expression).

5.2.2. EGFR phosphorylation in CRP and naïve lung cancer cells

Cisplatin induces EGFR phosphorylation at specific tyrosine residues [34, 67, 82, 84, 87, 90, 91, 93] and phosphorylated EGFR recruits downstream signalling molecules that are involved in various cellular processes such as proliferation, migration, and apoptosis [95]. We measured EGFR phosphorylation in cisplatin-resistant EGFR-mutant lung cancer cells. Phosphorylation was increased at Tyr845 in wild-type EGFR and EGFR missense mutant NSCLC CRP cells. We found increased phosphorylation at Tyr1173 and activated MAPK and PLCγ signalling in cisplatin-resistant EGFR missense mutant NSCLC cells. Her3 was phosphorylated at Tyr1289 in cisplatin-resistant EGFR-wild type and EGFR-mutant NSCLC cells, and Her4 was phosphorylated at Tyr1284 in cisplatin-resistant EGFR-wild type NSCLC cells (Figure 6 and Table 6).

Figure 6

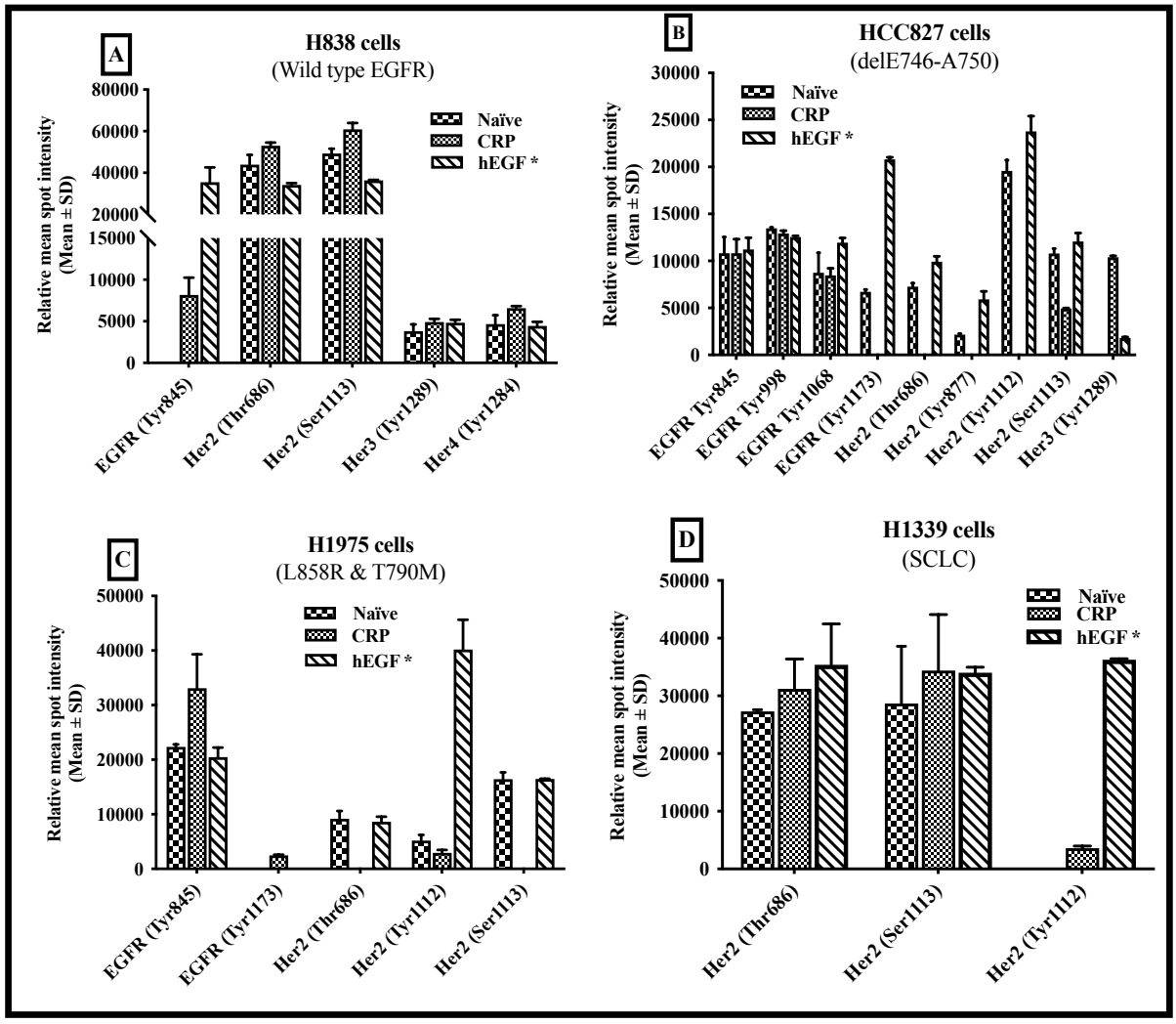

Figure 6 A–D: Cisplatin resistance induces EGFR phosphorylation. In EGFR-mutant lung cancer CRP cells (H838, HCC827, H1975, and H1339), EGFR phosphorylation was measured using a human EGFR family phosphorylation array. Dot intensities were quantified and represented as bar graphs (A–D). Naïve cells were stimulated with hEGF (100 ng/ml for 20 minutes) as a positive control. Data are expressed as mean ± SD. EGFR phosphorylation was different in CRP and naïve cells at different sites (A–D). (A) In H838 (wild type-EGFR) CRP cells, cisplatin induced the phosphorylation of Tyr845, Tyr686, Tyr1112, Tyr1113, Tyr1289, and Tyr1284 residues. (B) HCC827 (EGFR-activating mutation) CRP cells were phosphorylated at Tyr1289 of pHer3. (C) H1975 CRP cells were phosphorylated at Tyr845, Thr686, and Tyr1173 of EGFR1. (D) H1339 CRP cells showed elevated phosphorylation of Tyr1112 and Ser1113 residues and decreased phosphorylation at Tyr686 of Her2 compared with naïve cells. Data are expressed as mean ± SD from technical duplicates of two independent experiments (*P < 0.05, **P < 0.01, and ***P < 0.001).

Table 6: Lung cancer CRP cells showed differential oncogenic protein expression

Phosphorylation	Functional consequences	H838 (wild type)	HCC827 (delE746-A750)	H1975 (L858R &T790M)	H1339 (SCLC)
EGFR Tyr845	Receptor activation; Involves in STAT5b & c-Src signaling	↑	0	↑	-
EGFR Tyr998	Involved in RAS-ERK signaling activation	-	0	-	-
EGFR Tyr1068	Biomarker for the erlotinib sensitivity in vitro & preclinical	-	0	-	-
EGFR Tyr1086	MAP kinase signaling activation	-	-	-	-
EGFR Tyr1173	MAP kinase activation through PLCγ	-	-	↑	-
ErbB2 Tyr686	Not involved in tumour cell proliferation	↑	-	↑	↓
ErbB2 Tyr877	Regulation of ErbB2 biological activity	-	-	-	-
ErbB2 Tyr1112	MAPK/ERK kinase activation	↑	-	↓	↑
ErbB2 Tyr1173	MAPK/ERK activation through PLCγ	-	↓	↑	-
ErbB2 Ser1113	Activation of Src family	↑	↓	0	↑
ErbB3 Tyr1289	Activation of RAS-ERK signaling	↑	↑	-	-
ErbB4 Tyr1284	Activation of RAS-ERK signaling	↑	-	-	-

Note:([↑] =Increase, [↓] =Decrease, [-] = Not expressed and [0] =similar expression).

Some EGFR phosphorylation sites were not quantified because they were not affected by cisplatin resistance or because they were not expressed strongly enough for quantification in certain cells, including pEGFR1: Tyr992, Tyr1045, Tyr1148, Ser1046/1047 and Ser1070; pHer2-Tyr1221/1222, Tyr1248.

5.3. Cisplatin resistance altered the expression of EGFR downstream signalling proteins

EGFR signalling is intertwined with MAPK, AKT, Stat, and PLCγ downstream cell survival pathways [96], and dysregulation of these signalling proteins is involved in tumour progression by promoting cell proliferation, survival, and invasiveness. Understanding how these pathways are connected is crucial to defining their role in cisplatin resistance [85]. The key EGFR downstream signalling nodes and their mutations were quantified in cisplatin-resistant EGFR-mutant lung cancer (H838, HCC827, H1650, H1975, and H1339) cells. One of the objectives in this study was to provide a more comprehensive definition of EGFR downstream signalling networks that may regulate cellular responses to cisplatin resistance. Expression of EGFR downstream signalling nodes (Figure 7 and Table 7) was higher in CRP cells than naïve cells, and this difference was cell specific.

