

## Minireview

# Raf kinase as a target for anticancer therapeutics

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### Abstract

The Ras-Raf-MEK-ERK (ERK) pathway is a logical therapeutic target because it represents a common downstream pathway for several key growth factor tyrosine kinase receptors which are often mutated or overexpressed in human cancers. Although considered mainly growth-promoting, in certain contexts, this pathway also seems to be apoptosis-suppressing. Several novel agents targeting this pathway have now been developed and are in clinical trials. One of the most interesting new agents is BAY 43-9006. Although initially developed as a Raf kinase inhibitor, it can also target several other important tyrosine kinases including VEGFR-2, Flt-3, and c-Kit, which contributes to its antiproliferative and antiangiogenic properties. To date, encouraging results have been seen with BAY 43-9006, particularly in renal cell cancers which are highly vascular tumors. This review will provide an overview of the ERK signaling pathway in normal and neoplastic tissue, with a specific focus on novel therapies targeting the ERK pathway at the level of Raf kinase. [Mol Cancer Ther 2005;4(4):677–85]

### Introduction

Advances in our understanding of the basic molecular mechanisms underlying cell signaling have led to the identification of key pathways, such as the Ras-Raf-MEK-ERK (ERK) pathway which plays a critical role in many aspects of tumorigenesis. Novel anticancer agents targeting this signaling pathway are currently being evaluated and may prove to be more effective and less toxic than conventional cytotoxic therapies.

The ERK pathway plays a central role in regulating mammalian cell growth by relaying extracellular signals from ligand-bound cell surface tyrosine kinase receptors such as epidermal growth factor receptor (EGFR), HER-2,

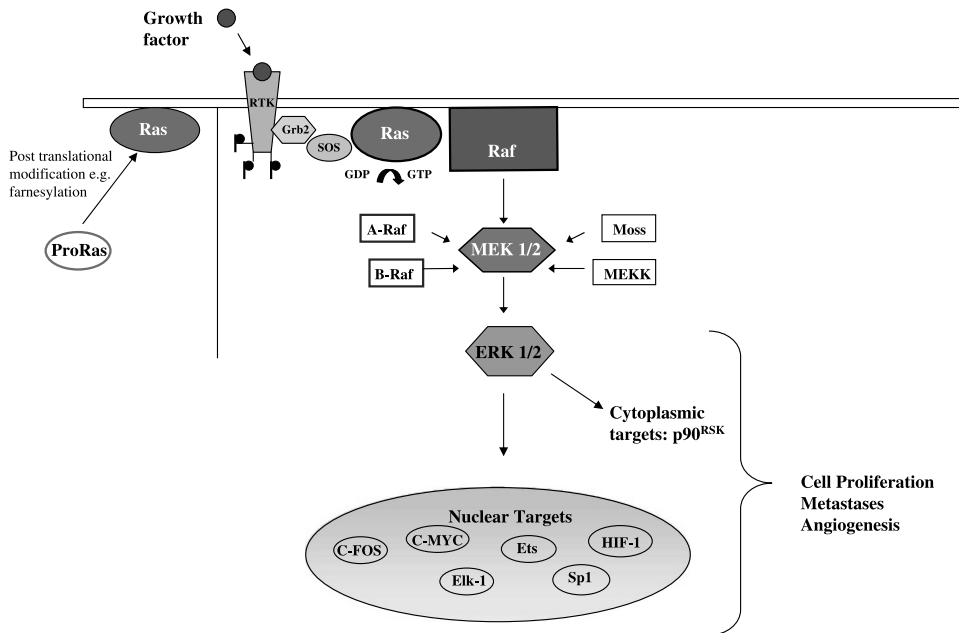
vascular EGFR (VEGFR), platelet-derived growth factor receptor (PDGFR), and MET to the nucleus via a cascade of specific phosphorylation events beginning with activation of Ras (1). The next critical step in this pathway involves activation of a family of serine threonine kinases known as Raf kinase. Raf kinase then phosphorylates and activates MEK1/2, which then phosphorylates and activates ERK1/2. When activated, ERK1/2 phosphorylates various downstream substrates involved in a multitude of cellular responses from cytoskeletal changes to gene transcription (ref. 2; Fig. 1). Aberrant signaling through the ERK pathway could thus promote: cell immortalization via telomerase induction, growth factor-independent proliferation and insensitivity to growth-inhibitory signals by cell cycle activation, autocrine signaling and inactivation of tumor suppressor genes, invasion and metastases via stimulation of cellular motility and extracellular matrix remodeling, angiogenesis through up-regulation of proangiogenic factors such as VEGF, avoidance of apoptosis by BAD inactivation and caspase inhibition, and resistance to radiation and chemotherapy by induction of the multidrug resistance gene (*MDR-1*; refs. 3, 4). Taken together, this would suggest that the ERK pathway is a logical target for novel therapeutic anticancer strategies.

