UNIVERSITA' DEGLI STUDI DI PARMA

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Biologia e Patologia molecolare

Ciclo XXVII

Combined use of Tyrosine Kinase Inhibitors (TKIs) and monoclonal antibodies (mAbs) as target therapy in Non-Small Cell Lung Cancer (NSCLC)

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SOMMARIO

Sia dal punto di visto biologico che istologico, il cancro al polmone è una neoplasia altamente complessa con diversi tipi e sottotipi istologici tra cui i più frequenti sono il carcinoma a piccole cellule (SCLC, 15%), il carcinoma polmonare non a piccole cellule (NSCLC, 85%). Nell'ambito del NSCLC si possono distinguere il carcinoma squamo cellulare (SQCC), l'adenocarcinoma (ADK) e il carcinoma a grandi cellule (LCK). Negli ultimi anni la ricerca oncologica ha fornito nuovi approcci terapeutici e sicuramente la terapia a target molecolare si è dimostrata una strategia innovativa. Due principali strategie sono state sviluppate per la terapia a target molecolare diretta verso i recettori della famiglia ErbB: inibitori tirosin chinasici (TKIs) come gefitinib ed erlotinib, e anticorpi monoclonali (mAbs), come cetuximab e trastuzumab. In pazienti affetti da NSCLC, sono state identificate molte aberrazione molecolari, tra cui alterazioni a carico di EGFR, BRAF, amplificazione/overespressione di HER2, riarrangiamenti di EML4-ALK, ROS e RET nell'istotipo adenocarcinoma, amplificazione/mutazione di FGFR, DDR2 o mutazioni di PI3KCA nel carcinoma a cellule squamose. Tutte queste aberrazioni sono state correlate ad una prognosi sfavorevole. Erlotinib è un trattamento efficace per i pazienti affetti da NSCLC ed è stato registrato come trattamento di seconda e terza linea del NSCLC indipendentemente dalla stato mutazionale di EGFR.

Gefitinib è stato registrato per la terapia di pazienti affetti da NSCLC avanzato recanti mutazioni attivanti nel dominio tirosin chinasico di EGFR; tra le più frequenti ricordiamo la mutazione L858R nell'esone 21 e la Del (746-750) all' esone 19. Sebbene le mutazioni in EGFR siano utili marker per predire l'efficacia degli inibitori tirosin chinasici, tuttavia non possono essere utilizzate come unico criterio per stabilire quali pazienti possano essere candidati alla terapia. Inoltre, sta diventando sempre più chiaro che anche i pazienti con EGFR wild-type possono beneficiare dell'attività dei TKI di EGFR.

Cetuximab è un anticorpo monoclonale (mAb) chimerico IgG1 che blocca il legame del ligando di EGFR, portando ad una diminuzione della dimerizzazione dei recettori, autofosforilazione, e conseguente riduzione dell'attivazione delle vie di segnalazione a valle. Inoltre il legame di cetuximab avvia l'internalizzazione e la degradazione del recettore causando un arresto del segnale. A differenza dei TKI di EGFR, cetuximab è in grado di stimolare il sistema immunitario, attivando cellule natural killer (NK) ed inducendo citotossicità cellulare anticorpo dipendente (ADCC). L'uso combinato di cetuximab con la chemioterapia è stato approvato dalla Food and Drug Administration (FDA) per il trattamento del tumore del colon-retto metastatico e della testa e del collo localmente avanzato.

Trastuzumab, registrato per il trattamento del carcinoma mammario HER2 positivo, è anche stato testato in studi di fase II in monoterapia ed in combinazione con la chemioterapia in pazienti affetti da NSCLC.

In oncologia clinica, l'acquisizione di fenomeni di resistenza agli inibitori tirosin chinasici è un fenomeno ben documentato in diversi tipi di tumori. Quasi tutti i pazienti affetti da NSCLC con mutazioni attivanti di EGFR trattati con TKI, dopo una prima risposta positiva, manifestano progressione della malattia entro 10-14 mesi dall'inizio della stessa. Il meccanismo più comunemente descritto di resistenza ai farmaci riguarda l'acquisizione di alterazioni genetiche aggiuntive in seno ad EGFR, la più frequente delle quali è la comparsa della mutazione T790M al dominio chinasico di EGFR, rappresentante circa il 50% dei casi di resistenza acquisita. Ad oggi, l'espressione di HER2 in NSCLC è in corso di valutazione, tuttavia, il recente ruolo di HER2 nell'acquisizione di resistenza ai TKI, riportato nel 12-13% dei pazienti, può rendere HER2 un potenziale bersaglio terapeutico non solo nel cancro alla mammella ma anche nel NSCLC.

T-DM1, trastuzumab emtansine, appartiene alla innovativa classe degli anticorpi coniugati a derivati citossici (ADC) e sfrutta la specificità di legame del mAb trastuzumab e le potenzialità citotossiche del chemioterapico DM1 (derivato della maitansina).

Diversi studi preclinici condotti su linee cellulari di diversi tipi di tumore, hanno indicato che l'associazione tra anticorpi monoclonali diretti contro EGFR/HER2 e TKI mostra una maggiore efficacia.

A tal fine, lo scopo della tesi è stato quello di esplorare il potenziale clinico di combinare erlotinib sia con cetuximab, sia con trastuzumab per migliorare l'efficacia della terapia a bersaglio molecolare di EGFR in linee cellulari di NSCLC con EGFR wild-type. Poichè HER2 rappresenta un nuovo bersaglio terapeutico nel NSCLC, è stato valutato se l'attività del T-DM1 è influenzata dall'espressione e/o dallo status mutazionale di HER2 e se la terapia con T-DM1 possa essere una nuova strategia per superare la resistenza ai TKI di EGFR in linee cellulari di NSCLC.

INDEX

Le parole più belle del mondo non sono:
"Ti amo"
ma
"È benigno".

Woody Allen,
Tratto da "Harry a pezzi", 1997

INTRODUCTION

1. LUNG CANCER

Carcinoma of the lung is currently the leading cause worldwide of death due to cancer. The disease has become an epidemic as incidence rates and lung cancer deaths have risen dramatically over the last century, correlating with an increase in cigarette consumption. The magnitude of the impact on mortality is indicated by comparing changes over time. More than 1.5 million new cases of lung cancer are now diagnosed annually worldwide [1,2]

In men, the highest incidence rates are observed in North America, East Asia, central-eastern and southern Europe. In less developed countries, the highest rates are seen in West Asia, South Africa, and the Caribbea. In women, the worldwide incidence rates of lung cancer are lower; the highest rates are seen in North America and in Northern Europe. [3]

1.1 EPIDEMIOLOGY and RISK FACTORs

In research carried out over the last half of the 20th century, many factors were causally associated with lung cancer and studies were implemented to identify determinants of susceptibility to these factors. Cigarette smoking was identified as the single most predominant cause of the lung cancer epidemic, but other causes were found, including workplace agents (eg, asbestos, arsenic, chromium, nickel, and radon) and other environmental factors (passive smoking, indoor radon, and air pollution) [4].

There is a consistent association between cigarette smoking and lung cancer as a cause of death. There are at least two ways that smoking is associated with lung cancer. First, polycyclic aromatic hydrocarbons, carcinogenic compounds present in tobacco smoke, induce mutations in the p53 gene that are crucial for cell cycle dysregulation and carcinogenesis [5]. G to T transversions within the gene have been linked to a molecular signature of tobacco mutagens in smoking-associated lung cancers. Second, the N-nitroso compounds are another major group of chemicals found in tobacco smoke, several of which are potent animal carcinogens. These compounds can be found in the urine of smokers [6]. Over the past few decades, the incidence of adenocarcinoma of the lung increased much more rapidly than that of squamous cell carcinoma in men and women. At the same time, filtered cigarettes with substantially reduced “tar” and nicotine yields have dominated the market [7]. The decrease in tars and the increase in nitrosamines appear to be the cause of the recent change of dominant cell type from squamous cell to adenocarcinoma.

The U.S. Environmental Protection Agency has determined radon to be the second leading cause of lung cancer after cigarette smoking [8]. The increased risk attributed to radon is from domestic exposure, due to diffusion of radon from the soil. High radon concentrations have been linked to an increased risk of lung cancer in underground miners [9]. More recent epidemiological studies of residential radon exposure also identify it as a risk factor for lung cancer. Inhaled radon can have a carcinogenic effect on the lung due to its emission of α particles upon decay and additionally it has a synergistic effect with tobacco smoke inhalation [10,11].

Occupational exposure to carcinogens accounts for 5% of all lung cancers in the United States. Asbestos accounts for a large number of these cases. Exposure to asbestos at high levels can cause lung cancer and mesothelioma. Because mesothelioma is so rare, asbestos-induced cases of lung cancer significantly outnumber cases of mesothelioma among asbestos-exposed workers [12].

Lung cancer could be one of the long-term adverse effects of cumulated exposure to ambient air pollution, such as emissions rich in various polycyclic aromatic hydrocarbon compounds, likely through oxidative stress, inflammation, induction of a procoagulatory state, and dysfunction of the autonomic nervous system. The proportion of lung cancers attributable to urban air pollution in Europe is estimated to be 11% [13].

1.2 CLASSIFICATION

Historically, lung cancers have been sub-divided by histology into **small-cell** (20%) and **non-small-cell lung cancers** (NSCLCs) (80%) [14], with NSCLC further classified into *squamous cell carcinoma (SCC)*, *large-cell carcinoma*, and *adenocarcinoma*. More than half of all lung cancers are adenocarcinomas [15]. While treatment advances have been made with the use of platinum-based chemotherapy, lung cancer remains the most frequent cause of cancer-related mortality worldwide and has a 5-year overall survival (OS) rate of just 16% for all stages [16]. Crucial to enhancing outcomes for patients with lung cancer is the ability to build a detailed profile of the disease, to guide treatment decisions and to enable the development of more effective therapeutic strategies. The last decade has seen a shift to a more molecular based classification, in which information about genetic alterations and protein expression level is considered alongside histology in order to better understand the pathogenesis of the disease [17]. In NSCLC, multiple genetic alterations have already been identified as therapeutic targets, including mutations of the *epidermal growth factor receptor* (EGFR) gene and rearrangements of the *anaplastic lymphoma kinase* (ALK) gene. Drugs designed specifically as inhibitors of these molecular targets have significantly extended the survival times for patients with NSCLC whose tumours harbour these mutations [18,19]. As novel molecular targets are discovered, and ultimately new therapies developed, we may edge ever closer to a personalized treatment approach in NSCLC and further extend survival for patients [20].

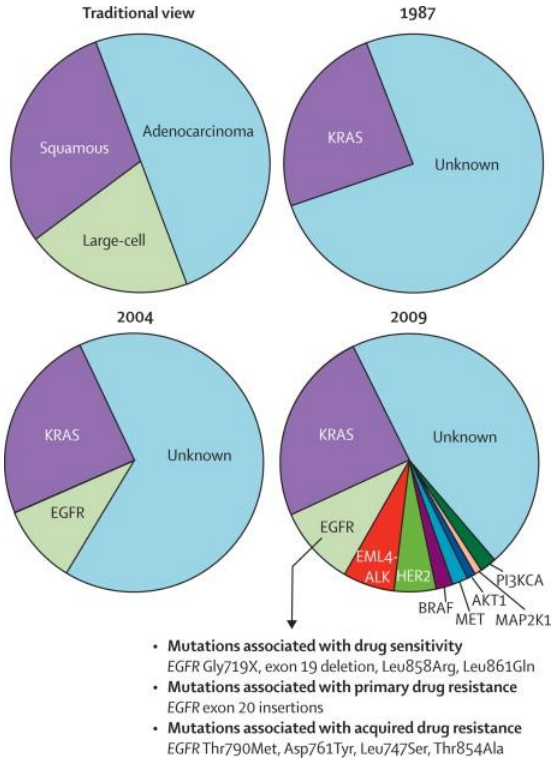


Figure 1. Evolution of knowledge in non-small-cell lung cancer

[Lancet Oncol 2011; 12: 175–180]

1.3 TREATMENTs

After the initial diagnosis of NSCLC, accurate TNM staging of lung cancer is crucial for determining appropriate therapy. Most patients with stages I to II NSCLC benefit from surgical resection, whereas patients with more advanced disease are candidates for nonsurgical treatment. Conventional clinical staging is most often performed with computed tomography (CT) of the thorax and upper abdomen. Nevertheless, CT imaging has limited sensitivity for microscopic metastatic disease and is frequently unable to discriminate between mediastinal lymph nodes that are enlarged owing to malignancy and those that are enlarged owing to benign reactive hyperplasia. In contrast, positron emission tomography (PET) with fluorine 18–labeled fluorodeoxyglucose has been shown to have greater sensitivity for the detection of metabolically active malignant disease and can lead to changes in initial staging and treatment plans for NSCLC [13].

Surgical resection is the standard of care for early stage NSCLC, with lobectomy considered the operation for optimal oncologic outcome. While this dictum has been questioned of late, remaining a controversial topic and an ongoing area of interest among surgeons, the role of operative resection overall is fairly clear. However, despite the efficacy of surgical intervention for early-stage lung cancer, only 16% of new lung cancer cases are diagnosed with localized disease that is potentially curable, thus contributing significantly to the outcomes from this disease [21,22].

For those patients who present with locally advanced or metastatic NSCLC, palliative chemotherapy is associated with only modest survival prolongation and indeterminate impact on quality of life. Platinum-based chemotherapy has become the standard of care; however, improvement in outcome from such pharmacologic agents has been modest at best, with reported 5-year survival advantages in the range of 4% – 15% [23]. The current first-line therapy for those patients with advanced NSCLC consists of a platinum agent – either cisplatin or carboplatin – administered in combination with one of several different chemotherapeutic drugs, including gemcitabine, paclitaxel, or vinorelbine. Response rates to these regimens are in the range of 17% – 32%. For those who fail platinum-based therapy, because of either intolerance or lack of response, current second-line treatment consists of docetaxel. Pemetrexed is an alternative second-line agent, with similar efficacy to docetaxel and potentially fewer side effects [24].

Patients with advanced NSCLC unresponsive to all lines of chemotherapy can, at times, be treated with radiation in order to decrease tumor size and associated symptoms. Goals of care,

at this point, are often turned toward psychosocial support and alleviation of discomfort. Aside from palliation in advanced disease, radiation may also be used concurrently with chemotherapy as definitive treatment for disease that is not amenable to surgical intervention, as adjuvant therapy for positive resection margins, or in treating nodal fields felt to be at high risk for regional recurrence [25].

In recent years, additional therapeutic options have come into the mix. Efforts have been put toward the recognition of specific driver mutations – those alterations that foster neoplastic transformation and contribute to tumor progression. Several driver mutations have been identified in subsets of patients with NSCLC, and, by focusing on specific molecular targets, new agents have been developed with the intent of treating the cancer cells with minimal toxicity to normal cells in the body. However, at this time, such options are limited and pertain to a very select group of patients.

2. GROWTH FACTORs

The role of growth factors-driven signaling in the pathogenesis of human cancer has been long established. Almost twenty years ago Mike Sporn and Anita Roberts elaborated the theory of autocrine secretion: cancer cells generally exhibit a reduced requirement for exogenously supplied growth factors to maintain a high rate of proliferation. This relaxation in growth factor dependency is due in part to the ability of tumor cells to produce high levels of peptide growth factors. It has been recognized that different mechanisms might contribute to amplify the signal driven by growth factors. For example, expression of a high number of receptors on the surface of tumor cells can increase their sensitivity to low concentrations of host- or tumor-derived growth factors. A direct correlation also exists between growth factors and cellular proto-oncogenes. In fact, several proto-oncogenes code for proteins that are either growth factors, or growth factor receptors, or proteins that are involved in the intracellular signal transduction pathway for growth factors. In addition, activated cellular proto-oncogenes may also control the endogenous production and/or the response of tumor cells to peptide growth factors. More recently, the involvement of growth factors in sustaining the survival of cancer cells and in promoting tumor-induced angiogenesis has been demonstrated, suggesting that growth factors contribute to tumor progression through different mechanisms [26].

2.1 ErbB FAMILY

The epidermal growth factor receptor (EGFR) and the EGF-family of peptide growth factors have a central role in the pathogenesis and progression of different carcinoma types. The EGF ligand/receptor system is also involved in early embryonic development and in the renewal of stem cells in normal tissues such as the skin, liver and gut. However, it is important to emphasize that the EGFR belongs to a family of receptors that encompasses three additional proteins, ErbB-2, ErbB-3 and ErbB-4. These proteins and the growth factors of the EGF-family form an integrated system in which a signal that hits an individual receptor type is often transmitted to other receptors of the same family [26,27].

The ErbB family of receptor tyrosine kinases (RTK) comprises four distinct receptors: the EGFR (also known as ErbB-1/HER1), ErbB-2 (neu, HER2), ErbB-3 (HER3) and ErbB-4 (HER4). All proteins of this family have an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase-containing domain [28]. ErbB receptors are activated by binding to growth factors of the EGF-family that are produced by the same cells that express ErbB receptors (autocrine secretion) or by surrounding cells (paracrine secretion). Proteins that belong to this family are characterized by the presence of an EGF-like domain composed of three disulfide-bonded intramolecular groups, which confers binding specificity, and additional structural motif such as immunoglobulin-like domains, heparin-binding sites and glycosylation sites. None of the EGF family of peptides binds ErbB-2 [29].

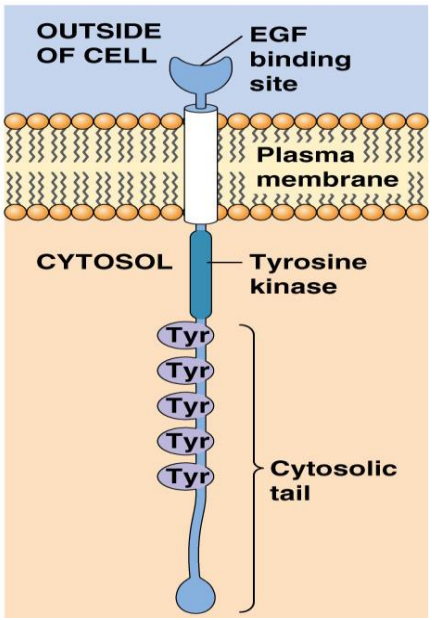


Figura 2. RTK structure

[EMBO J. 19, 3159–3167]

Binding of ligands to the extracellular domain of ErbB receptors induces the formation of receptor homo- or heterodimers, and subsequent activation of the intrinsic tyrosine kinase domain. All possible homo- and heterodimeric receptor complexes between members of the ErbB family have been identified in different systems. Receptor activation leads to phosphorylation of specific tyrosine residues within the cytoplasmic tail. These phosphorylated residues serve as docking sites for proteins containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains, the recruitment of which leads to activation of

intracellular signaling pathways [30,31].

All ErbB ligands and receptors induce activation of the RAS/RAF/MEK/MAPK pathway through either Grb2 or Shc adaptor proteins ErbB receptors also activate PI3K by recruitment of the p85 regulatory subunit to the activated receptors and induce activation of important tumorigenic processes, including proliferation, apoptosis, angiogenesis, and invasion [32,33].

The EGFR was originally identified as an oncogene because of its homology to v-ERBB, a retroviral protein that enables the avian erythroblastosis virus to transform chicken cells. Subsequently, EGFR overexpression was shown to be transforming in laboratory models, and EGFR gene amplification was reported in a wide range of carcinomas. Early studies by Mendelsohn and colleagues demonstrated that antibodies directed against EGFR block growth of A431 cells, demonstrating that EGFR signaling could drive cancer cell growth and setting the stage for clinical use of EGFR inhibitors [34].

The discovery that somatic mutations in the epidermal growth factor receptor (EGFR) gene are found in a subset of lung adenocarcinomas and are associated with sensitivity to the EGFR tyrosine kinase inhibitors (TKI) gefitinib and erlotinib has generated excitement among clinicians and researchers studying non–small cell lung cancer (NSCLC) [35-37]. These are point mutations in exons 18 (G719A/C) and 21 (L858R and L861Q) and in-frame deletions in exon 19 that eliminate four amino acids (LREA) just downstream of a critical lysine residue at position 745. The most common of these four drug-sensitive mutations are exon 19 deletions and the exon 21 L858R substitution, together representing 85% to 90% of EGFR mutations in NSCLC (Fig. 3) [38].

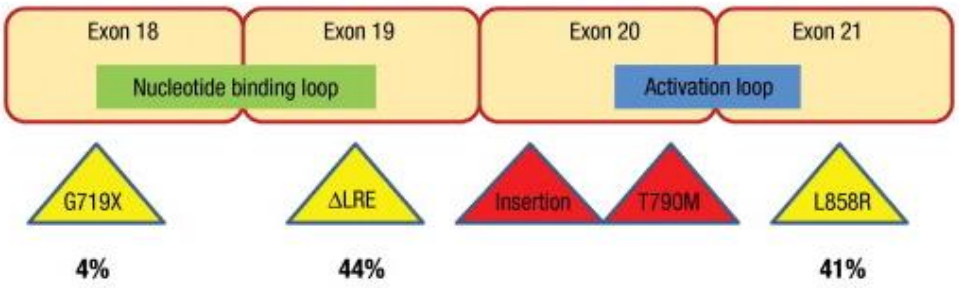


Figure 3. The Common EGFR Kinase Domain Mutations

G719X, ΔLRE, and L858R are associate with EGFR TKIs sensitivity. Exon 20 consists of the common EGFR mutations which correlate with EGFR TKIs resistance.

[Clinical Lung Cancer, Vol. 13, No. 4, 252-66 © 2012 Elsevier Inc.]

Thus far, three kinase domain mutations are associated with drug resistance: an exon 19 point mutation (D761Y) an exon 20 point mutation (T790M), and an exon 20 insertion (D770_N771insNPG). Within lung cancers, EGFR kinase domain mutations are more common in adenocarcinomas, East Asians, women, and never smokers. Mutations in EGFR may be more common in women [38].

The human HER2/neu oncogene is located on chromosome 17 and encodes a 185-kd transmembrane glycoprotein that has intrinsic tyrosine kinase activity and shares sequence homology with EGFR [39]. Among all four HER family proteins, HER2 has the strongest catalytic kinase activity. Although there is no known ligand that binds to HER2, the extracellular domain of HER2 remains in a constitutively active conformation, which makes HER2 a preferred partner for dimerization with other HER family proteins [40]. HER2-containing heterodimers function as the most active signaling complex of the HER family. In fact, the cytoplasmic kinase domain of HER3 does not have catalytic activity. Ligand-activated HER3 preferentially binds to HER2, and subsequent activation of HER2 cytoplasmic kinase activity is required for downstream PI3K/AKT signaling. Overexpression of HER2 in breast cancer leads to increased homodimerization (HER2-HER2) and heterodimerization (e.g. HER2-HER3), which initiates a strong pro-tumorigenic signaling cascade [41]. HER2 gene amplification and protein overexpression is observed in about 20% of breast cancers, which comprises a separate subset of breast cancer patients with a poorer prognosis before trastuzumab was introduced to clinical use [42]. Around 20% of advanced gastric cancers also exhibit HER2 amplification and/or overexpression. Unlike in breast cancer, studies to date have yielded inconsistent findings regarding the prognostic role of HER2 in gastric cancer [43]. Importantly, HER2 overexpression has now been described in a variety of tumors in addition to breast and gastric cancer. Other malignancies including, but not limited to, non-small cell lung cancer (NSCLC), ovarian cancer, salivary duct carcinoma, and pancreatic cancer, over-express HER2 protein and/or exhibit gene amplification in a variable percentage of cases, depending on the tumor. Additionally, mutations in HER2 have been described in a small subset of cancers of the breast, lung, ovary and colon [41]. In NSCLC, HER2 overexpression and/or mutation is found in around 5% of patients [39,44,45]. In one meta-analysis of over 4500 NSCLC patients, overexpression of HER2 was a poor prognostic marker for survival [45,46].

HER2 mutations were first described in NSCLC in 2004 (Figure 4) [47]. Since then, multiple studies analyzing more than 3000 NSCLC samples confirmed that around 2% of NSCLC patients harbor HER2 somatic mutations [44,48]. In a recent study, Arcila et al. identified HER2 mutations in 6% of 335 EGFR/KRAS/ALK-negative lung adenocarcinoma specimens [49]. Demographic distribution of HER2 mutations mimics that of EGFR mutations; HER2 mutations are found in up to 11% of Asian, non-smoking women with adenocarcinoma who do not harbor EGFR mutations [50]. Retrospective analysis of 27 patients with HER2 mutated NSCLC did not show a significant difference in survival from that of patients with EGFR-mutated or EGFR/ HER2-*wild-type* NSCLC [44]. Although rare, HER2 mutation may coexist with EGFR mutation [51,52]. This observation poses the intriguing question as to whether such patients would have a suboptimal tumor response to kinase inhibitors targeting EGFR alone, and emphasizes the need for comprehensive panels of molecular tests. HER2 mutations in NSCLC may be an important predictive marker for tumor sensitivity to an anti-HER2 agent. Cappuzzo et al. reported a patient with metastatic HER2-mutated (G776L) lung adenocarcinoma resistant to conventional cytotoxic chemotherapy, who had a favorable response to trastuzumab. Kelly reported a long-lasting favorable response to treatment containing trastuzumab and lapatinib in a patient with advanced HER2-amplified and mutated (L869R) NSCLC, who had failed conventional chemotherapy [51,53].

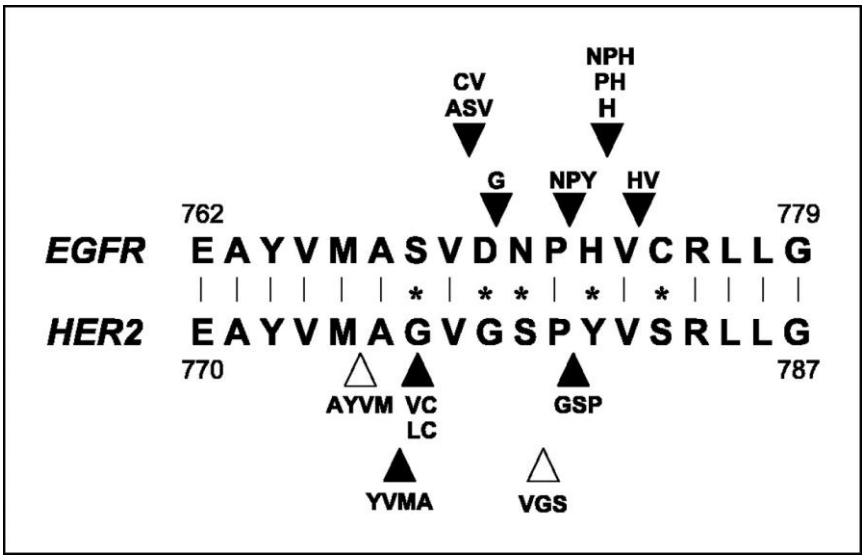


Figure 4. The common HER2 somatic mutations

[Cancer Res 2005;65:1642- 6]

In a recent study, Takezawa et al. have identified HER2 amplification as a new mechanism of acquired resistance to EGFR-TKIs in EGFR-mutant NSCLC tumors, occurring independently of the EGFR T790M secondary mutation. This observation could explain why the combination of afatinib/cetuximab induces responses in some but not all patients without T790M-mediated acquired resistance. If the 12% prevalence of HER2 amplification in this clinical setting is verified in future studies, this would place it as one of the most common acquired resistance mechanisms after the EGFR T790M mutation. Hopefully, this knowledge will eventually lead to improved therapeutic outcomes for patients with EGFR-mutant lung cancer (Figure 5) [54].

