

# Strategies for Overcoming Inherent and Acquired Resistance to EGFR Inhibitors by Targeting Downstream Effectors in the *RAS/PI3K* Pathway

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**Abstract:** Mutations in *K-Ras* are observed in approximately 40% of colon tumours. This has significant implications for predicting likelihood of response to the antibody-based EGFR inhibitors, cetuximab and panitumumab, with *K-Ras* mutant patients now clearly shown to be inherently resistant to these agents. Alternative treatment strategies for *K-Ras* mutant patients are therefore urgently needed. Farnesyltransferase inhibitors, developed to inhibit *K-Ras*, have to-date been largely unsuccessful. However, a number of agents which target signaling components in the MAPK and *PI3K* pathways downstream of mutant *K-Ras* are currently being evaluated in clinical trials and will be discussed. A further clinical concern is that *K-Ras* wild type patients who initially respond to EGFR inhibitors eventually develop acquired resistance to these agents and experience tumour progression. Studies from the use of related agents in other disease settings as well as pre-clinical studies provide important insights into mechanisms by which this may occur. While no evidence presently exists for somatic mutations as a basis for acquired resistance to EGFR inhibitors in colon cancer, several studies implicate upregulation and signaling via other Her family members, c-Met, IGFR and Src. Upregulation of the pro-angiogenic factor, VEGF, is also a possible mechanism of acquired resistance. This review discusses drugs currently in clinical trials that may potentially achieve more efficient and prolonged targeting of the EGFR pathway by overcoming these mechanisms of resistance.

**Keywords:** Epidermal growth factor receptor, colon cancer, acquired resistance, *K-Ras*, *PI3K*.

## INTRODUCTION

The development of colon cancer is the consequence of an accumulation of multiple genetic and epigenetic events that typically occur over the course of several years and even decades [1-4]. These changes result in the activation of pathways relevant to the tumour which impact on cell growth, differentiation and apoptosis. Targeted therapies were designed to inhibit signalling through specific pathways and consequently to affect tumour phenotype. While it is evident that the majority of colon tumours are dependent on the simultaneous activation of multiple signalling pathways, including Wnt/Apc/ $\beta$ -catenin, EGFR/*Ras*/MAPK, TGF $\beta$ /SMAD, p53 and PTEN/*PI3K*/AKT [5], it is also recognised that subgroups of tumours are especially dependent on a particular pathway, a phenomenon known as 'oncogene addiction'.

In the context of EGFR signalling, a subgroup of patients with NSCLC have activating mutations affecting EGFR which render them particularly dependent on this pathway and hence exquisitely sensitive to EGFR inhibitors [6]. Other tumours however, are less dependent solely on this pathway and derive varying degrees of benefit from the use of EGFR-targeted agents. In advanced colon cancer, two randomised studies comparing the EGFR-targeting antibodies, cetuximab or panitumumab, with best supportive care in chemorefractory patients showed a modest but clinically meaningful level of activity in unselected patients [7, 8], establishing the therapeutic benefit of inhibiting this pathway.

The signalling pathways downstream of EGFR include the *Ras*-MAPK, *PI3K*/AKT and STAT pathways [9, 10]. Constitutive activation of these pathways through mutation uncouples downstream signalling from upstream receptor inhibition, and hence constitutes a potential inherent resistance mechanism to EGFR-targeted antibodies.

## INHERENT RESISTANCE TO TARGETING THE EGFR PATHWAY

The failure of colorectal tumours with *K-Ras* mutations to respond to EGFR inhibitors requires the urgent development of alternative treatment strategies for this patient subgroup. In this regard, significant effort has been made to target either the mutant *K-Ras* protein itself or signalling mediators downstream of *K-Ras* such as components of the MAPK and *PI3K* pathways.

### Mutant *K-Ras*

Evaluation of randomised studies in advanced colorectal cancer now clearly demonstrates that *K-Ras* mutations, which are present in approximately 40% of patients, predict for intrinsic resistance to EGFR-targeted agents whether these agents are used in first-, second- or third- line settings [11]. Importantly, *K-Ras* mutations are purely predictive biomarkers, as the presence of a *K-Ras* mutation does not appear to impact on prognosis [12].

Binding of growth factor to its receptor, such as EGF, TGF $\alpha$  or amphiregulin to the EGFR, results in receptor dimerisation and phosphorylation. This in turn activates *Ras* guanine nucleotide exchange factors (*Ras*-GEFs) which catalyse the exchange of GDP for GTP on *Ras*. Normally, binding of GTP to *Ras* is transient, with specific *Ras*-GTPase activating proteins (*Ras*-GAPs) such as, NF1 and p120GAP, binding to *Ras* and activating its intrinsic GTPase activity, thereby returning *Ras* to its inactive GDP bound state [13, 14]. Mutations in codons 12 or 13 of *K-Ras* result in *Ras* mutants which are insensitive to *Ras*-GAPs, while mutations in codon 61 inhibit the intrinsic GTPase activity of *K-Ras* [13]. The consequence of these mutations is the failure to convert *Ras*-bound GTP into GDP, and subsequently in the constitutive activation of downstream signalling, independent of external signals (growth factor independence).