### Ras

One of the most frequently detected genetic alterations in cancer is in the *ras* oncogene family, which plays a pivotal role in the control of both normal and transformed cell growth. *Ras* genes were first identified in the 1960s as homologues to the viral oncogenes of transforming retroviruses. This gene family includes *N-ras* (neuroblastoma cell line), *H-ras* (Harvey murine sarcoma virus), and the alternatively spliced *K-ras* (Kirsten murine sarcoma virus), which is the type of *ras* most frequently activated in human cancers. Overactivation of wild-type *ras* and *ras* gene mutations, involving single amino acid substitution at codons 12, 13, or 61 leading to a constitutively active gene, have been shown to induce malignant transformation in many cancers (5). The highest incidence of *ras* alterations are seen in pancreatic cancer (90%), thyroid cancer (50%), colon cancer (50%), lung cancer (30%), and acute myeloid leukemia (30%); and in some settings, may be associated with a worse overall prognosis (6). In colon cancer, for example, the presence of *K-ras* mutations correlates with an increased risk of recurrence ( $P < 0.001$ ) and death ( $P = 0.004$ ), regardless of the disease stage (7). Similarly in lung cancer *K-ras* mutations, more common among women and smokers, may also be an important negative prognostic indicator (8).

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**Figure 1.** Overview of the Ras-Raf-MEK-ERK signaling pathway. General signaling events following binding of the receptor tyrosine kinase by ligand. Growth factor receptor binding protein 2 (*Grb2*) represent an adaptor molecule; SOS a GTP exchange factor. Sequential activation of Ras-Raf-MEK-ERK results in phosphorylation of both cytoplasmic (p90<sup>RSK</sup>:ribosomal 6 kinase) and nuclear targets (C-FOS, C-MYC, Elk-1, Ets, Sp1, and HIF-1) leading to cell proliferation, metastases, and angiogenesis.

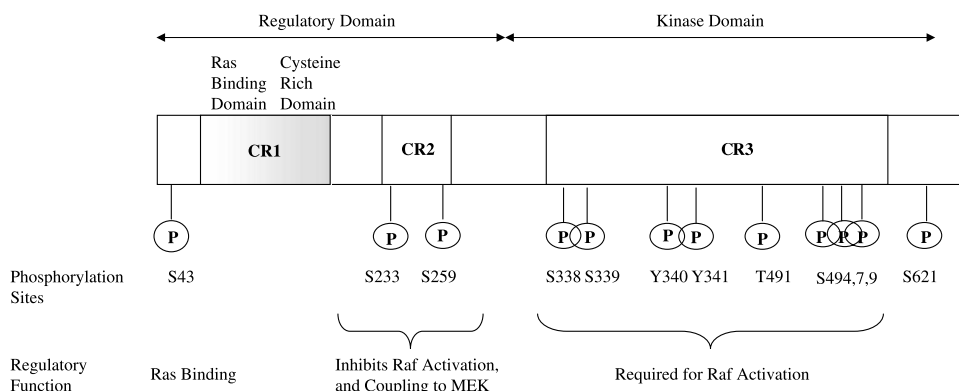
*Ras* genes are expressed in every cell type in a tissue-specific manner and encode a unique, but highly conserved, 21 kDa protein (p21<sup>ras</sup>), which has a high degree of homology at the protein level. Ras is synthesized as a propeptide in the cytoplasm and is dependent upon several posttranslational modifications such as farnesylation of its CAAX box motif (C, cysteine; A, aliphatic amino acid; X, other amino acid) to increase its hydrophobicity, and promote membrane association. When farnesylation is blocked by drugs such as the farnesyltransferase inhibitors, Ras is unable to anchor to the cell membrane and its function is impaired. Although farnesyltransferase inhibitors can block H-Ras membrane association and transformation, they lack potency against K-Ras or N-Ras likely due to redundancy of prenylation mechanisms in the latter (5).

Ras proteins are key intermediates in cell signaling, and are the prototypic members of a large family of G proteins which cycle between an active (GTP bound) or inactive

(GDP bound) state. Upstream, Ras becomes activated upon ligand binding to cell surface tyrosine kinase receptors such as EGFR, HER-2, VEGFR, PDGFR, and MET. Activated Ras then triggers a cascade of downstream phosphorylation events beginning with the most critical step-activation of Raf kinase.

