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Overcoming resistance to molecularly targeted anticancer therapies: rational drug combinations based on EGFR and MAPK inhibition for solid tumours and haematologic malignancies

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Abstract

Accumulating evidence suggests that cancer can be envisioned as a "signaling disease", in which alterations in the cellular genome affect the expression and/or function of oncogenes and tumour suppressor genes. This ultimately disrupts the physiologic transmission of biochemical signals that normally regulate cell growth, differentiation and programmed cell death (apoptosis). From a clinical standpoint, signal transduction inhibition as a therapeutic strategy for human malignancies has recently achieved remarkable success. However, as additional drugs move forward into the clinical arena, intrinsic and acquired resistance to "targeted" agents becomes an issue for their clinical utility. One way to overcome resistance to targeted agents is to identify genetic and epigenetic aberrations underlying sensitivity/resistance, thus enabling the selection of patients that will most likely benefit from a specific therapy. Since resistance often ensues as a result of the concomitant activation of multiple, often overlapping, signaling pathways, another possibility is to interfere with multiple, cross-talking pathways involved in growth and survival control in a rational, mechanism-based, fashion. These concepts may be usefully applied, among others, to agents that target two major signal transduction pathways: the one initiated by epidermal growth factor receptor (EGFR) signaling and the one converging on mitogen-activated protein kinase (MAPK) activation. Here we review the molecular mechanisms of sensitivity/resistance to EGFR inhibitors, as well as the rationale for combining them with other targeted agents, in an attempt to overcome resistance. In the second part of the paper, we review MAPK-targeted agents, focusing on their therapeutic potential in hematologic malignancies, and examine the prospects for combinations of MAPK inhibitors with cytotoxic agents or other signal transduction-targeted agents to obtain synergistic anti-tumour effects.

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Keywords

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1. Introduction

The term 'targeted therapy' refers to a new generation of cancer drugs designed to interfere with a specific molecular target, typically a protein, believed to have a critical role in tumour growth or progression (Scaltriti and Baselga, 2006; Baselga and Arteaga, 2005). The clinical success of the small-molecule tyrosine kinase inhibitor (TKI) imatinib mesylate (Gleevec®) in chronic myeloid leukaemia (CML) and gastrointestinal stromal tumours (GIST) has established a paradigm for the treatment of tumours whose growth is critically dependent on specific kinase targets. Indeed, CML is driven by the mutant kinase fusion protein BCR-ABL, which contains a constitutively activated ABL kinase, while GIST are caused by activating point mutations in the c-KIT or platelet-derived growth factor receptor (PDGFR) kinases. Imatinib effectively blocks the activity of all three kinases and produces dramatic clinical responses in all three clinical situations in a manner that correlates precisely with the presence of these mutations in the tumour (Sawyers, 2003). Encouraging clinical studies have opened the approval path to other targeted agents: the epidermal growth factor receptor (EGFR) inhibitor erlotinib in non small cell lung cancer (NSCLC) (Shepherd et al., 2005); the multi-kinase inhibitors sunitinib (Sutent®) in advanced renal cell cancer (Motzer et al., 2007); and the dual EGFR-HER2 TK inhibitor lapatinib (Tykerb®) in HER2-positive, Trastuzumab-resistant, advanced breast cancer (Geyer et al., 2006).

However, other compounds that specifically target protein kinases have been much less successful in the clinical, especially when combined with classical cytotoxic agents (Becker, 2004). These setbacks reflect a variety of factors, including a rush to get compounds into the clinic, a lack of validated biomarkers, insufficient characterization of patient populations appropriate for treatment, and oversight of pharmacodynamic and scheduling issues. One important point to keep in mind is that a single genetic alteration necessary and sufficient to drive the array of phenotypic hallmarks of malignancy is the exception rather than the rule in human tumours; their malignant behaviour is usually driven by the accumulation of several genetic and epigenetic aberrations (Fojo, 2007). Emerging evidence indeed indicates that clinically successful new therapeutic strategies will most likely rely on the selection of patients whose tumours harbour genetic aberrations that render them “addicted” to the constitutive activation of a certain pathway (and therefore exquisitely sensitive to the inhibition of that pathway), as well as on the mechanism-based manipulation of multiple, cross-talking pathways involved in growth and survival control (Broxterman and Georgopapadakou, 2005; Blum and Kloog, 2005).

Moreover, the therapeutic inactivation of an essential protein creates selective pressures for tumour cells to evolve mechanisms of resistance, in a manner similar to the extensively studied emergence of resistance in microorganisms after exposure to antimicrobial agents (Bardelli et al., 2003; Samuels et al., 2004).

In this review, we will focus on the molecular mechanisms of sensitivity/resistance to agents targeted at EGFR and mitogen-activated protein kinase (MAPK), with particular emphasis on EGFR/MAPK inhibition-based combination strategies, designed to achieve synergistic anti-tumour activity and to overcome resistance to the single agent.

2. Mechanisms of resistance to EGFR tyrosine kinase inhibitors

Resistance to targeted agents may occur by mechanisms similar to cytotoxic agent resistance (Broxterman et al., 2003) such as inactivating metabolism, poor absorption, reduced drug availability or defective immune system-mediated functions. An example is the acquired resistance to imatinib as a result of increased plasma activity of α1-acid glycoprotein, an acute phase reactant which binds the drug and reduces its availability to neutralize BCR-ABL kinase activity in the tumour (Gambacorti-Passerini et al., 2000). Most relevant causes of targeted drug resistance are:

1. specific mutations or loss of the target;
2. activation of alternative TK receptors that bypass the pathway targeted by the specific agent;
3. independent or constitutive activation of intracellular molecular effectors downstream to the target protein;
4. activation of tumour-induced angiogenesis (Figure 1).
5. constitutive EGFR activity.

All these mechanisms have been described as important determinants of the resistance to inhibitors of ErbB/HER family receptors, particularly EGFR and HER2. Therefore, the mechanisms of resistance to EGFR inhibitors may be considered a general paradigm for other molecularly targeted drugs.

2.1. Gene mutations and loss of the target

EGFR mutations were described in various human malignancies including breast, gliomas, prostate, lung and ovarian cancer several years ago (Garcia de Pallazzo et al., 1993). Among them, the most extensively characterized is the EGFR variant III (EGFRvIII), containing an in-frame deletion from exons 2 through 7 in the extracellular domain of EGFR (Lorimer, 2002), that prevents the mutated receptor from binding ligands and results in a constitutive EGFR activation. This mutation is the most frequently expressed EGFR genetic alteration in some cancers, such as glioblastomas (GBM), but it is also reported in breast cancer (Kuan et al., 2001; Li et al., 2004; Lorimer, 2002). GBM cell lines expressing this mutated variant EGFRvIII are relatively resistant to gefitinib; higher doses and longer exposure to this agent are necessary to significantly decrease EGFRvIII phosphorylation (Kuan et al., 2001). The protective activity of EGFRvIII may be due to phosphorylation of AKT, which gefitinib is unable to prevent in cells expressing EGFRvIII (Learn et al., 2004).

In the past two years several studies have correlated EGFR mutations with sensitivity or resistance to EGFR inhibitors (Arteaga, 2006). Specific somatic mutations in the EGFR kinase domain of selected patients with advanced and chemo-refractory NSCLC are associated with dramatic and longlasting clinical responses to the TKIs erlotinib and gefitinib, strikingly correlating with specific characteristics, such as the histological type adenocarcinoma, particularly in the bronchioloalveolar subgroup, the female sex, a never smoking history, and a Japanese/Asiatic ethnicity (Paez et al., 2004). More specifically, Lynch and colleagues (Lynch et al., 2004) observed heterozygous mutations, present in eight of nine patients responding to gefitinib, represented by in-frame deletions within exon 19 and amino acid substitutions within exon 21 of the TK domain. These mutated EGFR forms exhibit a longer EGFR activation upon ligand binding and hypersensitivity to erlotinib and gefitinib (Pao et al., 2004). Along with these mutations conferring hypersensitivity to EGFR TKIs, secondary mutations of EGFR gene in exon 20 were found, which lead to the substitution of methionine for threonine at position 790 (T790M) and confer resistance to gefitinib and erlotinib

(Kobayashi et al., 2005; Pao et al., 2005a). The crystallographic structure analysis of EGFR revealed that the threonine residue is located in the hydrophobic ATP-binding pocket of the catalytic region (Stamos et al., 2002) and is critical for the binding of small-molecule TKIs. Substitution of the threonine with a bulkier amino acid, such as methionine, could sterically interfere with the binding of gefitinib or erlotinib. In fact, the introduction of this type of amino-acid change in the EGFR gene causes resistance to EGFR anilinoquinazoline inhibitors, even in the T766 residue (Blencke et al., 2003).

A mutation in this specific pocket has been found in other TK receptors and correlated with resistance to specific targeted agents (Branford et al., 2002; Roumiantsev et al., 2002; Tamborini et al., 2004). Mutations of intracellular mediators of a specific receptor have also been found, as in the case of *KRAS. KRAS* mutations in NSCLC confer resistance to erlotinib and gefitinib and, interestingly, mutations in EGFR and *KRAS* seem to be mutually exclusive (Pao et al., 2005b).