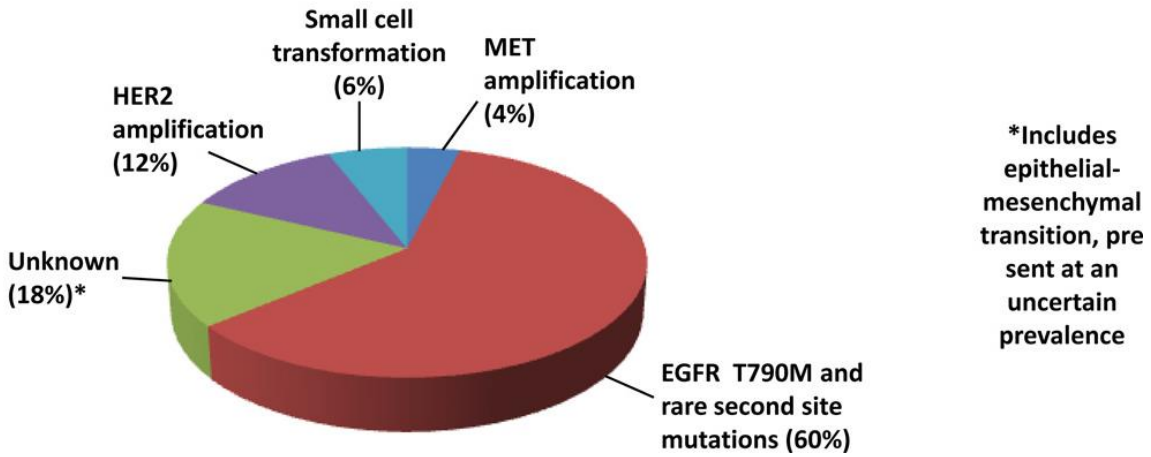


Figure 5. Frequency of mechanisms of acquired resistance to EGFR-TKIs in EGFR-mutant lung cancer

[Cancer Discovery 2012;2:922-933]

2.2 PATHWAYs

✓ RAS/RAF/MEK/MAPK pathway

The Mitogen-activated protein kinase (MAPK) pathway (Figure 6) encompasses different signaling cascades of which the RAS-RAF-MEK-extracellular signal-regulated kinase 1 and 2 (ERK1/2) is one of the most dysregulated in human cancer. This pathway regulates multiple critical cellular functions including proliferation, growth and senescence [55]. The Ras is an important component of the large family of GTPases. The ras genes are transforming oncogenes that have initially been recognized as murine sarcoma viruses by Jennifer Harvey (Harvey-Ras [H-RAS], and Werner Kirsten (Kirsten-Ras [K-Ras]) in 1960s. The association of activated and transforming RAS genes in human cancer was concurrently reported by several authors in 1982. Subsequent studies led to the identification of a third human RAS gene, designated NRAS in human neuroblastoma cells (Neuroblastoma-Ras [N-Ras]). The three human RAS genes encode four highly related 188 to 189 amino acid proteins, designated as H-RAS, N-RAS and K-RAS (K-RAS4A and K-RAS4B). RAS proteins function as binary molecular switches that control intracellular signaling pathways involved in fundamental cellular processes such as cell polarity, proliferation, differentiation, adhesion, migration, and apoptosis. RAS and RAS-related proteins are often dysregulated in cancers by activating mutations of RAS isoforms or its effectors in nearly one-third of all human cancers [56]. RAS activates several pathways, including the RAF-MEK-ERK/MAPK cascade, which transmits signals downstream and results in the transcription of genes involved in controlling several cellular mechanisms. The RAS family members are anchored to the cytoplasmic side of the plasma membrane by carboxyl-terminal farnesylation. This localization places the RAS in close proximity to adaptors, the growth factor receptor bound protein 2 (Grb2) and the nucleotide exchange factor son of sevenless (SOS), to mitigate the exchange of nucleotide guanosine diphosphate (GDP) bound to RAS with guanosine triphosphate (GTP) in the cytosol [56]. This exchange activates RAS conformationally, allowing its interaction with a number of downstream effectors. Accordingly, RAS communicates external cellular signals to the nucleus, and its altered activation leads to inappropriate cellular activities including enhanced cell growth, differentiation and survival and ultimately to cancer. The RAS-RAF-MEK-ERK pathway is activated by several known growth factors and cytokines that act through receptor tyrosine kinase signals and by activating mutations mainly in the RAS and RAF genes [55].

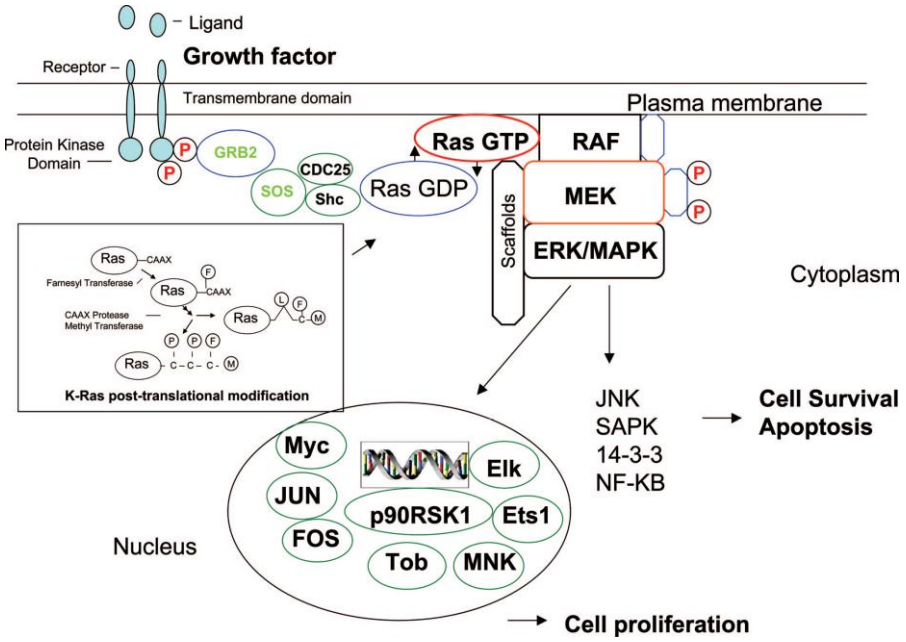


Figure 6. RAS-RAF-MAPK pathway

[Biochim Biophys Acta. 2007; 1773:1263–84]

RAS (H-, K-, N-isotypes) (guanine nucleotide-binding protein), is a single GTPase molecule related in structure to the Gα subunit of heterotrimeric G proteins. G proteins act as molecular switches and timers that cycle from inactive GDP-bound to active GTP-bound states [57]. In normal quiescent cells, RAS is bound to GDP and is inactive ("off" state), while upon extracellular stimuli, RAS bind to GTP ("on" state), which has an extra phosphate group than GDP. This extra phosphate holds the two switch regions in a "loaded-spring" configuration (switch I includes Threonine-35, switch II Glycine-60). Upon the release of this phosphate, the switch regions relax leading to conformational modifications and return to the inactivate state. Therefore, the activation and the inactivation of RAS and several other small G proteins are controlled by a cycling switching between the active GTP-bound and inactive GDP-bound forms [58].

The cyclic process of GDP/GTP is facilitated by guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs). The RAS intrinsic GTPase activity, hydrolyze the GTP into GDP. However, this process is inefficient and requires additional GAPs for binding, stabilizing, and accelerating the RAS catalytic activity. This is achieved by additional catalytic residues, "arginine fingers", where a H_2O molecule is positioned for nucleophilic attack on the gamma-phosphate of GTP, leading to the release of the inorganic phosphate molecule with a subsequent binding of RAS to GDP [58]. GEFs catalyze a "push and pull" process that unhinges the GDP from RAS by positioning close to the P-loop and the

magnesium cation binding site to block the interaction with the gamma phosphate anion. Acidic (negative) residues in switch II "pull" a lysine in the P-loop away from the GDP which "pushes" switch I away from the guanine. The contacts holding GDP in place break, leading to its release in cytoplasm. Because intracellular GTP is abundant relative to GDP, it predominantly re-enters the nucleotide binding pocket of RAS and reloads the spring. Thus, the GEFs and GAPs balance underlie and facilitate RAS activation and inactivation, respectively [58]. The RAS binding domain is found in many effectors and invariably binds to one of the switch regions. Activated RAS-GTP has a high affinity for numerous downstream effectors and other small GTPases such as arfaptin or second messenger systems such as adenylyl cyclase as well. Typically, ligand binding to receptor tyrosine kinases induces dimerization of the receptor and autophosphorylation of specific tyrosine residues in the C-terminal region. This generates binding sites for adaptor proteins e.g. growth factor receptor-bound protein 2 (GRB2), that recruit the GEF Sos at the plasma membrane, and in turn activates the membrane-bound Ras by catalyzing the GDP to GTP. In its GTP-bound conformation, RAS combines with RAF and mobilizes the inactive protein from the cytoplasm recruiting the RAF kinases (ARAF, BRAF and CRAF) to the plasma membrane [59]. Once the RAS-RAF complex is translocated to the cell membrane, RAS activates the serine/threonine kinase function of RAF isoforms. Upon activation of RAS, RAF acts as a MAP kinase kinase kinase (MAPKKK) to activate MEK1 and MEK2, which, in turn, catalyze the activation of the effector ERK1 and ERK2 kinases, and their translocation into the nucleus. Once activated, ERK1/ERK2 broadly phosphorylate several nuclear and cytoplasmic effector genes involved in diverse cellular responses such as cell proliferation, survival, differentiation, motility, and angiogenesis [58]. Although RAF can also be activated by RAS-independent activators, considerable experimental evidence indicates that the RAF-MEK-ERK cascade is a major mediator of RAS-induced oncogenesis. Recent data have clearly shown that RAS can activate other downstream signaling pathways including phosphatidylinositol 3-kinase (PI3K) and Rac and Rho proteins, associated with the regulation of the cytoskeleton and invasiveness of tumor cells. Through RAS, other signals may be activated such as p38 MAPK, and the stress-activated protein kinase pathway, c-Jun N-terminal [JNK] pathway. Several novel targeted drugs for this pathway have been developed and are currently being tested in clinical trials: sorafenib (multiple TKIs: Raf kinase, platelet-derived growth factor [PDGF] receptor, vascular endothelial growth factor receptor [VEGFR] 2 and 3 kinases, c-Kit), GSK 1120212 (selective inhibitor of MEK1), AS 703026 (selective inhibitor of MEK1/2), and AZD 6244 (selective inhibitor of MEK1/2) [60].

✓ PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway is a signal transduction pathway involved in the regulation of multiple cellular functions including cell proliferation, survival, differentiation, adhesion, motility and invasion. The pathway is switched on through activation of membrane receptors, including tyrosine-kinase receptors (RTKs), such as EGFR, HER2, IGFR-1, VEGFR and PDGFR. Activated receptor tyrosine kinases recruit to the cell membrane a complex including PI3Ks (Figure 7). PI3Ks phosphorylate the phosphatidylinositol (PtdIns) lipid substrates in the plasma membrane, generating PtdIns(3,4,5)P3, PtdIns (3,4)P2 and PtdIns3P, which interact with multiple effector proteins and transduce the signaling from the membrane to the cytoplasm. PI3Ks are divided into three subclasses on the basis of structure, regulation, and lipid substrate specificity. Class I PI3Ks are heterodimeric proteins formed of a p110 catalytic subunit and a p85 regulatory subunit primarily involved in the pathogenesis of human cancer [61,62]. Class I PI3Ks use PtdIns(4,5)P2 as substrate and can be activated by RTKs and G protein-coupled receptors, via phosphotyrosine binding of the Src homology 2 (SH2) domain present in the p85 regulatory subunit with the cytoplasmic domains of RTKs. Class I PI3Ks might be also activated by RAS, through the RAS-binding domain present in their catalytic subunits [61]. The tumor suppressor gene phosphatase and tensin homolog (PTEN) inhibits the PI3K signaling pathway by dephosphorylating PtdIns(3,4,5)P3 to PtdIns(4,5)P2. PtdIns(3,4,5)P3 at the plasma membrane recruits and activates a number of proteins that contain pleckstrin homology domains (PH domains). These proteins include AKT and phosphoinositide-dependent protein kinase-1 (PDK1).

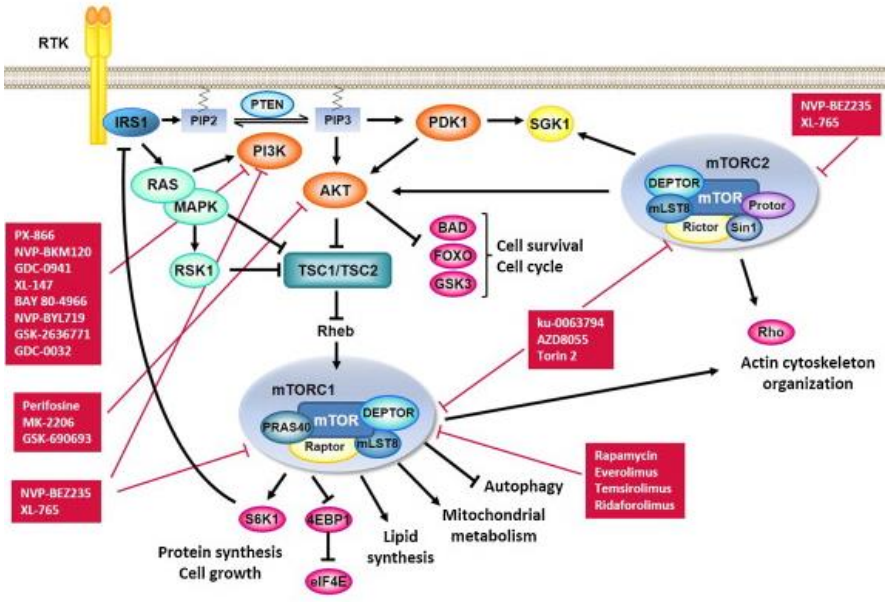


Figure 7. PI3K/AKT/mTOR pathway

[Biochemical Pharmacology 90 (2014) 197–207]

AKT is a serine-threonine protein kinase which exists in three isoforms: AKT1 and AKT2, ubiquitously expressed, and AKT3 which is predominantly expressed in the brain, kidney and heart. Activated AKT phosphorylates and inhibits downstream signaling proteins, including glycogen synthase kinase 3 (GSK3), forkhead box O (FOXO) transcription factors, and BAD, thereby suppressing apoptotic signals and stimulating cell cycle progression. In addition, AKT indirectly activates mTORC1, a master regulator of cell growth and metabolism, by phosphorylating and inhibiting the tuberous sclerosis complex 1/2 (TSC1/2).

Deregulation of PI3K/AKT/mTOR pathway is involved in lung tumorigenesis and it has been associated with high grade tumors (G3-G4) and advanced disease (stage III) [65]. Accordingly inhibitors of PI3K signaling have been suggested as potential therapeutic agents in NSCLC. Deregulation of this pathway occurs through a variety of mechanisms including activation of tyrosine kinase receptors upstream of PI3K, PIK3CA amplification as well as mutations in KRAS, PI3K or AKT, or loss of negative regulation by the tumor suppressor gene PTEN. These alterations are more common in squamous cell carcinoma (SQCLC) than in adenocarcinoma (ADK) of the lung. PI3KCA activating mutations in exons 9 (E545K) and 20 (H1047R), in the helical binding domain and the catalytic subunit of the protein, respectively, are reported in 3–10% of SQCLC and 0–2.7% of ADK [62]. A more frequent PI3KCA alteration is gene amplification, found in about 35% of SQCLC versus 7% of ADK [65]. Notably, PIK3CA mutations may occur in ADK concurrently with EGFR, KRAS, and ALK mutations. No oncogenic mutations have been found in PIK3CB (p110b) and PIK3CD (p110d), however, elevated expression of PI3Kb accompanied by a reduction/loss of PTEN has been recently identified in a subset of NSCLC more prevalent in SQCLC [62]. The loss of PTEN function may be caused by mutations, deletions or transcriptional repression via promoter hypermethylation. PTEN loss and PTEN mutations are reported in 8–59% and 3–10% of SQCLC, and 4–46% and 2–5% of ADK, respectively. AKT1 activating mutation is a rare event (1– 2% in SQCLC), but AKT1 and AKT2 overexpression is found in 19% and 32% of SQCLC, and in 16% and 12% of ADK [65]. Although recent studies (2010–2014) have analyzed the PI3K/AKT pathway in NSCLC and have reported frequent common alterations, the existence of a correlation between PI3K/AKT deregulation and grade or stage of the tumor, together with the different number of tumors analyzed in each study and the different methodologies used, may explain the high variability in the frequency of each alteration observed.

Several novel drugs interfere with the mTOR pathway at multiple levels. Everolimus is an oral mTOR inhibitor that was studied in a phase II trial of patients with advanced NSCLC who were previously treated with chemotherapy or EGFR inhibitors, or both. Other novel agents, such as PI3K inhibitors (GDC-0941, XL-147, PX-866, BKM 120) and dual PI3K and mTOR kinase inhibitors (XL-765, BEZ-235), have demonstrated efficacy in vitro and are now being tested in early-phase clinical trials. Furthermore, the novel drugs that inhibit this signaling pathway may be active despite an absence of *PIK3CA* mutation because dysregulation of the mTOR pathway can occur at multiple levels, such as *PTEN* loss, AKT activation, and other pathway alterations [60].

✓ JAK/STAT pathway

STAT proteins are a family of cytoplasmatic transcription factors that exist as inactive monomers and share remarkable homology with other members of the STAT family. STAT proteins interact with phosphotyrosine residues via their SH2 domain and, upon dimerization, translocate to the nucleus and induce the expression of specific target genes. In normal cells, STAT activation is transient, ranging from a few minutes to a few hours. In tumor tissues, however, constitutive activation of STAT proteins is present, especially for STAT-3. In fact, numerous studies have shown constitutive activation of STAT-3 and STAT-5 in a variety of human malignancies. Augmented activity of membrane-associated RTKs, such as EGFR, ERBB2, and platelet-derived growth factor receptor, promote persistent STAT-3 activation and subsequently induce oncogenesis and tumor progression (Figure 8) [66].

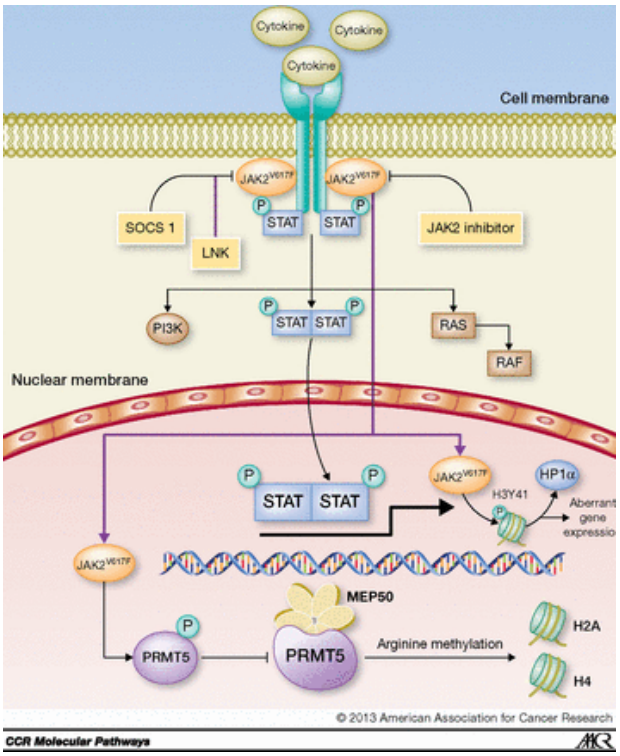


Figure 8. JAK/STAT pathway

[Cancer Res; 19(8); 1933–40. 2013 AACR]

3. TARGET THERAPY

Two major classes of EGFR-targeted therapies have been developed: small tyrosin kinase inhibitors (TKIs) and monoclonal antibodies (mAbs). Although both classes can target EGFR and HER2 respectively, they differ mechanistically and appear to differ in the clinical profile, too. TKIs such as gefitinib (Astra-Zeneca) and erlotinib (Roche, Nutley, N.J., USA) block the binding of adenosine triphosphate to the intracellular TK domain of EGFR, thereby blocking TK activity and subsequent intracellular signaling [67-69]. By contrast, mAbs such as cetuximab (ImClone) and trastuzumab (Roche) bind to the extracellular domain of EGFR/HER2 on the surface of tumor cells, thus preventing EGFR ligands from interacting and activating the receptor, as well as including receptor-ligand internalization [69]. Furthermore, lapatinib (GlaxoSmithKline) – a dual TKI – has been designed for dual suppression of the EGFR and HER2 signaling network and was approved by the US Food and Drug Administration (FDA) in 2007 for the therapy of breast cancer patients treated with first line capecitabine (Roche) [70].

3.1 TYROSIN KINASE INHIBITORs (TKIs)

✓ FIRST GENERATION EGFR-TKIs

First-generation EGFR TKIs such as ***erlotinib*** and ***gefitinib*** reversibly compete with adenosine triphosphate (ATP) binding at the tyrosine kinase domain of EGFR. This inhibits ligand induced EGFR tyrosine phosphorylation, EGFR activation, and subsequent activation of the downstream signaling networks (Figure 9) [71].

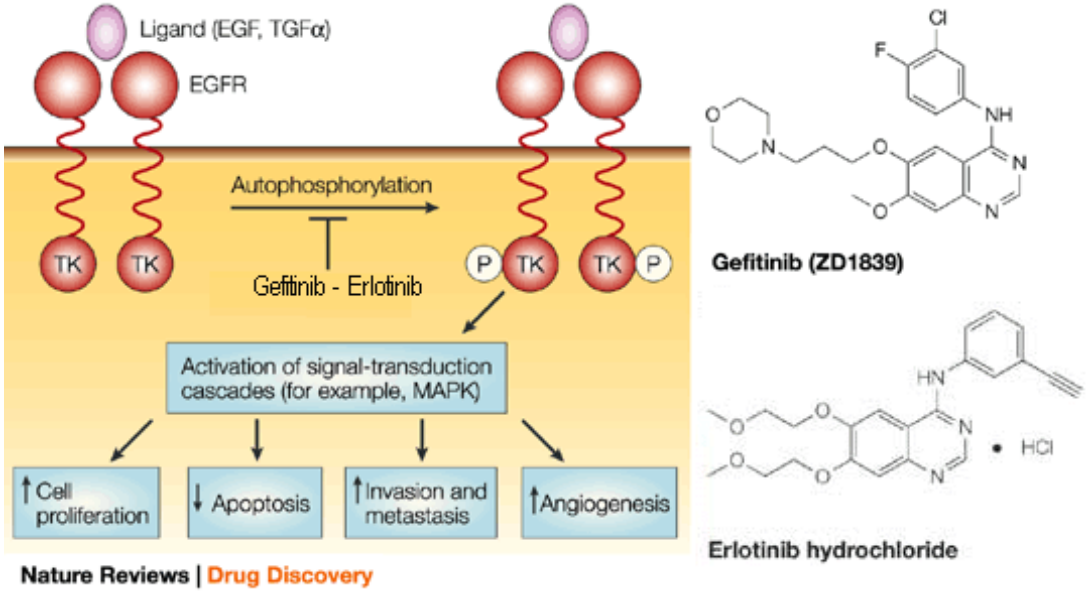


Figure 9. Structure of gefitinib/erlotinib and their *in vitro* inhibitory activity

[Nature Reviews Cancer 3, 556-557 (August 2003) | doi:10.1038/nrc1159, Nature Reviews Drug Discovery 4, 13-14 (January 2005) | doi:10.1038/nrd1612]

The IDEAL 1 [72] and IDEAL 2 [73] phase II trials were two of the first studies to test gefitinib in patients with stage IV NSCLC. These trials demonstrated that both 250 and 500 mg doses of gefitinib were equally active in an EGFR mutation-unselected patient population, resulting in response rates of approximately 20% and median progression-free survival of 2.7 and 2.8 months for the 250 and 500 mg doses of gefitinib, respectively. Because both doses showed equivalent results, the lower 250 mg dose was put forward for the registration phase III trials. A subset of patients treated with gefitinib demonstrated a very positive response, but it was unclear why that was the case. At that time, the implications of EGFR mutations were not understood, but we now know that most of these patients likely harbored an EGFR gene mutation [74].

The NCIC BR.21 phase III trial demonstrated that erlotinib prolonged survival in NCSLC following the failure of first-line or second-line chemotherapy [75]. This trial demonstrated a survival benefit in all patients regardless of whether their tumors had an EGFR gene mutation.

Why an EGFR inhibitor was efficacious in the absence of an EGFR mutation is unclear. This reflects the complexity of the EGFR mutation and other downstream signaling pathways, many of which are still to be delineated. As a result of the NCIC BR.21 trial, erlotinib was approved and became standard of care in the second or third line setting for patients with NSCLC [74].

In 2004, two articles were published in prestigious journals by Paez et al. and Lynch et al. [35,36]. Both publications demonstrated that patients who responded well to gefitinib had EGFR gene mutations, and the mutations were located in the region of the gene that encoded the tyrosine kinase domain. Although much discussion centered on whether the presence of the mutation should influence treatment decisions, clarity about the importance of EGFR mutations did not occur until the Iressa Pan Asian Study (IPASS) trial was completed, the mutation status of patients was analyzed, and the biomarker story became clear.
The IPASS trial was the study attributed to changing practice. The goal of the IPASS trial was to evaluate the benefit of gefitinib as compared to carboplatin/paclitaxel as first-line treatment for patients with advanced NSCLC [76]. Patients selected with this trial had favorable clinical characteristics and included Asian patients with adenocarcinoma, who were non smokers or former light smokers. Patients treated with gefitinib demonstrated superior progression free survival as compared to those treated with chemotherapy (HR 0.74, CI 0.65–0.85, P <0.001). Iressa Pan Asian Study demonstrated that EGFR was the most appropriate biomarker for the use of EGFR-TKI inhibitors in stage IV non-small cell lung carcinomas and with a significant improvement in PFS and quality of life, gefitinib became standard of care first line option for NSCLC patients with EGFR-mutated tumor [74].

The European Tarceva vs. Chemotherapy (EURTAC) trial was conducted in patients with EGFR mutation positive tumors, and was the first to demonstrate the benefits of an EGFR-TKI in a Caucasian population [77].

Lapatinib (figure 10) is a TKI with activity against EGFR and HER2. Like gefitinib and erlotinib, lapatinib is a reversible TKI. In preclinical studies, *wild-type* HER2 was found to be particularly sensitive to lapatinib.