**Raf Kinase**

Raf was the first identified and most characterized downstream effector kinase of Ras. The Raf serine threonine kinase family consists of three isoforms, Raf-1 (C-Raf), A-Raf, and B-Raf. Each isoform has three conserved regions in common: CR1, CR2, and CR3, and several regulatory phosphorylation sites therein (Fig. 2). CR1 is the Ras-binding domain, CR2 is the regulatory domain that negatively regulates Raf activity by Akt or protein kinase A phosphorylation at serine (S) residue S259, and CR3 is the kinase domain which when phosphorylated on S338, tyrosine (Y) Y340 and Y341, positively regulates



**Figure 2.** Structure and phosphorylation sites of Raf kinase proteins. CR, conserved region; P, phosphorylation sites; S, serine; Y, tyrosine.

Raf activity. Raf isoforms vary in their cell-specific expression, potency of kinase activity, and show both overlapping and unique regulatory functions as will be discussed below (9–12).

### Raf-1 (C-Raf)

Raf-1 is a 74 kDa mitochondrial protein, ubiquitously expressed in adult tissues, with highest expression in muscle, cerebellum, and fetal brain. Despite it being the most studied Raf isoform, its exact function remains poorly understood. Raf-1-deficient mice die midgestation due to widespread apoptosis, suggesting that Raf-1 may be antiapoptotic. This is supported by the findings that Raf-1 phosphorylates and inactivates BAD, phosphorylates and coimmunoprecipitates with Bcl-2, and also regulates BAG-1 and BAD expression in BCR-ABL-expressing cells. Mutations in Raf-1 have not been detected in human cancers (13, 14).

### A-Raf

The 68 kDa A-Raf gene product is mainly found in the urogenital tract. This isoform is the weakest activator of MEK, and can only activate MEK1 but not MEK2. Like Raf-1, A-Raf has a stable or transient localization to the mitochondria implicating it in the regulation of apoptosis. A-Raf deletion in mice results in postnatal mortality due to neurologic and gastrointestinal deficits, whereas overexpression promotes cytokine independence in hematopoietic cells. To date, however, no mutations in A-Raf have been found in human cancers (13, 15, 16).

### B-Raf

The alternatively spliced, 94 kDa B-Raf kinase is ubiquitously expressed but found mostly in testis and neuronal tissue. It is the strongest Raf kinase in terms of induction of MEK activity. In B-Raf<sup>-/-</sup> mice, ERK activation is significantly disrupted and mice die midgestation due to massive apoptosis of endothelial cells leading to hemorrhage (17). Unlike Raf-1 and A-Raf, more than 30 single-site missense activating mutations occurring mostly within the B-Raf kinase domain have recently been identified in human cancers, leading to considerable excitement about B-Raf as a potential therapeutic target.

Somatic mutations of B-Raf are found in 60% of malignant melanomas and occur with moderate to high frequency in papillary thyroid carcinomas, colorectal, and ovarian cancers, strongly implicating activation of B-Raf in tumorigenesis (18). Most of the mutations of B-Raf are clustered in two regions: the glycine-rich loop of the kinase domain, and within or immediately adjacent to the activation segment. A Glu for Val substitution at residue 600 (V600E, formerly designated V599E) accounts for 90% of B-Raf mutations, resulting in constitutive kinase activity which can transform NIH3T3 cells. B-Raf activating mutations have also been found at inhibitory Akt phosphorylation sites in the CR2 domain (19). Three B-Raf mutants show reduced kinase activity *in vitro*, but by

activating wild-type Raf-1, are able to preserve signaling to ERK. Preliminary evidence from Wan et al. (18) also suggests that the presence of B-Raf mutations may determine sensitivity to drugs which target the ERK pathway at the level of Raf kinase.

### Raf Kinase: Activation

The activation of Raf is a complex multistep process that begins with the recruitment of inactive Raf (complexed with 14-3-3 and heat shock proteins) from the cytosol to the plasma membrane by activated Ras. The effector domain of Ras, binds to Raf at the Ras-binding domain and cysteine-rich domains of the CR1 region (20). Once recruited to the cell membrane, Raf must then undergo several further modifications before being rendered active.