2.2. Activation of alternative TK receptors that bypass the pathway targeted by the specific agent

Cancer cells often simultaneously activate TK growth factor receptors of different families, such as insulin-like growth factor receptor-1 (IGF-1R), vascular endothelial growth factor receptors (VEGFRs), PDGFR (Board and Jayson, 2005), and c-MET (hepatocyte growth factor receptor), leading to activation of redundant and often overlapping signal transduction pathways that impact multiple cell functions (Samani et al., 2007; Takahashi et al., 1996; Morgillo and Lee, 2005). These receptors can maintain cell survival by replacing EGFR function.

In particular, signaling through the IGF-1R is an important alternative cell survival pathway (Samani et al., 2007), which leads to EGFR inhibitor resistance. IGF-IR transduces signals through insulin receptor substrate-1, which activates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and SHC, which activates the Ras/Raf/MAPK pathway. It is generally agreed that IGF-IR activation plays a key role in cell growth, establishment and maintenance of a transformed phenotype, cell survival and differentiation. IGF-R1 and its ligand insulin-like growth factor (IGF-1) are overexpressed in several cancers and their signaling pathway is altered in cancer cells (Nickerson et al., 2001; Samani et al., 2007). For instance, GBM cells with acquired resistance to the EGFR-TKI AG1478, display enhanced IGF-IR levels and sustained signaling through the PI3K-AKT pathway The combined targeting of IGF-1R and EGFR greatly enhanced apoptosis and reduced the invasive potential of these GBM resistant cells (Chakravarti et al., 2002). The correlation between IGF-1R activation and acquired resistance to EGFR blockade has been demonstrated also for breast and prostate cancer cell lines (Jones et al., 2004). MCF-7 breast cancer cells with acquired resistance to tamoxifen and to gefitinib (MCF-7 TAM/TKI-R) exhibit elevated levels of IGF-IR, PKC and AKT, but no detectable basal phospho-EGFR activity. Treatment of these cells with the specific IGF-IR inhibitor AG1024 resulted in a significant growth inhibition and in a reduced migratory capacity. Similarly, a gefitinib-resistant variant of androgen-independent human prostate cancer cell line DU145 (DU145/TKI-R) activates increased signaling via the IGF-1R pathway (Jones et al., 2004). Importantly, IGF-1R overexpression inversely correlates with response to anti-HER2 MAb Trastuzumab in breast cancer cells (Lu et al., 2005). Moreover, a physical association between HER2 and IGF-IR has been found in tamoxifen- and gefitinib-resistant MCF-7 cells (Balana et al., 2001). Similarly, a heterodimerization of EGFR and IGFR has been recently reported as main determinant of erlotinib resistance in NSCLC cell lines (Morgillo et al., 2006).

2.3. Independent or constitutive activation of intracellular molecular effectors downstream to the target protein

Activation of signalling pathways downstream of EGFR, is caused by gene amplification, overexpression of downstream effectors, such as PI3K/AKT, and/or loss or inactivating mutations of phosphatase and tensin homologue (PTEN), a lipid phosphatase that inhibits the PI3K/AKT pathway (Janmaat et al., 2003; Vivanco et al., 2002), all leading to a persistent activation of the PI3K/AKT and MAPK pathways and consequent development and maintenance of an EGFR resistant phenotype (see Figure 1). A hyperactive PI3K/AKT pathway has been also found in tumour samples from advanced gastric cancer or colorectal cancer patients failing EGFR-targeted therapy. Loss or reduction of PTEN expression occurs in some advanced cancers including GBM, melanoma, endometrial, breast, ovarian, renal cell, thyroid, and a small subset of NSCLC (Cully et al., 2006). The reconstitution of PTEN in PTEN-null cells is able to repress AKT and to inhibit tumour growth via induction of apoptosis or inhibition of cell proliferation (Li et al., 1998; Lu et al., 2004).

The lack of PTEN function in cancer cells is responsible for the resistance to HER2 inhibitor Trastuzumab and to EGFR TK inhibitors (Bianco et al., 2003). For instance, patients with PTEN-deficient breast cancers have significantly poorer responses to Trastuzumab-based therapy than those with normal PTEN (Nagata et al., 2004). Human breast cancer MDA-468 cells, lacking a functional PTEN protein, are relatively resistant to gefitinib treatment (Bianco et al., 2003) display an AKT activity independent from EGFR signals. The introduction of a functional PTEN results in a restored gefitinib-induced AKT inhibition and inhibition of cell growth and apoptosis (Bianco et al., 2003). These effects have been reproduced also with the other EGFR inhibitors, erlotinib and cetuximab. Intriguingly, the dual EGFR and HER2 inhibitor lapatinib has recently shown activity in inflammatory breast cancer patients overexpressing HER2 regardless of PTEN status.

Other signaling downstream to EGFR producing a constitutively activated pathway are Src, a non-receptor tyrosine kinase whose elevated levels correlate with poor prognosis in solid tumours (Aligayer et al., 2002; Dehm et al., 2004; Wiener et al., 2003) and MAPK, whose persistent activation is associated with resistance to EGFR inhibitors in NSCLC and breast cancer (Normanno et al., 2006). Also the signal transduction and activator of transcription (STAT) family, constitutively activated in breast or prostate cancers, is involved in dysregulation of cell cycle and apoptosis.

2.4. Activation of EGFR-independent, tumour-induced angiogenesis

The development of new blood vessels within a tumour mass is promoted by the production of several growth factors. Basic fibroblast growth factor (bFGF), VEGF and transforming growth factor (TGF)-β, secreted by cancer cells, have been identified as positive regulators of angiogenesis. VEGF has an endothelial-specific mitogenic activity exerted by binding to its TK receptors VEGFR-1 (Flt-1) and VEGFR-2 (flk-1/KDR), thereby inducing a signaling cascade and cellular responses (Ferrara et al., 2005). In cancer cells, the EGFR autocrine pathway partly controls the production of several proangiogenic growth factors, including VEGF (Gille et al., 1997; Goldman et al., 1993) and bFGF (Ciardiello et al., 2001).

The inhibition of EGFR activity by selective anti-EGFR agents often results in downregulation of VEGF and other angiogenic factors and of tumour-induced, VEGF-mediated angiogenesis (Ciardiello et al., 1996; Ciardiello et al., 2000; Perrotte et al., 1999; Petit et al., 1997). Viloria-Petit et al. Have demonstrated that an altered control of angiogenesis induces resistance to EGFR inhibitors *in vivo*. In fact, human A431 squamous cell carcinomas xenografted in SCID mice and treated chronically with three different anti-EGFR mAb, mR3, hR3 and cetuximab, eventually develop resistance to these mAb by increasing expression and secretion of VEGF

(Viloria-Petit et al., 2001). Transfection of VEGF into sensitive, parental A431 cells renders these cells significantly resistant to anti-EGFR mAb when injected in nude mice, demonstrating the causal role of deregulated overexpression of VEGF in the acquired resistance to anti-EGFR mAb (Viloria-Petit et al., 2001).

We have provided further evidences of the role played by the VEGF-dependent pathway in the resistance to EGFR inhibitors, generating models of human GEO colon cancer resistant to either small-molecule EGFR-TKI or to anti-EGFR MAb cetuximab (Ciardiello et al., 2004). Analysis of protein expression in samples from mice xenografted with these resistant tumours, revealed no major changes in the expression of EGFR, the EGFR ligand TGFα, Bcl-2, Bcl-XL, p53, MDM2 and AKT, but a 5–10-fold increase in the expression of cyclooxygenase-2 (COX-2) and of VEGF as compared with parental EGFR-inhibitor sensitive xenografts. Combined blockade of EGFR and VEGFR-2/KDR efficiently inhibits tumour growth for as long as five months. A recent study in colorectal cancer patients failing treatment with cetuximab, revealed higher tumour levels of COX-2 and VEGF, supporting our previous observations (Vallbohmer et al., 2005). These results confirm the notion that acquired resistance to EGFR antagonists might arise from enhanced VEGF expression rather than loss of expression or functional alteration of EGFR signalling.

2.5. Constitutive EGFR activity

Constitutive EGFR activity may be achieved in tumor progression without mutation of the EGFR itself or downstream pathway components. EGFR can be activated independently from the presence of ligands and this event, known as transactivation of the receptor, has important implications for cancer development and might by responsible for resistance to anti-EGFR drugs. EGFR, in fact, once produced as a transmembrane precursor, it is often cleaved by some proteases localized on the cell surface, which are able to generate soluble ligands (Harris et al., 2003). This mechanism is known as ectodomain shedding, it is driven from matrix metalloproteinases (MMPs) and disintigrin/metalloproteases (ADAM) and it could probably sustains a constitutive stimulation of the receptor and its downstream pathways, such as MAPK signalling (Luttrell et al., 1999). Some of these proteases are activated by other cell surface receptors called G protein coupled receptors (GPCRs), whose activation by specific agonists enables the EGFR transactivation in cancer cell (Prenzel et al., 1999). In primary breast tumors, high EGFR activity correlates with elevated levels of ADAM proteases (Borrell-Pages et al., 2003) and in prostate cancer altered expression of GPCRs and their ligands induces cancer development (Scher et al., 1995). It has recently been demonstrated that targeting some of these proteases, such as ADAM17 (know also as TNF-α–converting enzyme, TACE), might revert the malignant phenotype in breast cancer cell lines by preventing mobilization of EGFR ligands TGF-α and amphiregulin (Kenny and Bissel, 2007). Moreover, a strong correlation between TACE and TGFα expression is observed in human breast cancers, that is predictive of poor prognosis (Kenny and Bissel, 2007).