Potentially, compounds which restore the lost GTPase activity of mutant *K-Ras* should provide the greatest therapeutic benefit.

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However, the design of such “gain of function” compounds has not been possible to-date, with the challenge likened to that of the design of compounds that would lead to gain of function in mutant tumour suppressor proteins [13]. Another possibility could be the use of GTP-analogues, however, the high affinity with which mutant *K-Ras* binds to GTP, and the potential toxicities associated with such a generic strategy has discouraged the development of these agents. Finally, the advent of siRNA technology raises at least the theoretical possibility that mutant *K-Ras* could be specifically targeted using siRNAs designed to bind mutant but not wild type *K-Ras* mRNA. While evidence of the feasibility of such an approach has been demonstrated *in vitro* [15], significant challenges, including the method by which these agents would be delivered to tumour cells remain to be developed for the successful translation of this approach.

Instead, significant attention has been given to the development of agents that inhibit a key post-translational modification of *K-Ras*, farnesylation, catalysed by farnesyl transferase and required for targeting of *Ras* to the cell membrane prior to its activation. However, the farnesyltransferase inhibitor (FTI) R115777 (Zarnestra, tipifarnib) failed to improve survival compared with placebo in chemotherapy refractory advanced colorectal cancer [16]. Furthermore, the FTIs tipifarnib and lonafarnib, tested in phase III studies in combination with chemotherapy for pancreatic cancer, acute myeloid Leukaemia, and non-small cell lung cancer were also unsuccessful [8, 17, 18]. One explanation for the failure of FTIs is that inhibition of farnesyl transferase can still result in *K-Ras* (but not *H-Ras*) activation via an alternate, but related post-translational modification, geranylgeranylation [18]. A solution that has been explored is the dual inhibition of FTI and geranylgeranyltransferase (GGTI). However, while FTI/GGTI combinations induced a greater apoptotic response in *K-Ras* mutant cells than either agent alone *in vitro*, significant toxicity was associated with GGTI treatment *in vivo*, likely due to the large number of geranylgeranylated proteins that play key roles in normal physiology [19]. Reduced toxicities of newer generation, dual prenylation inhibitors (DPI) have been reported although their antitumour efficacy remains to be demonstrated [18].

Finally, biosynthesis of the isoprenoids farnesyl-PP and geranylgeranyl-PP occurs via the mevalonate pathway. Statins, which inhibit HMG-CoA reductase and mevalonate synthesis will also inhibit downstream isoprenoid synthesis and therefore potentially *Ras* activation. While trials involving administration of statins as single agents have not demonstrated activity, a recent phase II study of simvastatin in combination with chemotherapy (FOLFIRI) demonstrated a response rate of 46% and median time to progression of 9.9 months with no additional adverse events, in patients with metastatic colon cancer [20]. Interestingly, *in vitro* studies have demonstrated synergistic activity when statins are combined with EGFR-inhibitors (EGFRi) in several cell types including colon cancer cells [21], and a phase I trial testing the combination of the statin rosuvastatin with erlotinib is presently underway (NCT00966472).

### MAPK /BRAFF

Activating mutations in *BRAF* affect the MAPK signalling pathway and occur in a mutually exclusive manner with *K-Ras* mutations in colon cancer [22]. However, *BRAF* mutations occur less frequently than *K-Ras* mutations and are mainly confined to colon cancers with microsatellite instability, although approxi-

mately 5% of microsatellite stable colon cancers also harbor *BRAF* mutations [23]. Although the data are more limited, tumours with *BRAF* mutations also appear to have intrinsic resistance to EGFR inhibitors [24, 25]. However *BRAF* mutations are also prognostic, as microsatellite stable *BRAF* mutant colon tumours have shorter progression-free and overall survival times compared with *BRAF* wild type tumours [23].