MAPK signalling components promote survival or apoptosis depending on the level of phosphorylation, cell type, and duration of drug exposure in cisplatin-treated cells [97]. We found

that cisplatin resistance altered the expression of MAPK components in EGFR-mutant lung cancer cells in a cell-specific way. MEK was overexpressed in EGFR double-mutant NSCLC cells and wild type-EGFR SCLC cells. In contrast, expression was decreased in wild type-EGFR lung cancer cells. Phosphorylation was also affected by cisplatin resistance. MEK phosphorylation increased in EGFR-mutated cells and ERK phosphorylation was increased in wild type-EGFR and EGFR-mutated NSCLC cells. PI3K/Akt/mTOR signalling is activated in NSCLC [98] and can lead to chemotherapy resistance [19]. In the present study, cisplatin resistance enhanced pAkt activation at Ser473 in EGFR double-mutant cells. STAT3 activation is associated with cisplatin resistance in epithelial malignancies and STAT3 phosphorylation is higher in cisplatin-resistant lung cancer cells [82]. It has been shown that EGFR induces phosphorylation of Stat3 at Tyr705 [52, 99, 100]. In this study, we found that STAT3 phosphorylation at Tyr705 was higher in cisplatin-resistant EGFR-mutated cells than naïve cells. PLCγ1 phosphorylation at Ser1248 promotes tumour cell invasiveness and migration, and regulates calcium signalling [55, 101, 102]. In this study, PLCγ1 protein expression and phosphorylation at Ser1248 were increased in cisplatin-resistant EGFR double-mutant NSCLC and wild-type EGFR SCLC cells. Inhibiting PLCγ1 by preventing phosphorylation of Ser1248 has been shown to reduce migration in different cancer cells [55, 101]. c-MET expression is enhanced in cisplatin-resistant lung cancer cells and patient tumour tissues, which promotes cell migration, invasion, and tumour metastasis [83, 103]. In this study, c-Met expression was upregulated in cisplatin-resistant EGFR double-mutant NSCLC and wild-type EGFR SCLC cells. In addition, MET phosphorylation at Tyr1234/1235 was increased in cisplatin-resistant EGFR missense mutant cells.

EGFR mutations associated with lung cancer include the E746–A750 deletion and del L858R point mutation and these are associated with sensitivity to EGFR TKIs [104]. Cisplatin resistance did not alter the frequency of these mutations in HCC827 cells, nor the efficacy of EGFR TKIs.

Figure 7

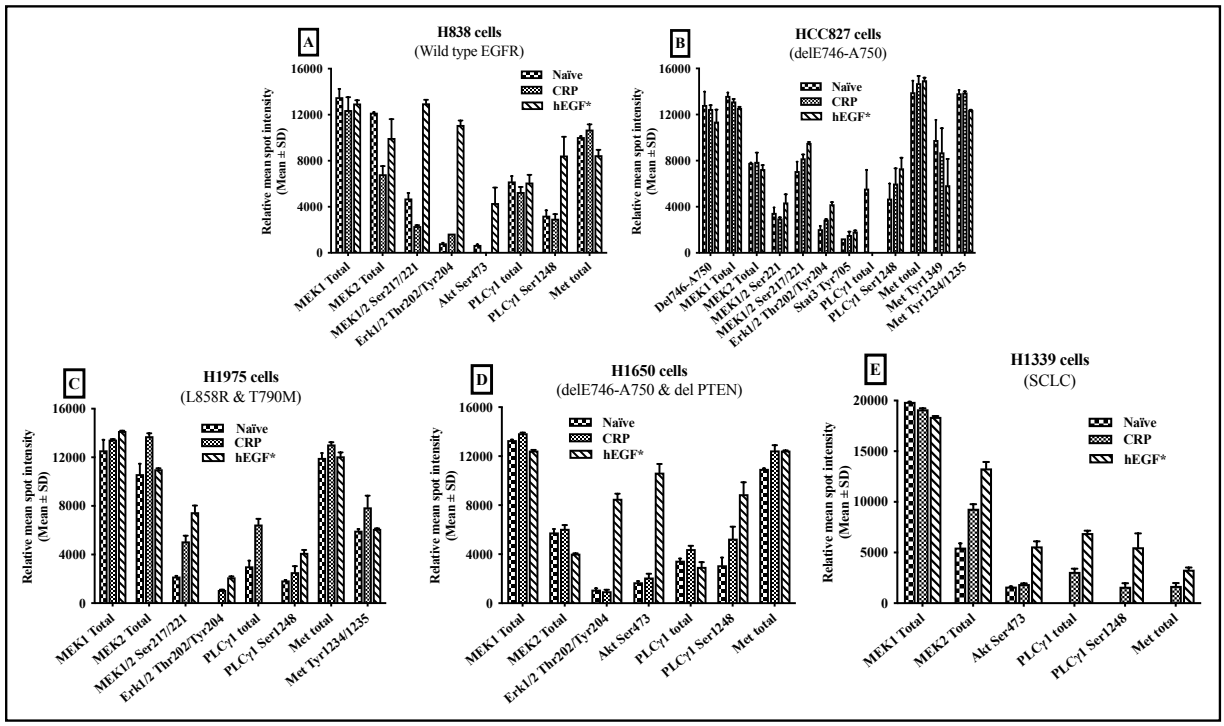

Figure 7A–E: Cisplatin resistance activates EGFR signalling in EGFR-mutant lung cancer cells. Expression of EGFR downstream signalling nodes in EGFR-mutant lung cancer (H838, HCC827, H1975, H1650, and H1339) cells was assessed using a PathScan EGFR signalling antibody array. Naïve cells were stimulated with hEGF (100 ng/ml for 20 minutes) as a positive control. Expression of EGFR downstream signalling components differed between CRP cells and naïve cells (Figure. 7A–E), and these differences were cell specific. (A) In H838 cells, the Thr202/Tyr204 residues of ERK were phosphorylated. (B) In HCC827 cells, phosphorylation was increased at Ser217/221 of MEK, Thr202/Tyr204 of ERK, Tyr705 of Stat3, and Ser1248 of PLCɣ1. Total Met protein expression was also enhanced. (C) H1975 cells showed enhanced expression of total MEK1/2 and enhanced phosphoryation of MEK at Ser217/221 and ERK at Thr202/Tyr204. These cells showed enhanced PLCɣ1 protein expression and phosphorylation at Ser1248. MET receptor expression and phosphorylation at Tyr1234/1235 was also increased in these cells. (D) H1650 cells showed enhanced expression of MEK1/2. The Akt pathway was also activated in these cells because of a PTEN gene deletion. Akt phosphorylation at Ser473 was also increased in CRP cells. H1650 cells showed enhanced PLCɣ1 protein expression and phosphorylation at Ser1248 and also elevated total MET receptor expression. (E) H1339 cells showed enhanced MEK1 and reduced MEK2 total protein expression and enhanced PLCɣ1 expression and phosphorylation at Ser1248. MET receptor expression was also elevated. Expression and phosphorylation of MEK, MET, ERK, Akt, Stat, and PLCɣ1 were altered in all CRP cells. Dot intensities were

quantified and presented as bar graphs. Data are expressed as mean ± SD (A–E). The EGFR Del746-A750 mutation was not altered in HCC827 cells (B). Data are expressed as mean ± SD from technical duplicates of two independent experiments (*P < 0.05, **P < 0.01, and ***P < 0.001).

Table 7: Aberrant expression of EGFR downstream proteins in cisplatin-resistant lung cancer cells

Oncoproteins	Functional consequences	H838 (Wild type)	HCC827 (DelE746-A750)	H1975 (L858R T790M)	H1650 (DelPTEN)	H1339 (SCLC)
MEK1 total	Activated RAS/RAF/MEK/Erk kinase pathway is a significant signalling node with a multitude of substrates and primarily transmits growth and proliferation signals.	↓	0	↑	↑	↓
MEK2 total		↓	0	↑	↑	↑
MEK1/2 Ser221		-	↓	-	-	-
MEK1/2 Ser217/221		↓	↑	↑	-	-
ERK1/2 Thr202/204		↑	↑	↑	0	-
Akt Ser473	Akt generates anabolic growth and survival signals.	-	-	-	↑	-
Stat3 Tyr705	Activated in response to EGFR stimulation & variety of cytokine receptors. Stat3 is an oncogene & a T.F	-	↑	-	-	-
PLCɣ 1 total	A second messenger & activated by EGFR. This hydrolysis to form IP3 (calcium mobilisation) and DAG (activates PKC).	↓	-	↑	↑	↑
PLCɣ 1 Ser1248		0	↑	↑	↑	↑
Met total	Induce cell scattering, migration, and invasion (EMT). Resistance to EGFR-therapies & contributing factor to tumour metastasis.	0	↑	↑	↑	↑
Met Tyr1234/1235		-	0	↑	-	-
Met Tyr1349		-	↓	-	-	-

Note:([↑] =Increase, [↓] =Decrease, [-] = Not expressed and [0] = similar expression).

Some EGFR downstream signalling nodes, phosphorylation sites, and mutations were not quantified because they were not affected by cisplatin resistance or did not have strong enough expression for quantification. These were: phosphorylation sites: pEGFR (Tyr669), pHER2 (Tyr1196), Tyr1221/1222; mutations: EGFR L858R mutation; downstream nodes: Akt Thr308 and PARP Asp214 cleavage.