3. EGFR-inhibition based combinations of targeted agents

3.1. Inhibition of EGFR and VEGF pathways

The tight connection between EGFR and VEGFR and the increased VEGF expression as escare pathway in the development and maintenance of anti-EGFR drug-resistant phenotype accounts for the rational combination of inhibitors targeting both signal transduction pathways. Several preclinical studies have provided the rational basis for such strategy, reporting an additive or even synergistic interaction (Datta et al., 1999; Hsuan et al., 1997; Jung et al., 2002). We have first demonstrated that an association of cetuximab with a human VEGF antisense (AS) 21-mer phosphorothioate oligonucleotide (VEGF-AS) in human GEO colon cancer resulted in a selective inhibition of growth factor production - including VEGF, bFGF and TGF - and of

neo-angiogenesis and a synergistic tumour growth inhibition in xenografted mice (Ciardiello et al., 2000). Combination of the VEGFR2 antibody DC101 and cetuximab significantly inhibited the growth of TMK-1 gastric cancer, decreased tumour vascularity and increased endothelial cell apoptosis (Jung et al., 2002).

On the basis of these encouraging data several clinical studies were initiated. Different approaches have been used to block EGFR and VEGF/VEGFR, including the combination of two specific agents and the use of multi-targeted drugs.

Combination of anti-EGFR mAb cetuximab with anti-VEGF mAb bevacizumab provided preliminary evidence of activity and increase in time to progression in colorectal cancer patients failing several lines of chemotherapy in a study known as Bond-2 (Saltz et al., 2005). Several phase II and III studies are now ongoing in colorectal cancer patients evaluating the combination of bevacizumab with either cetuximab or the other anti-EGFR mAb panitumumab. The combination of bevacizumab with the small-molecule TKI erlotinib is clinically investigated in renal cell, NSCLC, colorectal and pancreatic cancer with encouraging anti-tumour activity and safety data (Hainsworth et al., 2005; Herbst et al., 2005).

An alternative approach is the use of multi-target antagonists. AEE 788 and ZD6474/vandetanib are two examples of orally available inhibitors of both VEGFR and EGFR dependent pathways. Phase I/II clinical studies with ZD6474 have shown good tolerability, a specific side effect being QTc prolongation, and activity in NSCLC patients previously treated with chemotherapy. We have recently demonstrated that ZD6474 may synergize with cetuximab in preclinical models (Morelli et al., 2006). The combined blockade of EGFR and VEGF or VEGFR is thus a therapeutic strategy proven to be successful in different types of cancer (Morabito et al., 2006).

3.2. Combination of EGFR and mTOR inhibitors

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase downstream mediator in the PI3K/AKT signaling pathway that plays a critical role in regulating cell proliferation, growth, survival, invasion and angiogenesis (Figure 1). Moreover, activation of mTOR can occur independently from EGFR signaling trough non-PI3K/AKT pathways (Nobukuni et al., 2005;Shaw et al., 2004). Everolimus (RAD001) and temsirolimus (CCI-779) are rapamycin analogues that selectively inhibit mTOR function and have demonstrated promising activity in early clinical trials (Adjei et al., 2005a). Because EGFR and mTOR functions control linked signaling pathways, the combination of their specific inhibitors may represent a rational therapeutic strategy. Gefitinib and rapamycin in combination synergistically inhibit the growth of renal cell carcinoma lines, especially those without von Hippel-Lindau (VHL) mutations (Gemmill et al., 2005). Rapamycin is able to enhance the sensitivity of other TKI such as erlotinib, even in PTEN-deficient tumour cells. Combined EGFR/mTOR kinase inhibition inhibits PI3K pathway signaling, promoting cell death in PTEN-deficient tumour cells (Wang et al., 2006b).

Early clinical trials in patients with recurrent GBM have shown that either gefitinib or erlotinib in combination with the mTOR inhibitor sirolimus provide an encouraging percentage of objective response (Doherty et al., 2006). New multi-targeted agents directed against EGFR-dependent pathways and mTOR have been designed: the single agent PI-103 possess the unique capability of simultaneously blocking both PI3K/AKT and mTOR signaling, showing significant activity in GBM xenografts (Fan et al., 2006). Based on this preclinical evidence clinical trials of temsirolimus or everolimus in combination with EGFR TKI are now ongoing.

3.3. Inhibition of signaling from EGFR and Ras

The critical role of Ras in the transduction machinery of signaling from cell surface receptors to downstream molecular effectors and its relationship with development of resistance against EGFR antagonist explain the importance of Ras as a target of novel anticancer combinations (Blum and Kloog, 2005). Furthermore, Ras mutations induce its constitutive activation, producing persistent stimulation of tumour cell proliferation and inhibition of apoptotic cell death. It has been proposed that inhibition of Ras/Raf/MAPK signaling with farnesyl transferase inhibitors (FTI) may enhance the anti-tumour activity of EGFR inhibitors. Consistent with this hypothesis, AZD3409, a novel prenyl inhibitor active against both farnesyl transferase and geranyl-geranyl transferase, has shown potent growth inhibitory activity in tumour cells resistant to EGFR antagonists and synergism in combination with gefitinib. Combination of gefitinib with the FTI SCH66336 cooperatively inhibited the growth of NSCLC cells (Janmaat et al., 2006).

3.4. Multi-target agents targeting multiple signalling pathways

A multi-target inhibition approach that combines inhibitors of angiogenesis and the Ras/Raf/MAPK pathway and EGFR has been examined. Sorafenib (BAY 43-9006) is an oral multi-kinase inhibitor able to block several different targets, such as Raf kinase and VEGFR and PDGFR TKs (Wilhelm et al., 2004). Combining EGFR antagonists and sorafenib appears, at least theoretically, an interesting approach, able to inhibit growth factor signaling upstream at the level of EGFR and downstream at the level of Raf kinase. Moreover, inhibition of VEGFR, PDGF and Raf in endothelial and tumour cells may induce a strong simultaneous anti-angiogenic effect (Wilhelm et al., 2004). In preclinical studies, the combination of gefitinib with sorafenib resulted in tumour growth inhibition of A549 NSCLC xenografts with almost no toxicity (Carter et al., 2007). A phase I study on the combination of gefitinib and sorafenib has been conducted in patients affected by metastatic NSCLC. Among 30 evaluable patients, one partial response and 20 disease stabilizations were observed, with a median duration of 20.4 weeks (range 5.9–43.9 weeks) (Adjei et al., 2005b). The treatment was well tolerated, the most common drug-related adverse events being diarrhoea, fatigue and transaminase elevation. This combination strategy is now under further evaluation.

4. The MAPK pathway

Approximately twenty years after their initial discovery, at least four major MAPK families have been identified in mammalian cells: extracellular-signal regulated kinase (ERK-1/2), c-Jun-N-terminal kinase (JNK)-1/2/3, p38α/β/γ/δ, and ERK-5. They exert specific, albeit cross-talking, roles in the regulation of fundamental cellular functions. A detailed description of molecular themes underlying MAPK activation and function is beyond the scope of this review and has been covered by other recent overviews (Avruch, 2006; Chambard et al., 2006; Kondoh et al., 2006; Whitmarsh, 2006). Briefly, the basic MAPK module consists of three protein kinases that are sequentially activated by a phosphorylation cascade: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK (Lewis et al., 1998; Seger et al., 1995). A high degree of redundancy and overlap occurs upstream of MAP3K activation and the existence of a plethora of MAP3Ks reflects the exceptional variety of signals capable of recruiting these pathways, usually in combinatorial arrays. However, specificity progressively increases as signal transduction proceeds, so that little or no crosstalk exists between different modules at the MAPK level (Lewis et al., 1998; Seger et al., 1995).

Among the different MAPK modules, the Raf/MAPK/ERK kinase (MEK)/ERK is the most extensively studied and perhaps the most relevant to cancer pathogenesis and therapy (Kohno et al., 2006; McCubrey et al., 2006; Sebolt-Leopold et al., 2004a). This signaling module is activated by several extracellular stimuli that converge on the small G-protein Ras. It plays a

pivotal role in the control of cell proliferation, differentiation, and survival in response to the engagement of receptor tyrosine kinases, G protein-coupled receptors, and integrins (Lewis et al., 1998; Seger et al., 1995). Activated Ras, in turn, recruits the MAP3K Raf to the plasma membrane in a necessary, but not sufficient, activation step, allowing the mitogenic signal to proceed through the MEK/ERK module. MEK activation is a crucial step in signal transduction through the Raf/MEK/ERK cassette: MEK-1/2 belong to a small family of dual specificity kinases and catalyze the phosphorylation of ERK on both Ser/Thr and Tyr residues, allowing their full activation. This activation step is endowed with extremely high specificity, in that MEK is the only ERK kinase and ERK is the only MEK substrate identified thus far. Such high specificity has made MEK activation and enzymatic activity a prime target for pharmacological interventions directed against this MAPK module. ERK is the dominant multifunctional effector of the MAP3K/MAP2K/MAPK cassette: it directly phosphorylates many transcription factors including Ets-1, c-Jun, and c-Myc; phosphorylates and activates the 90 kDa ribosomal S6 kinase ($p90^{Rsk}$), leading to the activation of the transcription factor CREB; phosphorylates many proteins involved in cell cycle and apoptosis regulation (Figure 1); and may lead to activation of the NF-κB transcription factor (nuclear factor immunoglobulin κ chain enhancer-B cell) by phosphorylating and inactivating the inhibitor κB kinase (IKK) (Chambard et al., 2006; Lewis et al., 1998; McCubrey et al., 2006; Seger et al., 1995; Whitmarsh, 2006).