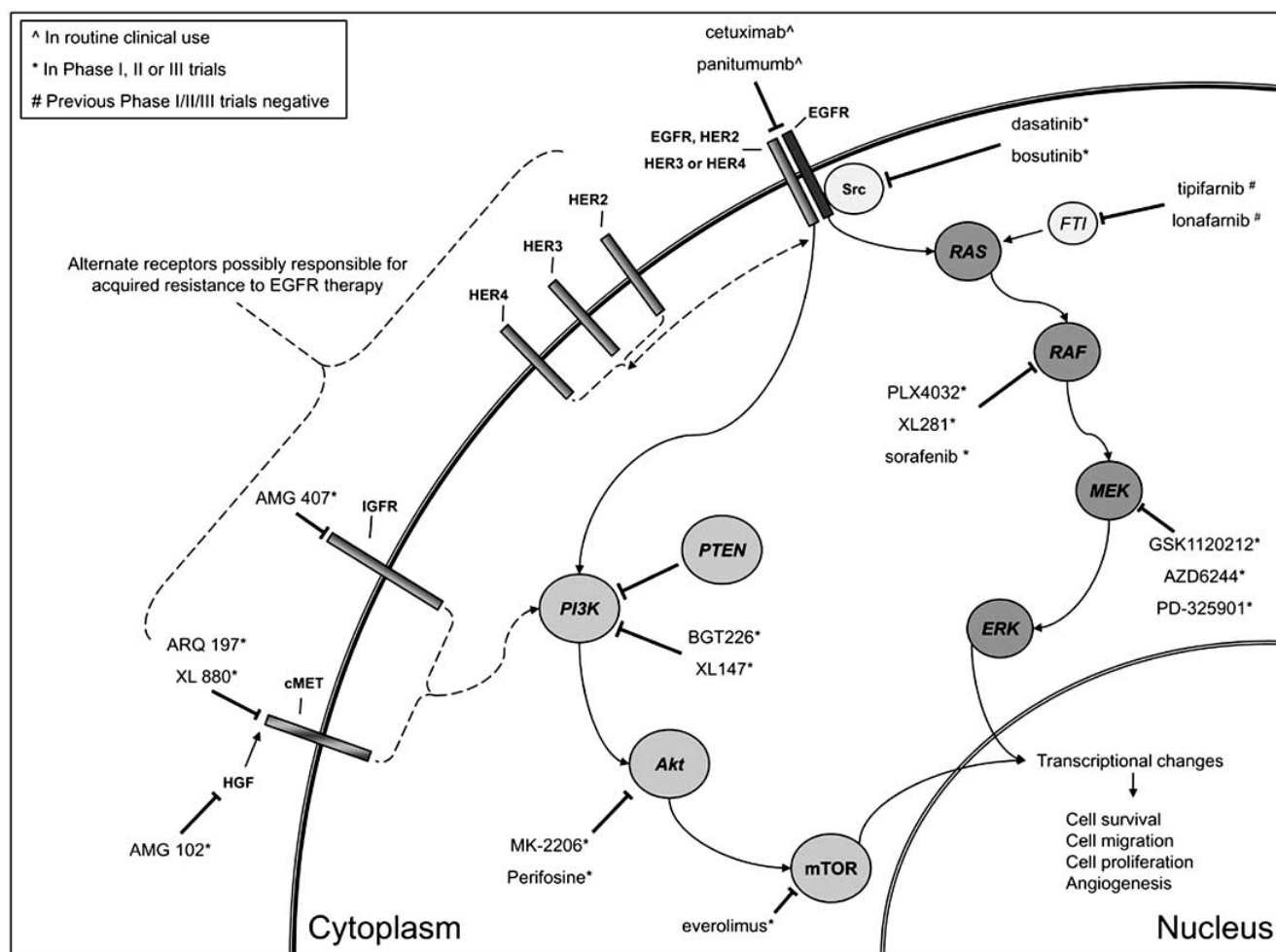
Activated *BRAF* phosphorylates MEK (MAPKK) which in turn phosphorylates and activates ERK (MAPK) (Fig. 1). Activated ERK translocates to the nucleus where it phosphorylates MSK1/2. This in turn modulates gene expression through phosphorylation and activation of Histone H3, and transcription factors such as AP-1 and CREB, which induce expression of pro-proliferative genes [26]. Inhibitors to a number of these signalling components have been developed and tested, although none have yet been approved for the treatment of colon cancer. Best developed among these is sorafenib, initially developed as a RAF inhibitor but now shown to also inhibit the VEGF and PDGF receptors and thereby inhibit angiogenesis, and currently approved for the treatment of renal cell cancer. A number of clinical trials are underway to test the efficacy of sorafenib in combination with existing agents in metastatic colon cancer, including a multicenter phase II study of sorafenib in combination with irinotecan for chemorefractory patients who have *K-Ras* mutations (NCT00343772) (Table 1).

Other *BRAF* inhibitors include PLX4032 from Plexxikon which is a highly specific inhibitor of V600E mutant *BRAF*. Trials of this compound in V600E mutant melanoma patients are producing highly encouraging response rates [27], while trials in the subset of colon cancer patients who harbour the V600E mutation are ongoing (NCT00405587). Notably however, ATP-competitive *BRAF* inhibitors have recently been shown to have 2 conflicting mechanisms of action depending on the genotype of the cells in which they are used [28-30]. While *BRAF* inhibitors effectively inhibit MAPK signalling and decrease tumour growth in *BRAF* V600E mutant tumours, in *BRAF* wild type / *K-Ras*-mutant tumours, *BRAF* inhibitors induce the opposite effect, activating MAPK signalling and promoting tumour growth [28-30]. This activation of MAPK signalling has now been shown to be due to *BRAF*-inhibitors inducing conformational changes and paradoxical activation of wild type *BRAF* and *CRAF* [30]. Binding of these inhibitors to wild type RAF isoforms induces *BRAF* and *CRAF* to homo and heterodimerise, interact with active GTP-bound *Ras*, and drive downstream MAPK signalling [28-30]. These recent findings emphasise the caution that needs to be exercised with the use of present generation of RAF inhibitors, particularly in the context of *Ras* mutations.