5.4. Impact of three generations EGFR TKIs on CRP cells at clinical concentrations

Various cancer cells increase EGFR expression to promote survival in response to cytotoxic chemotherapeutic agents, which makes them more susceptible to EGFR inhibition by EGFR TKIs [90]. In this study, cisplatin-resistant EGFR-mutant lung cancer cells were treated with EGFR TKIs (erlotinib, gefitinib, afatinib and rociletinib) at clinical doses (i.e., at concentrations of human plasma Cmax; summarised in Table 2). Cisplatin-resistant cells were less sensitive to EGFR TKIs except erlotinib in viability assays (Figure 8 and 9), with similar IC_{50} values to naïve

cells (Table 8). Apoptosis was similar in CRP and naïve cells after treatment with EGFR TKIs (Figure 10).

5.4.1. The inhibitory effects of first-generation EGFR TKIs on the survival of CRP cells

The effect of first-generation EGFR TKIs (erlotinib and gefitinib) on cell survival of cisplatin-resistant EGFR-mutant lung cancer cells (H838, HCC827, H1650, H1975, and H1339) is shown in Figure 8. Viable cell numbers did not differ between naïve and CRP cells one day after treatment with erlotinib and gefitinib, but there were significantly fewer viable naïve cells than viable CRP cells 2–3 days after treatment. Cisplatin resistance sensitised wild-type EGFR cells (H838 and H1339, which are insensitive to EGFR TKIs) and EGFR double-mutant cells (H1650, which carry a PTEN gene deletion and are resistant to first-generation EGFR TKIs) to erlotinib and also inhibited cell survival in HCC827 NSCLC cells carrying an EGFR-activating mutant.

Figure 8

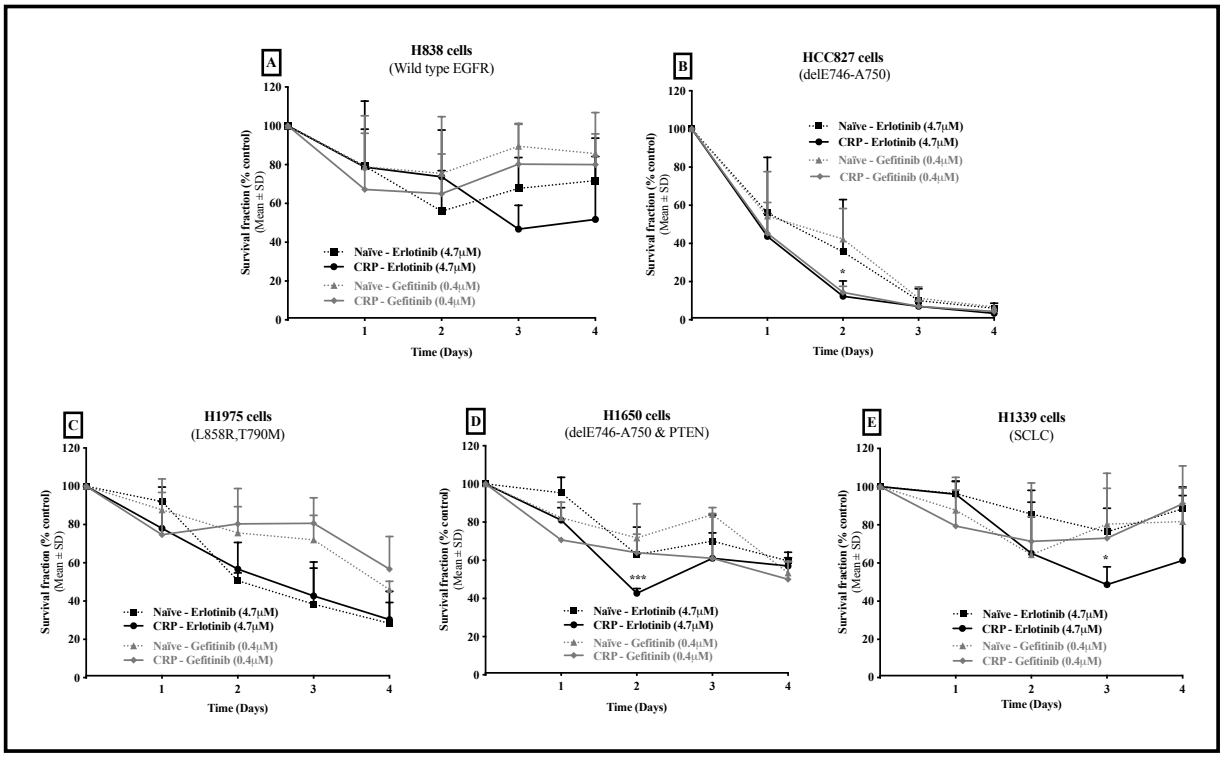

Figure 8 A–E: Viability of cisplatin-resistant EGFR-mutant lung cancer cell lines after treatment with erlotinib and gefitinib. Cell viability was assessed by trypan blue staining in cisplatin resistant and naïve H838, HCC827, H1650, H1975, and H1339 cells. Cisplatin-resistant cells (CRP cells) were incubated with erlotinib (4.7 μM) or gefitinib

(0.4 μM) for 1 to 4 days and survival were measured in every 24 hours for 4 days. Compared with naïve cells, the number of viable CRP cells treated with erlotinib did not differ significantly on the day one, but did decrease significantly from day 2 to day 3. Cisplatin-resistant wild-type EGFR (H838 and H1339) and EGFR double-mutant (H1650) lung cancer cells were sensitised to erlotinib. Erlotinib also inhibited cell survival in EGFR-activating mutant (HCC827) lung cancer cells compared with naïve cells. Data are expressed as mean ± SD from three independent experiments (n = 3; *P < 0.05, **P < 0.01, and ***P < 0.001).

5.4.2. The inhibitory effects of second generation - and third generation EGFR TKIs on survival of cisplatin-resistant EGFR-mutant lung cancer cell survival

We investigated the effect of second-generation (afatinib) and third generation (rociletinib) EGFR TKIs on cell survival in EGFR-mutant lung cancer cell lines (H838, HCC827, H1650, H1975, and H1339). Compared with naïve cells, the number of viable CRP cells treated with afatinib (62 nM) and rociletinib (360 nM) did not change (Figure 9). But afatinib reduced survival in cisplatin-resistant EGFR double-mutant H1975 cells (carrying a gatekeeper EGFR mutation and resistant to first-generation EGFR TKIs) and H1650 cells (carrying a PTEN gene deletion and resistant to first-generation EGFR TKIs) compared with naïve cells.

Figure 9

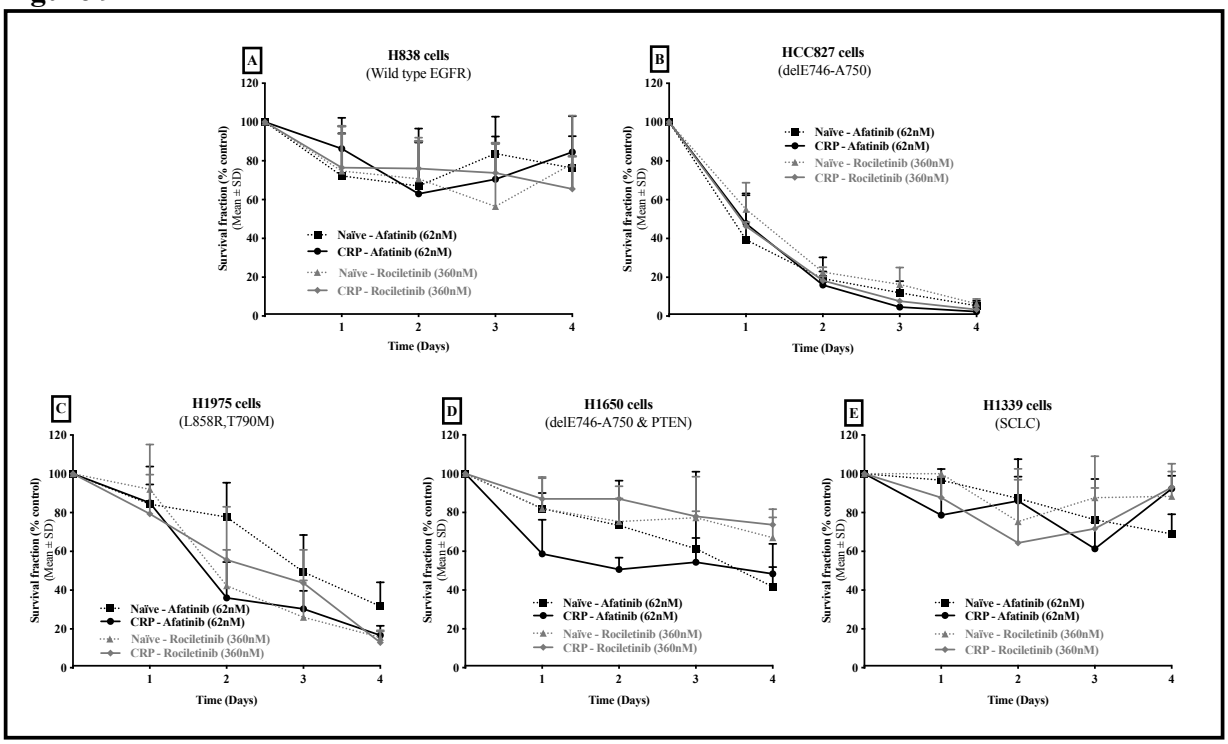

Figure 9A–E: Survival of cisplatin-resistant EGFR-mutant lung cancer cell lines after erlotinib and gefitinib treatment. Viable cells were assessed by trypan blue dye exclusion in cisplatin-resistant (CRP) and naïve H838, HCC827, H1650, H1975, and H1339 cells. CRP cells were incubated with afatinib (62 nM) and rociletinib (360 nM) for 1 to 4 days and survival were measured every 24 hours for 4 days. Afatinib and rociletinib did not affect the viability of CRP or naïve cells. Afatinib inhibited the survival of cisplatin resistant H1975 (C) and H1650 (D) cells but not naïve cells. Data are expressed as mean ± SD from three independent experiments (n = 3; *P < 0.05, **P < 0.01, and ***P < 0.001).