4.1. Targeting MEK for cancer therapy

The pivotal role played by the Raf/MEK/ERK module in the physiological regulation of many cellular processes, such as growth, proliferation, differentiation, survival, motility, and angiogenesis, provides the conceptual framework to understand the oncogenic potential of deranged signaling through this MAPK module (Avruch, 2006; Chambard et al., 2006; Lewis et al., 1998; McCubrey et al., 2006; Seger et al., 1995; Whitmarsh, 2006). Indeed, many cellular oncogenes, such as growth factor receptors and Ras, critically rely on activation of the Raf/MEK/ERK pathway to induce a transformed phenotype. In addition, members of this MAPK cascade, such as Raf and Mos, have been themselves identified as cellular oncogenes (Lewis et al., 1998; Seger et al., 1995). Although no naturally occurring MEK or ERK oncogenes have been identified, both proteins can efficiently transform mammalian cells to a neoplastic phenotype when expressed in a constitutively active form (Cowley et al., 1994; Mansour et al., 1994; Robinson et al., 1998) and disruption of their activation by pharmacological inhibitors severely impairs the transforming ability of many upstream-acting cellular oncogenes (Duesbery et al., 1999; Lewis et al., 1998; Sebolt-Leopold, 2000). As a result, constitutive MEK/ERK activation is detected in a significant proportion of a variety of human tumours, including breast, kidney, colon, pancreatic, thyroid and lung cancers, as well as GBM, and has recently emerged as a potential target for anticancer therapies (Kohno et al., 2006; Sebolt-Leopold et al., 2004a).

Not only is constitutive activation of the MEK/ERK module frequently observed in experimental and human tumours, but rapid ERK inactivation, as opposed to slower decay of the activity of other MAPK families endowed with pro-apoptotic activities such as the JNK and p38 families, may also be one of the factors underlying the massive apoptotic response elicited by several signal transduction-targeted agents, a phaenomenon referred to as “oncogene addiction” or “oncogenic shock”. Indeed, it has been recently suggested that rapid diminution of phospho-ERK, -AKT, and -STAT3/5 and delayed accumulation of the proapoptotic effector phospho-p38 MAPK may substantially contribute to cell death following the pharmacologic or genetic inactivation of several oncogenes, such as Src, BCR-ABL, and EGFR (Sharma et al., 2006a; Sharma et al., 2006b; Evan, 2006). These findings support the idea that the MEK/ERK signalling module may constitute a common therapeutic target downstream an array of diverse oncogenic genetic lesions.

As discussed above, the modular nature of the Raf/MEK/ERK cascade becomes less pleiotropic at the crossover point that is regulated by MEK. Indeed, no substrates for MEK have been identified other than ERK. This tight selectivity, coupled with the availability of monoclonal antibodies specific for the dually phosphorylated, active form of ERK, makes MEK inhibition exquisitely amenable to pharmacodynamic evaluation (Herrera et al., 2002; Sebolt-Leopold et al., 2003). In fact, phosphorylated ERK is the product of MEK activity and thus its *ex vivo* detection in tumour tissues (by either Western blotting or recently developed flow cytometric techniques (Bardet et al., 2006; Ricciardi et al., 2005)) could provide a direct measure of *in vivo* MEK inhibition. At the preclinical/early clinical stage, such pharmacodynamic assays are not only useful for optimizing the design of dosing regimens, but also offer the advantage of being able to correlate anti-tumour efficacy with inhibition of the biochemical target. All these reasons make MEK a very attractive target for anticancer drug development.

MEK inhibitors differ from most of the other currently available kinase inhibitors, in that they do not compete with ATP binding and therefore are endowed with an unusually high specificity towards their target (Delaney et al., 2002); indeed, none of these compounds significantly inhibit the activity of a large panel of protein kinases that include ERK1, JNK1 and p38 MAP kinases in an *in vitro* assay (Davies et al., 2000). Recently, crystal structures of MEK-1 and -2 have been determined as ternary complexes with Mg-ATP and PD184352-like inhibitors (see below), showing that both enzymes have a unique inhibitor-binding site located in an interior hydrophobic pocket adjacent to, but not overlapping with, the Mg-ATP-binding site (Ohren et al., 2004). Binding of MEK inhibitors to this hydrophobic pocket induces several conformational changes in unphosphorylated MEK, locking them into a closed and catalytically inactive conformation. Notably, the MEK inhibitor binding-site is located in a region where the sequence homology to other protein kinases is quite low. With the exception of MEK-2 (100% identical) and MKK-5 (81% identical), all other protein kinases share low sequence identity (60%–70%) with MEK-1 in the inhibitor-binding site, thereby explaining why PD184352-like MEK inhibitors are exceptionally specific for MEK-1, MEK-2, and MKK-5 (although to a much lesser extent), but do not inhibit many other protein kinases (Kohno et al., 2006; Ohren et al., 2004).

First-generation MEK inhibitors, such as PD98059 (Alessi et al., 1995; Dudley et al., 1995) and U0126 (Favata et al., 1998), have been extremely useful *in vitro* for establishing the role of the MEK/ERK module in a variety of biological processes. However, their unfavourable pharmacologic characteristics have largely precluded *in vivo* use and clinical testing (Kohno et al., 2006; Wang et al., 2006a). CI-1040 (PD 184352) was the first MEK inhibitor reported to inhibit tumour growth *in vivo* (Sebolt-Leopold et al., 1999) and, based on its anti-tumour activity in a variety of preclinical models of human cancer, was subsequently moved into clinical trials in patients with advanced solid tumours. In phase I and II studies of CI-1040, 77 and 67 patients with a variety of solid tumours were treated, respectively. Treatment was generally well tolerated and phosphorylated ERK levels, measured in tumour samples by quantitative immunohistochemistry, were found to be inhibited by an average of 71% (range, 46% to 100%), indicating promising on-target activity. However, the metabolic stability, bioavailability, and clinical activity (one partial response and 27 disease stabilizations) were considered insufficient to warrant further development in the tumour types tested and development of CI-1040 was terminated in favour of developing more potent and biopharmaceutically superior compounds (Kohno et al., 2006; Lorusso et al., 2005a; Rinehart et al., 2004; Wang et al., 2006a).

Two novel, orally bioavailable, MEK inhibitors (PD0325901 and ARRY-142886, a.k.a. AZD6244) endowed with increased potency against MEK (IC_{50}: 1–10 nM) and superior biopharmaceutical properties (including improved bioavailability, longer-lasting target suppression, and lower metabolic clearance) have recently been described (Kohno et al.,

2006; Sebolt-Leopold et al., 2004a; Sebolt-Leopold et al., 2004b; Wang et al., 2006a; Yeh et al., 2007). Both compounds have shown promising preclinical activity *in vitro* and *in vivo* against a broad spectrum of solid tumours and haematological malignancies (see below) and are currently in Phase I/II clinical testing (Adjei et al., 2006; Chow et al., 2005; Lorusso et al., 2005b; Menon et al., 2005). While effective suppression of ERK phosphorylation in either paired tumour biopsies or peripheral blood mononuclear cells has been demonstrated with both compounds, objective responses have been so far reported only with PD0325901 (2 partial responses in patients with malignant melanoma).

4.2. Molecular determinants of sensitivity/resistance to MEK inhibitors

The challenges we face in the design and interpretation of clinical trials of MEK inhibitors do not differ substantially from those faced with other anticancer agents, particularly signal-transduction inhibitors. Indeed, we do not presently know which tumour types will be most sensitive or which molecular alterations of the target or pathway are common to patients who benefit (or do not benefit) from treatment.

As mutations in *MEK* or *ERK* had not been described until recently (Rodriguez-Viciana et al., 2006), much attention has focused on mutations of *RAS* and *RAF*, long-known cellular oncogenes and immediate upstream activators of MEK, as possible molecular markers of sensitivity to MEK inhibition. Mutations in *RAS* and *RAF* (particularly *BRAF*) are common in human tumours and typically demonstrate mutual exclusivity, suggesting that either mutation might exert its oncogenic activity through common downstream proteins, such as the MEK/ERK kinase module (Kohno et al., 2006; McCubrey et al., 2006; Wang et al., 2006a). Using small-molecule MEK inhibitors in cells with *RAS* or *BRAF* mutations, Solit et al. have recently demonstrated that tumours with *BRAF* mutations display enhanced sensitivity to MEK inhibition when compared with wild-type cells and cells harbouring various *RAS* mutations (Solit et al., 2006). In addition, following treatment with MEK inhibitors, the growth of tumours in *BRAF*-mutant xenografts was completely suppressed, whereas *RAS*-mutant tumours were only partially inhibited. Extension of this study to the NCI 60 cell lines, for which a large body of data from inhibitor screening assays could be interrogated, yielded supportive information; the top-ranking compounds that scored on ^{V600E}*BRAF*-positive lines happen to represent predominantly MEK inhibitors with similar effectiveness as CI-1040 (Solit et al., 2006). From a molecular standpoint, recent data from Garnett et al. (Garnett et al., 2005) indicate that, even though a small fraction of *BRAF* mutations generates an enzyme that is impaired in its ability to activate the downstream MEK/ERK cascade, kinase-impaired mutants also work through the mitogenic cascade culminating in ERK activation. The mechanism is rescue of kinase-impaired mutant *BRAF* by wild-type C-RAF through a process that involves 14-3-3-mediated hetero-oligomerization and transactivation (Garnett et al., 2005; Rapp et al., 2006).