Further downstream of *BRAF* are MEK and ERK. MEK inhibitors presently in phase I and II trial are described in Table I. The outcomes of these trials are being eagerly awaited as they represent the most developed alternative treatment option for colon cancer patients with *K-Ras* mutant tumours. Fewer inhibitors of ERK, the signalling component furthest downstream in the pathway, have been developed and none are presently in clinical trials.

### PI3K

The impact of mutations affecting *PI3K* signalling on sensitivity to EGFR inhibitors is less clear. Mutations affecting the catalytic subunit of *PI3K*, *PIK3CA* characteristically result in activation of the pathway and occur in 15-30% of cases of advanced colorectal cancer [31, 32]. Studies *in vitro* have suggested that *PIK3CA* mutations may confer intrinsic resistance to EGFR-targeted antibodies



**Fig. (1). The EGFR pathway: therapeutic targets and new therapies.**

Possible strategies for treating KRAS mutant tumours include targeting molecules within the RAS/MEK/MAPK pathway, PI3K, AKT or mTOR. Possible strategies to prevent acquired resistance include combination therapy against alternate receptors including cMET, IGFR and alternate HER family members, or intracellular Src.

[33]. Three, non-randomised clinical studies have supported this finding [34-36], although a recent study observed no link between PIK3CA mutation and cetuximab response [37]. Cellular models and a retrospective review support the possibility that only mutations within the exon 20 kinase domain of PIK3CA are important in governing resistance to EGFR-targeted antibodies [38, 39]. Loss of PTEN can also induce activation of the PI3K pathway and has been linked to intrinsic resistance to EGFR-targeted antibodies [40-43]. However, a limitation of the evaluation of PTEN status has been the lack of concordance of PTEN expression between primary and metastatic tissue, with loss of PTEN expression in metastatic lesions shown to be a more reliable predictor of resistance to EGFR inhibitors [42].

Ras activation can also result in signalling through the PI3K pathway. This is suggested by the physical interaction that has been demonstrated between Ras and PI3K, and by the finding that mutating the residues in PIK3CA required for Ras-interaction, attenuates Ras-induced lung tumourigenesis [44, 45]. PI3K activation phosphorylates and activates the downstream signalling mediator, AKT, which in turn phosphorylates and activates a number of growth and

survival-promoting proteins including GSK3 $\beta$  and mTOR. Given Ras can activate PI3K, a possible limitation of targeting the MAPK pathway downstream of K-Ras is that mutant Ras continues to signal via cross talk with the PI3K pathway. Simultaneous inhibition of MAPK and PI3K signalling may therefore be required to fully negate the oncogenic activity of mutant Ras, as recently demonstrated in proof-of-principle experiments in a Ras-induced lung cancer mouse model [46]. In this regard, the development and testing of PI3K inhibitors is presently an active area of study, with a number of PI3K inhibitors presently in clinical trial (Table 1).

Inhibitors of signalling components downstream of PI3K, particularly AKT and mTOR (mammalian target of Rapamycin) are also being developed and may provide a further opportunity for targeting the EGFR signalling axis in colon cancer. The mTOR inhibitor, everolimus, inhibits growth of human cancer cell lines which are sensitive or resistant to EGFRi, when used alone or in combination with anti-EGFR drugs gefitinib or cetuximab. Furthermore, everolimus partially restores the ability of EGFRi to inhibit growth and survival in EGFR-resistant cancer cell lines [47]. There are currently two trials testing the combination of Cetuximab

**Table 1. MEK and PI3K Inhibitors Presently in Clinical Trial in Phase I or Phase II Studies in Colon Cancer (ClinicalTrials.gov)**