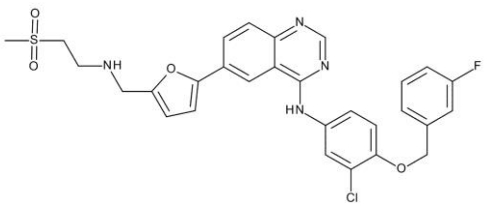


Figure 10. Structure of lapatinib

[Nature Reviews Drug Discovery 6, 431-432 (June 2007) | doi:10.1038/nrd2332]

Lapatinib has received FDA approval in combination with capecitabine for treating patients with advanced or metastatic breast cancer whose tumors overexpress HER2. However, because this agent failed to show substantial efficacy in NSCLC, clinical development was discontinued [78].

✓ ACQUIRED RESISTANCE TO FIRST GENERATION EGFR-TKIs

Several molecular mechanisms have been implicated in the development of resistance. Approximately 50% of cases of acquired resistance to EGFR TKIs are associated with the development of the secondary gatekeeper mutation T790M [79]. This mutation is believed to enhance resistance by sterically hinder the binding of TKIs to EGFR, although more recent data support that it increases the ATP binding affinity by approximately 10 fold, allowing ATP to compete with TKIs for the EGFR binding site. Rosell et al. have assessed T790M and TP53 mutations, the EML4-ALK translocation and BIM mRNA expression in pretreatment tumor samples of 95 patients from the EURTAC trial and correlated the findings with outcome. Concomitant T790M was found in 37.89%, but multivariate analysis failed to demonstrate T790M mutation as a prognostic factor for both PFS and OS. Other less frequent secondary mutations have been described and are also implicated in resistance to TKI treatment [80].

Amplification of the gene encoding the MET tyrosine kinase receptor is responsible for approximately 20% of cases of resistance to TKI treatment [81]. MET is a receptor for the Hepatocyte Growth Factor (HGF) and when activated MET stimulates cell–cell detachment, migration and invasiveness. Engelman et al. demonstrated that MET amplification confers resistance to TKI treatment by activating the Her-3 receptor resulting in the activation of phosphatidylinositol-3-kinase (PI3K) pathway thus overcoming the TKI blockade [82]. MET amplification is rare in untreated patients (approximately 3%) while it occurs in 20% of patients pretreated with EGFR TKIs [81].

Somatic KRAS mutations are observed in around 10–30% of NSCLC adenocarcinomas, are strongly associated with tobacco exposure and can result in EGFR independent activation of MAPK [83]. Such activating KRAS mutations predominantly occur in codons 12 (90% of patients) or 13, and are observed mutually exclusively to EGFR somatic mutants. NSCLCs harboring KRAS mutations are a negative predictive factor of response to TKIs treatment [84]. Other parallel signaling pathways can lead to resistance to TKI treatment. Insulin-like growth factor receptor 1 (IGFR-1) is a transmembrane receptor, that is involved in oncogenic transformation, growth and survival of cancer cells. IGF-1R activation can lead to the

triggering of two intracellular pathways; the PI3K pathway and the RAS-RAF-MAPK pathway, therein bypassing EGFR inhibition. In cell lines activation of IGF-1R is associated with resistance to TKIs. Furthermore in NSCLC patients IGF-1R increased gene copy number was associated with poor survival [85].

BRAF gene codes for a non-receptor serine/threonine kinase with a kinase domain structurally similar to other protein kinases of the HER family. BRAF mutations are found in approximately 1–3% of NSCLC and may result in constitutive signaling through the oncogenic RAS/RAF/MAPK pathway [86]

HER2 has no known ligand and is activated by creating heterodimers with other members of the HER family [39]. HER2 activation leads to activation of the PI3K/AKT/mTOR and the RAS/RAF/MAPK pathways. HER2 mutation in exon 20 has been observed in approximately 3–10% of adenocarcinomas and is associated with constitutive activation of the HER2 kinase that leads to the activation of the downstream pathways [50]. Patients with HER2 are considered to be resistant to TKIs treatment. HER2 and EGFR mutations are usually mutually exclusive [50].

The PI3K/AKT pathway represents a crucial growth and survival pathway for cancer cells [87]. Activation of this pathway can occur as a result of both amplification and mutations of the PIC3CA gene, that encodes p110a isoform, the main catalytic subunit of PI3K [87]. PIC3CA amplification is observed in approximately 12–17% of NSCLC patients, and has been associated with resistance to TKIs. Mutations are more rare (about 4% of NSCLC patients) and are not mutually exclusive with EGFR mutations.

Several new generation TKIs have been tested in NSCLC to overcame resistance, including second and third generation of EGFR-TKIs.

✓ SECOND GENERATION EGFR TKIs

Afatinib is second-generation EGFR-TKI, and block all HER family ligands, including EGFR, as well as HER2 and HER4. This agent form permanent covalent bonds with the target, irreversibly inhibiting ATP binding at the tyrosine kinase domain. As a result, second-generation TKIs are theoretically more effective in inhibiting EGFR signaling than first-generation erlotinib or gefitinib because the inhibition of EGFR signaling is prolonged for the entire lifespan of the drug-bound receptor molecule (Figure 11) [88].

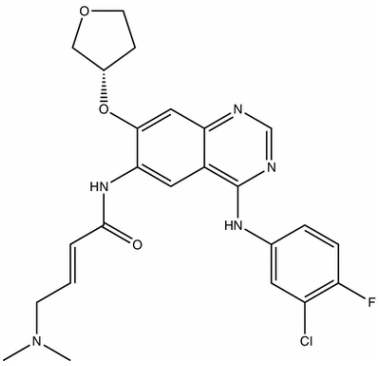


Figure 11. Structure of afatinib [Drugs (2013) 73:1503–1515]

LUX-Lung 1 was a phase 2b/3 randomized trial comparing afatinib to best supportive care in unselected patients who had received both a platinum doublet and 3 months of an EGFR TKI, gefitinib, or erlotinib [89]. Although progression-free survival was increased, the primary endpoint of overall survival was not. Because of this negative trial, the use of afatinib in patients with an acquired resistance to EGFR TKIs was not approved in any country except Japan. The pivotal afatinib trial is LUX-Lung 3 [90]. This phase III trial randomized 345 patients with NSCLC in the first-line setting who had EGFR mutation-positive tumors to receive either afatinib or cisplatin/pemetrexed. For this study, all EGFR mutations from codons 18–21 were analyzed. The LUX-Lung 6 trial, conducted in Asia, confirmed the value of afatinib in the population of patients with EGFR mutation-positive tumors [91]. This phase III, open-label trial randomized 364 NSCLC patients in a 2:1 fashion to receive afatinib or gemcitabine/cisplatin. The primary endpoint in this study was progression-free survival and secondary endpoints included objective response rate, disease control rate, patient-reported outcomes, and safety. A statistically significant improvement in progression-free survival was demonstrated between patients treated with afatinib as compared to those treated with chemotherapy, 11.0 vs. 5.6 months, respectively (HR 0.28, CI 0.20–0.39, P <0.0001). To date, none of the published randomized EGFR TKI trials have demonstrated a statistically significant improvement in overall survival. In the American Society of Clinical Oncology meeting in Chicago 2014, a pooled analysis of LUX-Lung 3 and LUX-Lung 6 was presented [92]. Although the pooling of clinical trial results in this way is controversial, the results are interesting. The pooled analysis showed an important improvement in overall survival in patients whose tumors had the most common EGFR mutations, Del-19 and Point 21 L858R. A highly anticipated trial is LUX-Lung 7. This phase III, open-label trial randomized 316 patients with EGFR mutation-positive advanced adenocarcinoma to receive either afatinib or gefitinib. The primary endpoint for the trial, which completed in July 2013, was overall survival. We await the results eagerly. Clinical trials with the third-generation EGFR TKIs are underway. These inhibitors work to selectively inhibit tumors that harbor the acquired T790M mutation.

✓ THIRD GENERATION EGFR TKIs

First generation TKIs, gefitinib and erlotinib, were found to be most effective in patients with advanced NSCLC whose tumors harbor recurrent somatic activating mutations occurring in the exons encoding the kinase domain of EGFR, i.e., small multinucleotide in-frame deletions in exon 19 (ex19del) and a point mutation in exon 21 leading to substitution of leucine for

arginine at position 858 (L858R) [35-37]. Unfortunately, most patients who respond to therapy ultimately develop disease progression after about 9 to 14 months of treatment. Preclinical modeling and analysis of tumor tissue obtained from patients after the development of disease progression have led to the identification of a number of mechanisms that mediate EGFR TKI resistance. It is now well established that acquisition of a second mutation in EGFR, resulting in substitution of threonine at the amino acid 790 to methionine (T790M), is the most common resistance mechanism and is detected in tumor cells from more than 50% of patients after disease progression [93]. The T790M mutation is believed to render the receptor refractory to inhibition by these reversible EGFR TKIs through exerting effects on both steric hindrance and increased ATP affinity. Current targeted therapeutic strategies for patients with acquired resistance are limited. Second-generation irreversible EGFR TKIs such as afatinib and dacomitinib are effective in untreated EGFR-mutant lung cancer [88]. However, as monotherapy, they have failed to overcome T790M-mediated resistance in patients [89], because concentrations at which these irreversible TKIs overcome T790M activity preclinically are not achievable in humans due to dose-limiting toxicity related to nonselective inhibition of wild-type EGFR. Furthermore, these inhibitors can drive resistance through acquisition of T790M in vitro and in patients, providing supportive evidence that they have low potency against T790M. One regimen that showed potential activity is afatinib plus the anti EGFR antibody cetuximab [89].

Third generation EGFR TKIs are designed to target T790M and EGFR TKI sensitizing mutations more selectively than wild-type EGFR. ***WZ4002*** was the first such agent to be published [94], although it has not progressed to clinical trials. A second agent closely related to the WZ4002 series, ***CO-1686***, has been recently reported [95] and is currently in early phase II clinical trials. HM61713 is another third generation agent that is currently in early phase I trials. ***AZD9291*** (figure 12) is a novel, irreversible, EGFR TKI with selectivity against mutant versus wild-type forms of EGFR. AZD9291 is a mono-anilino–pyrimidine compound that is structurally and pharmacologically distinct from all other TKIs, including CO-1686 and WZ4002 [96].

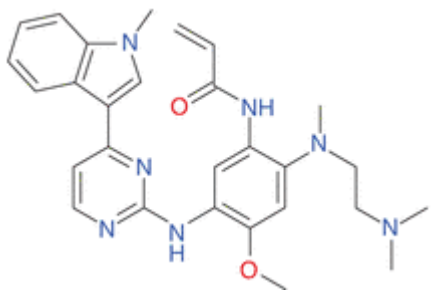


Figure 12. Structure of AZD9291

[Cancer Discovery 2014;4:1046-1061]

3.2 EGFR MONOCLONAL ANTIBODIES (EGFR-mAbs)

Cetuximab is a chimeric human-murine monoclonal IgG1 antibody against the extracellular domain of EGFR. Cetuximab binds to the receptor with higher affinity than its endogenous ligands and promotes its internalization with subsequent degradation. In addition to the direct inhibitory effects on tumor growth through EGFR blockage, cetuximab may also induce the activation of immune effector cells through antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. Cetuximab was investigated as a first-line treatment of patients with advanced NSCLC (Figure 13) [97]. The First-Line Erbitux in Lung Cancer (FLEX) study was conducted as a multinational randomized double-blind phase III clinical trial of 1125 patients with advanced NSCLC with EGFR-expressing tumors [98].

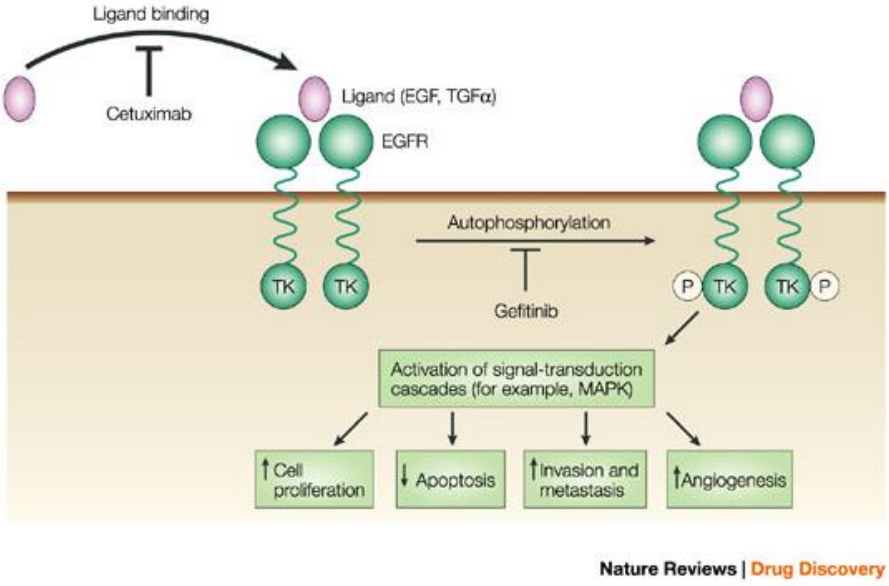


Figure 13. EGFR and the mode of action of cetuximab

[Nature Reviews Drug Discovery 3, 549-550 (July 2004)]

Patients were randomized to treatment with chemotherapy alone or chemotherapy plus cetuximab. However, from results of FLEX study, the justification for cetuximab in first-line combination therapy was questionable [60].

Two meta-analyses evaluated the efficacy and safety of cetuximab based therapy (CBT) in the setting of advanced metastatic NSCLC. The first meta-analysis analyzed 4 eligible randomized controlled trials (RCTs) that included 1003 and 1015 patients randomized to CBT and control intervention, respectively. The CBT arm demonstrated a 9% reduction in the risk of disease progression a 13% reduction in the risk of death and an approximately 50% increase in objective RR [99]. The other recent meta-analysis, from 10 RCTs involving 5936 patients, also demonstrated longer OS and higher RR in cetuximab plus platinum-based

doublet chemotherapy (PBDC) compared with PBDC alone [100]. Despite these marginal benefits, cetuximab is recommended as a category 2B in combination with platinum-based chemotherapy in National Comprehensive Cancer Network (NCCN) practice guidelines for advanced/metastatic NSCLC [60,101].

✓ DUAL INHIBITION OF EGFR IN EGFR-MUTANT NSCLCs

Patients with *EGFR*-mutant NSCLC who receive gefitinib or erlotinib, show dramatic tumor regression and derive a PFS advantage over chemotherapy. However, acquired resistance to erlotinib or gefitinib eventually develops in most patients [76,77] Currently, there are no targeted therapies approved for the treatment of patients with acquired resistance to erlotinib or gefitinib. At the time of acquired resistance to erlotinib or gefitinib, a second-site EGFR T790M mutation, which alters binding of first-generation EGFR TKIs to EGFR, can be identified in more than half of tumors [102]. Experiments in mice with L858R/T790M erlotinib-resistant tumors showed that the combination of **afatinib** with **cetuximab**, but not the individual drugs, induced near-complete tumor regression by depleting phosphorylated EGFR and total EGFR in tumors [103]. Moreover, animals treated with both drugs seemed to tolerate the regimen without difficulty.

On the basis of these preclinical observations, Janjigian et al. [104] have conducted a study to determine the maximum-tolerated dose (MTD) and to investigate the safety and preliminary efficacy of combined EGFR blockade with afatinib and cetuximab in patients with EGFR-mutant tumors and acquired resistance to erlotinib or gefitinib. A cohort of 126 patients with EGFR-mutant lung cancer was treated with the MTD of afatinib (40 mg oral daily) plus cetuximab (500 mg/m^2 intravenously every 2 weeks). This study demonstrated that a significant proportion of tumors in patients with acquired resistance to gefitinib/erlotinib remain dependent upon EGFR signaling for survival and confirms the preclinical hypothesis that dual EGFR inhibition is particularly meaningful in this patient population. Although the combination of afatinib and cetuximab was developed to overcome T790M-mediated resistance in preclinical models, response rates and PFS were similar in patients with and without T790M mutations. Interestingly, the duration of OR was longer in patients with T790M-negative tumors, which may be counterintuitive given the relative indolence of T790M-positive tumors [105]. The response rate and PFS data suggest that even tumor cells without T790M remain dependent upon the ErbB signaling axis for survival. Such dependence may be due to EGFR amplification, alone or in conjunction with T790M, which has been reported in cases of acquired resistance. Because afatinib also inhibits HER2,

another possibility is that such tumors harbored HER2 amplification, which can occur in patients with acquired resistance in the absence of T790M mutations [60]. Studies are ongoing to determine whether responses are correlated with EGFR or HER2 copy number as well as other reported rarer mechanisms of resistance to gefitinib/erlotinib. The antibody (cetuximab) blocks ligand binding and induces receptor degradation but alone is insufficient to inhibit the ligand-independent activity of the mutant receptors. The kinase inhibitor (afatinib) binds covalently to members of the ErbB family, blocking the tyrosine kinase activity of these receptors and resulting in reduced but incomplete inhibition of autophosphorylation and transphosphorylation of ErbB receptor dimers. This trial was the first study to demonstrate robust and durable clinical activity of a targeted treatment regimen in EGFR-mutant lung cancers with acquired resistance to erlotinib or gefitinib. At present, treatment options for patients with acquired resistance to first-generation EGFR TKIs are limited. Recently, third-generation EGFR mutant-specific TKIs (CO-1686 and AZD9291) have shown some promise in early-phase trials [96].

3.3 HER2 MONOCLONAL ANTIBODIES (HER2-mAbs)

Trastuzumab is a humanized monoclonal antibody that is directed against domain IV in the extracellular segment of ErbB2. Trastuzumab is approved for the treatment of breast cancer overexpressing ErbB2 as part of a regimen consisting of doxorubicin, cyclophosphamide, and either paclitaxel or docetaxel, with carboplatin and docetaxel, or as a single agent following anthracycline-based therapy. Trastuzumab is also approved for the treatment of metastatic breast cancer in combination with paclitaxel for first-line treatment of ErbB2-overexpressing disease or as a single agent for the treatment of ErbB2-overexpressing breast cancer in patients who have received one or more prior chemotherapeutic regimens [106].

The mechanism of action of trastuzumab is complex and is incompletely understood. Baselga reported that trastuzumab effectively inhibits the growth of ErbB2-overexpressing human breast cancer cells *in vitro* and *in vivo* [107]. The treatment of cancer cells with trastuzumab results in a modest down regulation of the ErbB2 receptor. Further cellular events resulting from ErbB2 inhibition include the accumulation of the CDK inhibitor p27 and cell cycle arrest. Trastuzumab induces ADCC in human patients and experimental animals. After binding to the cell surface of ErbB2-overexpressing tumor cells, the monoclonal antibody induces the clustering of Fc receptors (FcγRIIIa) on stromal immune natural killer (NK) cells that leads to tumor cell killing mediated by the release of perforin, granzyme, and cytokines

[108]. Trastuzumab also inhibits constitutive ErbB2 cleavage/shedding mediated by metalloproteases [107]. The cleavage of the extracellular domain from ErbB2 results in the formation of a 95-kDa membrane-bound carboxyterminal domain and a 110-kDa extracellular domain. Cleavage of the extracellular domain of ErbB2 results in the activation of the membrane-associated ErbB2 protein kinase domain. The ability of trastuzumab to inhibit ErbB2 cleavage may correlate with the clinical anticancer activity of the multifunctional ErbB2- targeting antibody (Figure 14) [106].

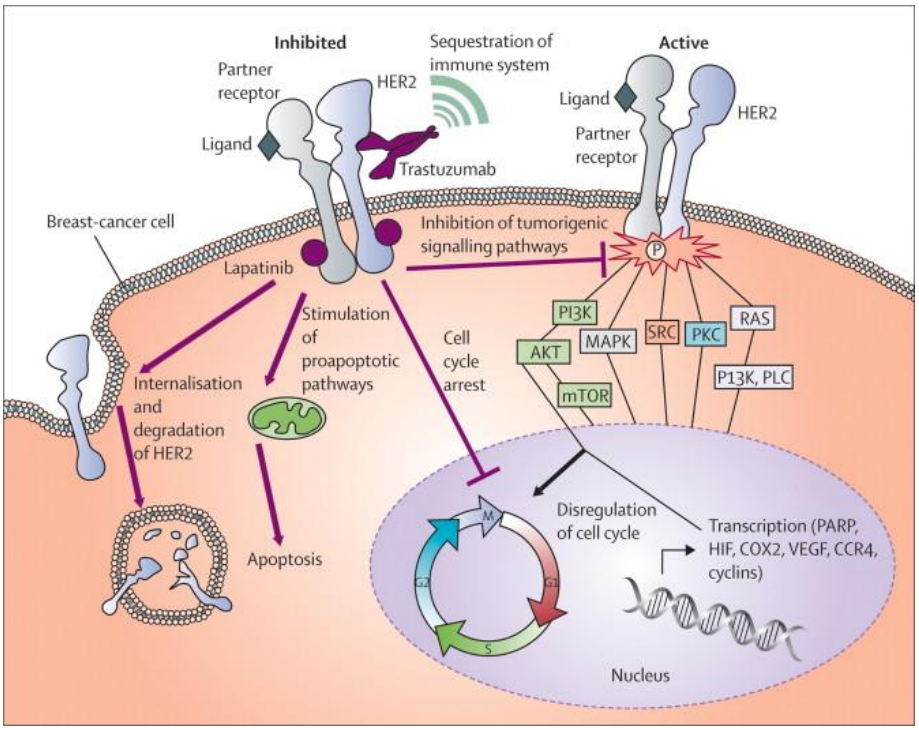


Figure 14. Mechanism of action of trastuzumab

[Lancet Oncol 2009; 10: 1179–87]

Slamon et al. have reported that the combination of trastuzumab with standard cytotoxic chemotherapy produces far better response rates than chemotherapy alone in patients with metastatic breast cancer that overexpress ErbB2 [109]. They reported that combination therapy was associated with a longer median time to disease progression (7.4 vs. 4.6 months), a higher rate of objective response (50% vs. 32%), a longer median duration of response (9.1 vs. 6.1 months), a lower rate of death at one year (22% vs. 33%), and longer median survival (25.1 vs. 20.3 months) [109].

As with all targeted anticancer therapies, primary and acquired resistances to trastuzumab are important problems. About 50–66% of ErbB2-positive breast cancer patients fail to respond to trastuzumab. The reasons for this primary resistance may be related to the many factors that participate in the pathogenesis of breast cancer besides ErbB2 over activity. After initially responding to trastuzumab patients generally relapse. The mechanisms for such resistance

include loss of the PTEN activity. Activation of alternative signaling pathways may also contribute to the development of trastuzumab resistance including those of the insulin-like growth factor receptor and the hepatic growth factor receptor (c-Met) [106,110].

3.4 ANTIBODY-DEPENDENT CELLULAR CYTO-TOXICITY(ADCC)

The antibody-dependent cell-mediated cytotoxicity is a well-recognized immune effector mechanism in which antigen-specific antibodies direct immune effector cells of the innate immunity to the killing of the antigen-expressing cancer cells [111]. The ADCC is a tripartite process and requires three components: 1) the expression of the target antigen on cancer cells, 2) the presence of the antigen-specific antibodies of the appropriate isotype and 3) Fc receptor-bearing effector cells. The antibodies bind to the antigens on the surface of the cell and its so-called crystallizable fragment (Fc) binds to the Fc receptor (FcR) of the effector cell. The binding cross-links FcRs on the effector cells. Consequently, the FcR-bearing effector cells become activated and trigger their functions, e.g., NK cells kill cancer cells and also release cytokines and chemokines (Figure 15). The NK cells secreted interferon-γ (IFN-γ) inhibit cell proliferation, increase cell surface expression of MHC antigens, and exert anti angiogenic activity. The ADCC provides a classical example of how innate and adaptive immune responses cooperate to protect host from malignancy and infections. The effector cells that may mediate ADCC include NK cells, monocyte-macrophages, polymorphonuclear leukocytes, especially neutrophils. Of these cells, NK cells constitute the principal ADDC effector cells. They bear low affinity type II (FcγRIIc; CD32c) and type IIIA (Fcγ RIIIa; CD16a) Fc receptors on their surface [112]. NK cells mainly kill their target cells via releasing cytotoxic granules, i.e., perforin, granulysin and granzymes. Macrophages engulf antibody coated tumor cells; releasing NO, reactive oxygen radicals and a variety of proteases. They may also kill target cells via Fas/FasL interactions [111]. It is not clear how neutrophils kill tumor cells. Neutrophils mainly mediate ADCC against cancer cells via FcαRI (CD89). Some authors have suggested that they transfer activated caspases to the target cell during conjugate formation [113]. CD16a plays a predominant role in NK cell-mediated ADCC. For this reason it has been dubbed as the ADCC receptor. The ADCC-mediated destruction of tumor cells as well as of virus-infected cells can be readily demonstrated in

vitro in the presence of NK cells and tumor or virus-specific antibodies of appropriate IgG isotypes. The process also occurs in vivo.