Alternatively, measurement of baseline levels of doubly phosphorylated ERK, the direct target of MEK enzymatic activity, in tumour biopsies and/or archived tumour tissue could be used to identify patients/tumour types in which the MEK/ERK module is constitutively active and that would potentially benefit from MEK inhibition-based therapeutic strategies. To this end, as well as for pharmacodynamic monitoring purposes, Western blot- and flow cytometry-based methods have been devised that allow the accurate and quantitative detection of phosphorylated ERK (pERK) in tumour tissue (Bardet et al., 2006; Ricciardi et al., 2005; Sebolt-Leopold et al., 2003). Though a mild association is seen between baseline pERK levels in archived tumour samples and subsequent stable disease, pERK inhibition in either peripheral blood mononuclear cells (PBMC) or in tumour tissues from patients receiving MEK inhibitor therapy has not correlated with clinical benefit (Wang et al., 2006a). Therefore, the presence of activated ERK, as well as the percentage of ERK inhibition, may not be sufficient in themselves as a guide to the anticancer effects of MEK inhibition. One possible explanation for the failure

of pERK reduction during MEK inhibitor therapy to predict clinical outcome is that tumour pERK levels are examined at pre-specified time points, and these data may reflect ERK activation at that time, but may not differentiate between short-lived mitogen-activation and sustained constitutive MAPK pathway activation. Kinetics consideration may also be of importance in determining the overall effect (apoptosis induction versus growth arrest) of MEK blockade in different clinical situations, as recently suggested by the "oncogenic shock" model (Sharma et al. 2006a; Sharma et al. 2006b).

An additional technical limitation lies within tumour sampling itself; tumour heterogeneity is likely to represent a key challenge in the attempt to quantitate the true ERK activation status of a given tumour. Unless biopsies are obtained from several different regions of a tumour, a true representation of the tumour profile may not be obtained, resulting in a false reading.

Potential mechanisms of resistance to MEK inhibition-based therapeutic strategies are currently similarly elusive. A molecular explanation for acquired resistance to imatinib has been provided by detailed studies characterizing its target, the BCR–ABL chromosome-translocation product. This fusion protein undergoes mutations in its kinase domain that change Thr-315 to an isoleucine residue (Branford et al., 2002). This hot spot in the ATP-binding site has been also identified in other kinases, such as EGFR and PDGFR, and might therefore undergo mutations that confer resistance to other drugs that target tyrosine kinases (see above). It is tempting to speculate that the non-ATP-competitive inhibitors of MEK that are now in clinical trials will not be subject to this type of resistance. The very absence of activating mutations, which rendered MEK an undesirable drug target to many researchers years ago, could ultimately allow this enzyme to be an effective therapeutic target.

Even though it is too early to tell whether clinical resistance to MAPK-pathway inhibitors will be encountered, as has been the case with other kinase inhibitors, preclinical data are starting to shed light on potential resistance mechanisms that may be operative in cancer cells exposed to MEK inhibitors. Recently, CI-1040–resistant clones were derived from the C26 mouse colon carcinoma cell line after long-term exposure to CI-1040 (Wang et al., 2005). The resistance of C26/CI-1040r cells was due to a combination of resistance to both growth inhibition and apoptosis in response to the drug; moreover, C26/CI-1040r cells exhibited elevated expression of activated KRAS. Consistently, KRAS expression was shown to increase in MEK inhibitor–resistant lines derived from *in vivo* experiments and overexpression of active *KRAS* in C26 parental cells also conferred resistance to CI-1040, suggesting high-level expression of active *KRAS* as a possible molecular mechanism for resistance to MEK inhibitors. In a subsequent report by the same group (Klein et al., 2006), MEK suppression by PD184161 (a MEK inhibitor structurally related to CI-1040) in preclinical models of hepatocellular carcinoma was only achieved in "naïve" tumours that had received a single drug dose, but not in tumours "conditioned" by multiple drug doses. Systemic efficacy of PD184161 was unlikely to be responsible for the lack of drug effectiveness, since MEK activity in the lung was effectively suppressed with PD184161 treatment after repeated dosing. While in this report the lack of growth inhibition appears to correlate with the lack of suppression of pERK levels, other signalling pathways could be involved in the growth of these tumours and different tumour types may behave differently (Collisson et al., 2003; Kramer et al., 2004; Sebolt-Leopold et al., 1999). Interestingly, our group has also recently observed the lack of effective pERK suppression in selected breast cancer and lymphoblastic leukaemia cell lines that are intrinsically resistant to growth inhibition induced by the MEK inhibitor PD0325901 (Milella, unpublished results).

The identification of relevant biomarkers and early response markers for the selection of patients most likely to derive the greatest clinical benefit from MEK-targeted therapies remains crucial to the clinical development of such agents. However, information currently available

does not allow to draw any definitive conclusion and biomarkers/predictive markers appear too premature to be the hinge driving MEK-directed therapeutic programs forward at this time.

5. The MEK/ERK pathway as a therapeutic target in haematological malignancies

Acute myeloid leukaemia (AML) is a deadly disease, resulting from the clonal expansion and accumulation of haematopoietic stem cells arrested at various stages of development (Löwenberg et al., 1999). Genetic aberrations that disrupt the function of haematopoietic transcription factors play a central role in leukaemogenesis; in addition to transcription factor fusion proteins, aberrant activation of the kinase-based signal transduction pathways that normally translate extracellular stimuli into appropriate homeostatic responses can powerfully contribute to leukaemogenesis by enabling leukaemic cells to grow autonomously and escape programmed cell death (Lee, Jr. et al., 2002; McCubrey et al., 2000; McCubrey et al., 2006; Steelman et al., 2004). A new paradigm is thus emerging, in which acute leukaemia could be modelled as comprising at least two mutational events: activation of a kinase-based signaling pathway, which confers proliferative and/or anti-apoptotic activity to haematopoietic cells without affecting differentiation, and a transcription factor fusion protein, which has a limited effect on cell transformation or proliferation, but impairs normal differentiation pathways (Deguchi et al., 2002; Frohling et al., 2005).

The MAPK pathway that proceeds from Ras and its downstream effector Raf to MEK and ERK, is a key integration point along the signal transduction cascades that emanate from receptor- and/or fusion protein-associated tyrosine kinases and links diverse extracellular stimuli to proliferation, differentiation, and survival (McCubrey et al., 2006; Rubinfeld et al., 2005). We and others have recently provided substantial evidence that the MEK/ERK signaling module is frequently deregulated in myeloid leukaemias and other haematological malignancies, as a result of genetic and epigenetic aberrations involving both receptor-associated and cytoplasmic tyrosine kinases, as well as inhibitory phosphatases (Milella et al., 2003; Milella et al., 2005). Constitutive activation of this MAPK module is particularly common in AML, where ERK phosphorylation/activation is detected (by Western blotting, *in vitro* kinase assay, or flow cytometry) in primary leukaemic blasts in 50% to 90% of patients (Bonati et al., 2000; Iida et al., 1999; Kim et al., 1999; Kornblau et al., 2001; Lunghi et al., 2001; Milella et al., 2001; Ricciardi et al., 2005; Towatari et al., 1997). Conversely, constitutive ERK activation is usually not detectable in CD34+ haematopoietic bone marrow progenitors from healthy donors or from leukaemic patients in complete remission (Bonati et al., 2000; Kim et al., 1999; Lunghi et al., 2001; Milella et al., 2001; Ricciardi et al., 2005; Towatari et al., 1997). Most interestingly, from a clinical standpoint, both retrospective (Kornblau et al., 2001) and prospective (Kornblau et al., 2006) analyses of pERK levels in primary blasts obtained at diagnosis from AML patients indicate that high pERK levels are an independent predictor of worse overall survival, as a result of a combination of lower remission rates, shorter remission durations, and higher relapse rates.

Limited information is available, at this time, on the presence and role of constitutive ERK signaling in acute lymphoblastic leukaemia (ALL). Constitutive ERK activation in ALL cell lines and in a limited number of clinical ALL specimens has been reported (Meng et al., 2003; Towatari et al., 1997), together with suggestion that elevated pERK levels may be prognostic for survival (Meng et al., 2003). We have recently analysed constitutive ERK phosphorylation by flow cytometry in the largest series (n=131) of primary adult ALL samples reported so far and found that approximately 30% of cases express pERK; constitutive pERK expression was significantly associated with higher WBC values and, most importantly, lower CR rates (Gregorj et al., 2007).