| Drug       | Manufacturer      | Phase | Target            | Condition                                    | ClinicalTrials.gov Identifier |
|------------|-------------------|-------|-------------------|--|-------------------------------|
| Sorafenib  | Bayer             | II    | <i>BRAF</i>       | Colon Cancer (combination with cetuximab)    | NCT00343772                   |
| PLX4032    | Plexxikon         | I     | <i>BRAF</i> V600E | Melanoma, Colon Cancer                       | NCT00405587                   |
| XL281      | Exelixis          | I     | <i>BRAF</i>       | Solid tumours including colon cancer         | NCT00451880                   |
| GSK1120212 | GlaxoSmithKline   | I     | MEK               | Solid tumours, lymphoma                      | NCT00687622                   |
| AZD6244    | AstraZeneca       | II    | MEK               | Colon cancer (combination with capecitabine) | NCT00514761                   |
| AS703026   | EMD Serono        | I     | MEK               | Solid tumours                                | NCT00982865                   |
| RDEA119    | Ardea Biosciences | I     | MEK               | Solid tumours                                | NCT00610194                   |
| GDC-0973   | Genentech         | I     | MEK               | Solid tumours                                | NCT00467779                   |
| TAK-733    | Millenium         | I     | MEK               | Solid tumours                                | NCT00948467                   |
| RO5126766  | Hoffman-La Roche  | I     | MEK               | Solid tumours                                | NCT00773526                   |
|            |                   |       |                   |  |                               |
| BGT226     | Novartis          | I/II  | <i>PI3K</i>       | Solid tumours, Her2 positive Breast Cancer   | NCT00600275                   |
| XL147      | Exelixis          | I     | <i>PI3K</i>       | Solid tumours, lymphoma                      | NCT00486135                   |
| SF1126     | Semafore          | I     | <i>PI3K</i>       |  | NCT00907205                   |
| PX-866     | ProIX             | I     | <i>PI3K</i>       | Solid tumours                                | NCT00726583                   |
| BEZ235     | Novartis          | I     | <i>PI3K</i>       | Solid tumours                                | NCT00620594                   |
| GDC-0941   | Genentech         | I     | <i>PI3K</i>       | Solid tumours                                | NCT00876109                   |
| XL765      | Exelixis          | I     | <i>PI3K</i>       | Solid tumours                                | NCT00485719                   |
|            |                   |       |                   |  |                               |
| Everolimus |                   | II    | mTOR              | CRC in combination with cetuximab            | NCT00593060<br>NCT00522665    |

and Everolimus in colon cancer which are currently recruiting patients (NCT00593060 and NCT00522665).

Evidence however also exists for the activation of *PI3K* signalling independent of *K-Ras*. Ligand-mediated activation of receptor tyrosine kinases such as EGFR has now been shown to directly activate *PI3K* via adaptor molecules such as Gab1 [45, 48]. Furthermore, in the context of colon cancer, mutations in both *Ras/BRAF* and *PI3K/PTEN* frequently co-exist [32], suggesting these two pathways function independently to provide colon cancer cells with a growth advantage. While the relative importance of *Ras*-dependent and independent *PI3K* activation remains to be fully dissected in colon cancer cells, addressing this issue may have important implications for patient treatment. For example, if *PI3K* is primarily activated by ligand binding to the EGFR, colon tumours with a *K-Ras* mutation but WT for *PI3K* could be treated with a MAPK inhibitor to inhibit hyperactive *Ras* signalling and an existing therapy such as cetuximab to inhibit ligand-mediated signalling via the *PI3K* pathway. Conversely, if *PI3K* activation occurs primarily via *Ras*, EGFR-targeting antibodies are not likely to provide added benefit and co-treatment of these tumours with a MAPK and a *PI3K* inhibitor is likely to be required. Notably, a phase II study testing the combination of sorafenib (RAF inhibitor) with cetuximab is presently underway (NCT00343772), although the study is not restricted to *Ras* mutant patients (Table 1).

### Oncolytic Viruses (Reovirus)

Reovirus serotype 3 is a naturally occurring virus found in the majority of humans where it is generally non-pathogenic [49]. However, it was observed that replication of human reovirus and cell lysis occurs preferentially in transformed cells with activated *Ras* signalling [49]. Mechanistically, this has been linked to inhibition of double-stranded RNA-activated protein kinase (PKR) phosphorylation in *Ras* mutant cells, an event which is required for inhibition of viral protein synthesis and replication [50]. Reovirus (Reolysin, Oncolytics) has therefore been developed as an alternative treatment option for patients with mutant *Ras* tumours [51], with its safety recently demonstrated in 2 phase I studies [52, 53]. A limitation however, is the development of immune sensitisation which while required for preventing systemic toxicity, reduces the anti-tumour efficacy of the virus. Immunosuppressive strategies which reduce neutralisation of the virus to allow for more efficient tumour targeting, but which retain sufficient antibody levels to protect normal cells are currently being developed [54].

Multiple strategies are therefore being developed for the treatment of *Ras* mutant tumours, most of which target downstream components of the MAPK signalling cascade. One point worthy of note is that it is likely that inhibitors that function downstream of mutant *Ras* may also be effective in *Ras* WT tumours. This is sug-

gested indirectly by the efficacy of anti-EGFR targeting Ab's in *Ras* WT tumours, which indicates that these tumours are still reliant on the MAPK signalling pathway, albeit through ligand-mediated canonical activation. Therefore, while agents that target downstream signalling components may be initially trialed in *Ras* mutant patients due to their limited treatment options, the possibility that these agents may also have efficacy in *Ras* WT patients should be explored.