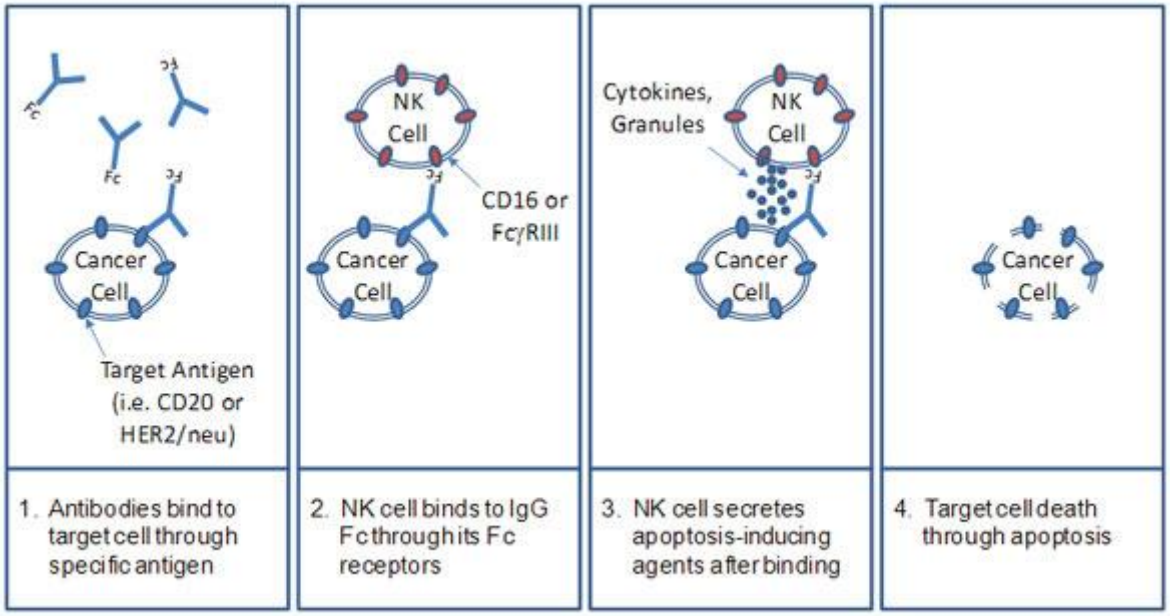


Figure 15. Antibody-dependent cellular cytotoxicity (ADCC)

[www.BioTech.com]

FcRs belong to the immunoglobin superfamily. They regulate cell activation, clearance of immune complexes (IC), homeostasis of circulating Ig and immune responses. FcγRs and FcαRs bind Fc region of IgG and IgA, respectively, and are involved in mediating ADCC. Depending on their affinity for the ligand, FcγRs can be divided into low or high affinity receptors. The high-affinity receptors bind monomeric IgG and are normally occupied by IgG in plasma. The low-affinity FcγRs bind aggregated IgG and IgG-containing IC. Like any other regulatory system, FcR system comprises activating and inhibitory receptors. The activating receptors occur as heterooligomeric complexes with a homodimer of a common γ chain in mice, and with a heterodimer of the γ and ζ chains in humans. The γ and ζ chains were originally identified as signaling elements present in the FcεR1 and the T cell receptor (TCR) complex, respectively. It has been demonstrated in mice that the γ chain is not only necessary for signal transduction by activating FcR, it is also necessary for efficient assembly and expression of these receptors on the cell surface. No activating FcRs are expressed on cell surface in the γ -chain knocked out (KO) mice. These mice express only inhibitory FcR (Fcγ RIIb; see below). These mice have proven useful in studying the role of activating FcRs in the efficacy of anti-tumor mAb [114]. Since γ chain associates with many activating FcRs, the disruption of an activating FcR increases the pool of available γ chains. This results in an increased expression of other activating FcRs. For example, FcεR1 KO leads to increased Fcγ RIII on mast cells and to enhanced systemic anaphylactic responses in mice [114]. The γ

chain has an immunoreceptor tyrosine-based activating motif (ITAM), D/ExxYxxL/Ix(6-8)YxxL/I, in its cytoplasmic tail. When cross-linked, the ITAM becomes tyrosine phosphorylated and recruits SH-2 domain-containing src and syk family tyrosine kinases. This cascade of phosphorylation leads to cell activation and causes triggering of its effector functions. So far only one inhibitory FcR, Fcγ RIIb, has been identified in humans and mice. It is the most widely expressed FcR and is present on all cells of the hematopoietic origin with the exception of NK cells and RBCs. It inhibits BCR-mediated proliferation, antibody secretion, and cytokine production in B cells. Fcγ RIIb KO mice mount 5–10 fold increased antibody responses to antigens and are more susceptible to peripheral cutaneous anaphylaxis to IgG due to deceased threshold of their mast cell activation [115]. It also inhibits FcR-mediated activation of macrophages. The inhibitory FcR is expressed on cell surface as a single chain, type I Ig-like molecule. It does not associate with the γ or ζ chains. The receptor bears an immunoreceptor tyrosine-based inhibitory motif (ITIM), I/V/L/SxYxxL/V, in its intracellular cytoplasmic tail. Upon cross-linking, the tyrosine in this motif is phsphorylated and recruits SH-2 domaincontaining phosphatases, which dephosphorylate several substrates involved in BCR and FcR-mediated cell activation. The receptor limits anti-tumor ADCC mediated by therapeutic mAb, and consequently, these antibodies are more effective against tumors in Fcγ RIIb KO mice [115].

Fcγ RIII is a 50–80 kDa glycoprotein. There are two distinct genes for this receptor and they are expressed in a cell type-specific manner. The A gene encodes Fcγ RIIIa (CD16a), which is a type 1 transmembrane glycoprotein expressed on NK cells, macrophages, monocytes, renal messangial cells, mast cells and a small fraction of circulating T cells. Recently it was demonstrated that about 10–20% of IL-2-activated $CD8^+$ T cells of the memory phenotype express CD16a. These cells may mediate ADCC via CD16 independent of activation via their TCR [116]. CD16a is a heterooligomeric complex with disulphide linked homodimers of γ chains or with a heterodimer consisting of γ and ζ chains. The latter chain becomes phosphorylated on tyrosine in ADCC mediated killing but not in natural killing in NK cells. The level of expression of CD16 on NK cells correlates with their ability to kill target cells in ADCC reactions. Other NK cell expressed molecules (CD2, CD18) may play a role in this killing by increasing conjugate formation between NK cells and target cells and via sending co-stimulatory signals [116]. It is noteworthy that stimulation of activated NK cells via CD16 alone induces activation-induced cell death. The CD16a expressed on NK cells and monocytes may vary in their affinity for IgG due to cell type specific glycosylation pattern of

the receptor [116]. The B gene encodes a version of CD16 (CD16b), which lacks the transmembrane region and cytoplasmic tail, and is anchored into its membrane via glycosyl phosphatidylinositol (GPI). The receptor acts as a sink and does not trigger neutrophil effector functions. The ADCC effector cells of the myeloid lineage mainly use these receptors to phagocytose Ig-coated pathogens and apoptotic cells. However, these cells may use other FcR and play a role in the antibody-mediated destruction of tumor cells. The CD16b has lower binding affinity for human IgG than CD16a.

In summary, ADCC plays an essential role in mediating anti-tumor effects of anti-cancer therapeutic mAb. FcR-bearing effector cells namely NK cells, monocytes, macrophages and neutrophils are recruited to the tumor sites by these mAb. These cells kill antibody-coated cancer cells. The inhibitory FcRs may dampen anti-tumor effects of the anti-cancer mAb. Cancers may develop different strategies to evade the mAb-mediated anti-tumor ADCC. Countering these strategies may include blockage of inhibitory receptors on FcR-bearing effector cells and/or augmenting their effector functions by recombinant cytokines.

3.5 ANTIBODY DRUG CONJUGATEs (ADCs)

Clinically useful, unconjugated monoclonal antibodies (mAbs) selectively recognize antigens that are preferentially expressed on or near tumor cells and exert their cytotoxic effects through mechanisms such as cell signaling, antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity [117]. However, the majority of these mAbs are used in combination with chemotherapy, and many others have demonstrated insufficient clinical activity. Therefore, significant effort has been devoted to empowering mAbs through various modifications [118]. One approach by which the activities of these mAbs have been enhanced is through conjugation with cytotoxic drugs, generating antibody-drug conjugates (ADCs) capable of antigen-specific delivery of highly potent cytotoxic drugs to tumor cells. An ADC must preferentially deliver the cytotoxic payload to tumor cells expressing the target antigen rather than to healthy tissue. This is accomplished by exploiting the specificity of a mAb targeting an antigen that is highly expressed on the surface of malignant cells. After binding the target antigen, the ADC-antigen complex is typically internalized and transported to intracellular organelles where release of the attached drug can occur. Upon release, the cytotoxic drug can interfere with various cellular mechanisms, leading to cell death (Figure 16) [119].

Two ADCs that have shown pronounced clinical activity at tolerated doses, brentuximab vedotin (ADCETRIS) and trastuzumab emtansine (T-DM1), target antigens with preferential expression on tumor cells. The normal cell surface antigen targeted by brentuximab vedotin, CD30, is strongly expressed on the Reed-Sternberg cells of Hodgkin lymphoma (HL) and on systemic anaplastic large-cell lymphoma (ALCL) tumor cells. Similarly, the HER2 antigen, targeted by T-DM1, is highly overexpressed on some metastatic breast cancer tumors compared to normal tissue. Both of these ADCs have shown meaningful clinical activities with manageable safety profiles [119].

The linker that connects the cytotoxic drug to the mAb is a key determinant of ADC activity. These linkers covalently couple the cytotoxic drug to the antibody, producing an ADC that should be relatively stable in circulation. Upon internalization, however, the linkers should facilitate efficient drug release [120]

Early ADC development focused on the use of readily available, clinically approved drugs such as doxorubicin, methotrexate, mitomycin, flurouracil, and vinka alkaloids [118]. These drugs have relatively low potencies, so access to solid tumors by macromolecules was inefficient and accumulation of the cytotoxic drug in target cells was poor. The low clinical activity exhibited by these early ADCs prompted the development of ADCs employing much

more potent cytotoxic drugs, which, though too toxic to use in an untargeted manner, have sufficient potency to be used in a more targeted manner. The majority of highly potent cytotoxic agents used in current ADCs are auristatins, maytansinoids, or calicheamicins. The cytotoxicities of auristatins and maytansinoids arise from binding to tubulin and the inhibition of polymerization, causing cell cycle arrest and subsequent apoptosis of the target cell [118,119].

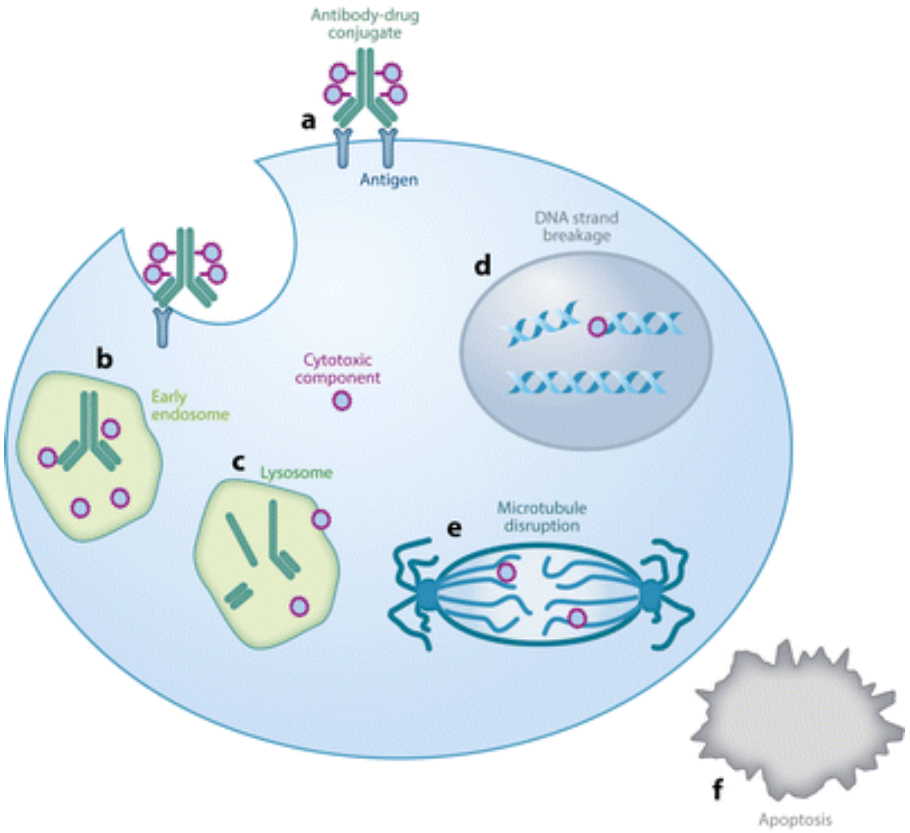


Figure 16. Generalized mechanism of action of antibody-drug conjugates (ADCs)

[Annu. Rev. Med. 2013. 64:15–29]

✓ TRASTUZUMAB EMTANSINE (T-DM1)

T-DM1 is comprised of the antibody trastuzumab, covalently linked through a thioether molecular bond to the microtubule inhibitor emtansine (DM1) [121]. The mechanism of action of T-DM1 is two-fold, both a direct antitumor effect from trastuzumab as well as a cytotoxic chemotherapy effect from the DM1 component. Trastuzumab retains all of its mechanisms of action, which were previously mentioned [122]. The most important aspect of using trastuzumab is that it can be used to deliver chemotherapy specifically to HER2-positive breast cancer tumor cells. By directly transporting cytotoxic agents to cancer cells, this both limits toxicity on other non-malignant tissue and allows for the utilization of more potent chemotherapy [123].

DM1 is a derivative of maytansine collected from plants and mosses, and primarily prevents tumor growth by inhibiting tubulin development of the mitotic spindle and cell division. This chemotherapeutic agent is not routinely used in oncology practices due to the side effect profile caused by a small therapeutic window, as DM1 is approximately 100 times more potent than vincristine. The linker of the antibody and cytotoxic drug is very important. If the link is too unstable, then the drug will be delivered to the blood stream and peripheral tissues. If the link is too strong, then it may not be able to be cleaved. An average of 3.5 DM1 moieties is linked to each trastuzumab molecule in T-DM1 [124].

Briefly, T-DM1 binds to HER2 with high affinity. After internalization of the receptor – T-DM1 complex, intracellular release of DM1-containing moieties from T-DM1 is thought to occur via lysosomal degradation, resulting in the inhibition of cell division and cell growth and eventually culminating in cell death (Figure 17) [125].

Lewis Phillips et al did much of the preclinical work on T-DM1; they determined *in vitro* and *in vivo* efficacy, pharmacokinetics, and toxicity of T-DM1 [124]. T-DM1 was evaluated in normal cell lines (MCF10A breast epithelial cells) and tumor cell lines (breast carcinoma BT 474, SK-BR-3, MCF7, MDA-MB-468, MDA-MB-361, and HCC1954; NSCLC Calu-3; and ovarian carcinoma line SK-OV-3). The cell lines with HER2 overexpression (BT-474, SK-BR-3, and MCF7) responded well to T-DM1; while the cell lines without HER2 overexpression (MDA-MB-468) were unaffected. The cell cycle was also analyzed and found to be arrested in the G2-M phase. In HER2-overexpressiong breast cancer mouse models, complete tumor regression was seen for the duration of the study. T-DM1 was also active in trastuzumab-refractory tumors [124]. In a preclinical study, Junttila et al. reported that T-DM1 did not affect the mechanism of action of trastuzumab itself [122].

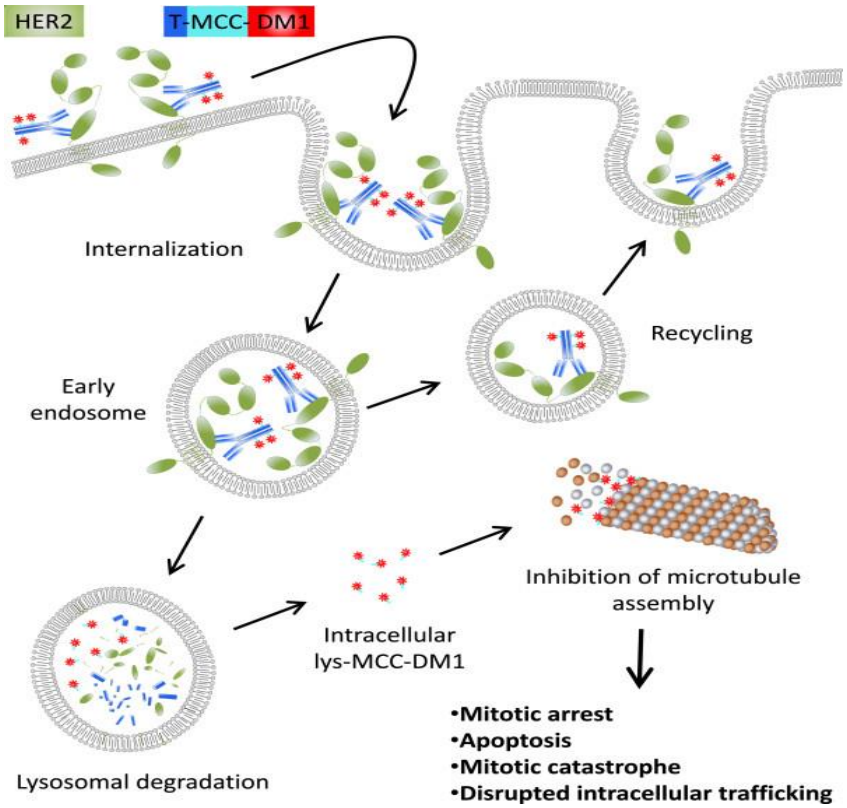


Figure 17. Structure and mechanisms of action of T-DM1

[Breast Cancer Research 2014, 16:209]

After the extensive preclinical work, Phase I clinical trials have been conducted to characterize the pharmacokinetics, safety, and maximum tolerated dose (MTD) of the drug (Table 1). Krop et al. conducted a Phase I study in 24 patients with HER2-positive MBC whose disease had progressed on earlier trastuzumab-based therapy [126]. Beeram et al. released further data in 2012 from the above mentioned multicenter, dose-escalation study. Additional patients were enrolled at the MTD. Twenty-eight patients were included in the analysis [127].

Table 1. Summary of Phase I Clinical Trials

Authors	Year	Number of patients	Study design	Dose escalation	Dosing frequency	Grade 3 or 4 adverse events	Maximum tolerated dose
Krop et al.	2010	24	Phase I single-arm	0.3–4.8 mg/kg	Every 3 weeks	Thrombocytopenia, pulmonary hypertension	3.6 mg/kg every 3 weeks
Beeram et al.	2012	28	Phase I single-arm with additional patients added later at maximum dose	1.2–2.9 mg/kg	Weekly	Thrombocytopenia	2.4 mg/kg weekly

Based on the preliminary efficacy results presented in the Phase I clinical trials, T-DM1 moved on to Phase II development. The MTD of T-DM1 (3.6 mg/kg every 3 weeks) from the 2010 published paper was selected as the recommended Phase II dose. There have been three main Phase II clinical trials published to date (Table 2).

Burris et al conducted a Phase II clinical trial with 112 patients with HER2-positive MBC with tumor progression after prior HER2-directed therapy [128]. Another Phase II clinical trial was conducted by Krop et al. in which 110 patients with HER2-overexpressing MBC with prior exposure to trastuzumab, lapatinib, an anthracycline, a taxane, and capecitabine [129].

Table 2. Summury of Phase II Clinical Trials

Author	Year	Number of patients	Study design	Study population	Objective response rate	Progression-free survival	Grade 3 adverse events
Burris et al.	2010	112	Phase II single-arm	HER2-positive MBC who had tumor progression after prior HER2-directed therapy or received prior chemotherapy	25.90%	4.6 months	Hypokalemia (8.9%), thrombocytopenia (8.0%), and fatigue (4.5%)
Krop et al.	2012	110	Phase II single-arm	HER2-positive MBC who had prior treatment with trastuzumab, lapatinib, an anthracycline, a taxane, and capecitabine	34.50%	7.3 months	Thrombocytopenia (9.1%),fatigue (4.5%), and cellulitis (3.6%)

Given the results in the Phase II clinical trials, the Phase III clinical trials were conducted to characterize the value of T-DM1 in clinical practice. To date, there is one Phase III clinical trial with published results utilizing T-DM1.

The EMILIA trial was a randomized and multicenter Phase III trial in which 991 patients with HER2-positive locally advanced breast cancer or MBC who were previously treated with trastuzumab and a taxane were included [130]. Patients were randomly assigned to T-DM1 (3.6 mg/kg intravenously) or lapatinib (1.250 mg orally daily) plus capecitabine (1.000 mg/m^2 orally twice a day, days 1 to 14). The authors concluded that T-DM1 significantly prolonged PFS and OS with less toxicity.

Based on the EMILIA clinical trial, the FDA approved T-DM1 on February 22, 2013 with the name of ***Kadcyla***®. It was approved for patients with HER2-positive MBC who previously received trastuzumab and a taxane (separately or in combination). Patients should have either:

received prior therapy for metastatic disease, or developed disease recurrence during or within 6 months of completing adjuvant therapy [131].

To date, six Phase III clinical trials are ongoing. These include the MARIANNE study, TH3RESA study and KATHERINE study (Table 3) [132-134].

Table 3. MARIANNE, TH3RESA and KATHERINE Phase III Clinical Trials

ClinicalTrials.gov identifier	Phase	Name	Status	Sponsor	Number of patients	Study design
NCT01120184	III	MARIANNE	Ongoing, not recruiting anymore	Hoffmann-La Roche	1,095	T-DM1 with pertuzumab or T-DM1 with pertuzumab–placebo (blinded for pertuzumab), versus the combination of trastuzumab plus taxane (docetaxel or paclitaxel) in patients with HER2-positive progressive or recurrent locally advanced or previously untreated MBC.
NCT01419197	III	TH3RESA	Ongoing, notrecruiting anymore	Hoffmann-La Roche	604	T-DM1 in comparison with treatment of the physician's choice in patients with metastatic or unresectable locally advanced/recurrent HER2-positive breast cancer.
NCT01772472	III	KATHERINE	Currently recruiting	Hoffmann-La Roche	1,484 (estimated)	T-DM1 versus trastuzumab as adjuvant therapy in patients with HER2-positive breast cancer who have residual tumor present in the breast or axillary lymph nodes following preoperative therapy. Radiotherapy and/or hormone therapy will be given in addition if indicated.

AIM

Two main strategies targeting ErbB receptors have been developed: small-tyrosin kinase inhibitors (TKIs) such as gefitinib and erlotinib, and monoclonal antibodies (mAbs) such as cetuximab and trastuzumab. In NSCLC patients, many molecular aberration have been identified including EGFR, BRAF, HER2 amplification/mutations, EML4-ALK, ROS and RET rearrangements in adenocarcinoma, FGFR amplification/mutations, DDR2 or PI3KCA mutation in squamous cell carcinoma. All of these aberrations have been correlated with a poor prognosis [135,136].

Erlotinib is an effective treatment for NSCLC patients and has been registered as a second and third-line treatment of NSCLC regardless of EGFR mutation status [75].

Gefitinib has been registered for the therapy of advanced NSCLC harbouring activating EGFR mutations in the tyrosine kinase domain, the most frequent being L858R in exon 21 and Del (746–750) in exon 19 [35,36]. Although mutations in EGFR are useful predictors for the activity of EGFR-TKI, they cannot be used as the only criterion to determine who should receive anti-EGFR therapy and it is becoming increasingly clear that even patients with EGFR wild-type can benefit from EGFR-TKI [74,76].

Cetuximab is a chimeric IgG1 monoclonal antibody (mAb) that blocks ligand binding to EGFR, leading to a decrease in receptor dimerization, autophosphorylation, and activation of signaling pathways. In addition the binding of cetuximab initiates EGFR internalization and degradation which leads to signal termination. Moreover, unlike EGFR-TKIs, cetuximab can induce antibody dependent cellular cytotoxicity (ADCC) activity, an important immunologic antitumour effect. Cetuximab in combination with chemotherapy has been approved by the FDA for the treatment of metastatic colorectal cancer and of locally advanced head and neck cancer [97,98].

Trastuzumab, registered for the treatment of HER2 positive breast cancer, has also been tested in phase II trials as a single agent and in combination with cytotoxic chemotherapy for patients with NSCLC [106].

The acquisition of resistance to tyrosine kinase inhibitors (TKIs) in clinical oncology is a well documented phenomenon that applies to several types of cancers. Almost all NSCLC patients with activating EGFR mutations treated with EGFR-TKI, after an initial response, experience disease progression within 10 to 14 months from the beginning of the therapy. A commonly described mechanism of drug resistance involves additional genetic alterations within the EGFR itself, the most frequent being the T790M mutation accounting for approximately 50% of cases of acquired resistance [82,93]. The potential clinical relevance of HER2 expression in NSCLC is currently under evaluation, however, the recent role of HER2 amplification in the

acquisition of resistance to TKI, reported in 12-13% of patients, may render HER2 a potential target not only in breast cancer but also in NSCLC [60].
T-DM1, trastuzumab emtansine, is an antibody-drug conjugate composed by the microtubule polymerization inhibitor DM1 (derivative of maytansine) linked with a stable thioether linker to trastuzumab [124].

Several preclinical studies on cell lines from different tumour types, indicated that the association between EGFR/HER2 mAbs with TKIs displays an increased efficacy [137].

The aim of my thesis was to explore the potential of combining erlotinib with either cetuximab or trastuzumab in order to improve the efficacy of EGFR targeted therapy in EGFR *wild-type* sensitive NSCLC cell lines. Because HER2 represents a relatively new therapeutic target for NSCLC, I evaluated whether T-DM1 activity is affected by HER2 expression/mutation status and may overcame EGFR-TKI resistance in NSCLC cell lines.

METHODs

1. CELL COLTURE

The human NSCLC cell lines used in this study were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and banked at early passage (P2). Furthermore, the cells we culture, are regularly verified on the basis of cell morphology and never cultured for more than 3 months. The PC9, HCC827, HCC827 GR5 and H1781 cell lines were kindly provided in 2013 by Dr P. Jänne (Dana-Farber Cancer Institute, Boston MA, USA). The PC9/HER2c1 was kindly provided in 2013 by Dr.William Pao (Vanderbilt-Ingram Cancer Center, Nashville, Tennessee). All cells were cultured as recommended and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

As previously reported [138] cells showing by proliferation assays IC_{50} for erlotinib < 1 μM were considered sensitive (H322, H292, Calu-3, PC9, HCC827) while cell lines with IC_{50} > 5 μM (H1299, A549, H1703, Calu-1, HCC827 GR5) were considered resistant.

2. DRUGs

Erlotinib, gefitinib, cetuximab, trastuzumab, rituximab and vinorelbine were from inpatient pharmacy. RAD001, NVP-BKM-120 and NVP-BYL-719 were from Novartis. T-DM1 was supplied from Genentech Inc. (South San Francisco, CA) through a Materials Transfer Agreement. Stock solutions of 20 mM drugs were prepared in dimethylsulfoxide (DMSO) (with the exception of mAbs), stored at −20 °C and diluted in fresh medium for use. The final concentration of DMSO never exceeded 0.1% v/v.

3. WESTERN BLOT ANALYLIS

Procedures for protein extraction, solubilization, and protein analysis by 1-D PAGE are described elsewhere [139]. Fifty μg of proteins from lysates were resolved by SDS-PAGE and transferred to PVDF membranes.

Antibodies against EGFR; HER2; p-HER2 Tyr1221/1222; p70S6K; p-p70S6K $^{Thr421/Ser424}$; Akt; p-Akt ^{Ser473}; p44/42 MAPK; p-p44/42 MAPK; caspase-7 and 9; cyclin A and B1; Rb; p-Rb were from Cell Signaling Technology (Beverly, MA). Antibody against cytochrome-c (7H8) was form Santa Cruz Biotechnology Inc. (Dallas, TX). Antibodies against actin was from Sigma–Aldrich (St Louis, MO). Antibody against GAPDH was from Ambion (Austin, TX). HRP-conjugated secondary antibodies were from Pierce (Rockford, IL) and chemiluminescence system (ImmobilionTM Western Cemiluminescent HRP Substrate), was from Millipore (Temecula, CA).

4. CELL SURFACE PROTEIN ISOLATION

Calu-3 cells were grown in T75 flasks and treated with 0.5 μM erlotinib for 24 h. Cells were incubated with EZ-LINK Sulfo-Biotin (Pierce) for 2 h at 4°C with gentle rotation. The reaction was stopped by washing twice with 25 mM Tris–HCl (pH 7.5) in PBS (phosphate-buffered saline) and cells were scraped into ice-cold lysis buffer (50 mmol/l HEPES, pH 7.0, 10% glycerol, 1% TritonX-100, 5 mmol/l EDTA (ethylenediaminetetraacetic acid), 1 mmol/l $MgCl_2$, 25 mmol/l NaF, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 0.5 mmol/l orthovanadate, and 1 mmol/l phenylmethylsulfonyl fluoride). Lysates were centrifuged at 15000 g for 20 min at 4°C, and supernatants were removed and assayed for protein concentration using the DC Protein assay (Bio-Rad, CA, USA). A volume of 500 μl of lysis buffer containing equal amount of proteins was incubated with UltraLink Immobilized NeutrAvidin protein (Pierce) for 2 h at 4°C with gentle rotation, washed three times with lysis buffer before suspension in SDS (sodium dodecyl sulfate)-loading buffer and then resolved by SDS-PAGE.