A-Raf and Raf-1 exist in an inactive state, phosphorylated at residues S259 and S621 and bound to the highly conserved chaperonin protein 14-3-3, which maintains Raf in an inactive conformation. Raf activation, therefore first requires dephosphorylation of S259 and S621 which dissociates 14-3-3 from Raf, and then phosphorylation at residues S338, Y340, and Y341 (20). In contrast, B-Raf becomes immediately activated upon translocation to the plasma membrane. This is likely because B-Raf is constitutively phosphorylated on serine (S445), which corresponds to a regulated phosphorylation site (S338) on Raf-1, and because a regulatory tyrosine residue (Y341) is replaced by an aspartic acid residue (D448) which, because of its negative charge, mimics phosphorylation (21). Since B-Raf activation is comparatively easier, this may explain why B-Raf is the strongest activator of downstream MEK and also why B-Raf is a preferred target for mutational activation in human cancers (22).

Activated Raf then induces a downstream signal transduction cascade beginning with the activation of MEK, by phosphorylation at S218 and S222 which increases MEK activity by more than 7000-fold (23). Increased MEK activity has been seen in a variety of leukemias, and *in vitro*, constitutively activated MEK has shown both transforming ability and antiapoptotic effects, suggesting that MEK may be another important target in this pathway (24). Two novel MEK inhibitors CI-1040 (PD 184352) and PD 0325901 are currently in clinical trials. Phase I studies with CI-1040 showed encouraging results, but the recently reported phase II study in patients with non-small cell lung cancer, breast, colon, and pancreatic cancers showed that though CI-1040 was well tolerated, it failed to show sufficient antitumor activity to warrant further development (25, 26). Unfortunately, this study did not assess posttreatment changes in tumor pERK, and so the level of target inhibition was not known. PD 0325901, a second-generation MEK inhibitor, which has recently entered into clinical trials, has a >50-fold increased potency against MEK and improved bioavailability, therefore, it may show better results than CI-1040 (26).

Downstream of MEK lies two kinases, ERK1 and ERK2, which are activated by phosphorylation on residues T183 and Y185. Phosphorylation results in dimerization of ERK

with other ERK proteins, inducing nuclear translocation and activation of a variety of nuclear targets. ERK also has a key cytoplasmic target which is p90<sup>RSK</sup> whose substrates include an array of transcription factors including cyclic AMP-response element binding protein, C-MYC, Ets, activator protein-1, and nuclear factor- $\kappa$ B, all of which have diverse cellular effects (27). The ERK signaling cascade thus couples signals from cell surface receptors to transcription factors which regulate gene expression. Depending on the stimulus and cell type, this pathway can transmit signals which modulate cell proliferation, differentiation, angiogenesis, or apoptosis.

### Ras-Raf Signaling and Cancer

Constitutive activation of the ERK pathway is frequently seen in human cancers and is often due to overexpression or mutation of upstream receptor tyrosine kinases such as EGFR, PDGFR, or VEGFR, increased expression of growth factor ligands, or mutational activation of Ras and its downstream effectors (28). A prime consequence of this constitutive activation seems to be the promotion of tumor growth, invasion, angiogenesis, and metastasis.

Signaling through the ERK pathway may lead to increased tumoral expression of growth factors and cytokines, which serves to further stimulate this pathway in an autocrine fashion and promote cell proliferation in the absence of growth factors (29). The ERK pathway may also play an important role in cell cycle progression by inducing cyclin D and E expression, phosphorylating and inducing the degradation of the cell cycle inhibitor p27<sup>kip</sup>, and by interacting with the tumor suppressor gene RB (30). Another feature of ERK signaling relevant to tumorigenesis is its ability to regulate cell motility, extracellular matrix remodeling, and the production of key angiogenic factors such as VEGF (22). In addition, activation of this pathway may promote resistance to anticancer therapies by inactivating caspase and BAD proteins as well as down-regulating p53, which are all necessary for treatment-induced apoptosis—the normal response to genetic damage (30). Taken together, the ERK pathway is clearly critical to tumorigenesis and represents an attractive therapeutic target, particularly in tumors where it is a dominant oncogenic drive.

Drugs targeting the ERK pathway at the level of Raf may be particularly useful because Raf is the key activator of the ERK pathway, whereas other upstream targets such as the growth factor ligands, receptor tyrosine kinases or even Ras, have many other potential effectors. In addition, constitutively active forms of Raf exhibit transforming activity comparable to Ras and are themselves sufficient to transform some cells. Interestingly, mutations of B-Raf and K-Ras are often found in the same tumor types, but in a mutually exclusive fashion, suggesting that B-Raf and K-Ras may provide an equivalent or at least a redundant oncogenic stimulus in cancer pathogenesis (31). Furthermore, dominant-negative mutants of Raf can impair Ras transforming activity, confirming that inhibition of Raf is a viable therapeutic approach.