5.4.3. Effect of EGFR TKIs on CRP lung cancer cell proliferation

Cisplatin-resistant EGFR-mutant lung cancer cell lines were treated with log concentrations of EGFR TKIs (erlotinib, gefitinib, afatinib, and rociletinib) ranging from 0.01 to 100 μM for 72 hours and cell proliferation was measured. Dose-response curves were generated and IC_{50} concentrations were calculated. The results are summarised in Table 8.

Table 8: The IC_{50} (μM) of EGFR TK inhibitors in CRP of lung cancer cells

Cell line	H838 (Wild type)		HCC827 (DelE746-A750)		H1975 (L858 & T790M)		H1650 (delPTEN)		H1339 (SCLC)	
Compound	Naïve	CRP	Naïve	CRP	Naïve	CRP	Naïve	CRP	Naïve	CRP
Erlotinib	37 ± 12	71 ± 29	0.23 ± 0	2.3 ± 1	6.4 ± 1	7.6 ± 2	1.6 ± 1	2.3 ± 2	>100	>100
Gefitinib	23 ± 4	22 ± 6	1.8 ± 2	13.7 ± 7	22 ± 6	14 ± 2	7 ± 2	12 ± 3	33 ± 12	23 ± 8
Afatinib	3.2 ± 0.8	3.2 ± 0.9	0.4 ± 0	5.4 ± 3	2.9 ± 0.6	2.4 ± 1	4 ± 3	3 ± 1	3 ± 1	2.6 ± 1
Rociletinib	1.7 ± 0.6	2.3 ± 0.9	0.22 ± 0	0.04 ± 0	2.5 ± 0.6	0.8 ± 0.4	3 ± 2	4 ± 2	9 ± 4	8 ± 3

Table 8: The inhibitory effects of EGFR TKIs on the proliferation of cisplatin-resistant EGFR-mutant lung cancer cells. Naïve and CRP cell lines were treated with log concentrations of EGFR TKIs for 72 hours and cell proliferation was measured using the CellTiter-Blue cell viability assay. The mean growth inhibition (IC_{50}) elicited by erlotinib, gefitinib, afatinib, and rociletinib was not significantly different between CRP and naïve lung cancer cells. Data are expressed as mean ± SD from three independent experiments (n = 3; *P < 0.05, **P < 0.01, and ***P < 0.001).

5.4.4. Effect of EGFR TKIs on CRP cell apoptosis

Induction of apoptosis in cisplatin-resistant EGFR-mutant lung cancer cells was examined by flow cytometry. Cells were incubated with EGFR TKIs (erlotinib, gefitinib, afatinib, and

rociletinib) at clinical concentrations for 24 hours. After drug exposure, apoptotic cells were stained and quantified (Figure 10). The number of apoptotic naïve or CRP cells did not increase after 24 hours of drug incubation. However, erlotinib-induced apoptosis in cisplatin-resistant wild-type EGFR cells (H838, normally insensitive to EGFR TKIs) and EGFR double-mutant H1975 cells, which carry gatekeeper EGFR mutation and resistant to first-generation EGFR TKIs. EGFR double mutant H1650 cells carry a PTEN gene deletion and are resistant to erlotinib, but gefitinib increased apoptosis in these cells. All tested EGFR TKIs enhanced apoptosis in cisplatin-resistant HCC827 cells, which carry an EGFR-activating mutation and are extremely sensitivity to EGFR TKIs.

Figure 10

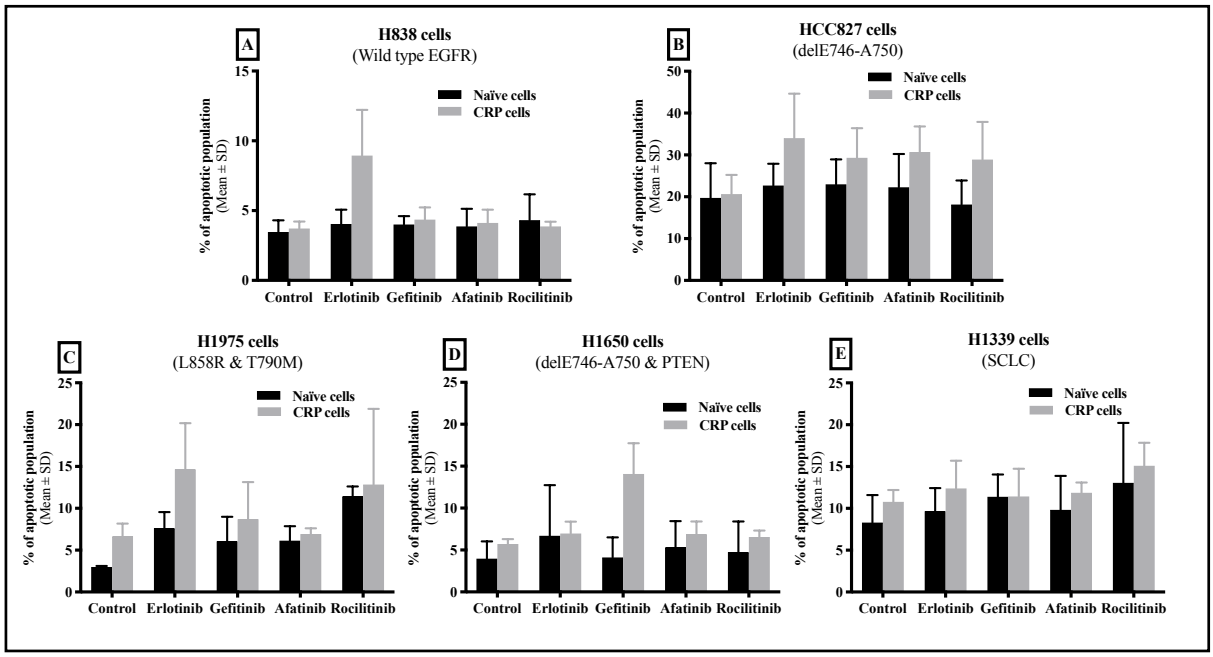

Figure 10 A–E: Apoptosis in cisplatin-resistant EGFR-mutant lung cancer cell lines in response to EGFR TKIs. Cisplatin-resistant (CRP) and naïve H838, HCC827, H1650, H1975 and H1339 cells were incubated with erlotinib (4.7 μM), gefitinib (0.4 μM), afatinib (62 nM), and rociletinib (360 nM) for 24 hours. After drug exposure, apoptotic cells were stained with annexin V and PI and quantified by flow cytometry. Erlotinib-induced apoptosis in cisplatin-resistant H838 and H1975 cells (A and C), and gefitinib enhanced apoptosis in H1650 cells (D). All tested TKIs enhanced apoptosis in HCC827 cells after 24 hours of drug incubation. Data are expressed as mean ± SD from three independent experiments (n = 3; *P < 0.05, **P < 0.01, and ***P < 0.001).

5.5. Effect of EGFR TKIs on cellular cytoplasmic calcium levels

Calcium (Ca^{2+}) is a universal cellular messenger and is regulated by multiple proteins in the cytosol [2, 61]. Intracellular calcium concentration plays a role in chemo-resistance of lung cancer cells [2, 59, 70, 105, 106]. In this study, we quantified calcium levels in the cytoplasm of cisplatin resistant HCC827 and H1975 NSCLC cells after treatment with cisplatin (1 µg/ml) for 3 hours (Figure 11A). Cytoplasmic calcium levels did not change in cisplatin-resistant or naïve NSCLC cells.

We also measured cytoplasmic calcium levels in HCC827 and H1975 NSCLC cells after 24 hours of treatment with erlotinib (4.7 µM), gefitinib (0.4 µM), afatinib (62 nM), and rociletinib (360 nM). Calcium levels were not altered in cisplatin-resistant or naïve NSCLC cells after 24 hours of drug incubation (Figure 11B and 11C).