5. FLOW CYTOMETRY

One million of NSCLC cell lines were incubated, for one hour at room temperature, with Isotype control Monoclonal Mouse IgG1/R-PE (Ancell IRP, Bayport, MN, USA) or PE mouse anti-Human EGFR (BD Biosciences, San Josè CA) or PE mouse anti-Human HER2 (BD Biosciences, San Josè CA) to determine HER-2 protein membrane levels as previously described [140]. After the incubation the analysis was performed using an EPICS-XL flow cytometer. Mean fluorescence intensity (MFI) values were converted in units of equivalent fluorochrome (MEF) using the FluoroSpheres 6-Peak Kit (Dako, CA, USA).

6. QUANTITATIVE real-time PCR

Total RNA was isolated by the TRIzolW reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed as previously described [141]. The transcript levels of EGFR gene were assessed by Real-Time qRT-PCR on an iCycler iQ Multicolor RealTime PCR Detection System (Bio-Rad, Hercules, CA, USA). Primers and probes included: EGFR-F (5'-GCCTT GACTGAGGACAGCA-3'), EGFR-R (5'-TTTGGGAAC GGACTGGTTTA-3'), EGFR-probe (5'-FAM CTTCCTCC3'DQ); PGK1-F (5'-GGAGAACCTCCGCTTTCAT-3'), PGK1-R (5'-CTGGCTCGGCTTTAACCTT-3'), PGK1-probe (5'-FAM GGAGGAAG-3'DQ); RPL13-F (5'-ACAGCTGCTCAGCTTCACCT-3'), RPL13-R (5'-TGGCAG CATGCCATAAATAG-3'), RPL13-probe (5'-FAM CAGTGGCA-3'DQ); HPRT-F (5'

TGACCTTGATTTATTTTGCATACC-3'), HPRT-R (5'-CGAGCAAGACGTTCAGTCCT-3'), HPRT-probe (5'-FAM GCTGAGGA-3'DQ). The amplification protocol consisted of 15 min at 95°C followed by 40 cycles at 94°C for 20s and at 60°C for 1 min. The relative transcript quantification was calculated using the geNorm algorithm for Microsoft ExcelTM after normalization by expression of the control genes [phosphoglycerate kinase1 (PGK1), ribosomal protein L13 (RPL13) and hypoxanthine-guanine-phosphoribosyltransferase (HPRT)] and expressed in arbitrary units (a.u.).

7. ANALYSIS OF CELL PROLIFERATION AND CELL CYCLE

Cell viability was evaluated by tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium- bromide] (MTT) assay and by crystal violet staining as previously described [141]. Data are expressed as percent inhibition of cell proliferation versus control cells. Distribution of the cells in the cell cycle was determined by PI staining and flow cytometry analysis as described elsewhere [142].

8. DETECTION OF APOPTOSIS

Apoptosis was assessed by morphological study: stained (Hoechst 33342, propidium iodide) or unstained cells were observed using light-, phase-contrast- and fluorescencemicroscopy. Western blot analysis was performed to evaluate cleavage products of caspase-7 and caspase-9. Cytosolic and mitochondrial fractions for cytochrome-*c* detection were generated using a digitonin-based subcellular fractionation technique as previously described [143].

9. DETERMINATION OF PROTEASOME ACTIVITY

Cells were lysed in 20 mM Tris–HCl pH 7.5, containing 10% glycerol, 5 mM ATP and 0.2% NP-40. Extracts were centrifuged at 12,000 X g for 10 min at 4 °C. Chymotrypsin-like (CTL), activity of the proteasome was assayed in Proteasome Assay Buffer, containing 25 mM Hepes pH 7.5, 0.03% SDS, 0.05% NP-40, with 50 mM fluorogenic peptide substrate, after 1 h of incubation at 37 °C, in the absence and in the presence of erlotinib 1 µM and MG262 0.5 µM. The assay of CT-L activity is based on the detection of the fluorophore 7- Amino-4-methylcoumarin (AMC) after cleavage from the labeled substrates Suc–Leu–Leu–Val–Tyr–AMC. The free AMC fluorescence was quantified using a 380/460 nm filter set in a LS 50 B

luminescence spectrometer (Perkin-Elmer). Proteasome activity was calculated as the difference between the total activity of crude extracts and the remaining activity in the presence of 0.5 μM MG262 [144].

10. ISOLATION AND CULTURE OF NK CELLs and ADCC ASSAY

Human PBMC were isolated from buffy coat of healthy donors by using a Lympholyte-H density gradient centrifugation (Cederlane Burlington, Ontario, Canada). Highly purified $CD56^+$ natural killer (NK) cells were obtained by magnetic separation using the NK Cell Isolation Kit and the autoMACS Separator (Miltenyi Biotec, Cologne, Germany) according to the user manual. Purified NK cells were resuspended in culture medium (RPMI 1640 without phenol red and supplemented with heat inactivated 10% FCS, 50 U/ml penicillin, 50 U/ml streptomycin, 2 mmol/l glutamine) plated and preincubated at 37°C for up to 18 h in the presence of human Interleukin-2 (IL-2, 100 U/ml, Miltenyi Biotec). Antibody-dependent cell-mediated cytotoxicity (ADCC) was measured with the CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI, USA) according to manufacturer's instructions. After 4 hours the lactate dehydrogenase (LDH) release was determined and the percentage of cytotoxicity was calculated after correcting for background absorbance values according to the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental - Effector spontaneous - Target spontaneous}}{\text{Target maximum - Target spontaneous}} \times 100$$

11. TUMOR XENOGRAFTs

All experiments involving animals and their care were performed with the approval of the Local Ethical Committee of the University of Parma, in accordance with the institutional guidelines that are in compliance with national (DL116/92) and international (86/609/CEE) laws and policies. Balb/c-Nude female mice (Charles River Laboratories, Calco, Italy) 6 weeks old, were housed in a protected unit for immunodeficient animals with 12-hour light/ dark cycles and provided with sterilized food and water ad libitum [139,140]. 200 μl of matrigel (BD Biosciences) and sterile PBS (1:1) containing Calu-3 cells were subcutaneously injected on the right flank of each mouse. Ten days after cells injection, animals were randomized. Tumor xenografts were measured three times per week using a digital caliper and tumor volume was determined using the formula: (length × $width^2$)/2. At the end of the

experiments, mice were euthanized by cervical dislocation and tumors weighted and collected for immunohistochemical and histological analysis [140,145].

12. ISOLATION AND *IN VITRO* EXPANSION OF NEOPLASTIC CELLS FROM TUMOR XENOGRAFTs

After tumor excision, part of each nodule was utilized for the re-isolation of tumor injected cells. The tumoral nodules were excised in sterile condition and processed separately for each group under hood at laminar flow. After removal of surrounding epidermal and connective tissue, one-half of each nodule was fixed in 4% formaldehyde, while the remain part was washed-out with a saline buffer and then processed for the cell isolation.

Nodules were firstly minced with scissors and then put into a collagenase/dispase solution (C/D, ROCHE). At the end of enzymatic digestion, the tissue fragments were removed using a nylon filter and the cell suspension was centrifuged at 240 X g for 5 minutes.

Cell suspension was seeded in 6 well plates at 37°C-5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1%, P/S and 1% NEAA.

Twenty-four hours after plating the debris and the non-adherent cells were removed by washing twice with PBS; fresh culture medium was then added to cell culture and then changed twice a week.

13. MORPHOMETRIC and IMMUNOHISTO-CHEMICAL ANALYSIS OF TUMOR XENOGRAFTs

Formalin fixed samples were embedded in paraffin. On each tumor serial sections of 5 μm thickness were stained with Haematoxylin and Eosin (H&E), Masson's Trichrome and subjected to immunohistochemistry. A morphometric analysis was performed on the entire section in order to evaluate the volume of neoplastic tissue, connective tissue and vascular interstitium. To better define the fraction occupied by neoplastic cells, sections were stained with pancytokeratin antibody (PanCK monoclonal mouse, 1:500, o.n. 4°C, Dako) revealed through biotin-streptavidin-DAB system (Dako). The volume fraction of fibrosis and vascular interstitium was assessed on Masson's Trichrome stained samples. To this end, the number of points overlying each tissue components was counted and expressed as percentage of the total number of points explored. All these morphometric measurements were obtained with the aid of a grid defining a tissue area of 0.23 mm^2 and containing 42 sampling points each covering

an area of 0.0052 mm^2. Combining the entire tumor volume with the above morphometric measurements, the total volume occupied by neoplastic cells, connective tissue and vascular interstitium was computed for each sample. Moreover, immunohistochemical analysis of HER2 was performed on each tumor of each experimental group. Sections were stained with HER2 antibody (monoclonal mouse clone 4B5, Ventana, USA) and revealed through biotin streptavidin-DAB system. This analysis was performed on the entire tumor by an optical microscope (Olympus, BX60- 100X magnification) to evaluate the area occupied by cell expressing HER2 and their intensity. The latter was expressed as Integrated Optical density (IOD), as detected using a software for image analysis (Image Pro Plus, Media Cybernetics, USA). In addition, the nuclear expression of the phosphorylated form of Histone H3 (pH-H3, rabbit poyclonal, 1:100, Millipore, USA) on Pancytokeratinpos cells was detected by immunofluorescence to document mitotic figures. For all tested epitopes negative controls were represented by immunostaining the sample with an irrelevant antibody or by exposing the sections only to the secondary antibody.

14. STATISTICAL ANALYSIS

Statistical analyses were carried out using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Results are expressed as mean values ± standard deviations (SD) for the indicated number of independent measurements. Differences between the mean values recorded for different experimental conditions were evaluated by Student's t test, and P values are indicated where appropriate in the figures and in their legends. A P value <0.05 was considered as significant. Bliss interaction was calculated as previously described [130]. For in vivo studies comparison among groups was made using analysis of variance (two-way ANOVA repeated measures) followed by Bonferroni's post-test.

RESULTs

1. DIFFERENTIAL EFFECTS OF ERLOTINIB ON EGFR AND HER2 EXPRESSION IN SENSITIVE AND RESISTANT NSCLC CELL LINES

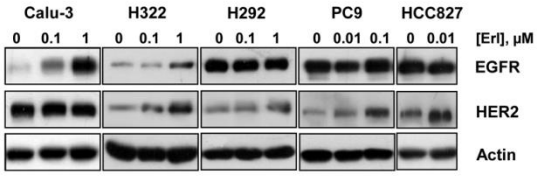
Firstly, we evaluated the effect of erlotinib on total EGFR and HER2 protein levels in sensitive NSCLC cell lines (Calu-3, H322 and H292 cell lines carrying wild-type EGFR; PC9 and HCC827 carrying EGFR E746-A750del mutation, as reported in methods section) and in resistant cell lines (A549, H1299, H1703 and Calu-1 intrinsically resistant carrying *wild-type* EGFR; HCC827GR5 with MET amplification as mechanism of acquired resistance to TKI, as reported in methods section) [82]. As shown in Figure 1A, erlotinib induced accumulation of EGFR protein in Calu-3 and H322 cells while HER2 accumulated in H322, H292, PC9 and HCC827 cells in a dose-dependent manner. The EGFR/Actin and HER2/ Actin ratios obtained after treatment with erlotinib were calculated and values expressed as fold differences versus control (Figure 1B). In contrast, EGFR and HER2 protein accumulation was not observed in any cancer cell line with intrinsic resistance to EGFR inhibitors until the concentration of 10 μM. Indeed the ratios EGFR/Actin or HER2/Actin were similar or even lower than those calculated in untreated cells (Figure 1C)

and similar results were obtained with gefitinib (not shown). A representative Western blotting of resistant H1299 cell line is reported in Figure 1D. The different effect of TKIs on HER2 expression between sensitive and resistant NSCLC cell lines was confirmed in the HCC827 parental and in the HCC827GR5 resistant clone treated for 48 h with gefitinib (Figure 1E).

2. ERLOTINIB INCREASES THE CELL SURFACE EXPRESSION OF EGFR AND HER2 IN ERLOTINIB SENSITIVE NSCLC CELL LINES

EGFR and HER2 expression on the plasma membrane was quantified by flow cytometry in sensitive EGFR wild-type NSCLC cell lines Calu-3, H322 and H292 after exposure to 1 μM erlotinib for 24 h. The drug enhanced surface expression, calculated as molecules of equivalent soluble fluorophore, of EGFR in Calu-3 (Figure 2A) and H322 (Figure 2C, 2D) and of HER2 in H292 (Figure 2B) and H322 (Figure 2C, 2D) cell lines.

A



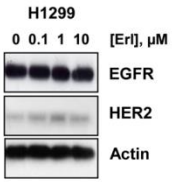
B

Sensitive NSCLC cell lines	EGFR	RAS	EGFR/Actin	HER2/Actin
CALU-3	WT	WT	5.8	1
H322	WT	WT	2.4	2.5
H292	WT	WT	1	1.6
PC9	Mut (E746A750del)	WT	1	2.1
HCC827	Mut (E746A750del)	WT	1	1.5

C

Resistant NSCLC cell lines	EGFR	RAS	EGFR/Actin	HER2/Actin
HCC827GR5	Mut (E746A750del)	WT	1	1.1
A549	WT	G12S	1.2	1
H1299	WT	Q61K	1	1
H1703	WT	WT	0.9	0.9
Calu-1	WT	G12S	0.6	0.7

D



E

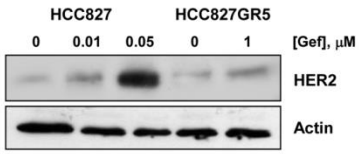


Figure 1. Erlotinib induces EGFR and HER2 protein accumulation only in sensitive NSCLC cell lines. (A) Calu-3, H322, H292, PC9 and HCC827 cell lines were treated with the indicated concentrations of erlotinib for 48 h. At the end of the drug treatment cell lysates were immunoblotted to detect the indicated proteins. The immunoreactive spots were quantified by densitometric analysis, ratios of EGFR/Actin and HER2/Actin were calculated at 1 µM erlotinib for Calu-3 H322 and H292 or 10 nM for PC9 and HCC827 and values are expressed as fold increase versus control (B). (C) HCC827GR5, A549, H1299, H1703, Calu-1 cell lines were treated with 1 µM erlotinib for 48 h and at the end of treatment cell lysates were immunoblotted to detect the indicated proteins. The immunoreactive spots were quantified by densitometric analysis, ratios of EGFR/Actin and HER2/Actin were calculated and values are expressed as fold increase versus control. (D) Representative Western blotting of resistant H1299 cell line exposed to increased concentration of erlotinib. (E) HCC827 parental cell line and HCC827GR5 resistant clone were treated with the indicated doses of gefitinib and processed as above. The results are from representative experiments. Each experiment, repeated three times, yielded similar results.

In H322 cell line, the increase in EGFR and HER2 surface expression was dose and time dependent (Figure 2C, 2D). Western blot analysis of isolated cell surface membrane proteins (inset Figure 2A) confirmed the increase of EGFR in erlotinib treated Calu-3 cells. Exploiting the ability of cetuximab and trastuzumab to bind EGFR and HER2, we used these mAbs as primary antibodies for flow cytometry analysis. By this approach, as shown in Figure 3, we confirmed that the surface density of cetuximab and trastuzumab-binding sites, respectively, on Calu-3 (Figure 3A), H322 (3B) and H292 (3C) cells were increased after 1 µM erlotinib treatment. These results suggest that erlotinib enhanced cell surface expression of EGFR or HER2 on sensitive NSCLC cells, leading to an increase of mAbs binding to cancer cell surface.

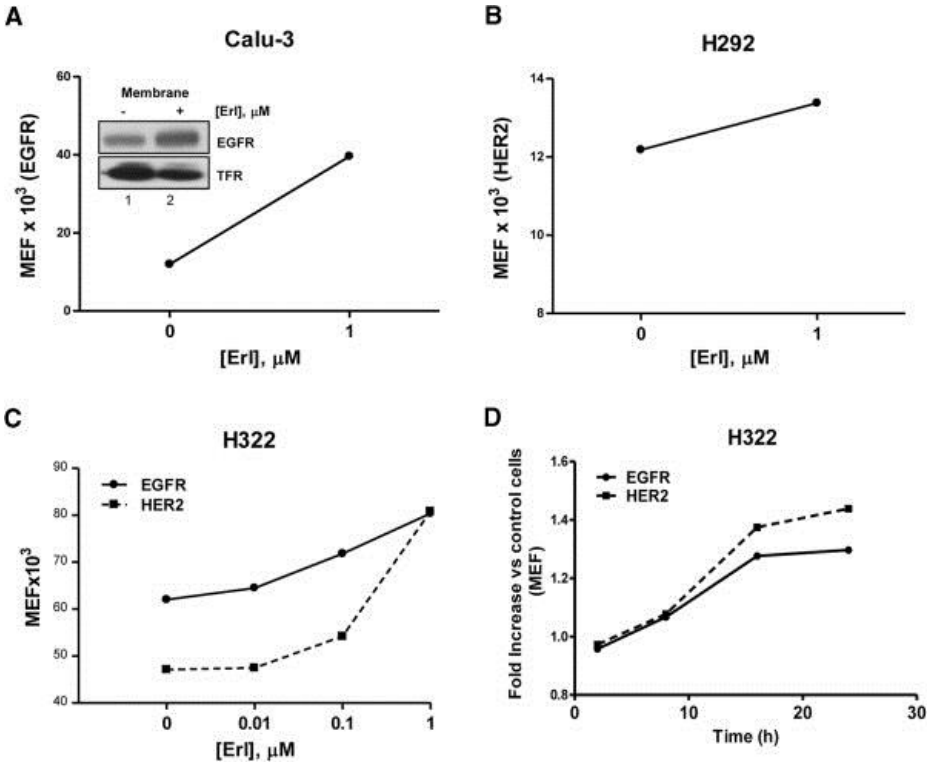


Figure 2. EGFR and HER2 increase at the plasma-membrane level. Calu-3 (A) and H292 (B) cell lines were treated with 1 μM erlotinib for 24 h, H322 cell line was treated with increasing concentration of erlotinib (C) or with 1 μM erlotinib for the indicated period of time (D). At the end of the treatment, cell surface expression of EGFR and/or HER2 were evaluated by flow cytometry and the quantification is reported as Molecules of Equivalent Fluorophore [MEF] or as fold increase versus untreated control cells (D). Inset Figure 2A: Western blot analysis of EGFR protein membrane level in Calu-3 after treatment with 1 μM erlotinib for 24 h. Whole cells were labeled with biotin and membrane bound proteins were pulled down with neutrAvidin beads. The results are from representative experiments. Each experiment, repeated three times, yielded similar results.

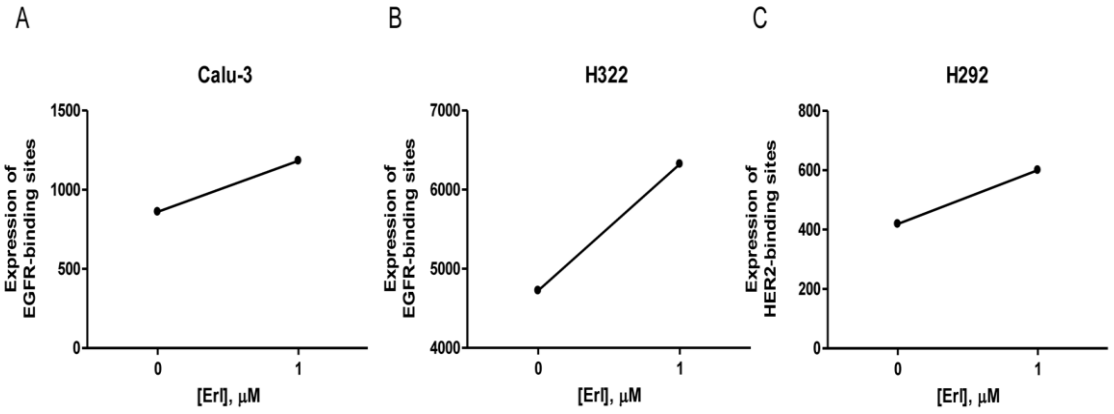


Figure 3. Figure 3 Erlotinib induces the increase of cetuximab and trastuzumab binding sites. Calu-3, H322 and H292 cell lines were treated with erlotinib for 24 h. Binding sites were assessed by flow cytometry using cetuximab (Calu-3, H322) and trastuzumab (H292) as primary antibodies followed by PE-anti-human IgG exposure. Binding sites quantification is reported as Molecules of Equivalent Fluorophore [MEF]. The results in A, B, C are from representative experiments. Each experiment, repeated three times, yielded similar results.

3. ERLOTINIB INDUCES EGFR PROTEIN STABILIZATION

The possibility that the higher EGFR level observed in Calu-3 cells exposed to erlotinib was due to protein stabilization or increased synthesis was then explored. As shown in Figure 4A, EGFR level increased after 2 h of erlotinib treatment and reached a plateau after 24 h. Furthermore, the maximum level was maintained during time in the presence of the drug. However, after 48 h of erlotinib removal, EGFR expression was reduced to level comparable to untreated cells (Figure 4B). Calu-3 were also treated with erlotinib in the presence of specific inhibitors of mRNA (Actynomicin D) and protein (Cycloheximide) synthesis. As shown in Figure 4C, the erlotinib- induced EGFR protein increase was neither influenced by Actynomicin D nor Cycloheximide treatment indicating that the higher level of EGFR after erlotinib treatment could be ascribed to post-transcriptional mechanisms such as protein stabilization. Moreover, we analyzed EGFR transcript level by real time PCR after erlotinib treatment (Figure 4D). Erlotinib did not affect EGFR mRNA level when compared to untreated cells. With the aim to clarify why the increased level of EGFR was induced only in sensitive cells, we then tested the effect of EGFR inhibitors (gefitinib, erlotinib, cetuximab, lapatinib) and of inhibitors of MAPK and PI3K/ AKT/mTOR signaling transduction pathways on EGFR accumulation in Calu-3 cell line. Gefitinib, erlotinib, lapatinib significantly inhibited the phosphorylation of p70S6K and p44/42 and induced a significant increase in EGFR protein level (Figure 4E). The MEK inhibitor U0126 strongly enhanced EGFR expression, in contrast no increase in the EGFR level was observed after incubation with the inhibitors of PI3K/AKT/mTOR pathway tested (NVP-BKM-120 and NVP-BYL-719 PI3K inhibitors and RAD001 mTOR inhibitor).

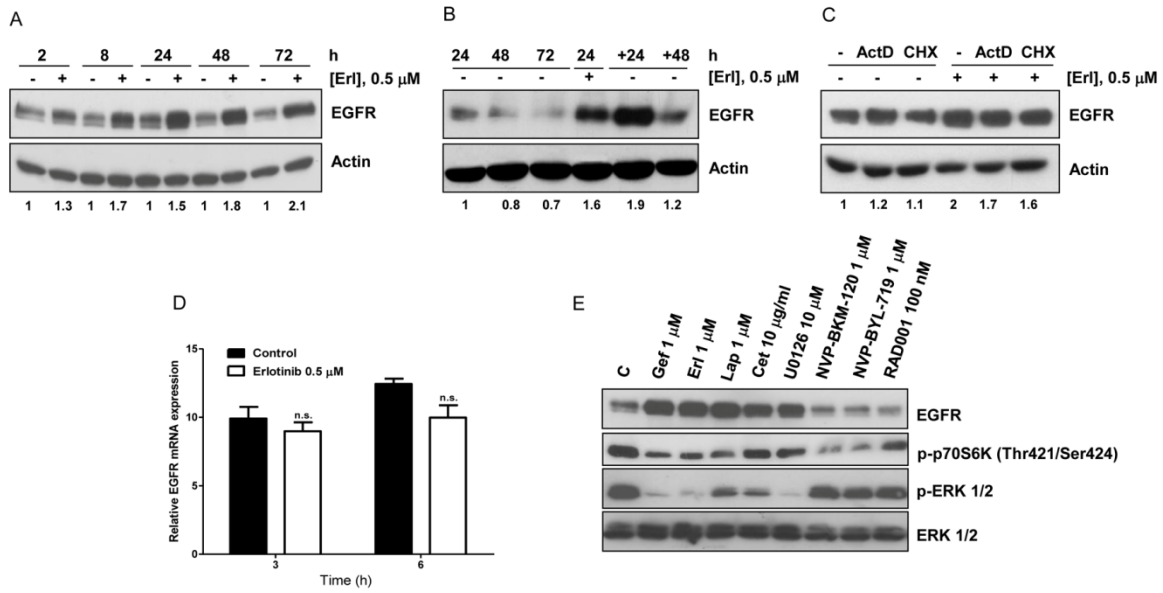


Figure 4. Erlotinib induces EGFR protein accumulation through protein stabilization. (A) Calu-3 cells were treated for the indicated period of time with 0.5 μM erlotinib. At the end of drug treatments cell lysates were immunoblotted to detect EGFR protein. (B) Calu-3 cells were treated with 0.5 μM erlotinib for 24 h then the drug was removed and drug-free medium was added for further 24 and 48 h. Then, cell lysates were immunoblotted to detect the EGFR protein levels. (C) Calu-3 cells were treated for 24 h with erlotinib 0.5 μM, in the absence/presence of 0.1 μg/ml actynomicin D and 2 μg/ml cyclohexymide. At the end of the experiment, cell lysates were immunoblotted to detect the indicated proteins. The immunoreactive spots were quantified by densitometric analysis, ratios of EGFR/Actin were calculated and values are expressed as fold increase versus control. (D) Calu-3 cells were exposed to 0.5 μM erlotinib for the indicated period of time and the EGFR mRNA was detected by RT-PCR. The mean values of two independent measurements (± SD) are shown. (E) EGFR, p-P70S6K, p-P44/42 and P44/42 were detected by Western blotting in Calu-3 cells untreated or treated for 24 h with 1 μM gefitinib, erlotinib and lapatinib, 10 μg/ml cetuximab, 10 μM U0126, 1 μM NVP-BKM-120 and NVP-BYL-719 and 100 nM RAD001. The results are from representative experiments. Each experiment, repeated three times, yielded similar results.

4. EFFECTS OF ERLOTINIB ON PROTEASOMAL ACTIVITY

To better clarify whether protein stabilization was the cause of the higher EGFR level in Calu-3 cells after treatment with erlotinib, we performed experiments to study the proteasomal activity. Proteasome is a multimeric proteinase complex responsible for the degradation of abnormal, unfolded or oxidatively damaged proteins. In mammalian cells, 80–90% of protein degradation occurs via the proteasome pathway. Calu-3 and H1299 cell lines were treated with erlotinib 1 μM for 24 h. The proteasome inhibitor MG262 0.5 μM was used as positive control (Figure 5). In sensitive Calu-3 cell line, erlotinib was able to inhibit the proteasomal activity of about of 45%, while in the resistant H1299 cell line the inhibition was less then 10%. This result shown that in Calu-3 cell line, the reduction of the proteasomal activity may induce an accumulation and stabilization of EGFR protein levels.