### Targeting Raf

Several strategies have been developed that specifically target Raf kinase. These include inhibitors of Raf kinase activity such as BAY 43-9006, antisense oligonucleotides such as ISIS 5132 and LeRafAON, Raf destabilizers such as geldanamycin, and Ras-Raf interaction inhibitors such as MCP-1. Of these, only BAY 43-9006 has shown activity against B-Raf, which as discussed previously is the only Raf kinase in which activating mutations, commonly occurring in melanoma, papillary thyroid, ovarian and colon cancers, have been found.

### Raf Kinase Inhibitors

#### BAY 43-9006

BAY 43-9006 is one of the most promising agents of the class of Raf kinase inhibitors, and has moved into phase II and III clinical trials. BAY 43-9006, is an orally available, novel bi-aryl urea compound that prevents tumor growth by combining two anticancer properties: namely, the inhibition of proliferation by targeting the ERK pathway, and the inhibition of angiogenesis by targeting the receptor tyrosine kinases VEGFR-2 and PDGFR- $\beta$  and their associated signaling cascades (32). Although BAY 43-9006 was initially developed as a Raf kinase inhibitor, it has since been shown to have activity against many receptor tyrosine kinases involved in tumorigenesis and angiogenesis including VEGFR-2, VEGFR-3, PDGFR- $\beta$ , Flt-3, c-Kit, and p38 $\alpha$  (refs. 32, 33; Table 1). In cellular mechanistic assays, BAY 43-9006 reduced basal phosphorylation of the ERK pathway in a panel of breast, melanoma, pancreatic, and colon tumor cell lines.

*In vivo*, BAY 43-9006 showed potent and dose-dependent antitumor activity in several xenografts models. Examples include: HCT-116 and DLD-1 colon cancer models, A549 and NCI-H460 non-small cell lung cancer models, and MIA PaCa-2 pancreatic cancer model, all of which contain K-Ras mutations; SK-OV-3, an ovarian cancer cell line containing wild-type Ras and intact Raf, but overexpressing epidermal growth factor and HER-2 receptors resulting in a constitutively active ERK signaling pathway; COLO-205 and HT-29 colon cancer models which contain B-Raf mutations; and the MDA-MB-231 breast cancer model which contains both B-Raf and K-Ras mutations (Fig. 3). This would suggest that BAY 43-9006 may be of therapeutic value in tumors dependent upon signaling through the ERK pathway. BAY 43-9006 has also shown activity against larger (400mg-1g) colon and ovarian tumors as well as some residual activity after it was discontinued. When combined *in vivo* with gemcitabine, cisplatin, irinotecan, vinorelbine, paclitaxel, or gefitinib, antitumor efficacy was seen without significant increase in the toxicity associated with the chemotherapeutic agents (ref. 32; Fig. 3).

Phase I studies with BAY 43-9006, both as a single agent and in combination with cytotoxic agents, have been completed. In general, toxicities with BAY 43-9006 were mild to moderate and included reversible skin rash, hand-foot



**Table 1. BAY 43-9006 inhibits the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor angiogenesis**

|  | IC <sub>50</sub> (nmol/L)<br>± SD (n)* |
|--|--|
| Biochemical assay <sup>†</sup>   |  |
| Raf-1 <sup>‡</sup>   | 6 ± 3 (7)                              |
| BRAF wild-type <sup>§</sup>  | 22 ± 6 (7)                             |
| V599E BRAF mutant <sup>¶</sup>   | 38 ± 9 (4)                             |
| VEGFR-2  | 90 ± 15 (4)                            |
| mVEGFR-2 (flk-ss1)   | 15 ± 6 (4)                             |
| mVEGFR-3   | 20 ± 6 (3)                             |
| mPDGFR-β   | 57 ± 20 (5)                            |
| Flt-3  | 58 ± 20 (3)                            |
| c-KIT  | 68 ± 21 (3)                            |
| FGFR-1   | 580 ± 100 (3)                          |
| ERK-1, MEK-1, EGFR,<br>HER-2, IGF1R-1, c-met, PKB, PKA,<br>cdk1/cyclinB, PKC, PKC, pim-1 | 1 > 10,000                             |
| Cellular mechanism <sup>○</sup>  |  |
| MDA MB 231 MEK<br>phosphorylation (human breast)   | 40 ± 20 (2)                            |
| MDA MB 231 ERK 1/2<br>phosphorylation (human breast)                                     | 90 ± 26 (7)                            |
| BxPC-3 ERK 1/2 phosphorylation<br>(human pancreatic)                                     | 1,200** ± 165 (2)                      |
| LOX ERK 1/2 phosphorylation<br>(human melanoma)  | 880** ± 90 (2)                         |
| VEGFR-2 phosphorylation<br>(human, NIH 3T3 cells)  | 30 ± 21 (3)                            |
| VEGF-ERK 1/2 phosphorylation<br>(human, HUVEC)   | 60** ± 26 (2)                          |
| PDGFR-β phosphorylation<br>(human HAoSMC)  | 80 ± 40 (3)                            |
| mVEGFR-3 phosphorylation<br>(mouse, HEK-293 cells)                                       | 100 ± 80 (2)                           |
| Flt-3 phosphorylation<br>(human ITD, HEK-293 cells)                                      | 20 ± 10 (2)                            |
| Cellular proliferation   |  |
| MDA MB 231 (10% FCS)   | 2,600 ± 810 (3)                        |
| PDGF-BB HAoSMC<br>(0.1% bovine serum albumin)  | 280 ± 140 (5)                          |