Figure.11

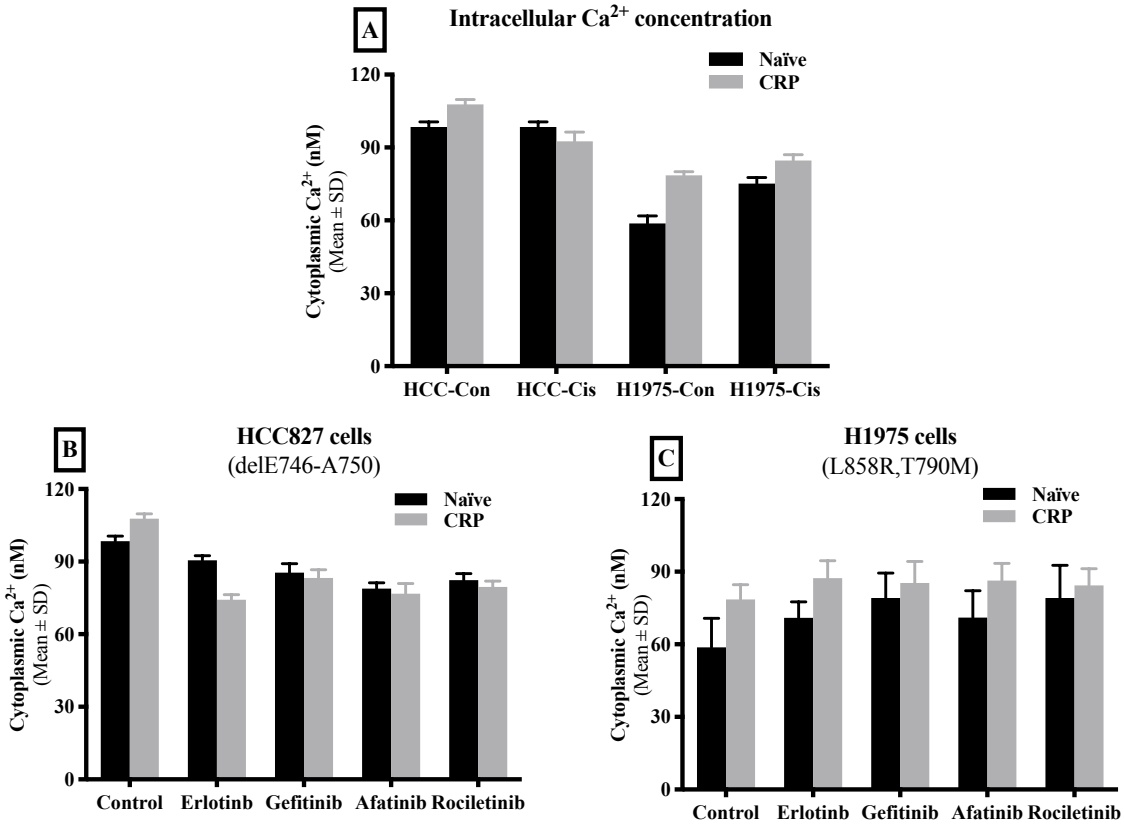

Figure 11 A–C: Impact of cisplatin and EGFR TKIs on cellular cytoplasmic calcium levels. (A). Intracellular calcium levels in NSCLC cell lines after cisplatin treatment. There no significant difference in calcium levels between cisplatin resistant and naïve HCC827 and H1975 cells. (B and C) Intracellular calcium levels in NSCLC cell lines after treatment with EGFR TKIs (erlotinib [4.7 μM], gefitinib [0.4 μM], afatinib [62 nM], and rociletinib [360 nM]) for 24 hours. All tested EGFR TKIs did not affect intracellular calcium levels in HCC827 and H1975 cells. Erlotinib reduced calcium levels slightly more in cisplatin resistant HCC827 cells compared with corresponding naïve cells (B). Data are expressed as mean ± SD from three independent experiments (n = 3; *P < 0.05, **P < 0.01, and ***P < 0.001).

6. Discussion

Cell culture models provide ways to study the mechanisms of cisplatin resistance in EGFR-mutant lung cancer cells and are useful for examining the effect of EGFR TKIs at clinical concentrations. EGFR antagonists are prescribed to patients with advanced-stage tumours carrying EGFR mutations, or after cisplatin treatment [89]. We used a clinically isogenic cell model to generate cisplatin resistance in a panel of EGFR-mutant lung cancer cells. Our findings indicate that cisplatin resistance activates the EGFR receptor in a ligand-independent manner, triggering EGFR downstream signalling. It also sensitised wild-type EGFR cells to erlotinib treatment.

Cisplatin is the standard of care for lung cancer and most tumours become resistant to cisplatin, which limits its clinical use [107]. Cisplatin resistance manifests in different ways, even in the same cell type, and it is difficult to determine which of the underlying mechanisms are the most crucial [108, 109]. Cisplatin activates the cell survival pathway by triggering EGFR signalling in different cancer cells [33, 66, 67, 82, 110-112]. Chronic exposure to cisplatin induces stem cell marker expression and alters apoptotic signals in NSCLC cells [113, 114]. It also elevates PD-L1 expression in head and neck squamous cell carcinoma cells [84, 115]. Ca^{2+} homeostasis is altered in cisplatin-treated and low-level cisplatin-resistant NSCLC and SCLC cells [70, 83]. Our study aimed to elucidate how cisplatin resistance affects EGFR-associated changes in downstream signalling and how EGFR TKIs affect cisplatin-resistant EGFR-mutant lung cancer cells at clinical doses. This information can be used to identify novel therapeutic combinations for patient survival.

We induced cisplatin resistance in a panel of EGFR-mutant lung cancer cells by exposing naïve cell lines to the same cisplatin concentration (1 µg/ml) as the patient plasma C_{max} to mimic the clinical condition [70]. We characterised these cells in terms of their viability, proliferation, apoptosis, and colony formation following treatment with 1 µg/ml cisplatin. The generated cells

were resistant to cisplatin in terms of their viability, proliferation, colony formation, and apoptosis response to cisplatin.

6.1. EGFR activation and altered downstream signalling in cisplatin-resistant cells

Understanding how EGFR TKIs associate with EGFR and how they influence conventional alkylating agent-based therapy resistance could help identify which patients would benefit most from TKI treatment [116]. Exogenous stimuli and cisplatin activate the EGFR via an autophosphorylation mechanism, which triggers downstream signalling events in a ligand-independent way [34]. This can promote cancer initiation, tumour progression, and drug sensitivity [2, 25, 59, 70]. Mutation or overexpression of EGFR has been identified as an oncogenic driver for many tumour types, making it an attractive target for anti-cancer therapies [117]. EGFR was the first growth factor receptor to be introduced as a target for cancer therapy, and antagonists of EGFR are effective in current clinical practice [28].

Sequential treatments with cisplatin followed by recovery periods activate EGFR signalling. In this study, we measured EGFR expression, phosphorylation, and downstream signalling using a receptor antibody phosphorylation array. Cisplatin resistance stimulates the overexpression of Her2 in wild-type EGFR lung cancer cells and Her2 conveys pro-survival signals via PI3K and MAPK signalling cascades in NSCLC patients [30]. Cisplatin-resistant wild-type NSCLC cells overexpressed Her3 and Her4, and Her3 was reported to be a primary mediator of the PI3K/AKT cell survival signalling pathway in different cancer cells [96, 118-125]. Her4 also plays a vital role in HER2-dependent cell proliferation in lung and ovarian cancer cells [126-130].

In this study, we showed that EGFR is phosphorylated at Tyr845 in cisplatin-resistant EGFR wild-type and EGFR double-mutated NSCLC cells, and that this phosphorylation involves Src activation. In agreement with our findings, Raimbourg et al. (2017) and others have reported that phosphorylation of EGFR at Tyr845 in cisplatin-treated NSCLC cells requires Src kinase [84, 131, 132], and that EGFR phosphorylation activates EGFR signalling [88]. We found increased

phosphorylation of Her2 at Tyr1173 and activated MAPK and PLCγ signalling in cisplatin-resistant NSCLC cells with an EGFR missense mutation. Her-2 upregulation induces cell migration by modulating PLC-γ1 activation in MDA-MB-468 cells and PLCγ promotes migration of tumour cells [133, 134]. Her3 is phosphorylated at Tyr1289 in cisplatin-resistant wild-type and mutated NSCLC cells and the HER3-PI3K-AKT cascade is involved in chemo-resistance in ovarian cancer. Cisplatin activates Her3 in ovarian cancer cells, and this effect is blocked by EGFR inhibitors [118, 130]. Her4 is phosphorylated at Tyr1284 in wild-type cisplatin-resistant NSCLC cells, and HER4 can inhibit proliferation and promote differentiation in human breast cancer cells [127]. In addition, activating HER4 mutants promotes the survival of NIH 3T3 cells in the absence of serum [128].