### ACQUIRED RESISTANCE TO TARGETING THE EGFR PATHWAY

Despite advances in the selection of colorectal cancer patients for treatment with anti-EGFR antibodies, WT-*Ras* patients who initially respond to these agents inevitably progress after a few months of treatment. Understanding the mechanisms of acquired resistance to EGFRi could guide rationale drug development and be used to maximise the effect of EGFR-targeted therapy. However, limited clinical data are available to-date which provide definitive mechanistic insight into the basis of acquired resistance, nor is there data from large randomised trials that would suggest alternative therapeutic strategies that would prolong the successful use of EGFR-*Ras*-MAPK targeted therapy in colon cancer. However, lessons into possible mechanisms of acquired resistance may be learned from experience with similar agents in other disease settings, particularly lung and breast cancer. Furthermore, a series of *in vitro* studies have recently been reported which also provide insight into possible mechanisms of acquired resistance.

Collective consideration of the data available from these two sources points to two fundamental mechanisms of acquired resistance: acquisition of secondary mutations within the EGFR pathway as observed in patients who become refractory to tyrosine kinase inhibitors of EGFR in lung cancer [55], or activation of compensatory signalling pathways such as *PI3K*/mTOR [56, 57], Src [58], cMET [59], IGFR [60] and other HER family members [61].

### Lessons from Clinical Trials of Targeting EGFR/Her2 in Lung and Breast Cancer

In NSCLC patients, exquisite sensitivity to the small molecule EGFR tyrosine kinase inhibitors (TKI), gefitinib and erlotinib, is observed in those patients who harbour activating mutations of EGFR, 90% of which are in exon 19 and 21 of the ATP binding pocket [62, 63]. These activating mutations enhance signalling and reliance on the EGFR pathway, but also confer increased binding and fit of the TKI within the ATP pocket [64]. Patients with these mutations therefore have significantly higher response rates and progression free survival than is seen with standard chemotherapy, to those patients who lack the mutation [65]. However, patients treated with EGFR TKIs typically develop resistance after several months treatment, which in approximately 50% of cases is due to the acquisition of secondary mutations within the EGFR [55, 66], with the T790M mutation in exon 20 being the most common [6, 67, 68]. This resistance mutation reduces the potency of ATP-competitive kinase inhibitors such as erlotinib, but may be overcome using newer irreversible inhibitors that use covalent binding [69, 70]. More recently, it was shown that the T790M mutation is detectable in the original NSCLC biopsy specimen in 38% of patients [71]. This mutation was present in a very small percentage of

tumour cells in the original biopsy, with a likely explanation being that treatment with EGFR TKIs selects for the emergence of the pre-existing T790M subpopulation.

In colorectal cancer, no studies to-date have reported the acquisition of resistance through secondary mutations. EGFR mutations are rare in untreated colon cancer patients [72], suggesting this may not be a major target for mutation, although the possibility cannot be discounted. Likewise, acquisition of mutations in *K-Ras* and *PI3K*, or loss of PTEN expression would be worthy of examination in WT tumours which have developed resistance. The increasing ability to detect and isolate Circulating Tumour Cells (CTCs), which could supplant invasive biopsies that have prohibited much work *in vivo* in this area, coupled with the advent of modern sequencing technologies provides a unique opportunity to comprehensively address these possibilities.

Lessons can also be learnt from the targeting of Her2, a surface receptor in the same receptor family as the EGFR. Trastuzumab (herceptin) is a monoclonal antibody that targets the Her2 receptor in breast cancer and there are several parallels to the use of EGFR-targeting antibodies in colon cancer. For example, several studies have shown that mutations within the *PIK3CA* pathway or loss of PTEN expression, contributes to inherent resistance to trastuzumab [59, 73, 74]. Insights gained from the use of this agent may therefore provide clues to possible mechanisms of acquired resistance to EGFR-targeting antibodies in colon cancer. In regards to mechanisms of acquired resistance to trastuzumab, it is notable that as for EGFR-targeting antibodies in colon cancer, no studies reporting acquired resistance due to acquisition of new mutations have been reported. This suggests that in contrast to the genetically based acquired resistance mechanisms observed in lung cancer patients treated with small molecule EGFR inhibitors, non-genetic resistance mechanisms may be more prevalent in driving acquired resistance to antibody-based EGFR inhibitors. Consistent with this notion, acquired resistance due to the formation of alternative Her2 signalling complexes and activation of subsequent downstream signalling has been postulated as a possible mechanism of trastuzumab resistance [75]. This is supported by pre-clinical studies where EGFR inhibitors such as cetuximab can overcome trastuzumab resistance in breast cancer xenografts [76], and by clinical findings where the small molecule dual inhibitor of EGFR and Her2, lapatinib, has efficacy in treating women who have acquired resistance to trastuzumab [77]. Notably, the combination of trastuzumab and lapatinib has also now been shown to be superior to single agent treatment in a recently completed phase III trial [78]. Collectively, these findings indicate that upregulation of alternate HER family members is a mechanism of acquired resistance to HER2 signalling in breast cancer, establishing the possibility that similar mechanisms may be involved in acquired resistance to EGFR targeting Ab's in colon cancer.