Constitutive - and functionally relevant - activation of the MEK/ERK axis has been also recently reported in other haematological malignancies, including myelodysplastic syndrome (MDS) (Steensma et al., 2003), hairy cell leukaemia (Kamiguti et al., 2003), and NK-type lymphoproliferative disease of granular lymphocytes (Epling-Burnette et al., 2004).

5.1. Preclinical activity of MEK inhibitors in haematological malignancies (Table 1)

Collectively, the above-reported data indicate that constitutive activation of the MEK/ERK MAPK module frequently occurs in human acute leukaemias (particularly in AML), but not in normal haematopoietic progenitors, suggesting that it could be exploited for therapeutic purposes in haematopoietic malignancies. Indeed, pharmacological MEK inhibitors, such as PD98059, U0126, and CI-1040, have shown substantial growth inhibitory and pro-apoptotic activity *in vitro* in both cell line models and primary samples of CML (Chu et al., 2004; Kang et al., 2000), AML (including the acute promyelocytic leukaemia, APL) (Baines et al., 2000; James et al., 2003; Kerr et al., 2003; Lunghi et al., 2003; Milella et al., 2001; Morgan et al., 2001), ALL (Meng et al., 2003), and MDS (Steensma et al., 2003). In most studies, MEK inhibitor-induced growth inhibition is due to a combination of inhibition of cell cycle progression and induction of apoptosis, which take place preferentially in cell lines and primary samples with high levels of constitutive ERK phosphorylation, but not in pERK-negative leukaemias or CD34+ haematopoietic progenitors from healthy donors (Milella et al., 2001). The differential sensitivity of leukaemic cells with constitutive MEK/ERK activation to pharmacological MEK inhibitors (namely CI-1040) has also been recently demonstrated both *in vitro* and *in vivo* in murine models of Raf-1-driven AML (Konopleva et al., 2005).

More recently, we have analyzed the molecular and functional effects of the novel MEK inhibitor PD0325901 in cell line models of different haematopoietic malignancies (Milella, manuscript in preparation): the growth of AML cell lines with known constitutive ERK activation (OCI-AML3, OCI-AML2, HL-60, and, to a lesser extent, NB4) was found to be exquisitely sensitive to MEK inhibition by PD0325901, with IC_{50}s in the low nanomolar range. As mentioned above for other MEK inhibitors, growth inhibition was due to a combination of G_1 cell cycle arrest and induction of apoptosis, which were also observed in response to PD0325901 in a substantial proportion of freshly isolated blasts from patients diagnosed with AML. Genetic aberrations potentially resulting in the “addiction” of transformed cells to MEK activity were also explored in the murine FDC-P1 model transfected with different oncogenes: in this model, constitutive activation of Fms, Ras, Raf, MEK, IGF-1R, and STAT5a conferred hypersensitivity to MEK inhibition, resulting in apoptosis induction at sub-nanomolar concentrations of PD0325901. Phosphoprotein and gene expression profiling of OCI-AML3 cells exposed to PD0325901 revealed extreme selectivity of the drug for its target (with a 5- to 8-fold reduction in ERK1/2 phosphorylation at 10 nM) and marked modulation of downstream targets, particularly genes and proteins involved in cell cycle regulation (such as cyclin D3, cyclin E, and cdc25A).

Recent data obtained using ARRY-142886 indicate that MEK inhibition also induces potent growth-inhibitory and pro-apoptotic effects *in vitro* in multiple myeloma (MM) models, both cell lines and primary cultures in the presence or absence of bone marrow stromal cells. The effects are due, at least in part, to the downregulation of autocrine and paracrine cytokine loops and adhesion molecules mediating stromal cells’ anti-apoptotic activity. Interestingly, the expression of the c-*MAF* oncogene, which is overexpressed in approximately 50% of MM, and its downstream targets integrin β7, CCR1, and cyclin D2, were profoundly downregulated by ARRY-142886 in MM models exposed to hypoxia and/or IL-6 (Tai et al., 2006a; Tai et al., 2006b).

Overall, these results strongly support the hypothesis that constitutive ERK activation in AML blasts (and possibly in other premalignant/malignant haematological disorders) is crucial to

their ability to proliferate and survive default apoptosis induction in the absence of specific survival factors or in response to death stimuli. Not only is this constitutive activation crucial, but it also confers a high sensitivity to inhibitors of the MEK/ERK pathway that could be exploited for therapeutic purposes. Conversely, recent data indicate that in normal haematopoietic progenitors the activation of the MEK/ERK module is not only dispensable for expansion, proliferation and self-renewal, but could rather mark the transition from proliferation to maturation, thereby limiting the proliferative potential of self-renewing stem cells (Bonati et al., 2002; Fichelson et al., 1999) and thus providing the basis for a highly selective anti-leukaemic activity of MEK inhibitors.

5.2. Prospects for MEK inhibition-based combinations with synergistic anti-leukaemic activity

Although exceptions occur, the bulk of evidence indicates that constitutive activation of the MEK/ERK signaling module increases the apoptotic threshold of leukaemic and other cancer cells, consistent with its ability to regulate the expression and function of multiple anti-apoptotic players through transcriptional and non-transcriptional mechanisms (Figure 2). In particular, MAPK signaling may favour cell survival both at the mitochondrial level, through regulation of the expression and function of pro- and anti-apoptotic Bcl-2 family members (BAD, Bim, Bcl-2, Mcl-1, etc.), and at the cytosolic caspase activation level, through regulation of the expression of caspase inhibitors of the IAP family (survivin, XIAP) and the recently described direct phosphorylation and inactivation of pro-caspase-9 (McCubrey et al., 2006;Milella et al., 2005). However, at concentrations close to the IC_{50} for ERK enzymatic activity, MEK inhibitors have cytostatic rather than cytotoxic effects and higher doses are required to efficiently trigger apoptosis (Milella et al., 2001), suggesting that other parallel cytoprotective pathways that help maintain cell viability may be operative in cancer cells (Lee, Jr. et al., 2002;Steelman et al., 2004). Nevertheless, one of the most intriguing features of MEK inhibitors as potential anti-cancer agents is their ability to lower leukaemic cells' apoptotic threshold, setting the stage for increased sensitivity to the pro-apoptotic action of classical cytotoxic drugs, ionizing radiation, and other biological agents that modulate apoptosis (Dent et al., 2001;Milella et al., 2005). Together with their amenability to pharmacodynamic evaluation and negligible systemic toxicity, these apoptosis-sensitizing actions make MEK inhibitors an ideal starting point to build pharmacological combinations with synergistic anti-leukaemic effects.

5.3. MEK inhibition-based combinations with cytotoxic agents

Several lines of evidence indicate that the activity of the MEK/ERK module may be particularly critical in regulating chemosensitivity in leukaemic cells. Consistent with a cytoprotective action of the MEK/ERK pathway, MEK blockade by pharmacological inhibitors, strikingly increases Ara-C cytotoxicity (Jarvis et al., 1998; Milella et al., 2001; Yu et al., 2001b), at least in part through enhanced cytosolic release of cytochrome *c* and Smac/DIABLO, but not loss of mitochondrial membrane potential, thereby implicating activation of apoptotic pathways that may differ from those triggered by Ara-C alone (Yu et al., 2001b). Our own findings indicate that only cell lines with constitutive ERK activation were sensitized to Ara-C-induced apoptosis, suggesting that the observed effect may depend on intrinsic rather than on Ara-C-stimulated ERK activity (Milella et al., 2001). Another critical aspect is the sequence-dependent potentiation of Ara-C cytotoxicity by MEK inhibitors. Indeed, Ara-C followed by PD98059 substantially potentiated Ara-C-induced apoptosis, whereas the reverse sequence had a slight protective effect (Milella et al., 2001). This concept also applies to the reported ability of MEK inhibitors to enhance apoptotic cell death induced by chemotherapeutic agents that disrupt microtubule integrity, such as vinblastine, colchicine, and paclitaxel, in different cellular models of cancer, including leukaemia (MacKeigan et al., 2000; Stadheim et al., 2001; Townsend et al., 1998; Wang et al., 1998; Yu et al., 2001a). At least with regard to

paclitaxel, in fact, pre- and co-treatment with PD98059 fail to increase or even oppose paclitaxel-induced apoptotic cell death (Huang et al., 1999; Lieu et al., 1998), whereas sequential exposure to paclitaxel followed by PD98059 or CI-1040 potently enhance apoptosis (Wang et al., 1998; Yu et al., 2001a). The sequence-dependent effects observed for both Ara-C and paclitaxel may be explained by the cell cycle-inhibitory activity of MEK/ERK blockers; indeed, in addition to lowering the apoptotic threshold, MEK blockade also causes cell cycle arrest at the G_1/S boundary in those cells that critically rely on this signalling module for their proliferation, thereby preventing incorporation of nucleoside analogs, such as Ara-C, into newly synthesized DNA and entry of cells into the paclitaxel-sensitive G_2/M phase of the cell cycle.