These studies have shown that different signalling pathways downstream of EGFR are activated in many cancers, and that autophosphorylation of a tyrosine residue in EGFR activates specific downstream signalling molecules [85]. Aberrantly active EGFR promotes conventional downstream signalling pathways, and cisplatin resistance activates alternative cell survival signals and second messengers (PLCγ).

ERK1 plays a predominant role in the cellular response to cisplatin, whereas MEK1 and MEK2 are redundant [46, 135]. Cisplatin resistance altered the expression of MAPK components in EGFR-mutant lung cancer cells. MEK was overexpressed in NSCLC cells with EGFR mutations and in SCLC cells expressing wild-type EGFR. In contrast, MEK expression was decreased in wild-type EGFR lung cancer cells. MEK phosphorylation was increased in EGFR-mutated cells and ERK phosphorylation was increased in wild-type and mutated EGFR NSCLC cells. Preventing ERK1/2 activation improves sensitivity to cisplatin in both cisplatin-sensitive and cisplatin-resistant NSCLC cells [135]. The combined use of a MEK inhibitor and PD-L1Ab may have clinical relevance in cisplatin-resistant lung cancer patients [136]. The PI3K/AKT cell survival pathway is linked to the EGFR pathway by the docking protein GAB1, which recruits

PI3K in response to EGFR stimulation [137]. Cisplatin resistance enhances Akt phosphorylation at Ser473 in EGFR double-mutant cells. Cisplatin-induced activation of Akt contributes to chemo-resistance in cancer cells and AKT phosphorylation at Ser473 plays a role in PI3/Akt signalling and cell survival in lung cancer cells [138]. Targeting the PI3K/AKT/mTOR pathway may overcome drug resistance and restore sensitivity to agents that are well tolerated in NSCLC patients [139]. JAK/STAT activation is a critical mediator of chemotherapy resistance in NSCLC cells and promotes oncogenesis through enhanced proliferation, angiogenesis, and immune escape [16, 52]. We found that STAT3 phosphorylation at Tyr705 is increased in cisplatin-resistant EGFR-mutated cells. This is in agreement with previous findings that cisplatin phosphorylates STAT3 at Tyr705 in early-stage ovarian cancer to promote cancer cell proliferation [99]. Inhibiting STAT3 signalling enhances cisplatin-mediated apoptosis in different cancer cells [16].

PLCγ1 has pro-oncogenic and pro-metastatic properties, and these functions change depending on the situation. PLCγ-mediated signalling is involved in tumour cell invasiveness induced by growth factor receptors [37, 54-56]. The expression and phosphorylation of these growth factor receptors are upregulated in cisplatin-resistant cancer cells [55]. Upregulation of PLCγ1 protein expression and phosphorylation at Ser1248 promotes migration of cisplatin-resistant EGFR double-mutant NSCLC cells and wild-type EGFR SCLC cells. Phosphorylation of PLCγ1 at Ser1248 promotes tumour cell invasiveness and migration, and regulates calcium signalling in head and neck squamous cell carcinoma cells [55, 101, 102]. Inhibiting PLCγ1 with U73122 reduces migration and inhibits phosphorylation of PLCγ1 at S1248 in head and neck squamous cell carcinoma cells [55, 102]. HGF/c-MET signalling plays an essential role in acquired resistance to cytotoxic anti-cancer agents [140]. Met is a marker for chemotherapy sensitivity, EGFR TKI sensitivity, and EMT properties in tumours [141]. c-Met expression was upregulated in cisplatin-resistant EGFR double-mutant NSCLC cells and wild-type EGFR SCLC cells. In

addition, phosphorylation of MET at Tyr1234/1235 was increased in cisplatin-resistant EGFR missense mutant cells. The amplification of c-Met expression in cancer cells is sensitive to c-Met inhibitors, and downregulating c-MET alters resistance to cytotoxic anti-cancer agents in NSCLC cells [140, 142-144].

6.2. Effect of EGFR TKIs on cisplatin-resistant cells

A concern in clinical oncotherapy is how cisplatin resistance influences the responsiveness of cancer cells to EGFR TKIs [89]. It has been shown that overexpression and constitutive phosphorylation of Her2 and Her3 alters HER ligand expression and activates EGFR signalling; these changes might contribute to increased sensitivity to gefitinib in chemoresistant tumour cell lines [124].

Cisplatin resistance increases the sensitivity of wild-type EGFR lung cancer cells, EGFR-mutant lung cancer cells, and EGFR double-mutant NSCLC cancer cells to erlotinib and inhibits cell survival in EGFR-activating mutant NSCLC cells. Erlotinib sensitisation in cisplatin-treated wild type-EGFR cells may be caused by the overexpression of EGFR2–4 and phosphorylation of Erb1 at Tyr845 [84]. Furthermore, erlotinib may reduce phosphorylation of EGFR and MET in PC-9 and PC-9/hHGF cells, suggesting that muted EGFR trans-activates MET signalling [145]. Afatinib inhibited the survival of cisplatin-resistant EGFR double-mutant cells compared with naïve cells. Treatment with other EGFR TKIs (gefitinib, afatinib, and rocilitinib) had similar effects on naïve and cisplatin-resistant cells.

In this study, cisplatin resistance did not affect the IC_{50} of EGFR TKIs (erlotinib, gefitinib, afatinib, and rocilitinib). The apoptotic potential of cisplatin-resistant cells exposed to erlotinib was increased in EGFR-wild-type and EGFR-mutant cells. This is in agreement with previous studies reporting a similar phenomena in cisplatin-treated EGFR-wild-type NSCLC cells [84]. We showed that gefitinib enhances apoptosis in cisplatin-resistant EGFR-mutant cells, in agreement with previous findings in chemo resistant tumour cell lines [124]. In this study, all

tested EGFR TKIs increased apoptosis in cisplatin-resistant EGFR-mutant NSCLC cells. These cells are extremely sensitive to EGFR TKIs because of a mutation on exon 19 [43]. However, afatinib and rocilitinib did not influce apoptosis in cisplatin-resistant EGFR-mutant lung cancer cells. Extrapolating these cell culture findings to the clinical setting, this may indicate that cisplatin can shorten the duration of subsequent EGFR TKI therapy, but may not affect the clinical response to this treatment.

6.3. Influence of cisplatin resistance on intracellular calcium concentration

The dysregulation of intracellular calcium homeostasis contributes to carcinogenesis by increasing proliferation, decreasing apoptosis, promoting dedifferentiation, enhancing metastasis, and inducing chemotherapy resistance. Drug resistance is regulated by calcium signalling, and altered Ca^{2+} homeostasis in the ER of lung cancer cells correlates with drug resistance [2, 59]. The cytotoxic effect of a number of anti-cancer agents is coupled to cellular calcium overload [38]. However, only few studies have investigated the role of intracellular Ca^{2+} homeostasis in cisplatin resistance [70].

In this study, cisplatin resistance did not change intracellular calcium levels in NSCLC cells. Ca^{2+} homeostasis is altered in chemoresistant lung cancer cells, and intracellular calcium is reduced in low-level resistant EPLC and SCLC lung cancer cells [70]. In taxol-resistant human lung adenocarcinoma cells, the resting $[Ca^{2+}]c$ was decreased [38]. In our study, EGFR TKIs did not influence intracellular calcium levels in cisplatin-resistant NSCLC cells compared with their corresponding naïve cells.

7. Conclusion

We have described a cisplatin-resistant phenotype in different lung cancer cell lines. Cisplatin resistance affects EGFR expression and ligand phosphorylation and activates EGFR downstream signalling. In addition, cisplatin triggers pro-survival signalling pathways (e.g. MAPK, Akt, Stat) and activates the second messenger PLCγ1 in lung cancer cells. We found that cisplatin-resistant cells had enhanced survival, migration, and invasion potential, which indicates that they are prone to metastasis. Episodic cisplatin treatment sensitises wild-type EGFR NSCLC and SCLC cells to erlotinib. Cisplatin resistance did not alter the cell growth inhibition (IC_{50}) of erlotinib, gefitinib, afatinib, and rociletinib.

The development of chemo-resistance has not been well defined, but a better understanding of these processes may reveal promising strategies for combination therapy. A variety of specific inhibitors (e.g. for MAPK, Akt, Stat, JAK, PLC, and Met) are available, and many are being tested in clinical trials for the treatment of several cancers. Tumour-specific oncoprotein expression in individual patients should be considered when deciding upon combination treatment with cisplatin. This individualised therapeutic approach could reduce toxicity and enhance disease-free survival. Further investigations into potential combination therapies to improve anti-tumour responses are essential.

Understanding the impact of cisplatin resistance on intracellular Ca^{2+} homeostasis in cancer cells may reveal novel combination therapies for cisplatin-resistant patients. In this study, cisplatin resistance did not change intracellular calcium levels.