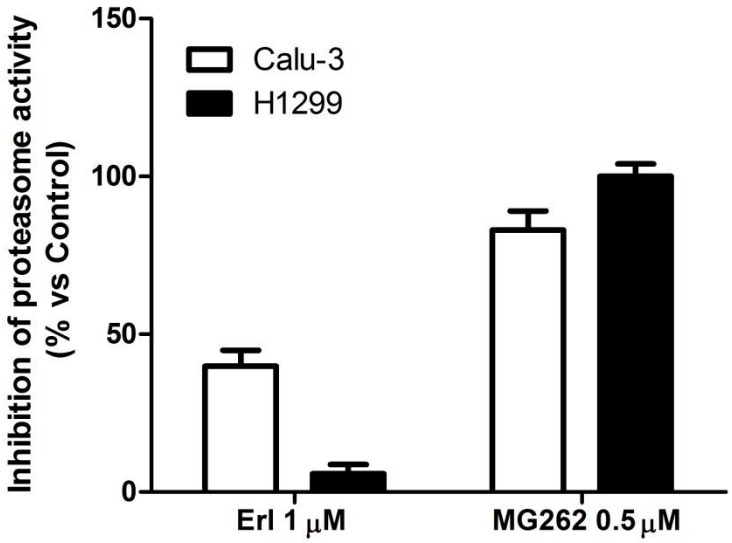


Figure 5. Erlotinib induce an inhibition of proteasomal activity. Calu-3 and H1299 cell lines were treated with erlotinib 1μM and MG262 0.5 μM. At the end of experiment, the activity was quantified using a 380/460 nm filter set in a LS 50 B luminescence spectrometer (Perkin-Elmer). The results are reported as inhibition of proteasome activity (% vs Ctrl).

5. EFFECTS OF ERLOTINIB AND CETUXIMAB COMBINED TREATMENT ON NSCLC CELL GROWTH AND ADCC

We then investigated the effect of targeting EGFR by both the TKI erlotinib and the mAb cetuximab in a cell viability assay (Figure 6). We treated Calu-3, H322 and H1299 cells with erlotinib, cetuximab (doses ranged from 1 to 50 μg/ml) or the combination based on the schedule erlotinib 24 h followed by the combination of erlotinib with cetuximab for 72 h. As expected Calu-3 (Figure 6A) and H322 (Figure 6B) cells were responsive to erlotinib and cetuximab treatment, whereas H1299 (Figure 6C) cells were resistant to both the single regimens. Comparing the experimental combination points with that expected by the Bliss criterion, an additive effect was observed only in the Calu-3 cells. In fact, in the H322 cells we failed to observe any improvement treating cells with the combined treatment and H1299 remained resistant. Moreover, cell death, evaluated by morphological analysis, caspase-3 activation and cleavage, was negligible under any of the tested treatments at all the time points analyzed (not shown) suggesting that the combined erlotinib-cetuximab treatment exerted a cytostatic and not a cytotoxic effect. Since the engagement of immune component system is one of the main mechanisms of the activity of specific mAbs directed to ErbB family members in vivo, we examined whether erlotinib could enhance cetuximabor

trastuzumab-mediated ADCC by NK cells. As shown respectively in Figure 7 A-B cetuximab-dependent cytotoxicity in the presence of IL-2 activated NK cells was higher in Calu-3 and H322 cells previously treated with erlotinib compared with cells treated with cetuximab alone. Similarly, trastuzumab-dependent cytotoxicity was higher in H322 and H292 cells (Figure 7 C-D) previously treated with erlotinib compared with cells treated with trastuzumab alone. On the contrary, the combination of erlotinib with cetuximab did not significantly modify the mAb dependent cytotoxicity in H1299 resistant cancer cells (Figure 7E).

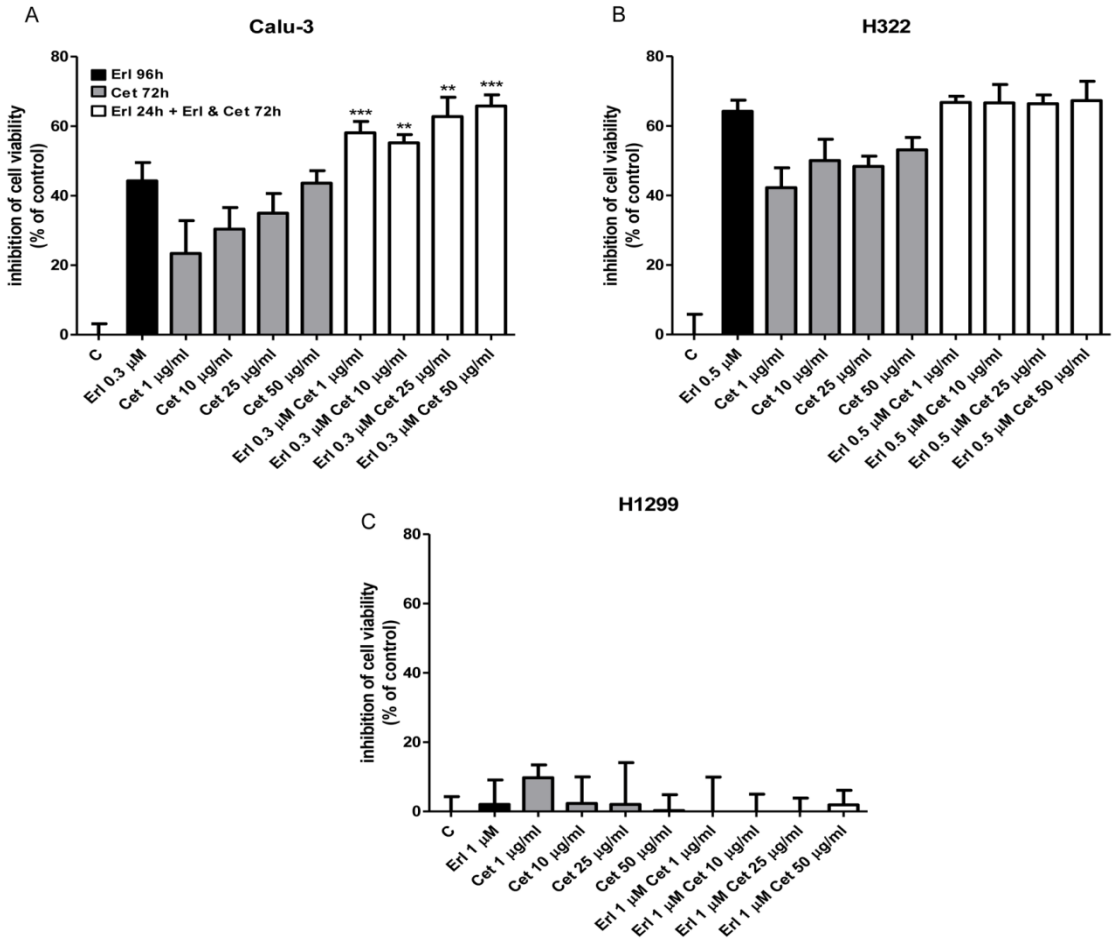


Figure 6. Effect of erlotinib and cetuximab combination on cell viability in NSCLC cell lines. Calu-3, H322 sensitive cells (A, B) and H1299resistant cells (C) were exposed to the indicated concentrations of erlotinib for 96 h or cetuximab for 72 h and to erlotinib for 24 h followed by the combination of erlotinib and cetuximab for 72 h. After the treatments, cell viability was assessed by MTT assay. Data are expressed as percent inhibition of cell viability versus control cells and are mean values of three separate experiments (**P < 0.01 ***P < 0.001).

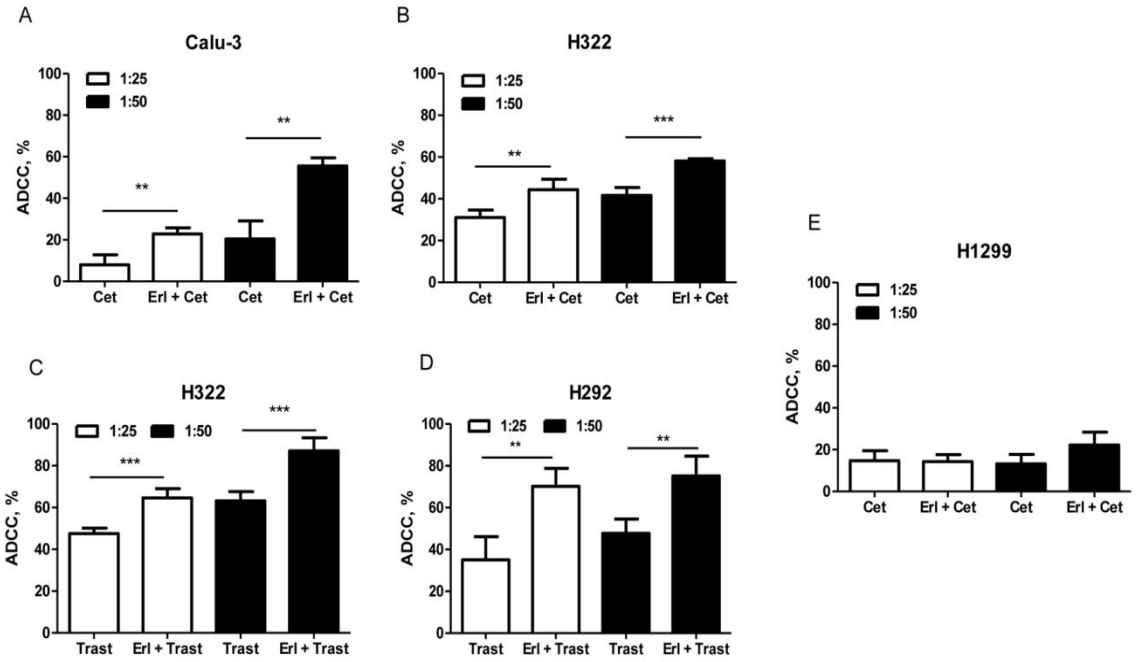


Figure 7. Erlotinib potentiates antibody dependent cell cytotoxicity. The indicated human NSCLC cell lines were treated with 1 μM erlotinib for 24 h. After the treatment with erlotinib, 10 μg/ml Cetuximab (A, B, E) or Trastuzumab (C, D) were added to cancer cells seeded with 100 U/ml IL-2 activated-NK cells at the ratio of 1:25 and 1:50. After 4 h LDH release was determined as described in Methods section. The results are from representative experiments. The experiment, repeated three times, yielded similar results (**P < 0.01 ***P < 0.001).

6. EFFECT OF ERLOTINIB AND CETUXIMAB ON CALU-3 XENOGRAFTs

To extend our results *in vivo*, we tested the combination of erlotinib with cetuximab in a Calu-3 xenograft model (Figure 8). When tumours were well established (14 days post-injection, average volume of 300 mm^3) mice were randomized into four treatment groups receiving erlotinib alone, cetuximab alone, the combination, or vehicles as described in the Methods section. Drug treatments were well tolerated, and no signs of toxicity were detected during the study. The treatment with either erlotinib or cetuximab as single agent delayed tumour growth. However, the significance of the treatment versus the control was observed only with cetuximab as single agent or in combination. Interestingly, the treatment with the combination of erlotinib plus cetuximab significantly inhibited tumour growth when compared to both the single agent treatments. The histologic analysis of tumour samples showed that the subcutaneous injection of Calu-3 strikingly reproduced within four weeks the morphological features of human adenocarcinoma (Figures 9A, 9B1-4, 9C-1). Neoplastic epithelial cells clearly expressed cytokeratin (Figure 9C-2) and were organized in secretory glands surrounded by cellularized collagen as evidenced by Masson's trichrome staining (Figure 9C-4). Regressive phenomena and changes in size of neoplastic glands together with intense

stromal reaction were observed in histologic samples of tumours from treated mice. Interestingly, cetuximab clearly resulted in dense inflammatory periglandular infiltrates mostly composed of lymphocytes (Figure 9C-3). Thus, the real impact of treatment on tumour mass within the nodules was assessed by the morphometric analysis of tissue composition. By this quantitative approach, in agreement with gross anatomic measurements, we documented that the combination of erlotinib with cetuximab was the most effective treatment on tumour growth inhibition (Figure 9D). This contention was further supported by the immunofluorescence analysis of Ki67 labelling on tumour tissues at the end of the experimental protocol (Figure 10). Erlotinib was able to reduce proliferation of neoplastic cytokeratin^{pos} cells only in association with cetuximab whereas cetuximab had a negative impact on cycling cells also as individual agent. The TUNEL assay indicated that, according with in vitro data, apoptosis was not a significant ongoing cellular event implicated in the effect of different treatments. We have calculated that 0.026+/−0.016% neoplastic cells were undergoing apoptosis in untreated tumours. Similar low numbers were obtained after Erlotinib or Cetuximab single treatment whereas Erl + Cet increased the amount of TUNEL positive neoplastic cells although reaching a rate of 0.12+/−0.03%. However, we cannot exclude that apoptotic cell death may have contributed to the positive effect of tumor shrinkage at earlier times after drug administration. Thus, these experimental observations suggest that targeting EGFR by the combination of small molecules and antibodies increases the in vitro and in vivo antiproliferative activity of both individual agents and seems to be a potent therapeutic strategy against NSCLC.

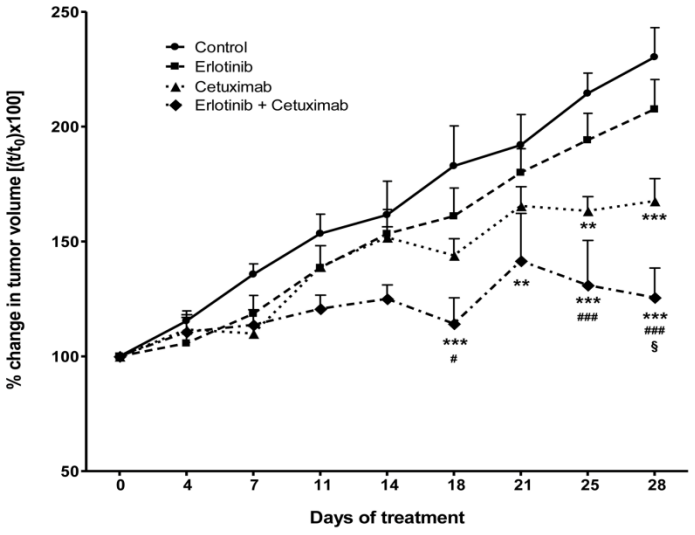


Figure 8. Antitumour activity of erlotinib and cetuximab on Calu-3 xenografts. Calu-3 cells were suspended in matrigel and sterile PBS (1:1) and implanted s.c. (right flank) on female BALB/c-Nude mice. Tumours were allowed to establish growth after implantation for 14 days, and the treatments started when tumours reached an average volume of 300 mm^3. Vehicle, erlotinib (25 mg/Kg, orally 5 days/week), cetuximab (2 mg/Kg i.p. twice weekly), or erlotinib plus cetuximab were administered for the duration of the study. Data are expressed as percent change in tumour volume ± SEM of 6 mice per group. (**p < 0.01, ***p < 0.001 vs control; #p < 0.05, ###p < 0.001 vs erlotinib; §p < 0.05 vs cetuximab; two-way ANOVA followed by Bonferroni post-test).

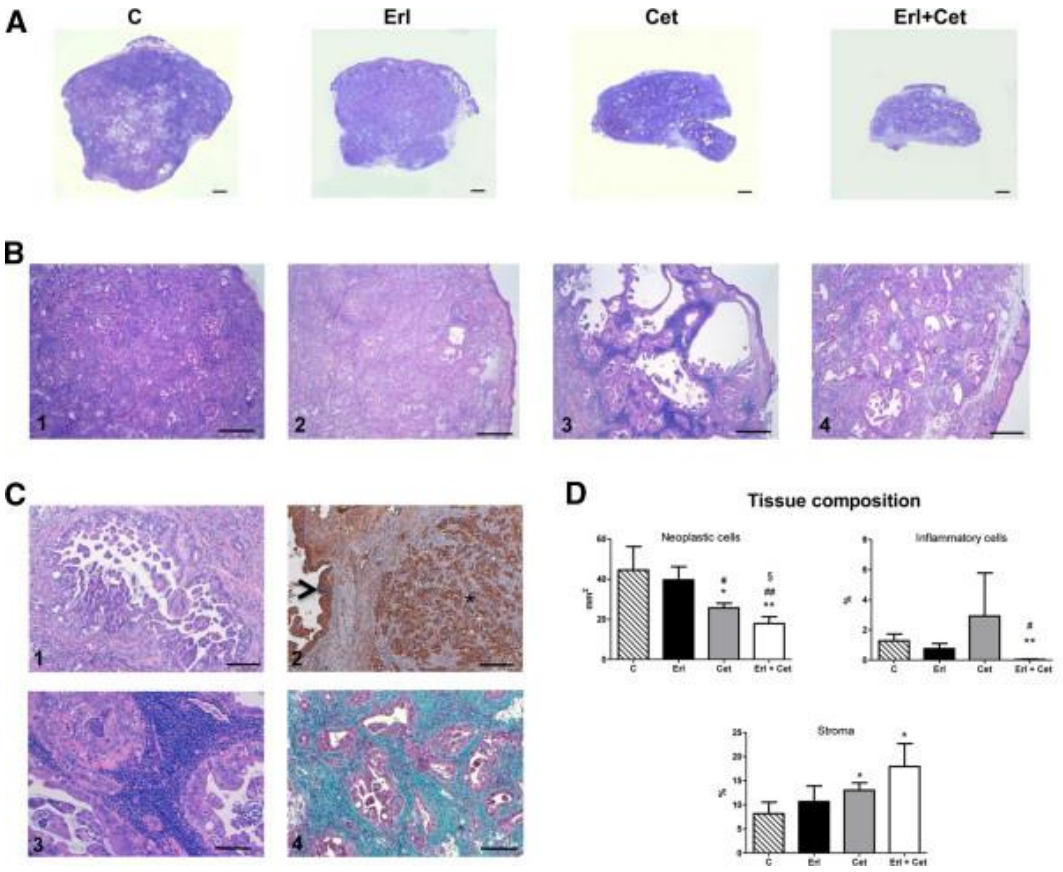


Figure 9. Hystological analysis of tumours. A: Selected examples of H&E stained sections of the entire subcutaneous xenografted tumour induced by Calu-3 injection in untreated (C) and erlotinib (Erl), cetuximab (Cet) or erlotinib + cetuximab (Erl + Cet) treated BALB/c nude mice (scale bars: 1 mm). Higher magnification of the same samples are shown on corresponding panels in B (scale bars: 500 μm). C: representative morphological details of the control neoplastic epithelium (1, H&E) expressing cytokeratin (2,* brown-immunoperoxidase) that also depicts the epidermis (arrowhead). The presence of inflammatory interstitial cells in a cetuximab treated tumour (3, H&E) and the intense collagen deposition (bluish) surrounding neoplastic glands (purple) in a Erl + Cet treated tumour (4, Masson's trichrome) are shown (scale bars: 100 μm). D: Bar graphs illustrating the quantitative measurements of neoplastic, inflammatory cells and stromal compartments composing the tumours. (*p < 0.05, **p < 0.01, vs control; #p < 0.05, ##p < 0.01 vs erlotinib; §p < 0.05 vs cetuximab).

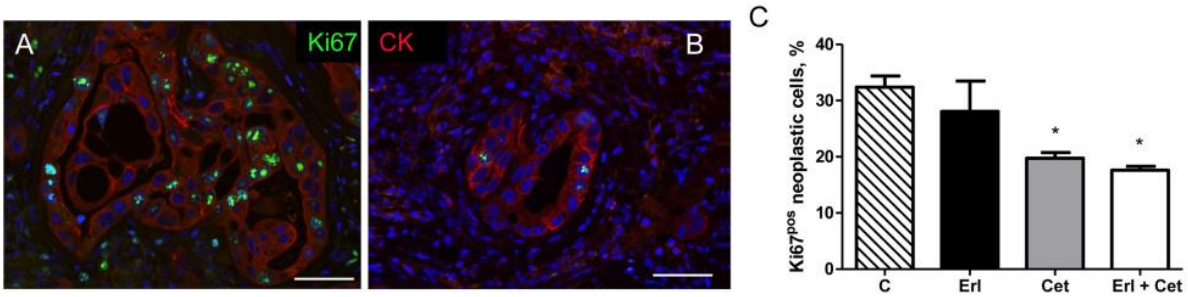


Figure 10. Immunohistochemical analysis of cellular proliferation. Immunofluorescence images of Ki67 (green) nuclear labelling of cytokeratin (CK, red) positive neoplastic cells in sections of xenografted tumours from an untreated (A) and Erl + Cet treated (B) BALB/c nude mouse. C: bar graph illustrating the effect of the different treatments on the percentage of cycling (Ki67pos) neoplastic cells within the tumour. CTRL: untreated, ERL: erlotinib, CET: cetuximab, COMB: erlotinib + cetuximab. * p < 0.01 vs CTRL.

7. HER2 EXPRESSION LEVEL IN HUMAN NSCLC CANCER CELL LINES

In two recent papers, Takezawa [54] and Yu [146] have demonstrated that HER2 represents a relatively new therapeutic target for NSCLC. The potential clinical relevance of HER2 expression in NSCLC is currently under evaluation, however, the recent role of HER2 amplification in the acquisition of resistance to TKI, reported in 12-13% of patients, may render HER2 a potential target not only in breast cancer but also in NSCLC.

The total level of HER2 protein was detected by immunoblotting on cell lysates in a panel of NSCLC cell lines (H1781, H3255, H322, H1299, H1975, Calu-6, H596, H460, A549, PC9, HCC827 and Calu-3). As shown in Figure 11A, HER2 expression varied widely among the analyzed cell lines, ranging from barely detectable levels in Calu-6 to high levels in Calu-3. The latter was an expected finding, due to known amplification of HER2 in Calu-3 cell line [147]. Considering that trastuzumab and T-DM1 have a common targeted receptor on the cell surface, we quantified HER2 expression levels on the plasma membrane by flow cytometry. Indeed, the ability of the antibody to interact with its target is strictly related to the presence of the receptor on the cell surface. As reported in Figure 11B, Calu-3 and H3255 cells displayed the highest levels of HER2 at the plasma membrane. The total level of HER2 in H3255 was similar to that observed in other cell lines such as H460 and A549 (Figure 11A) indicating that the total level of proteins detected on cell lysate is not a good predictor of HER2 level on plasma membrane.

8. EFFECT OF T-DM1 AND TRASTUZUMAB TREATMENT ON CELL VIABILITY OF NSCLC CELL LINES WITH DIFFERENT HER2 EXPRESSION

Based on the previous analysis, the effect of T-DM1 and trastuzumab on cell viability was focused on NSCLC cell lines expressing different cell surface levels of HER2: Calu-3 (very high), H3255 (high) and Calu-6 (low). T-DM1 showed strong anti-proliferative effect in HER2 over-expressing Calu-3 cells (IC_{50} = 0.40 ± 0.08 μg/ml, Figure 11C), whereas no

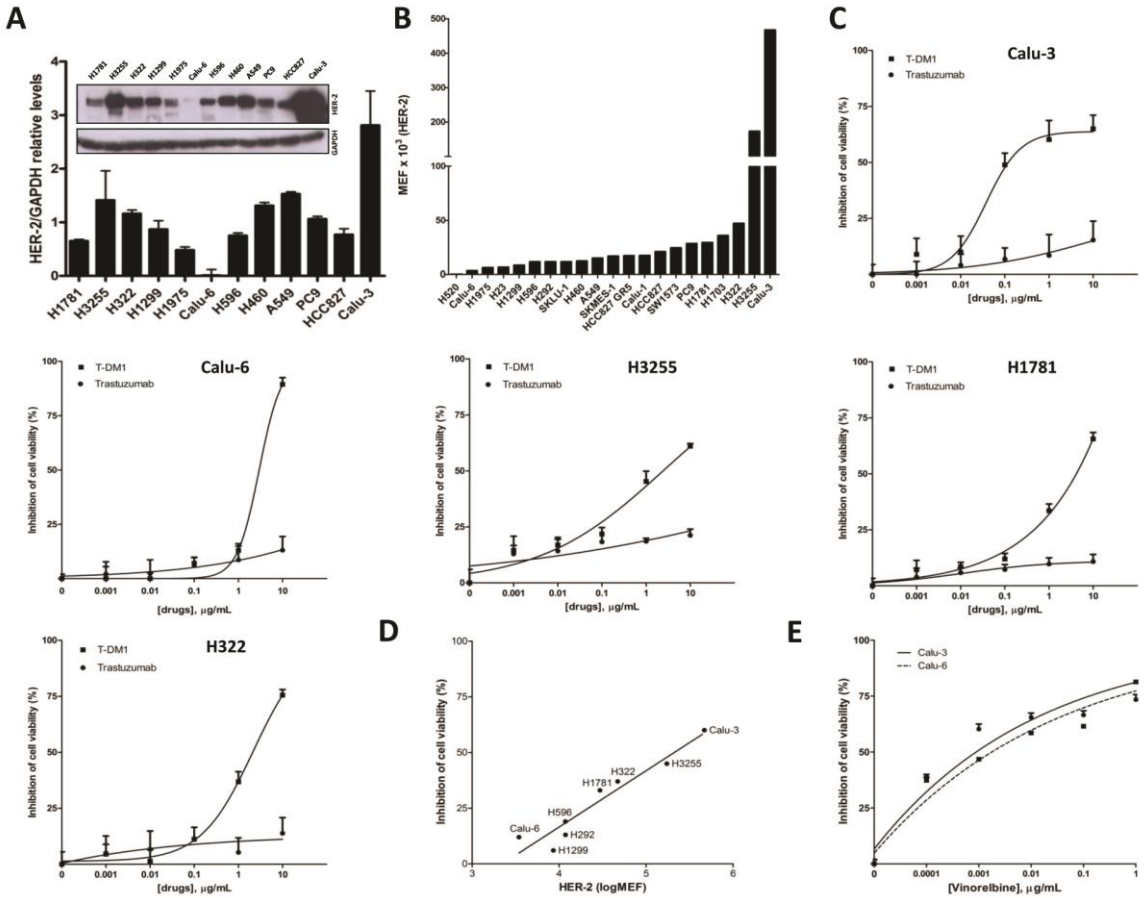


Figure 11. HER2 levels and effects on cell viability of T-DM1, trastuzumab and vinorelbine in NSCLC cell lines. (A) Densitometric quantification of total HER2 protein level, detected by Western blotting, was calculated using Quantity One software. Three different Western blot experiments were performed on total cell lysate of the indicated NSCLC cell lines. A representative Western blot analysis is reported as inset. (B) HER2 protein levels on cell surface was quantified by flow-cytometry and expressed as molecular equivalent of fluorochrome (MEF) as described in Methods section. (C) Calu-3, Calu-6, H3255, H1781 and H322 cells were exposed to increasing concentrations (0.001, 0.01, 0.1, 1 and 10 µg/ml) for 72 h and then cell viability was assessed by MTT assay. (D) Percent of inhibition of cell viability induced by T-DM1 at 1 µg/ml as a function of HER2 level (E) Calu-3 and Calu-6 cell viability inhibition curves after treatment with increasing vinorelbine concentrations. Data are expressed as mean + SD of three different experiments.

effects were detected on Calu-6 cell line, with low levels of HER2 on the plasma membrane. The inhibition observed at 10 µg/ml was related to a non-specific toxic effect of T-DM1, as previously reported in MCF-7 HER2 negative breast cancer cell line [148]. Intermediate results were seen on H3255 cells. Trastuzumab, administered at the same dosages of T-DM1, did not show notable effect on cell growth in any of the cell lines tested. H1781 cell line, harbouring mutated HER2 (G776insV_G/C), was also included in this study in order to investigate whether this mutation influenced the anti-proliferative effect of T-DM1. As shown in Figure 11C, T-DM1 at 1 µg/ml induced about 35% inhibition of cell viability. A similar inhibition profile was observed in T-DM1 (1 µg/ml) treated H322 cells, displaying comparable levels of HER2 on cell surface (Figure 11B), suggesting that H1781 sensitivity to the drug is not affected by the mutated receptor. We then analyzed the percentage of inhibition of cell viability induced by 1 µg/ml T-DM1 on eight different cell lines, as a

function of HER2 level on plasma membrane and, as shown in Figure 11D, we confirmed that sensitivity to T-DM1 is strictly correlated to HER2 expression on the cell surface. To exclude the hypothesis that different effects of T-DM1 could be ascribed to dissimilar sensitivity to the microtubule polymerization inhibitor DM1, we treated two cell lines harbouring high and low levels of HER2 on the plasma membrane, respectively Calu-3 and Calu-6, with increasing concentration of Vinorelbine, an anti-mitotic drug, which acts by a similar mechanism of action of the maytansinoid DM-1. Vinorelbine inhibited viability of Calu-3 and Calu-6 cells in a comparable manner (Figure 11E).