Recent evidence suggests that MEK inhibition may also increase anthracycline-mediated cytotoxicity: in fact, daunorubicin and PD98059 displayed additive effects in daunorubicin-sensitive samples from AML patients, while PD98059 significantly increased daunorubicin-induced apoptosis in resistant samples, suggesting that MEK blockade can restore daunorubicin cytotoxicity in drug-resistant AML cells (Mas et al., 2003). Consistent with these results, cell lines that have been rendered resistant to anthracycline-induced cell death display strong constitutive activation of the MEK/ERK pathway and become hypersensitive to MEK inhibition (McCubrey, unpublished results). Finally, a synergistic pro-apoptotic interaction between 2-chloro-2'-deoxyadenoosine (CdA) and MEK inhibitors has been recently reported in cell line models of B-cell chronic lymphocytic leukaemia (B-CLL) (Smal et al., 2007).

5.4. MEK inhibition-based combinations with other signal transduction inhibitors/apoptosis modulators

Even more intriguing is the ability of MEK inhibitors to synergistically induce apoptosis in leukemic cells when combined with an array of different signal transduction inhibitors and/or apoptosis modulators. Among these, 7-hydroxystaurosporine (UCN-01), a PKC/Chk1 inhibitor endowed with potent pro-apoptotic activity, particularly in haematopoietic cells, has been recently shown to result in the activation of the MEK/ERK MAPK module, when used at marginally toxic concentrations (Dai et al., 2001); under these conditions, simultaneous MEK blockade by different inhibitors, such as CI-1040, PD98059, and U0126 synergistically triggered mitochondrial damage, caspase activation, DNA fragmentation, and apoptosis in multiple lymphoid and myeloid cell lines and in drug-sensitive and – resistant myeloma cell lines and primary samples (Dai et al., 2001; Dai et al., 2002), suggesting that this combination strategy could have a broad applicability in haematological malignancies. At a molecular level, MAPK activation by UCN-01 is partly dependent on Chk1 activity, while the pro-apoptotic effect of combined UCN-01 and MEK inhibitors appears to require both Chk1 inhibition and cdc2 activation (Pei et al., 2006).

In preclinical models of CML, both sensitive and resistant to the pro-apoptotic action of imatinib mesylate (Gleevec®), as well as in primary CML samples, a highly synergistic potentiation of apoptosis induction has been recently reported in response to combined treatment with imatinib or the dual Abl/Src kinase inhibitor dasatinib (BMS-354825) and different MEK inhibitors, such as CI-1040, PD98059 and U0126 (Nguyen et al., 2007; Yu et al., 2002). These findings are further supported by recent evidence indicating that imatinib exposure causes a dose-dependent increase in MAPK activation in CD34+ primary CML cells and that combined treatment with imatinib and MEK inhibitors results in significantly increased growth inhibition and apoptosis of CML progenitors (Chu et al., 2004). Similar results (i.e. synergistic induction of apoptosis in cell line models of CML and CD34+ progenitors from CML patients) have been recently reported using combinations of either the histone deacetylase inhibitor suberanoylanilide hydroxamic acid (SAHA) or the heat shock protein-90 antagonist 17-dimethylaminoethylamino-17-demethoxygeldanamycin (DMAG)

and MEK inhibitors (U0126 or CI-1040), which caused substantial apoptosis in CML cell lines and primary samples, while relatively sparing CD34+ progenitors from normal bone marrow (Nguyen et al., 2006; Yu et al., 2005).

Another intriguing combination strategy that appears to exert synergistic anti-leukaemic effects involves the use of arsenic trioxide (ATO); recent evidence indeed indicates that the combination of MEK inhibitors with ATO has the capacity to synergistically enhance ATO-induced apoptosis in both APL and AML cell lines and primary blasts by a novel mechanism that involves modulation of the balance between pro- and anti-apoptotic p73 isoforms (TAp73 and ΔNp73, resp.; (Müller et al., 2006)), induction of the pro-apoptotic p53/p73 target gene p53AIP1, and dephosphorylation of BAD (Lunghi et al., 2004; Lunghi et al., 2005; Lunghi et al., 2006).

As mentioned above, MEK inhibitors have mostly cytostatic rather than cytotoxic effects. Intriguingly, although ERK may regulate Bcl-2 anti-apoptotic functions at a posttranslational level (namely by phosphorylation) (Breitschopf et al., 2000; Deng et al., 2000; Konopleva et al., 2002), we have shown that MEK inhibition does not affect Bcl-2 protein expression (Milella et al., 2001; Milella et al., 2007). We therefore speculated that, even in the presence of a MEK inhibition-induced decrease in the levels of other anti-apoptotic players (such as Mcl-1 and survivin), above-threshold levels of Bcl-2 could maintain cell viability and prevent apoptosis. If this is the case, simultaneous MEK blockade and downregulation of Bcl-2 expression or function should synergistically trigger apoptotic cell death. Indeed, we have recently demonstrated that simultaneous inhibition of Bcl-2 (by either small-molecule inhibitors, such as HA14-1, or antisense oligonucleotides) and MAPK function (by CI-1040) results in a highly synergistic reduction of cell viability and induction of apoptosis in AML cell lines with constitutive ERK activation (Milella et al., 2002). Moreover, CI-1040 synergistically potentiated HA14-1-mediated reduction in the clonogenic growth of primary AML samples in semisolid clonogenic assays and circumvented the protection from HA14-1-mediated apoptosis conferred by forced Bcl-2 overexpression (Milella et al., 2002).

Putative molecular mechanisms underlying the pro-apoptotic synergism between Bcl-2 and MEK inhibitors have been recently identified using the novel small-molecule inhibitor of the BH3-domain-mediated heterodimerization between pro- and anti-apoptotic Bcl-2 family members ABT-737 (Konopleva et al., 2006). Indeed, this agent effectively kills acute myeloid leukaemia blasts, progenitors and stem cells by disrupting the Bcl-2/Bax complex and causing Bak-dependent, but Bim-independent, activation of the intrinsic apoptotic pathway. However, Bcl-2 phosphorylation and Mcl-1 overexpression induce render myeloid cells resistant to the pro-apoptotic effects of ABT-737. By inhibiting both Bcl-2 phosphorylation and Mcl-1 expression, MEK inhibitors are able to overcome resistance to ABT-737 in AML cells, with the combination acting synergistically in an unprecedented manner (combination index < 0.1) (Konopleva et al., 2006).

The above-mentioned anti-apoptotic cross talk between Bcl-2 and the MEK/ERK module may also explain the pro-apoptotic synergism observed in M3 and non-M3 AML cells with the combination of retinoids and MEK blockade. In fact, we have shown that MEK inhibition by CI-1040 strikingly potentiates the pro-apoptotic effects of all-*trans* and 9-*cis* retinoic acid (ATRA and 9-*cis* RA, respectively) in AML cell lines with constitutive activation of the MEK/ERK pathway (Milella et al., 2007). This pro-apoptotic interaction is strongly synergistic (with combination indexes ranging from 0.4 to <0.2) and appears to involve both RAR and RXR receptors. Neither increased differentiation nor modulation of death-inducing ligand/receptor pairs appear to play a major role; instead, ATRA efficiently decreases Bcl-2 expression (Andreeff et al., 1999), whereas MEK inhibition downregulates downstream caspase inhibitors, such as survivin (Carter et al., 2001; Milella et al., 2001), resulting in the

simultaneous inhibition of complementary survival pathways, with synergistic effects on leukaemic cell viability. Consistent with this hypothesis, enforced Bcl-2 expression partially inhibits and significantly delays apoptosis induced by the combination of retinoids and CI-1040 (Milella et al., 2007).

6. Concluding remarks

As more targeted anti-cancer agents move forward into the clinic, offering renewed hope to our patients, clinicians and scientists are faced with new challenges. Primary and acquired resistance remains the most significant obstacle to the successful fulfilment of targeted agents' therapeutic promise. The identification of genetic and/or epigenetic lesions that render individual tumours "addicted" to certain pathways and the design of predictive tests to identify those patients with the highest probability to derive benefit from their therapeutic manipulation remains a top priority. This is well exemplified by the dramatic and unprecedented objective response rate (~ 90%) obtained with erlotinib monotherapy in NSCLC patients whose tumours harbour an activating EGFR mutation (Paz-Ares et al., 2006). While molecularly "tailored" therapy of individual tumours remains the most ambitious goal, building novel, mechanism-based combinations that have the potential to bypass escape mechanisms and overcome resistance to single-pathway inhibitors in relatively unselected patient populations appears already within our reach and may result in substantial therapeutic advances in the near term. Again, combined EGFR and VEGF(R) targeting constitutes a good example of a promising combination of targeted agents that has already shown to be feasible in a clinical setting (Hainsworth et al., 2005; Herbst et al., 2005).

Clinical development of MEK inhibitors is at its dawn. While clinical experience is limited, an impressive amount of preclinical data is accumulating, which suggest that haematological malignancies, particularly AML, may be exquisitely sensitive to MEK inhibition, provided that patients with constitutive activation of the MEK/ERK pathway are prospectively identified. Recent clinical data suggest that constitutive activation of multiple signaling pathways is the rule rather than the exception in AML, adding up to convey an increasingly adverse prognosis (Kornblau et al., 2006). However, the ability of MEK inhibitors to sensitize leukemic cells to apoptosis induced by a wide array of conventional and molecularly targeted anti-cancer agents raises the hope that combinations with synergistic anti-leukemic effects could be successfully developed for therapeutic purposes. Interestingly, the mechanism of action of certain combinations, such as the combination of MEK inhibitors and retinoids (Milella et al., 2007), appears to be entirely different from that of individual agents, suggesting that they may be usefully applied to patients potentially resistant to single-pathway inhibition.