A further mechanism of acquired resistance to trastuzumab may be via upregulation of pro-angiogenic factors such as VEGF [79]. Notably, in a mouse breast cancer model of acquired resistance to trastuzumab, upregulation of VEGF was observed, and tumours were responsive to the VEGF-targeting antibody, bevacizumab [76]. Similar observations have now been made in pre-clinical colon cancer models of acquired resistance to EGFRi [80].

### Compensatory Pathways

Further information on acquired resistance has been derived from the generation of cell lines resistant to EGFR-targeting thera-

pies through continuous culture in the presence of EGFRi. Analysis of these cell line models, as well as the parallels with EGFR/Her2 targeting in lung and breast cancer has indicated that compensatory upregulation of alternate signalling pathways such as c-Met [81], other Her family members, IGFR and Src is a major form of acquired resistance to EGFRi.

### C-Met and Other HER Family Members

C-Met is a transmembrane receptor tyrosine kinase which is activated upon binding of its ligand, hepatocyte growth factor (HGF). HGF binding induces receptor dimerisation and increases kinase activity of the receptor, triggering downstream signalling via the MAPK and *PI3K* pathway and ultimately in cell proliferation, migration, and angiogenesis [82]. Evidence for upregulation of c-Met signalling as a mechanism of acquired resistance to EGFR-targeting therapeutics is provided from several studies [57].

First, amplification of the c-MET oncogene has been observed in EGFR mutant lung cancers which develop resistance to EGFR inhibitors but which have not developed secondary mutations within the EGFR [59]. Second, Engelman *et al* demonstrated that gefitinib-resistant NSCLC cell lines express increased amounts of c-Met, and inhibition of c-Met restores sensitivity to gefitinib [59]. The authors also demonstrated that c-Met signalling drives resistance by activating Her3, and increasing signalling via the *PI3K*-Akt pathway [59]. Similar findings were observed by Wheeler *et al.*, where cetuximab resistant NSCLC cell lines were shown to have increased levels of Her2, Her3 and c-Met, with subsequent heterodimerisation of these receptors, and increased downstream signalling via the *PI3K*-Akt pathway [61, 83]. The role of c-Met in acquired resistance to cetuximab in colon cancer cells is therefore worthy of investigation. Significantly, antibody-based therapeutics which target HGF (AMG-102), and small molecule inhibitors which target c-Met (ARQ 197, GSK1363089/XL880), are presently in phase I and II clinical trials (NCT00788957, NCT00558207, NCT00726323).

### IGFR

Insulin Growth Factor 1 (IGF 1), and Insulin Growth Factor 2 (IGF 2) are synthesised in the liver in response to human Growth Hormone (GH). They act as ligands for the Insulin Growth Factor Receptor (IGFR) [84], which is expressed on all human cells apart from hepatocytes and mature B cells. The IGFR is a transmembrane receptor tyrosine kinase which is autophosphorylated upon binding IGF1 and 2, in turn causing phosphorylation of the proteins IRS-1, -2, -3, -4, and Src Homology and Collagen protein (SHC), which in turn activates *PI3K*/AKT signalling.

A role for IGFR in acquired resistance to EGFR targeting therapies is suggested by a study in A431 cells where exposure to increasing amounts of gefitinib resulted in a gefitinib-resistant cell line with increased signalling via the IGF-1R/IRS-1/*PI3K* pathway [85]. The authors also demonstrated that gefitinib resistance was associated with the loss of IGF binding proteins which negatively regulate IGFR signalling, and importantly, gefitinib sensitivity was restored upon IGF-1R inhibition [85]. Notably, gefitinib-resistant cells were also resistant to Erlotinib and Cetuximab, suggesting that IGF-1R upregulation may confer resistance to both antibody and small molecule TKI EGFR inhibitors. Finally, the combinatorial inhibition of IGF-1R and EGFR has been shown to enhance the efficacy of EGFR inhibitors in a variety of cancer cells *in vitro* [86-

89]. As for c-Met, antibody-based therapies which target the IGFR such as AMG 479, are currently in clinical trials including a phase II study in combination with the EGFR-targeting antibody, panitumumab, in advanced colon cancer (NCT00788957).

### Src

Src is the prototypical member of the Src Family Kinase (SFK) group, which comprises 9 non-receptor tyrosine kinases that share similar structures and functions. Src interacts with the EGFR, and is essential for cell signalling via this pathway [90, 91]. Although Src can activate EGFR in the absence of EGF ligand [92], its normal role is to bind and phosphorylate the Y845 residue of EGFR in response to EGF stimulation, increasing MEK and MAPK activity [93, 94].