9. EFFECT OF T-DM1 AND TRASTUZUMAB TREATMENT ON CELL CYCLE DISTRIBUTION, SIGNAL TRANSDUCTION, CELL DEATH AND ADCC

To determine the effect of T-DM1 on cell cycle, Calu-3 treated with 1 μg/ml T-DM1 or Trastuzumab for 24 h were analyzed by flow cytometry. As shown in Figure 12A, T-DM1 caused an increase in the proportion of cells in G2-M phase with a decrease in G1 and S phases, whereas no alterations on cell cycle distribution were detected in cells treated with trastuzumab. The arrest of Calu-3 in G2-M phase of the cell cycle, as a result of T-DM1 exposure, was also supported by increased levels of Cyclin B1 as measured by western blot analysis (Figure 12B) whereas pRb and Cyclin A were unchanged. We then tested the effect of T-DM1 and trastuzumab on phosphorylation status of HER2, AKT and p42-44 MAPK in Calu-3 cell line. Differently of trastuzumab, T-DM1 significantly inhibited the phosphorylation of AKT and p42-44 after 24 h of treatment (Figure 12C) with a decrease in HER2 total level and phosphorylation at 48 h.

T-DM1 (1 μg/ml) exerted a significant cytotoxic effect already after 24 h of treatment, with appearance of floating dead cells (Figure 13A). By contrast trastuzumab did not modify cell proliferation nor induced cell death up to 72 h of treatment. As shown in Figure 13B, 48 h exposure of Calu-3 cells to T-DM1 at 0.1 and 1 μg/ml induced the activation of caspases-7 and −9 and the release of cytochrome-*c* into the cytoplasm (Figure 13C) indicating that the intrinsic pathway is involved in T-DM1-triggered apoptotic cell death. Vinorelbin was used as internal control. A lower activation of caspases and a weak release of cytochrome-*c* was also

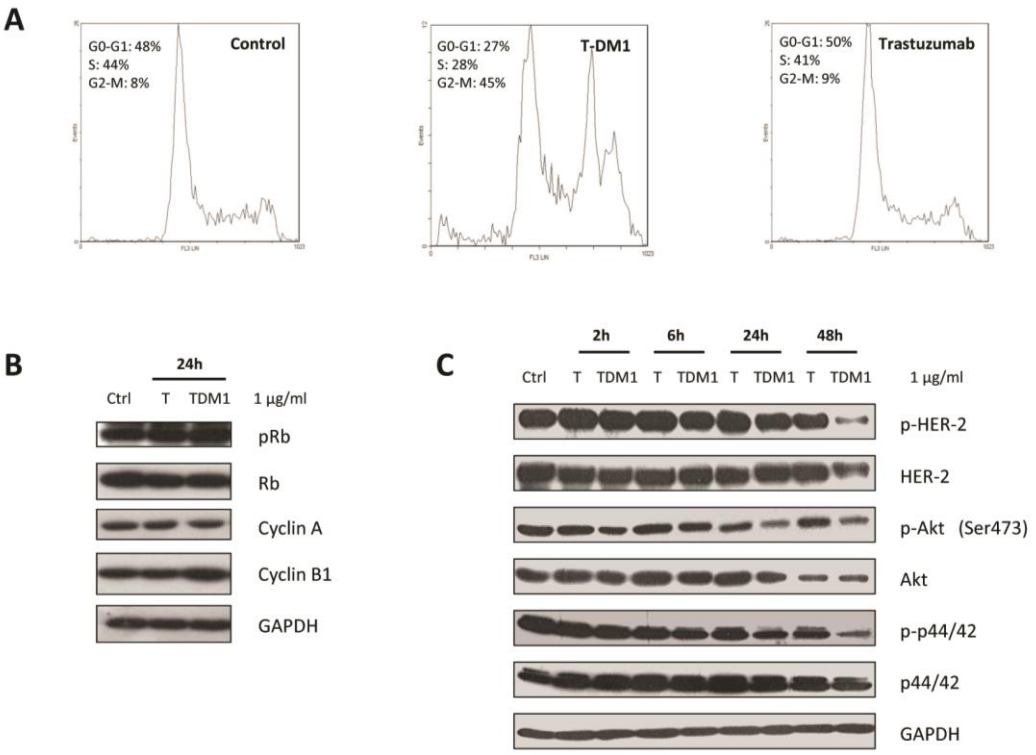


Figure 12. Influence of trastuzumab and T-DM1 on cell cycle phase distribution and cell signalling. (A) Calu-3 cells were cultured in the absence of drugs or treated either with T-DM1 or trastuzumab (1 µg/ml). After 24 h cells were stained with propidium iodide an cell-cycle-phase distribution was determined by flow cytometry analysis. Cell-cycle distributions were analyzed as described in Methods section and data were expressed as percentage of distribution in each cell-cycle phase. Immunoblot analysis on protein involved in cell cycle regulation (B) or signaling pathways (C) were conducted on cell lysates obtained after treatment with trastuzumab or T-DM1 (1 µg/ml) for the indicated period of time.

induced by trastuzumab treatment even if no significant cell death was observed (Figure 13A). Since antibody-dependent cell-mediated cytotoxicity (ADCC) is one of the main mechanisms of action of specific mAbs directed to ErbB family members in vivo [149], we examined whether the capability to activate natural killer (NK)-mediated ADCC is preserved by T-DM1. As shown in Figure 13D, T-DM1-dependent cytotoxicity in the presence of IL-2 activated NK cells was similar to trastuzumab-dependent cytotoxicity in Calu-3 overexpressing HER2. In the low HER2 expressing H1299 cells, neither T-DM1 nor trastuzumab significantly induced mAb-dependent cytotoxicity.

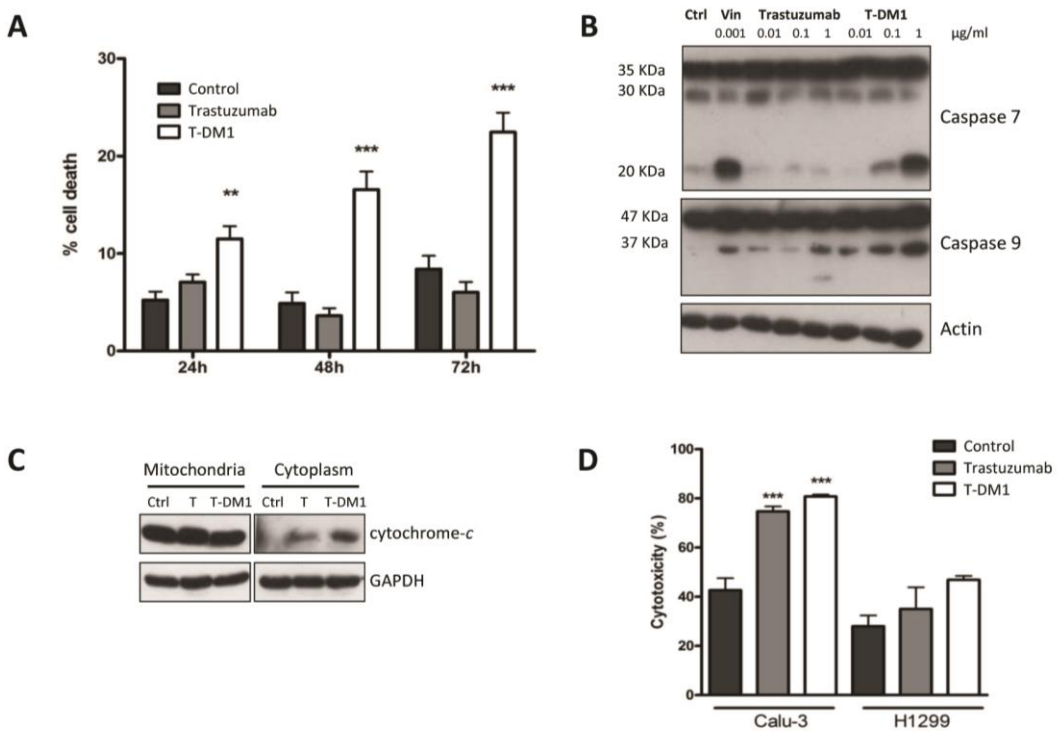


Figure 13. Effect of trastuzumab and T-DM1 on cell death and antibody dependent cell cytotoxicity. (A) Dead cells were counted after 24, 48 and 72 h of exposure to trastuzumab or T-DM1 (1 µg/ml) and the percentage of dead cells was calculated. (**p < 0.01, ***p < 0.001 versus control, one-way ANOVA followed by Tukey's post-test). (B) Caspases 7 and 9 activation were detected by immunoblotting on cell lysates obtained after 48 h of Calu-3 exposure to increasing concentration of trastuzumab or T-DM1. Vinorelbine 0.001 µg/ml was used as positive control. (C) Cytochrome c was detected in the cytoplasm by immunoblotting after 48 h of treatment with T-DM1 1 µg/ml as described in Methods section. (D) Trastuzumab (1 µg/ml) or T-DM1 (1 µg/ml) were added to Calu-3 and H1299 cells seeded with 100 U/ml IL-2 activated-NK cells, at the ratio of 1:50. After 4 h lactate dehydrogenase release was quantified as described in Methods section and data expressed as percentage of cytotoxicity. The results are from representative experiments. The experiment, repeated three times, yielded similar results (***P < 0.001, one-way ANOVA followed by Tukey's post-test).

10. EFFECT OF DUAL INHIBITION OF HER2 IN HER2-MUTATED H1781 CELL LINE

As discussed elsewhere, dual HER2-targeted therapy combining small molecule kinase inhibitors with an anti-HER2 antibody may be associated with better outcomes compared to either agent alone [150,151]. Capuzzo et al. reported a patient with metastatic HER2-mutated (G776L) lung adenocarcinoma resistant to conventional cytotoxic chemotherapy, who had a favorable response to trastuzumab [51]. To study the dual inhibition of HER2, H1781 cell line harbouring mutated HER2 (G776insV_G/C) was treated with lapatinib and/or afatinib alone or in combination with T-DM1 1µg/ml. As reported in Figure 14, comparing the experimental combination points with that expected by the Bliss criterion, an additive effect was observed. In fact, no significant differences between experimental and theoretical points were observed.

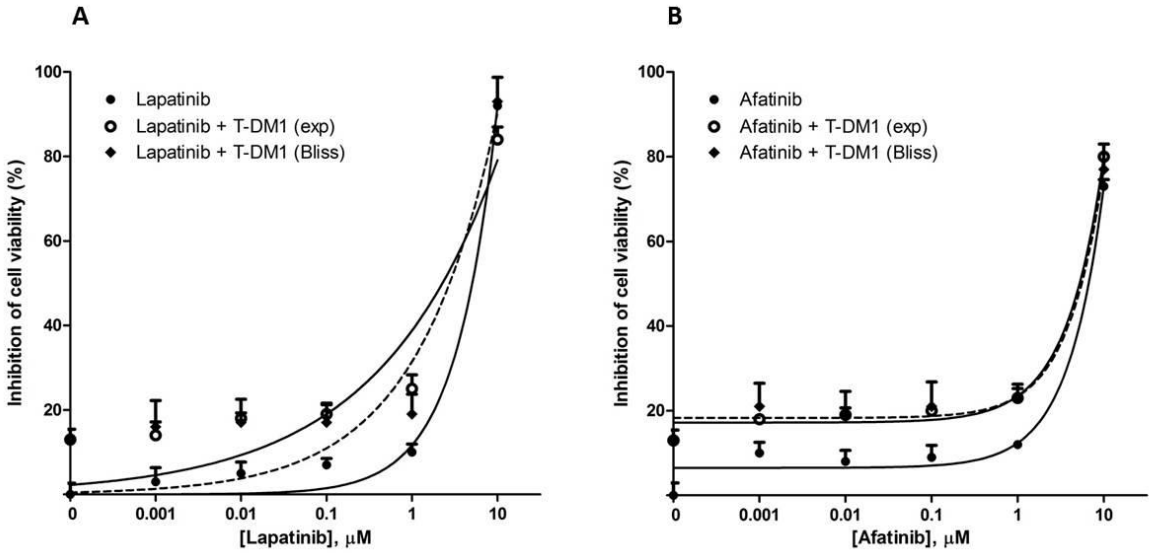


Figure 14. Effect of dual inhibition of HER2 in HER2-mutated H1781 cell line. Curves of growth inhibitory effects of lapatinib (A) and afatinib (B) and their combined treatments with T-DM1 versus theoretical Bliss additivity curve are reported. Cells were treated with the drugs for 72 h and then cell number was assessed by MTT assy. Data are expressed as percent inhibition of cell proliferation versus control cells. The experiments, repeated three times, yielded similar results.

Darren et al. [96] have demonstrate that AZD9291, a novel third generation EGFR-TKI, and its metabolite AZD5104 exhibit moderate activity against H1781 cell line. These results, suggest that combining TKIs with mAbs in HER2-mutated NSCLC cell lines may have a potential clinical relevance, overall further studies should be conducted to better clarify the effect of dual HER2 inhibition.

11. EFFECT OF T-DM1 ON EGFR-MUTANT PC9 CELL LINE RESISTANT TO GEFITINIB FOR HER2 OVEREXPRESSION

As previously reported [54] and independently confirmed by our laboratory, the clone PC9/HER2c1 (a generous gift from Dr. William Pao), obtained by stably transfection of PC9 cells with HER2 expression vector, is more resistant to gefitinib than parental cells. HER2 expression on plasma membrane was 10 time higher in the clone compared to the parental cell line (data not shown). Based on these results we tested the effect of T-DM1 on PC9/HER2c1 and in the parental PC9 cells. As shown in Figure 15A, HER2 overexpression significantly enhanced the efficacy of T-DM1 with 40% inhibition of cell viability at 1 μg/ml in the PC9/HER2c1 clone. With respect to PC9 cells, the clone showed a marked increase in AKT,

p70S6K and p42-44 activation. After 48 h of treatment with T-DM1 a reduction in AKT and p70S6K phosphorylation was observed (Figure 15B) suggesting that T-DM1 might improve gefitinib treatment. In Figure 15C the dose–response curves of gefitinib in the presence of a fixed concentration of T-DM1 (0.1 μg/ml) are shown. Comparing the experimental combination points with that expected by the Bliss criterion, an additive effect was observed. In fact, no significant differences between experimental and theoretical points were observed.

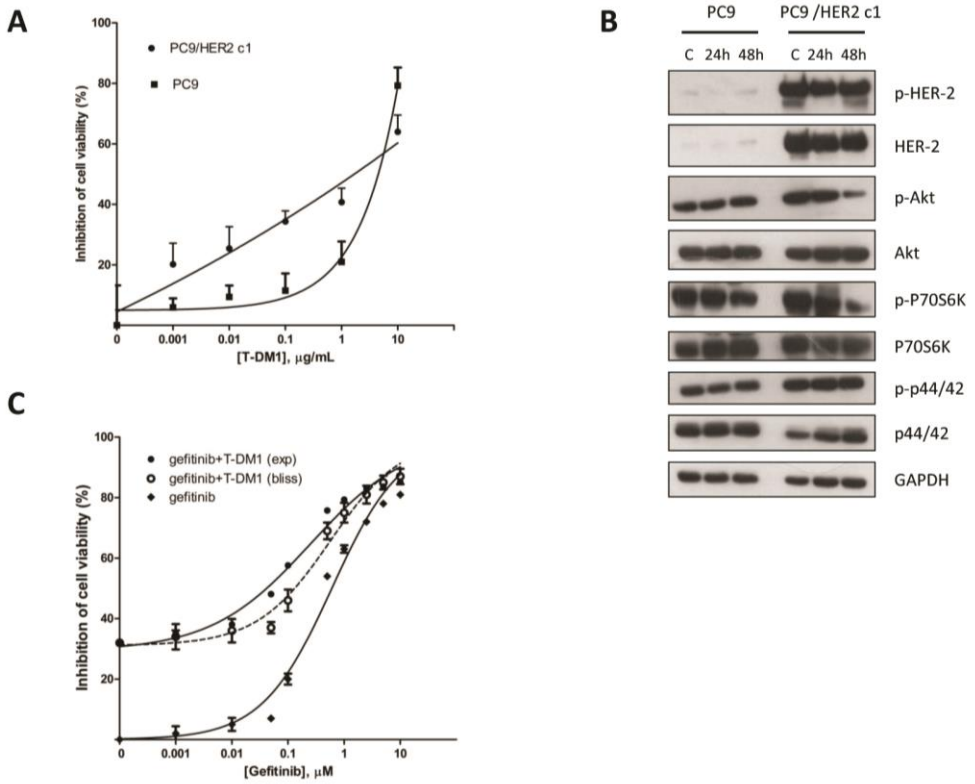


Figure 15. Effect of T-DM1 on EGFR-mutant PC9 cell line become resistant to gefitinib for HER2 overexpression. (A) PC9 and PC9/HER2 c1 cells were exposed to increasing concentrations of T-DM1 for 72 h and then cell viability was assessed by MTT assay. Data are expressed as mean + SD of three different experiments. (B) Immunoblot analysis of proteins of signalling transduction pathways were conducted on cell lysates obtained after treatment with T-DM1 (1 μg/ml) for 24 or 48 h. (C) Curves of growth inhibitory effects of gefitinib and combined treatment gefitinib plus T-DM1 versus theoretical Bliss additivity curve are reported. Cells were treated with the drugs for 72 h and then cell number was assessed by MTT assy. Data are expressed as percent inhibition of cell proliferation versus control cells. The experiments, repeated three times, yielded similar results.

12. *IN VIVO* ACTIVITY OF T-DM1 IS DEPENDENT ON TUMOR SIZE AND HER2 EXPRESSION

It has been reported that cell density can influence the expression of EGFR in breast cancer [79] and in pancreatic cancer cell lines [152] and that surface expression of HER2 is regulated post-transcriptionally in mammary epithelial cells by the culture cell density [153]. We

investigated the dependence of HER2 membrane protein expression on cell density as well as the effect of T-DM1 on cells seeded at different densities. Confluent Calu-3 cells exhibited a significant decrease of HER2 at cell surface level detected by immunohistochemistry (Figure 16A ii), as compared to cells seeded at low density (i). At low density, more than 80% of cells showed a strong surface expression of the receptor whereas in almost confluent Calu-3 cultures a significant downregulation of HER2 was observed (nearly 35%). Consequently, as reported in Figure 16B, the inhibition of cell viability induced by T-DM1 at 1 μg/ml was markedly decreased with increasing cell density. We performed an in vivo experiment, aimed to determine whether T-DM1 efficacy might be affected by tumor size and structural organization. Tumors were clearly visible in all mice inoculated with 4×10^6 or 8×10^6 Calu-3 with a mean volume of 161 ± 15 mm^3 (Figure 16C) and 370 ± 50 mm^3 (Figure 16D) respectively. Trastuzumab (15 mg/Kg intraperitoneal) or T-DM1 (15 mg/Kg intravenously) were given every six days. T-DM1 or trastuzumab treatments in animals carrying tumors of small size were able to strongly inhibit tumor growth compared to vehicle treated mice (Figure 16C). Treatment with T-DM1 not only inhibited tumor growth, but a reduction of tumor dimension was observed in five out of six mice. On the other hand, when treatments were performed on larger tumors, only T-DM1 was able to significantly reduce tumor growth compared to control group, whereas no significant effect was seen with trastuzumab (Figure 16D). We did observe neither rapid tumor shrinkage nor long term complete response in T-DM1 treated animals. T-DM1 in vivo efficacy was confirmed by the reduction in weight of tumors excised at sacrifice, compared to control (Inset to Figure 16C and D). To define at tissue level the response of small and large tumors to T-DM1, a morphometric analysis of neoplastic tissue composition was performed. To this purpose, the fractional volume occupied by $PanCK^{pos}$ cells was assessed (Figure 17A,B). Quantitatively, compared to control in small tumors a 67% and 73% reduction of neoplastic epithelial cells within the nodules was observed with trastuzumab and T-DM1, respectively (A). In large tumors, the amount of neoplastic tissue was reduced by 16% and 37%, respectively, with trastuzumab and T-DM1 compared to control (B). Importantly, both drugs significantly decreased mitotic index in small and large tumors, however, the antiproliferative activity of T-DM1 was superior to that of trastuzumab (data not shown). To gain insights on the mechanism underlying these effects of T-DM1, we sought to determine whether the results obtained in vitro on the different sensitivity to T-DM1 according to cell density and HER2 expression had an in vivo counterpart. To this hand, sections of small and large tumors were immunostained with HER2 antibodies (Figure 17C,D). Again, differences in the surface expression of the HER2 was

striking, as neoplastic cells composing small tumors (C) showed higher extent and intensity of HER2 immunolabelling than large tumors (D). Thus, our data clearly document that the anti-tumor potency of T-DM1 is strictly dependent on HER2 expression, which in turn is intrinsically modulated among neoplastic cells and their structural organization. Characteristic and atypical mitotic figures and giant multinucleated cells were detected both morphologically on $HER2^{pos}$ cells (Figure 17E,F,G,H) and by the nuclear labelling of PH-H3 in PanCK expressing cells (Figure 17I,J,K) both in small (Figure 17E,F,G,H) and large tumors (not shown).

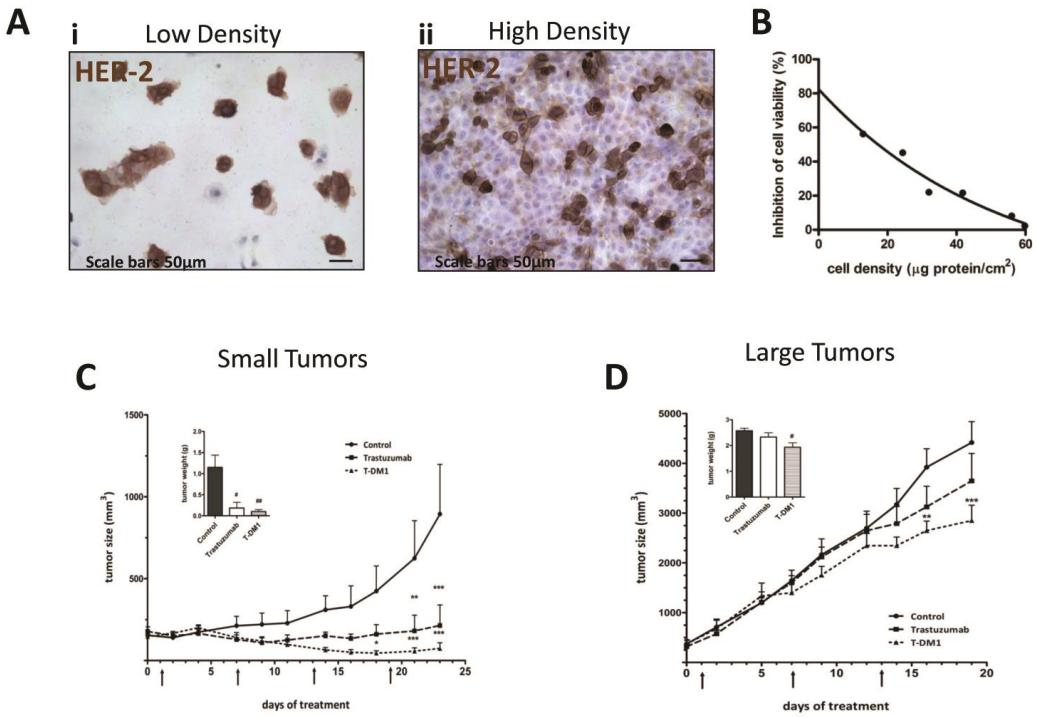


Figure 16. Cell density in vitro and tumor size in vivo influenced HER2 expression and efficacy of T-DM1. (A) Calu-3 cells were plated at low (10^4cells/cm^2) (i) and high ($8x10^4$cells/cm^2) (ii) density and after 24 h membrane HER2 protein expression was evaluated by immunohistochemistry. (B) Calu-3 cells were plated at different density and exposed for 72 h to T-DM1 1 µg/ml and then cell number was assessed using crystal violet staining as described in Methods section. Percent inhibition of cell proliferation versus control cells was plotted as function of cell density. The experiments, repeated three times, yielded similar results. 4×10^6 (C) or 8×10^6 (D) Calu-3 cells were subcutaneously implanted on BALB/c-Nude mice. At the beginning of the treatments average tumor volumes were 161 ± 15 mm^3 and 370 ± 50 mm^3 respectively. In both settings vehicle, trastuzumab (15 mg/Kg i.p.) or T-DM1 (15 mg/Kg i.v.) were administered every six days as pointed (arrows). Tumor sizes were measured three times per week and data expressed as volume + SEM (n = 6 mice per group). (**p < 0.01, ***p < 0.001 vs control; two-way ANOVA followed by Bonferroni's post-test). After sacrifice tumors were excised and weighted (# p < 0.05 ##p < 0.01; one-way Anova followed by Tukey's post-test).