NOTE: Reproduced with permission from Wilhelm et al. (32).

\*IC<sub>50</sub> mean ± SD (n = number of trials).

<sup>†</sup>Kinase assays were carried out at ATP concentrations at or below K<sub>m</sub> (1–10 μmol/L).

<sup>‡</sup>Lck activated NH<sub>2</sub>-terminal-truncated Raf-1.

<sup>§</sup>NH<sub>2</sub>-terminal-truncated BRAF (wild-type).

<sup>¶</sup>NH<sub>2</sub>-terminal V599E-truncated BRAF (mutant).

<sup>○</sup>Cellular mechanism assays (receptor tyrosine kinase autophosphorylation and RAF/MEK/ERK pathway) were done in 0.1% bovine serum albumin using phosphospecific antibodies or 4G10 for VEGFR-3.

\*\*Activated phospho-ERK 1/2 was quantitated with phospho-ERK 1/2 immunoassay (Bio-Plex; Bio-Rad, Hercules, CA).

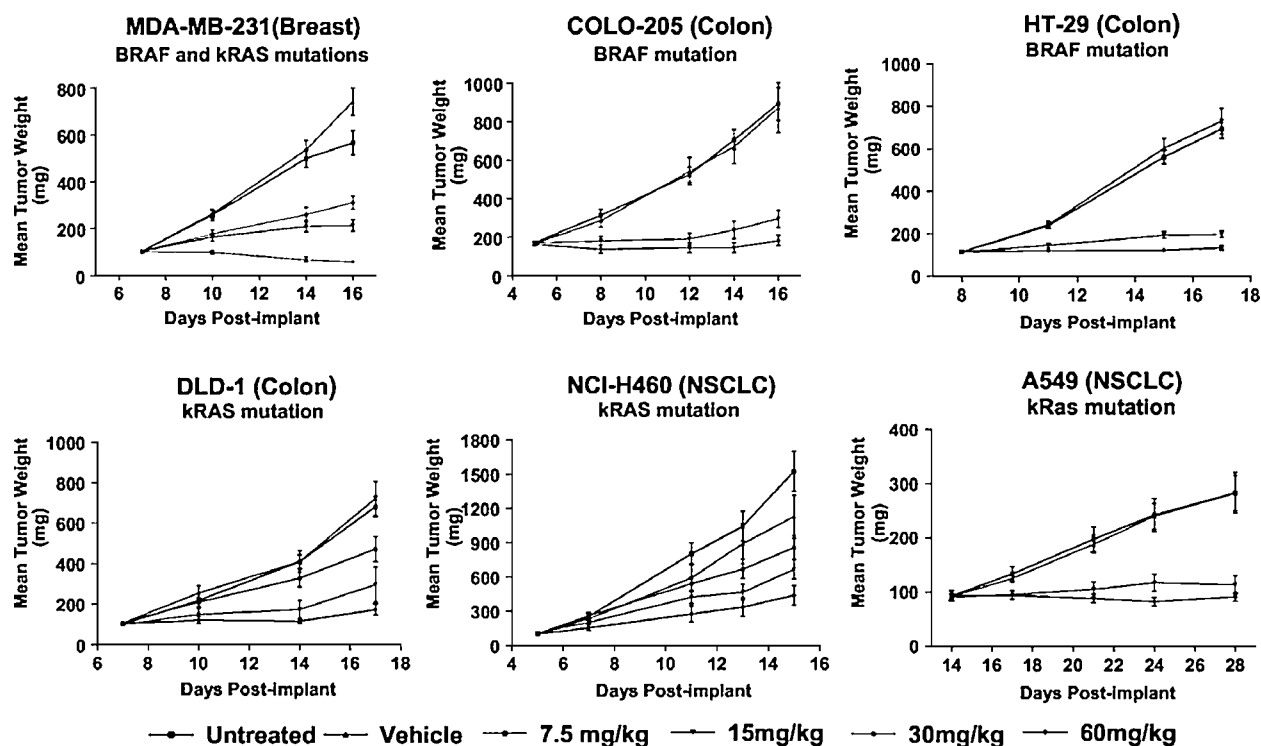
syndrome, diarrhea, and fatigue. The dose-limiting toxicities were diarrhea, skin toxicity, nausea, and hypertension all occurring at doses of greater than 600 mg b.i.d. (34, 35). BAY 43-9006 has shown activity in several tumor types, as measured by disease regression but also by biomarker studies. In a phase I trial by Crump et al. (36), in myelodysplastic syndrome/acute myeloid leukemia patients, assessment of Flt-3 mutational status was done.