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9. Abbreviations

Ag: Antigen
AKT: Protein kinase B
ALK: Anaplastic lymphoma kinase
ATPase: Adenosine triphosphatase
Bcl-2: B-cell lymphoma 2
Bmi-1: B-cell-specific Moloney murine leukaemia virus integration site
BSA: Bovine serum albumin
BIRC5: Baculoviral inhibitor of apoptosis repeat-containing 5/survivin
$[Ca^{2+}]$: Calcium concentration
$[Ca^{2+}]i$: Intracellular calcium concentration
$[Ca^{2+}]c$: Cytoplasm free calcium
$[Ca^{2+}]ER$: Endoplasmic reticulum calcium concentration
c-met: Tyrosine-protein kinase Met or hepatocyte growth factor receptor (HGFR)
c-kit: Mast/stem cell growth factor receptor/ tyrosine-protein kinase kit or CD117
CTLA-4: Cytotoxic T-lymphocyte-associated protein 4/CD152
CTR1: Copper transport protein
CRP: Cisplatin-resistant phenotype
CO_2: Carbon dioxide
DAPP1: Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide
DDR2: Discoidin domain receptor 2
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DYRK1B: Dual-specificity Y-phosphorylation-regulated kinase 1B
EDTA: Ethylenediaminetetraacetic acid
EGFR: Epithelial growth factor receptor
ER: Endoplasmic reticulum
Her: Epidermal growth factor receptor
ERCC1: Excision repair cross-complementing rodent repair deficiency
ERK: Extracellular signal-related kinases
EZH2: Enhancer of zeste homolog 2
FAK: Focal adhesion kinase
FBS: Foetal bovine serum
FDG: 18-fluorodeoxyglucose
FER: Feline sarcoma
FHIT: Fragile histidine triad
FGFR: Fibroblast growth factor receptors
FITC: Fluorescein
GBM: Glioblastoma multiforme
GSH: Glutathione
GST: Glutathione S-transferase
Grb2: Growth factor receptor-bound protein 2
GPCR: G-protein-coupled receptor
HER: Human epidermal growth factor receptor
HGF: Hepatocyte growth factor
HB-EGF: Heparin-binding EGF-like growth factor
hMCA: Human monoclonal antibody
ID3: DNA-binding 3

IFN: Interferon
IGF: Insulin growth factor
IGFR: Insulin-like growth factor receptor
IMRT: Intensity-modulated radiation technique
ITK: Interleukin-2-inducible T-cell kinase
JNK: c-Jun N-terminal kinase
KEAP1: Kelch-like ECH-associated protein 1
KRAS: Kirsten rat sarcoma
LAG-3: Lymphocyte-activation gene 3
LC: Lung cancer
LCC: Large-cell carcinoma
MAPK: Mitogen-activated protein kinase
MEK: Mitogen-activated protein kinase
MET/HGFR: Hepatocyte growth factor receptor
mTOR: Mammalian target of rapamycin
MRP2: Multidrug resistance-associated protein 2
MMR: Mismatch repair
MLH1: MutL homolog 1
miR: microRNA
NK: Natural killer
NSCC: Non-squamous cell carcinoma
Nrg: Neuregulin
NSCLC: Non-small-cell lung cancer
PARP1: Poly ADP-ribose polymerase-1
PDGFRA: Platelet-derived growth factor receptor A
PDL1: Programmed death-ligand 1
P38MAPK: P38 mitogen-activated protein kinases
PDGF: Platelet-derived growth factor
PMCAs: Plasma membrane Ca^{2+}-ATPases
PI: Propidium iodide
PBS: Phosphate buffered saline
pEGFR: Phosphorylated epidermal growth factor receptor
pSTAT: Phosphorylated signal transducer and activator of transcription proteins
PD: Pharmacodynamic
PIK3CA: Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha
PK: Pharmacokinetic
PM: Plasma membrane
PTEN: Phosphatase and tensin homolog
RB1: Retinoblastoma protein
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RPMI-1640: Roswell Park Memorial Institute
SAA: Salvianolic acid A
SAPK: Stress-activated protein kinases
SF: Scatter factor
SCLC: Small-cell lung cancer
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel
SERCA: Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SMOCs: Second messenger-operated channels

SH2: Src homology 2
STAT: Signal transducer and activator of transcription proteins
Src: Proto-oncogene tyrosine-protein kinase Src
SOS: Son of sevenless
SCC: Squamous cell carcinoma
SR: Sarcoplasmic reticulum
TMEM205: Transmembrane protein 205
TP53: Tumour protein 53
VDAC: Voltage-dependent anion channel
VEGF: Vascular endothelial growth factor
XAF1: X-linked inhibitor of apoptosis factor 1
5-FU: 5-Fluorouracil
O.D: Once daily
BID: bis in die (twice daily)
M: Molar
mM: Millimolar
μM: Micromolar
nM: Nanomolar
pM: Picomolar
g: Gram
mg: Milligram
μg: Microgram
ng: Nanogram
L: Litre
ml: Millilitre
μl: Microliter
nl: Nanolite

10. Appendix-I: Publication

1. Venu Pamidiboina, Fei Tian, Kiefl Rosemarie, Marina Schaule, Pandey Dharmendra, Amanda Tufman and Rudolf Maria Huber. (2020) Cisplatin resistance associated EGFR signalling changes in lung cancer cells and the efficacy of EGFR TKIs on Cisplatin-Resistant Phenotype cells. (Under Communication process).

2. Venu Pamidiboina, Fei Tian[1], Kiefl Rosemarie, Marina Schaule, and Rudolf Maria Huber Cisplatin-Resistant Phenotype: Characterization, adaptation to EGFR signalling and sensitivity to EGFR inhibitors in NSCLC cells.
   Journal of Thoracic Oncology. April 2018, Volume 13, Issue 4, Supplement, Page S34

3. Venu Pamidiboina, Fei Tian[1], Kiefl Rosemarie, Marina Schaule, and Rudolf Maria Huber Impact of epidermal growth factor receptor (EGFR) inhibitors in cisplatin-resistant non-small-cell lung cancer (NSCLC) cell lines in parallel to naïve cells at therapeutic doses.
   Pneumologie. (2015) 69 - A70

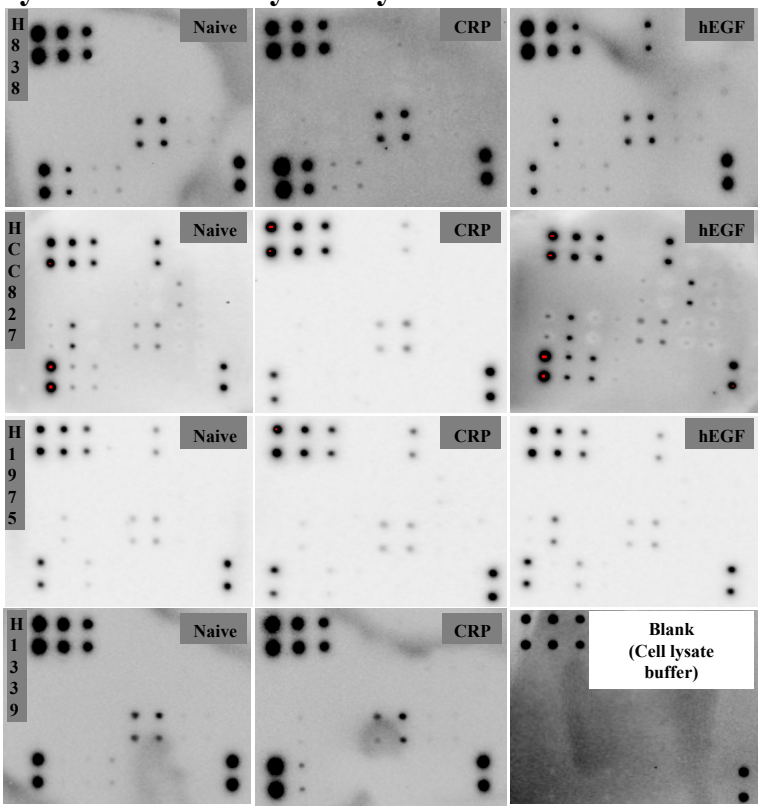
11. Appendix-II: Raw Data

11.1. Human EGFR Phosphorylation Antibody Array

EGFR Phosphorylation Antibody Array map

1	P1	P2	P3	Blank	Negative	EGFR (Tyr845)	EGFR (Tyr992)	EGFR (Tyr1045)	EGFR (Tyr1068)
2	P1	P2	P3	Blank	Negative	EGFR (Tyr845)	EGFR (Tyr992)	EGFR (Tyr1045)	EGFR (Tyr1068)
3	Blank	Blank	Blank	Blank	EGFR (Tyr1086)	EGFR (Tyr1148)	EGFR (Tyr1173)	EGFR (Tyr1046/1047)	EGFR (Tyr1070)
4	Blank	Blank	Blank	Blank	EGFR (Tyr1086)	EGFR (Tyr1148)	EGFR (Tyr1173)	EGFR (Tyr1046/1047)	EGFR (Tyr1070)
5	ErbB2 (Tyr877)	ErbB2 (Tyr1112)	ErbB2 (Tyr1121/1122)	ErbB2 (Tyr1248)	ErbB2 (Tyr686)	ErbB2 (Ser1113)	ErbB3 (Tyr1289)	ErbB4 (Tyr1284)	Blank
6	ErbB2 (Tyr877)	ErbB2 (Tyr1112)	ErbB2 (Tyr1121/1122)	ErbB2 (Tyr1248)	ErbB2 (Tyr686)	ErbB2 (Ser1113)	ErbB3 (Tyr1289)	ErbB4 (Tyr1284)	Blank
7	EGFR	ErbB2	ErbB3	ErbB4	Blank	Blank	Negative	Blank	P4
8	EGFR	ErbB2	ErbB3	ErbB4	Blank	Blank	Negative	Blank	P4