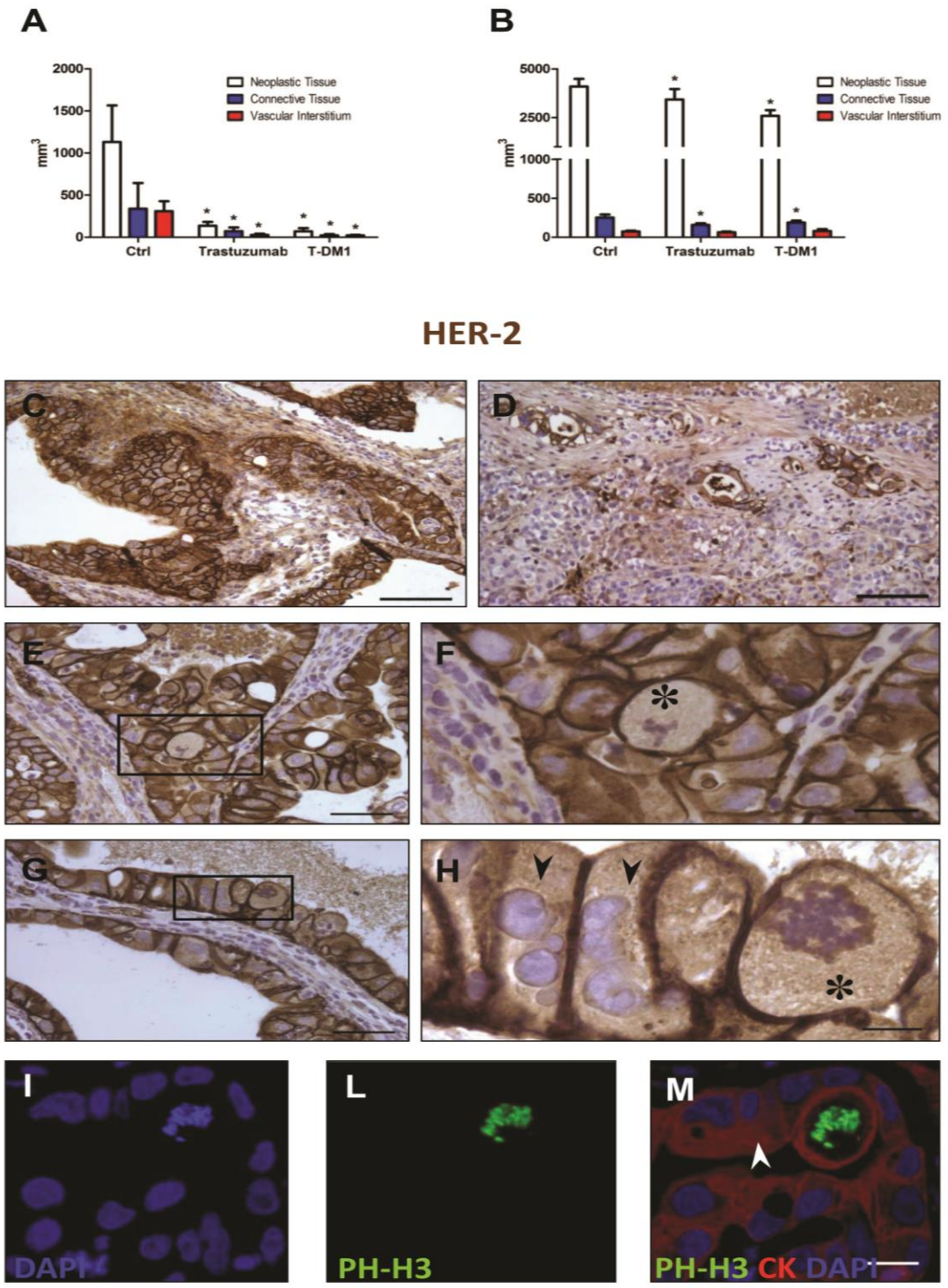


Figure 17. Neoplastic tissue composition and HER2 expression in small and large tumors. Quantification of tissue composition in small (A) and large (B) tumour xenografts in untreated (CTRL) and Trastuzumab or T-DM1 treated mice (*p < 0.05, vs control) was evaluated as described in Methods Section. C-H: Immunoperoxidase staining of xenografts by anti-HER2 antibodies. The sharp difference in HER-2 expression (brownish) by neoplastic cells composing small (C) and large (D) tumours is apparent. E and G illustrate sections of small T-DM1 treated tumour xenografts in which black rectangles include a microscopic field shown at higher magnification in F and H, respectively, to document giant mitotic figures (✻) on HER2 labeled cells. Arrows indicate polynucleated HER2 positive neoplastic cells. The lower panels show the specific immunofluorescent labeling of metaphase chromosomes (I) by phospho-Histone H3 (PH-H3, green, J) on a large cytokeratin (CK, red, K) positive cell. Arrow points to a giant polynucleated neoplastic cell. Scale bars: C, D = 100 μm; E, G = 50 μm; F, I,L,M=20μm-H=10μm.

13. ACQUIRED RESISTANCE TO T-DM1

Most patients with breast cancer treated with T-DM1 eventually progress, and some HER2-positive breast cancers are primarily non-responsive or are only minimally responsive to T-DM1. Understanding the mechanisms of acquired resistance to T-DM1 is important for further development of therapies. Except for low HER2 expression in cancer, the clinical, biological and pharmacological factors that are related to poor efficacy of T-DM1 are incompletely understood. Yet, factors that are strongly implicated in the biological mechanism of action of T-DM1 are good candidates for having a role in resistance to T-DM1. DM1 and its metabolites (lysine-MCC-DM1) need to accumulate in cancer cells to reach a concentration that exceeds the threshold to evoke cell death. Expression of HER2 on cancer cells is essential for T-DM1 efficacy. Not surprisingly, retrospective analyses of two phase II trials (TDM4258g and TDM4374g) carried out in advanced breast cancer revealed that patients with HER2-positive cancer (IHC 3+) had more frequent responses to T-DM1 than patients who had HER2-normal cancer; in TDM4258g the objective response rates were 34% and 5%, respectively, and in TDM4374g, 41% and 20%, respectively [154]. A high rate of HER2-T-DM1 complex internalization may result in high intracellular concentrations of DM1, and deceleration of the endocytosis rate might cause loss of sensitivity to T-DM1. Since DM1 release in the cytosol occurs only following proteolytic degradation of the trastuzumab part of the TDM1 complex in the lysosomes, efficient lysosomal degradation is essential. Expression and activity of lysosomal enzymes may vary between tumors and even cancer cells, and is influenced by several factors such as tumor necrosis factor-α, lysosomal vacuolar H+-ATPase (V-ATPase), and Bax inhibitor-1. All of these factors may thus affect cancer sensitivity to T-DM1 [154]. MDR1 (also known as P-glycoprotein) is an ATPdependent transporter that mediates efflux of drugs and toxins from the cell. Tumor MDR1 expression is associated with poor response to chemotherapy in many types of cancer. DM1 and other maytansinoids are substrates of MDR1, and MDR1 expression is linked with a maytansine-resistant cancer phenotype. Lewis et al [155] demonstrated that the presence of the HER3 ligand neuregulin-1β (NRG-1β,heregulin) suppressed the cytotoxic activity of T-DM1 and this effect being reversed by pertuzumab.

In our *in vivo* investigations, we observed that a mouse with small tumor (#11) (Figure 18A) who had received T-DM1 treatment, after an initial response had become less sensitive to T-DM1. Indeed starting from 20 days of treatment the tumor size increased.

To investigate the potential mechanism of acquire resistance to T-DM1 treatment, after tumor excision, part of each nodule was utilized for the re-isolation of tumor injected cells. Calu-3

from control nude mice (Ctrl) and Calu-3 from #11 were cultured and treated with T-DM1 for 72 h to assess cell viability. As reported in Figure 18B, cells from #11 were less sensitive to T-DM1 treatment compared to Ctrl cells. Furthermore, in parallel we performed a western blot analysis to detect the total amounts of HER2. Figure 18B (inset) shows a reduction of amount of total HER2 protein level in cells derived from #11 compared to the Ctrl cells.

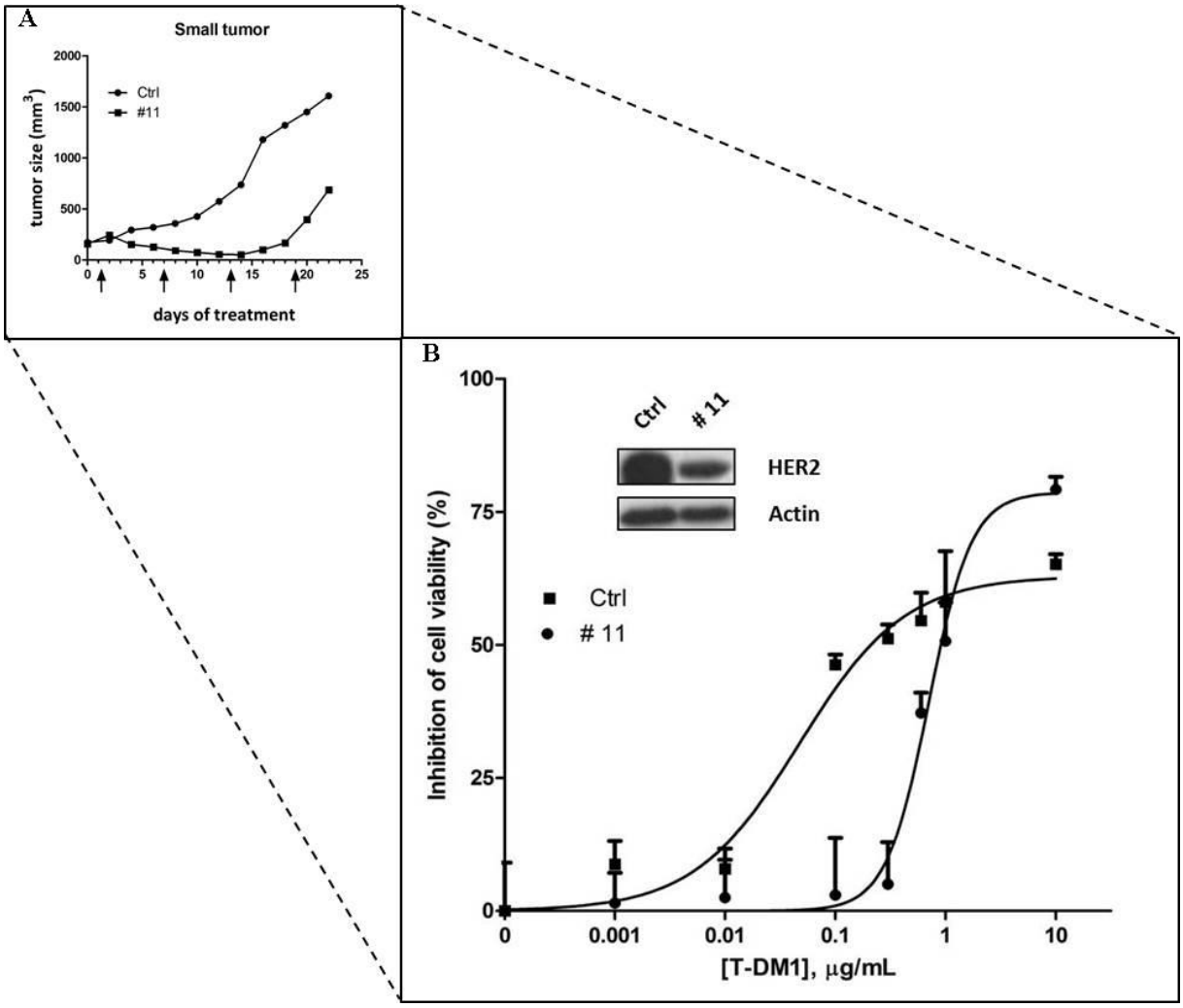


Figura 18. Acquired resistance to T-DM1 treatment. (A) Tumor size (mm^3) of a control nude mice (Ctrl) and a nude mice treated with T-DM1 (15 mg/Kg i.v.) (#11). (B) Calu-3 cells from Ctrl and Calu-3 cells from #11 were exposed to increasing concentrations of T-DM1 for 72 h and then cell viability was assessed by MTT assay. Data are expressed as mean + SD of three different experiments. (Inset) Immunoblot analysis of total HER2 protein level was conducted on cell lysates.

As mentioned above, and demonstrated in our experiments, expression of HER2 in cancer cells is essential for T-DM1 activity. The reduction of HER2 protein levels in #11 could be the cause of lower sensibility to T-DM1 in *in vivo* experiments. However further studies should be conducted to better clarify the acquired resistance to T-DM1.

DISCUSSION

&

CONCLUSIONs

The potential for dual-agent molecular targeting of the ErbB family, has been clearly demonstrated in preclinical models and confirmed on the clinical setting for HER2-targeting agents in breast cancer. However, little is known about this therapeutic strategy in other tumour types. In our current study we demonstrated that the combination of erlotinib with cetuximab or trastuzumab may enhance the antitumour activity of EGFR-TKI in NSCLC cell lines harbouring wild-type EGFR and in xenograft models. The efficacy of the association between an EGFR/ HER2 mAbs with TKIs has been documented in preclinical studies in several cell lines originating from different tumour types [137]. In EGFR wild-type H292 and A549 NSCLC cell lines, the combination of either gefitinib or erlotinib with cetuximab was reported to enhance growth inhibition in comparison to single treatment, particularly in the H292 gefitinib sensitive cell line [156]. In the A549 cell line, expressing both EGFR and HER2, the combination of gefitinib with trastuzumab significantly inhibited cell growth and proliferation [157]. In Calu-3 xenograft models, the combined treatment of erlotinib and pertuzumab showed an enhanced antitumour activity [158]. A correlation between cetuximab efficacy and EGFR expression has been reported in preclinical studies [159] and confirmed in clinical trials. Thus, the phase III FLEX study involving patients with advanced NSCLC showed a strong correlation between high tumour EGFR overexpression and the efficacy of adding cetuximab to platinum based first-line chemotherapy [98]. The combination of a TKI and a mAb was explored as a potential strategy to overcome acquired resistance to first generation EGFR-TKIs. Kim and colleagues demonstrated that the combination of lapatinib with cetuximab overcame gefitinib resistance due to the secondary T790M mutation in NSCLC by inducing enhanced cytotoxicity both in vitro and in vivo [160]. Furthermore, the association of cetuximab with afatinib has been shown to be effective to overcome T790M-mediated drug resistance [103]. However, the combination of erlotinib with cetuximab did not lead to a significant radiological response in NSCLC patients with clinically defined acquired resistance to erlotinib indicating that such strategy is not sufficient to overcome acquired resistance to erlotinib patients [161]. The mechanisms leading to an enhanced activity of combining a TKI with a monoclonal antibody have been ascribed, in other cancer cell models, either to a more efficient inhibition of TK receptors [156] or to an increased targeted receptors on plasma membrane induced by TKIs [162,163]. Scaltriti et al. showed that lapatinib enhanced the effects of trastuzumab by inducing HER2 stabilization and accumulation at the cell surface of breast cancer cell lines [146], and Mimura et al. reported that lapatinib induced accumulation of HER-2 and EGFR on esophageal cancer cell lines evoking trastuzumab- and cetuximab- mediated ADCC [163]. ADCC, one of the killing mechanism of the immune

system mediated by Natural Killer cells, plays a pivotal role in the anti-cancer effects exerted by mAbs. Therefore, increasing the ADCC activity is an important objective in the development of novel therapeutic approaches. It has been recently demonstrated that the EGFR inhibitors gefitinib and erlotinib enhance the susceptibility to NK cell mediated lysis of A549, NCI-H23 and SW-900 lung cancer cell lines [164] by the induction of ULBP1 (a ligand of the NK cell activation receptor NKG2D). These data indicate that EGFR blockade could not be the only mechanism of action of EGFR inhibitors in vivo. The efficacy of these inhibitors in lung cancer may be at least in part mediated by increased susceptibility to NK activity. Moreover, cetuximab serves as a potent stimulus for NK functions including INF-gamma production [165] and is also associated with a complement –mediated immune response [166]. We demonstrated that erlotinib induces an accumulation of EGFR and/or HER2 protein at the plasma membrane level only in TKI sensitive NSCLC cell lines whereas, in resistant cells (both, intrinsic or MET amplification-mediated acquired resistance), this enhancement was not observed. The anti-tumour effect of drug combination was more evident in ADCC experiments compared with cell viability experiments. In the Calu-3 xenograft model, the combined treatment resulted in a lower rate of tumour growth, suggesting the involvement of NK activity as a determinant factor to improve the efficacy of the combined treatment. Moreover, regressive phenomena and changes in size of neoplastic glands together with intense stromal reaction were observed in histologic samples of tumours from mice treated with cetuximab alone or the combination. The reason why EGFR inhibitors such as gefitinib, erlotinib or lapatinib induce EGFR accumulation only in sensitive cells could be ascribed to their ability to inhibit both signal transduction pathways downstream EGFR and proteasomal activity. The constitutive activation of signaling pathways downstream of EGFR (i.e. presence of RAS mutations) is indeed a recognized mechanism of resistance against reversible EGFR-TKIs [167]. The inhibition of the MAPK pathway might represent a link between EGFR inhibition and EGFR accumulation since U0126, a well known MEK1/2 inhibitor, induced EGFR accumulation in Calu-3 cells, while none of PI3K/AKT/mTOR inhibitors tested was effective. A correlation between MAPK pathway and protein degradation by the ubiquitin system was described for the pro-apoptotic BH3-only protein BIM, indeed in the absence of MAPK activation, BIM protein accumulated in the cell promoting activation of apoptotic cell death [168].

The first ADC targeting the HER2 receptor is T-DM1(Kadcyla®), which is a conjugate of trastuzumab and a cytotoxic moiety (DM1, derivative of maytansine). T-DM1 carries an

average of 3.5 DM1 molecules per one molecule of trastuzumab. Each DM1 molecule is conjugated to trastuzumab via a non-reducible thioether linker (N-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate; SMCC, MCC after conjugation). T-DM1 has shown substantial antitumor efficacy in preclinical studies and clinical trials. T-DM1 has superior activity compared with trastuzumab on trastuzumab-sensitive breast cancer cell cultures and tumor xenografts. Importantly, T-DM1 is effective in in vitro and in vivo models of trastuzumab-resistant breast cancer, and in trastuzumab and lapatinib cross-resistant breast cancer models. A key clinical trial to investigate the efficacy and safety of T-DM1 in the treatment of breast cancer was the EMILIA study, where 991 patients previously treated for locally advanced or metastatic breast cancer with trastuzumab and a taxane were randomly assigned to receive either single agent T-DM1 3.6 mg per kilogram of body weight intravenously 3-weekly or lapatinib plus capecitabine. The median progression-free survival (PFS) was 9.6 months with T-DM1 versus 6.4 months with the control regimen. These data led to approval of T-DM1 by the US Food and Drug Administration (FDA) in February 2013 for the treatment of patients with HER2-positive MBC who had previously received trastuzumab and a taxane [154].

In a recent paper, Takezawa et al. [54] have identified that HER2 plays a significant role in mediating sensitivity of EGFR -mutant lung tumors to anti-EGFR therapy. The authors identify HER2 amplification as a new mechanism of acquired resistance to EGFR-TKIs in EGFR -mutant NSCLC tumors, occurring independently of the EGFR T790M secondary mutation.

One of the major findings of our study is that targeting HER2 with Trastuzumab-DM1, developed to improve the treatment of HER2 positive breast cancer, may offer a new therapeutic approach in lung cancers expressing HER2 even when resistant to EGFR TKIs. We also demonstrated that HER2 is highly expressed in low density NSCLC cells in vitro and in small tumors in vivo by which mechanism T-DM1 exerts a stronger efficacy. The involvement of HER2 in lung carcinogenesis has been known for many years but clinical research was slowed down after the negative outcome of the initial clinical trials with trastuzumab plus chemotherapy in patients with HER2-immunohistochemistry positive NSCLC [169,170]. HER2 protein overexpression are reported in 2-9% (IHC 3+) and 20% (IHC 2+) and gene amplification are reported in 2-20% of NSCLC [46]. Moreover, HER2 amplification was present in 13% of the cases at the time of resistance to EGFR TKI [146]. The role of HER2 overexpression and amplification in lung cancer remains controversial. In a

meta-analysis of 40 studies in NSCLC, HER2 overexpression assessed by IHC was associated with poor prognosis, specifically in adenocarcinomas [136]. Conversely, HER2 amplification determined by FISH had no prognostic role [136]. HER2 mutations are present in about 2-4% of NSCLC, especially in women, never-smokers, Asian patients and in adenocarcinomas without EGFR or K-RAS mutations [49]. In a population of EGFR/K-RAS/ALK-mutation negative patients, HER2 mutations can reach up to 6% [49]. T-DM1 has been extensively studied in preclinical models of breast cancer [122,124] These studies demonstrated that T-DM1 has dual mechanisms of action: selective delivery of DM1 to the HER2-positive tumor cells and activation of antibody-dependent cellular cytotoxicity. T-DM1 demonstrated activity in both trastuzumab and lapatinib resistant HER2 positive cancer models [122]. T-DM1 was effective also in gastric cell lines [148] and antiproliferative properties have been reported in ovarian SK-OV-3 cell line and in the NSCLC Calu-3 cell line and xenografts [155]. In a recent review Landi and Cappuzzo [171] hypothesized that T-DM1 could play an important role even in NSCLC, and underlined the need of a proper investigation of the real impact of T-DM1 in lung cancer. All the above observations prompted us to investigate, in a panel of NSCLC cell lines with different levels of HER2 expression or carrying HER2 mutation, the effect of T-DM1 on cell proliferation and survival.

In agreement with previously reported data in breast cancer models [124], we documented that also in NSCLC cell lines T-DM1 efficiently inhibited proliferation with arrest in G2-M phase. Moreover, T-DM1 induced cell death by apoptosis in cells with a significant level of surface expression of HER2 while cells with low level of HER2 failed to respond to the drug. Interestingly, trastuzumab did not inhibit cell proliferation irrespective of HER2 expression. H1781 cell line, harbouring mutated HER2 (G776insV_G/C), was also included in our study.

The effect of T-DM1 in this cell line was presumably related to HER2 level and not affected by the presence of the mutation. HER2 mutations in NSCLC may be an important predictive marker for tumor sensitivity to an anti-HER2 agent. Capuzzo et al. reported a patient with metastatic HER2-mutated (G776L) lung adenocarcinoma resistant to conventional cytotoxic chemotherapy, who had a favorable response to trastuzumab [51]. Kelly reported a long-lasting favorable response to treatment containing trastuzumab and lapatinib in a patient with advanced HER2-amplified and mutated (L869R) NSCLC, who had failed conventional chemotherapy [53]. Recently, Yan reported a 53-year-old patient with left-side lung adenocarcinoma and a HER2 exon 20 aberration (insertion 774–775 AVYM). The patient had disease metastatic to the brain and right lung. She received trastuzumab combined with lapatinib and bevacizumab after her disease had progressed on two lines of systemic

chemotherapy [41,150]. To investigate the dual inhibition of HER2 in H1781 cell line we tested the effects of lapatinib and/or afatinib alone or in combination with T-DM1. We observed that both lapatinib plus T-DM1 or afatinib plus T-DM1 have an additive effect compared to the single agent. Antibody-dependent cytotoxicity assay performed with NK cells demonstrated that T-DM1 retained the activity of trastuzumab as previously reported in breast and gastric models [122,148]. Moreover, we demonstrated that T-DM1 is able to inhibit the growth of a EGFR mutant cell line in which HER2 overexpression confers resistance to gefitinib. Therefore, targeting HER2 with T-DM1 might represent a potential approach to overcome EFGR-TKI resistance. Our *in vitro* and *in vivo* experiments documented that, respectively, low cell density and small xenografted tumors were associated with higher HER2 expression and thereby greater T-DM1 sensitivity. Thus, the present investigation strongly support the contention that HER2 expression in NSCLC is regulated by the tumor mass and its structural organization which in turn condition the efficacy of T-DM1. Furthermore, we suggest that the expression level of HER2, determined by immunohistochemistry, might represent a predictive factor of response to T-DM1 in tumors carrying wild type or mutant HER2 receptor.

Finally, it is well known that some patients after treatment with T-DM1 partial progressed. Except for low HER2 expression in cancer, the clinical, biological and pharmacological factors that are related to poor efficacy of T-DM1 are incompletely understood. Yet, factors that are strongly implicated in the biological mechanism of action of T-DM1 are good candidates for having a role in resistance to T-DM1. In our *in vivo* experiments we observed an acquired resistance to T-DM1 treatments. In order to study this potential mechanism of acquired resistance, we isolated and cultured Calu-3 cell line from tumor xenografts. We observed that Calu-3 cells derived from a nude mice who prior received T-DM1 treatment, were less sensitive compared to Calu-3 derived from a control nude mice. This response was probably ascribable to a reduction of HER2 protein levels. However, more studies are needed to investigate the acquire resistance to T-DM1.

In conclusion, in this thesis I explored the potential of combining erlotinib with cetuximab or trastuzumab in improving the efficacy of EGFR targeted therapy in EGFR wild-type erlotinib-sensitive NSCLC cell lines. The results indicate that erlotinib, through ERK inhibition, increases surface expression of EGFR and/or HER2 only in erlotinib sensitive NSCLC cell lines and in turn leads to increased susceptibility to ADCC both in vitro and in xenografts models. These data prompt future adequate clinical trials that will give the ultimate proof of

the utility of this combined treatment for the care of NSCLC patients carrying EGFR-wild type that are sensitive to TKIs.

In order to study HER2, a relatively new therapeutic target in NSCLC, our results indicate that targeting HER2 with T-DM1 may offer a new therapeutic approach in HER2 over-expressing lung cancers. To date, a phase 2 study of Trastuzumab Emtansine in patients with HER2 IHC-Positive, locally advanced or metastatic Non-Small Cell Lung Cancer (*NCT02289833*) is ongoing [172]. This Phase 2, multicenter, single-arm, two cohort study designed to evaluate the efficacy and safety of trastuzumab emtansine as single-agent in patients with HER2-positive locally advanced or metastatic non-small cell lung cancer (NSCLC). Patients are enrolled into two separate cohorts, depending on HER2 status: HER2 immunohistochemistry (IHC) 2+ or HER2 IHC 3+. Patients in both cohorts will be treated with an intravenous dose of 3.6 mg/kg trastuzumab emtansine on Day 1 of 21-day cycles. The primary end-point will be to define the Objected Response Rate (ORR) and the second end-points the Overall Survival (OSS), Progression Free Survival (PFS), Duration of Response (DOR), Serum and Plasma concentration of T-DM1 and the Incidence of Adverse Events. The unit of Oncology of the University Hospital of Parma is one of the center of the multicenter trial.

Dual-agent molecular targeting through T-DM1 may be a promising therapy also in HER2 positive lung cancer even in tumors which had developed resistance to EGFR-TKIs and in HER2 mutated patients.

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PS. Probabilmente dopo questo ennesimo pezzo di carta mi aprirò qualche locale, tipo un ristorante e una pasticceria, qualora volessi darmi una mano sei la benvenuta!! Ahahah

GRAZIE A TUTTI
UN BACIO

DANIELE