Although the overall response rate was low, as defined by improvement in blood and bone marrow leukemic blast counts, one patient with a Flt-3 mutation had a complete remission lasting 3.5 months, which suggests that this drug may be active in patients enriched for Flt-3 mutations. However, this was a small study and further research is needed to clarify this. When used in combination with other agents, BAY 43-9006 is well-tolerated with evidence of antitumor efficacy. Phase I/II studies are therefore ongoing, evaluating the following combinations: BAY 43-9006 and gemcitabine in advanced ovarian and pancreatic cancers (37); BAY 43-9006 and doxorubicin in patients with hepatocellular carcinoma (38), BAY 43-9006 and oxaliplatin in colorectal cancer (39), and BAY 43-9006, carboplatin, and paclitaxel in melanoma (40).

Preliminary results from a large randomized discontinuation phase II study, involving 484 patients with multiple tumor types, of which 39 patients had melanoma, and 203 had renal cell cancer (RCC), are becoming available. In melanoma, BAY 43-9006 may be particularly effective because activating mutations of B-Raf occur in approximately two-thirds of cases, leading to constitutive activation of the ERK pathway, which is required for cell proliferation. Treatment of xenografts, which have oncogenic B-Raf, with BAY 43-9006 *in vivo* has been shown to inhibit tumor growth (41). Ahmad et al. (42) recently presented their data on the patients with stage IV refractory melanoma who were enrolled in the randomized discontinuation phase II trial, and treated with BAY 43-9006 at a dose of 400 mg b.i.d. Among 39 melanoma patients, there were seven with stable disease and one with unconfirmed partial response. Five patients thus far have had mutational analysis done on their tumors, and no B-Raf mutations have been found. However, the correlation between the presence of B-Raf mutations and antitumor efficacy of BAY 43-9006 is yet to be determined and mutational analysis on the remaining samples is ongoing.

The most encouraging data for BAY 43-9006 emerge from the RCC setting. In the randomized discontinuation phase II study, which enrolled 203 RCC patients, 63 were evaluable for response at the initial 12-week assessment. Among these, there were 25 responders, 18 patients with stable disease, and 15 patients with progressive disease (43). These results have led to a large, randomized controlled phase III multicenter trial of BAY 43-9006 as second-line of therapy in patients with RCC.

BAY 43-9006 also seems to possess antitumor activity in the RCC setting. This is likely attributable to the fact that in addition to its anti-Raf kinase effects, it is also a potent inhibitor of VEGFR-2, and RCC is known to be particularly dependent on VEGF-mediated angiogenesis. Many sporadic and hereditary RCC are due to mutations in the von Hippel-Lindau gene, which results in the secretion of high levels of VEGF (33, 43). Interestingly, other novel antiangiogenic agents, such as bevacizumab, PTK 787, and SU11248, which specifically target VEGF-mediated angiogenesis, but do not have anti-Raf kinase properties, have also shown activity in RCC (44).



**Figure 3.** BAY 43-9006 shows broad oral antitumor efficacy in a panel of human tumor xenografts model. MDA-MB-231, Colo-205, HT-29, DLD-1, NCI-H460, and A549 tumor cells were implanted s.c. in the flank of athymic mice. Treatment in each experiment was initiated on the day shown when all mice had tumors ranging in size from 75 to 150 mg. BAY 43-9006 was given p.o. at 7.5 to 60 mg/kg, q.d.  $\times$  9. There was no lethality and no increase in weight loss in any treated group relative to the corresponding control group. Daily oral administration of BAY 43-9006 at 30 to 60 mg/kg produced complete tumor stasis during treatment in five of the six models (reproduced with permission from Wilhelm et al., ref. 32).

Together, these results suggest that the antiangiogenic properties of BAY 43-9006, in some settings such as RCC, may be particularly important and contribute to its promising therapeutic potential.