Notably, the requirement of Src for EGFR-dependent signalling is increased in cell lines that have developed resistance to cetuximab. NSCLC cell lines resistant to cetuximab have increased levels of active SFKs, resulting in hyperphosphorylation of EGFR Y845 [60]. Although SFKs become active by interacting with many receptors including PDGFR and EGFR, targeting the EGFR with siRNA or an EGFR TKI resulted in decreased SFK activity, suggestive that the primary mechanism of SFK activation in resistant cell lines is via its interaction with EGFR [60]. In NSCLC cell lines resistant to cetuximab, treatment with the Src-inhibitor, dasatinib, can re-sensitise cells to cetuximab [60], and reduce downstream signalling via the *PI3K* pathway. Notably, the effect of dasatinib on SFK activity and cell growth was significantly greater in the resistant line, suggestive of dependence on Src. Difi colon cancer cells with acquired resistance to cetuximab (Difi5) also show significantly higher SrcY416 phosphorylation at baseline, and show a preferential decrease in Y845 EGFR compared to its parental line in response to dasatinib treatment, suggestive of a greater role of Src in the resistant cell line [95]. The combination of the Src inhibitor, PP2, and cetuximab also preferentially increased apoptosis in the resistant cell line as compared to the parental line [95].

Src has also been suggested to facilitate acquired resistance to cetuximab in NSCLC cells by facilitating translocation of the EGFR to the nucleus [96]. Nuclear EGFR has been shown to promote cell proliferation by interacting with transcription factors such as STATs and E2F1 and promoting expression of pro-proliferative genes including cyclin D1 and aurora A [97-100], as well as by inducing PCNA phosphorylation and stabilisation [101]. In the cetuximab-resistant NSCLC cells, dasatinib treatment resulted in decreased nuclear EGFR levels and increased membrane levels, and resensitisation of cells to cetuximab [96, 102]. Trials of the combination of cetuximab and dasatinib are planned for colon, head and neck and lung cancer (NCT00835679, NCT00501410).

### VEGF

In addition to cell proliferation and survival, EGFR signalling can also regulate angiogenesis through modulating the production of pro-angiogenic factors such as VEGF [47]. Upregulation of proteins which promote tumour angiogenesis may therefore also be a mechanism of acquired resistance to EGFR treatment. In support of this, overexpression of VEGF in A431 cells renders the cells resistant to EGFR antibody-based therapy *in vivo* [80]. The same authors also demonstrated that A431 xenografts with acquired resistance to anti-EGFR antibodies express significantly higher levels of VEGF compared to parental controls. Likewise, GEO colon cancer

cells rendered resistant to EGFR inhibitors in an *in vivo* xenograft system demonstrated increased VEGF expression and their growth could be inhibited by the VEGFR-1 tyrosine kinase inhibitor, ZD6474 [56]. These findings highlight the importance of angiogenesis in mediating tumour response to EGFRi *in vivo*, and in acquired resistance to these agents. While a potential clinical application of these findings could be the combinatorial use of EGFR inhibitors with anti-angiogenic therapies, increased toxicity when combining EGFR and VEGF targeted therapies has been observed [103, 104], which may limit the routine clinical use of this combination.

## SUMMARY

Factors such as *K-Ras* and *BRAF* mutations govern *de novo* resistance to EGFR-targeted therapy in colon cancer. Less is known about those features of colon cancer cells that govern acquired resistance to these agents, but information from other tumour types and pre-clinical models is now accumulating which provide suggestions for further treatment strategies to prolong response in initial responders. No evidence presently exists for the development of mutations as a mechanism of acquired resistance to EGFRi in colon cancer, although comprehensive mutation screening studies need to be performed before this can be definitively concluded. More evidence presently exists for the increased signalling via alternative pathways as the primary acquired resistance mechanism, including upregulation of other HER family members, IGFR, c-Met and Src. Fortunately, a number of agents which target these alternate pathways are already in clinical development, providing the hope that alternate treatment options may be available for patients who develop resistance to EGFR-targeting therapies, and for the more prolonged and productive use of EGFR treatment.

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## ABBREVIATIONS

|             |   |  |
|-------------|---|--|
| EGFR        | = | Epidermal Growth Factor Receptor           |
| EGFRi       | = | Epidermal Growth Factor Receptor inhibitor |
| TKI         | = | Tyrosine Kinase Inhibitor                  |
| NSCLC       | = | Non Small Cell Lung Cancer                 |
| MAPK        | = | Mitogen-Activated Protein Kinase           |
| VEGF        | = | Vascular Endothelial Growth Factor         |
| IGFR        | = | Insulin-Like Growth Factor Receptor        |
| <i>PI3K</i> | = | Phosphatidylinositol-3-Kinase              |
| TGF         | = | Transforming Growth Factor                 |

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