EGFR Phosphorylation Antibody Array membranes



4.2.1. PathScan EGFR Signalling Antibody Array

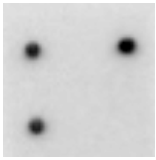
Targets of Sub-array-A

Positive control	EGFR Total	EGFR Total	EGFR Tyr669	EGFR Tyr669	Positive control
EGFR Tyr845	EGFR Tyr845	EGFR Tyr998	EGFR Tyr998	EGFR Tyr1068	EGFR Tyr1068
EGFR L858R mutant	EGFR L858R mutant	EGFR E746-A750 Del	EGFR E746-A750 Del	MEK1 Total	MEK1 Total
MEK2 Total	MEK2 Total	MEK1/2 Ser221	MEK1/2 Ser221	MEK1/2 Ser217/221	MEK1/2 Ser217/221
Positive control	Stat3 Tyr705	Stat3 Tyr705	Negative control	Negative control	Negative control

Targets of Sub-array-B

Positive control	Positive control	HER2 Total	HER2 Total	HER2 Tyr1196	HER2 Tyr1196
HER2 Tyr1221/1222	HER2 Tyr1221/1222	Met total	Met total	Met Tyr1349	Met Tyr1349
Met Tyr1234/1235	Met Tyr1234/1235	PLCγ1 Total	PLCγ1 Total	PLCγ1 Ser1248	PLCγ1 Ser1248
Akt Thr308	Akt Thr308	Akt Ser473	Akt Ser473	Erk1/2 Thr202/Tyr204	Erk1/2 Thr202/Tyr204
Positive control	PARP Asp214 Cleavage	PARP Asp214 Cleavage	Negative control	Negative control	Negative control

Blank



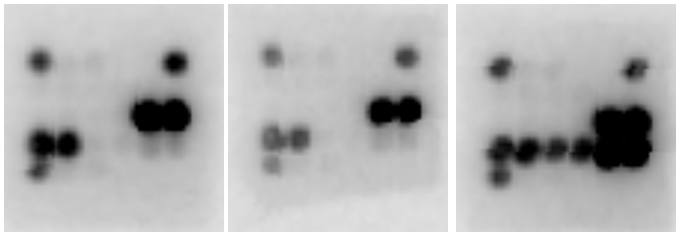
Array-A

Array-B

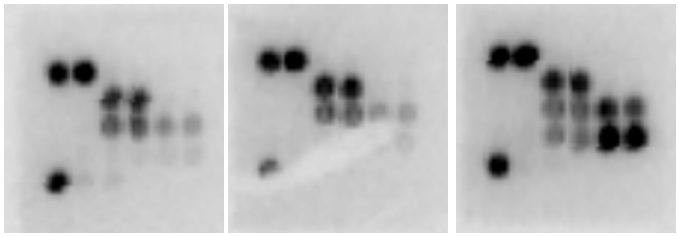
H838_ Wild type EGFR

Naive **CRP** **EGF**

Array-A



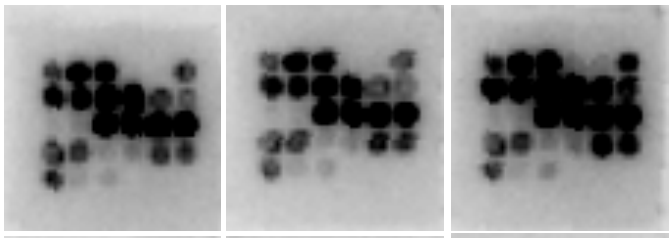
Array-B



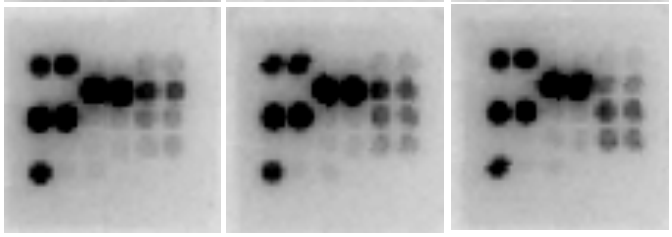
HCC827_[del E746-A750]

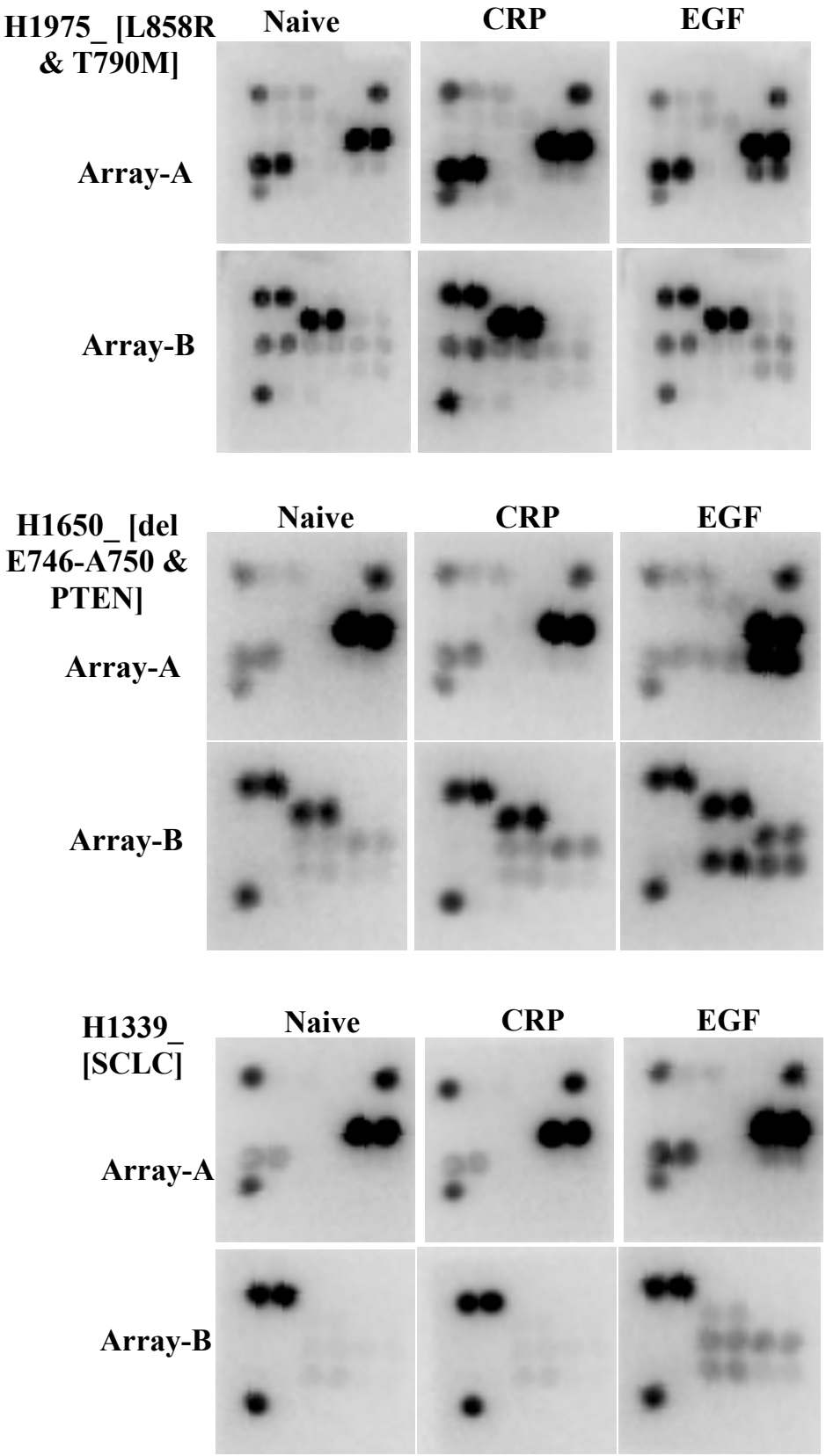
Naive **CRP** **EGF**

Array-A



Array-B




H1975_ [L858R & T790M]
Naive
CRP
EGF
Array-A
Array-B
H1650_ [del E746-A750 & PTEN]
Naive
CRP
EGF
Array-A
Array-B
H1339_ [SCLC]
Naive
CRP
EGF
Array-A
Array-B