In summary, substantial progress has been made in the identification of molecular mechanisms of sensitivity/resistance to targeted anti-cancer agents and novel strategies to overcome resistance are being developed. Deeper insights into the molecular mechanisms of action of signal transduction inhibitors, alone or combined with other agents, and extensive preclinical/early clinical modelling will be of paramount importance for the full realization of their therapeutic potential.

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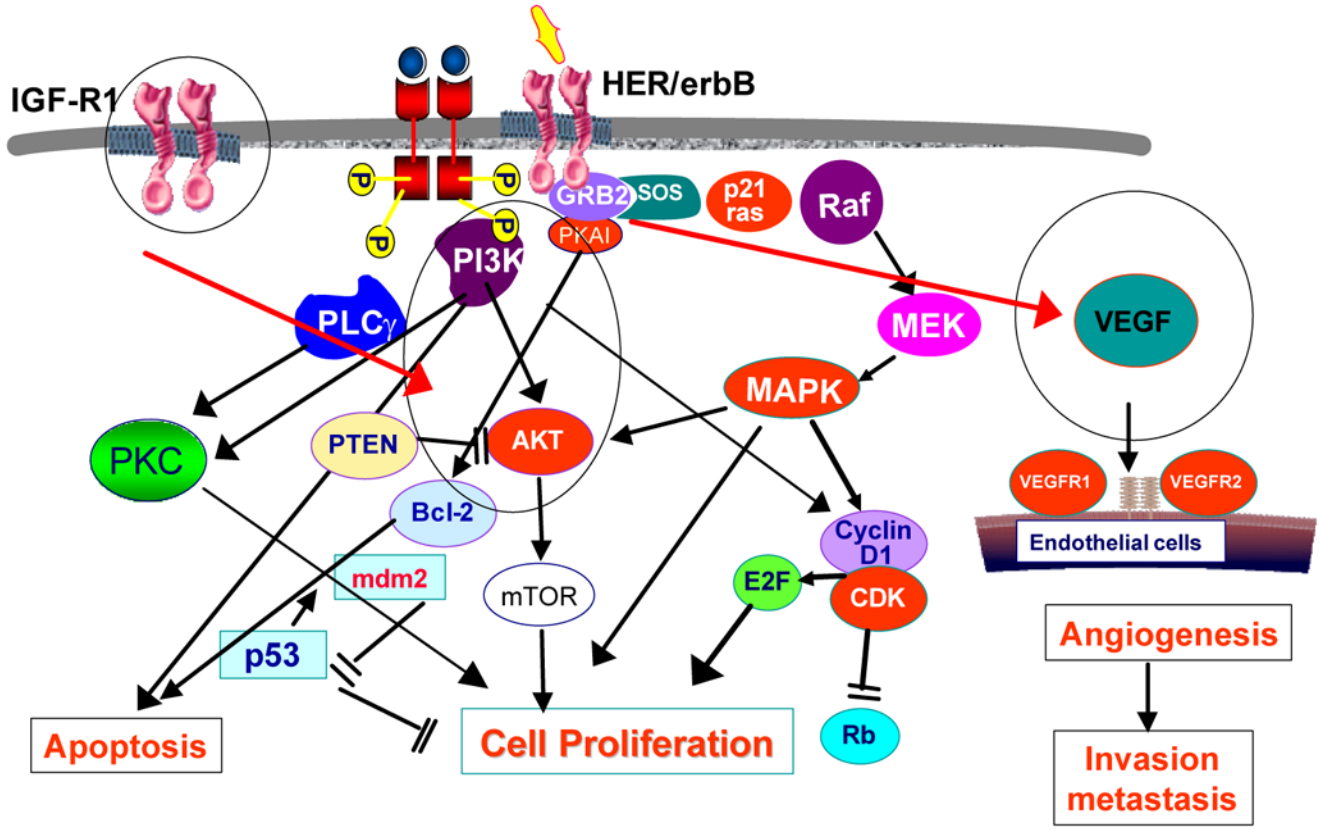

Figure 1.
IGFI-R, PI3K/Akt and VEGF are “escape pathways” responsible of the resistance to EGFR-targeted therapies.

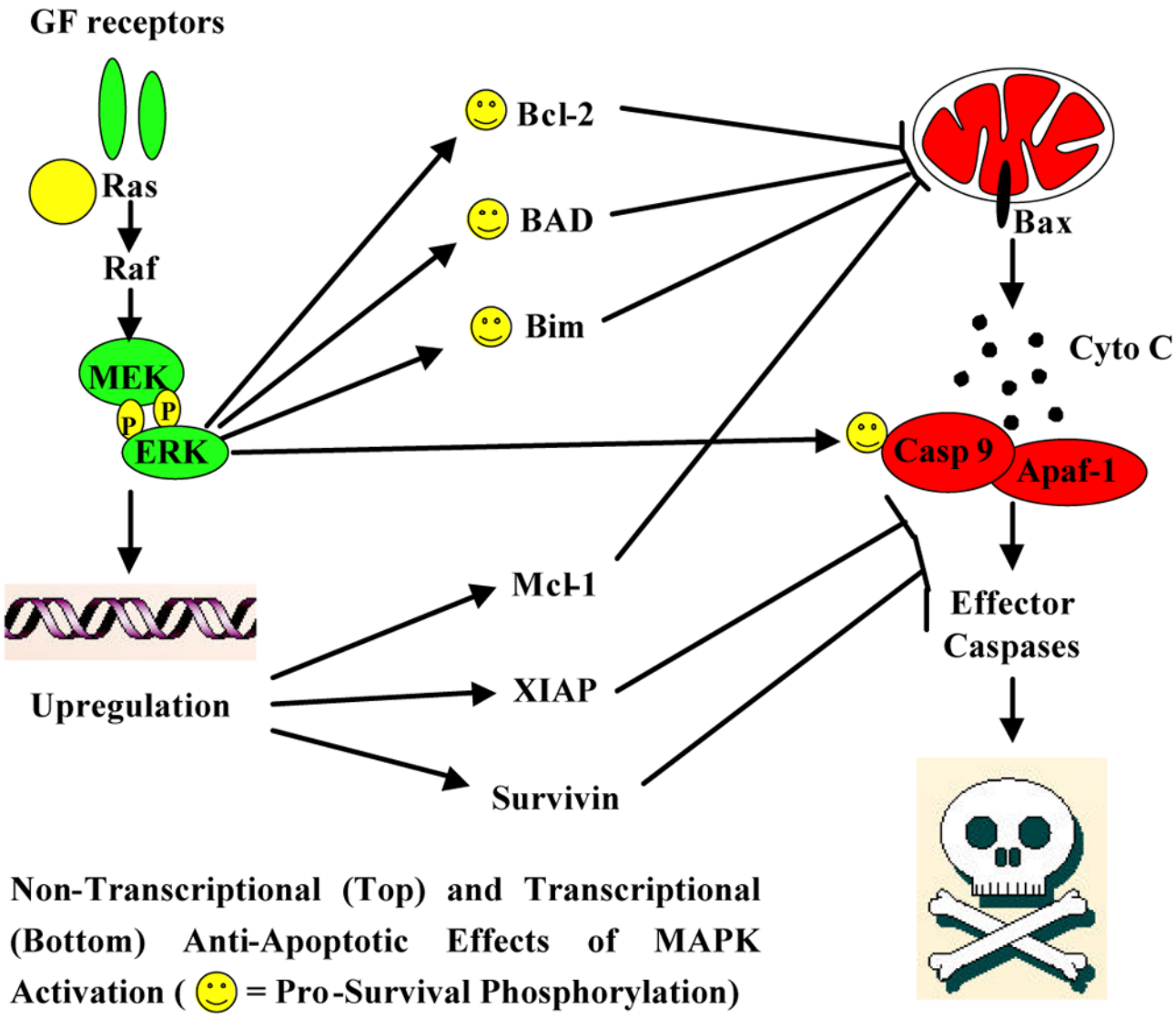

Figure 2.
Non-transcriptional and transcriptional anti-apoptotic effects of MAPK activation.

Table 1

MEK inhibitors and their activity in haematologic malignancies.

Inhibitor	Disease models in which preclinical activity was demonstrated	Synergistic activity in combination	Clinical development status	Key references
PD98059	CML AML ALL MM	Yes	-	Chu et al. 2004; Morgan et al. 2001; Milella et al. 2001; Lunghi et al. 2003; Lunghi et al. 2004; Meng et al. 2003
U0126	CML AML	Yes	-	Chu et al. 2004; Morgan et al. 2001; James et al. 2003
CI-1040 (PD184352)	AML ALL MDS MM	Yes	Phase II (discontinued)	Milella et al. 2001; Meng et al. 2003; Steensma et al. 2003; Lunghi et al. 2004
PD0325901	AML	Unknown	Phase I/II (solid tumours)	Milella (manuscript in preparation)
AZD6244 (ARRY-142886)	MM	Unknown	Phase I/II (solid tumours)	Tai et al. 2006a, 2006b

CML, chronic myeloid leukaemia; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; MDS, myelodysplastic syndrome; MM, multiple myeloma.