### Antisense Oligonucleotides

Antisense oligonucleotides are synthetic nucleic acids designed to hybridize to a selected region within a target mRNA transcript which leads to subsequent degradation by RNase H or steric inhibition of translation, ultimately inhibiting synthesis of the target protein (45). Second-generation phosphorothioate antisense oligonucleotides compare favorably against their first-generation predecessors with greater resistance against degradation by nucleases, longer plasma, and tissue half-lives, better affinity for their mRNA targets, and improved therapeutic indices.

#### ISIS 5132

ISIS 5132, a 20-nucleotide phosphorothioate 2'-deoxynucleotide is a potent, and specific antisense inhibitor that targets the 3'-untranslated region of Raf-1 mRNA, inhibiting tumor cell growth both *in vitro* and *in vivo*. Three phase I trials (87 patients total) and two phase II studies (31 patients total) of ISIS 5132 have been reported. The phase I trials examined continuous and intermittent i.v. dosing schedules, and defined modest (grades 2 and 3) transient

and nonrecurrent thrombocytopenia as the principal hematologic toxicity (45). Hemolytic anemia and acute renal failure with anasarca were dose-limiting in one of these studies (46). In phase II trials in patients with hormone-refractory prostate cancer, colorectal cancer, and ovarian cancer, ISIS 5132 failed to show clinically significant anti-tumor activity, although protracted stable disease in some patients may suggest a cytostatic effect (47–49). Further development of ISIS 5132 has been halted due to the lack of clinical responses. However, to overcome degradation and improve intracellular delivery, a liposomal formulation of ISIS 5132, LERafAON, is currently under investigation.

#### LERafAON

Preclinical studies of LERafAON, transfected into human tumor cells, show inhibition of Raf-1 expression, delayed tumor growth, increased apoptosis, and enhanced sensitivity to radiation and other anticancer agents. LERafAON also exhibited favorable safety and pharmacokinetic profiles in rodent and primate model systems (50). In combination with docetaxel and ionizing radiation, LERafAON was significantly more efficacious than single agents or dual treatment in athymic mice with hormone-refractory prostate cancer (51, 52). A phase I trial of LERafAON in patients with advanced solid tumors treated with eight weekly i.v. infusions showed dose-independent hypersensitivity and dose-dependent thrombocytopenia

at a dose of 6 mg/kg/week, the latter toxicity being dose-limiting (53). Another phase I study examined LERafAON in combination with palliative radiotherapy and showed that a dose of 2 mg/kg given twice weekly was well tolerated with steroid and antihistamine premedication (54).

Although antisense oligonucleotide technology is conceptually attractive and may permit highly selective inhibition of specific gene products, encouraging preclinical results have not yet translated into significant clinical benefits. There are several important limitations to this approach. The chemical structure of antisense oligonucleotides, independent of the target mRNA sequences, could cause class-related toxic effects such as thrombocytopenia, activation of the complement and coagulation cascades, hypotension, and transaminitis. The delivery of sufficient amounts of active moiety into tumor tissues remains a concern. Hence, modifications of antisense oligonucleotide backbones or alternate forms of drug delivery such as liposomal encapsulation are actively being investigated. Ironically, another potential limitation of antisense oligonucleotide technology may occur as a result of their unique specificity for target mRNA sequences, in situations where the tumorigenicity of the target is uncertain. The antisense oligonucleotide targeting Raf-1, ISIS 5132, may be an example of such a caveat. Recent RNA interference studies in human melanoma cells bearing B-Raf mutations suggest that targeted interference of Raf-1 does not significantly inhibit downstream phosphorylation of MEK, and does not appreciably alter the biological properties of these cells, compared with interference of B-Raf (55).

## Raf Kinase Destabilizers

### Geldanamycin

Another novel anti-Raf approach involves destabilizing the Raf kinase protein with drugs such as geldanamycin. Geldanamycin is a benzoquinone ansamycin that binds to heat shock protein 90 (hsp90), disrupting the Raf-1-hsp90 multimolecular complex. This leads to Raf-1 destabilization and its degradation via cellular proteolytic mechanisms such as the proteasome-mediated pathway. Several other key cellular proteins might also undergo degradation and this seems to contribute to the anticancer activity of geldanamycin. Preclinical studies with geldanamycin have been encouraging and confirm both decrease in Raf-1 and phosphorylated Raf-1. Phase I and II trials with 17 AAG, a less clinically toxic analogue of geldanamycin, and a phase I trial of the second-generation geldanamycin analogue, 17-DMAG, are currently ongoing (56, 57).

## Ras-Raf Interaction Inhibitors

### MCP1

MCP1 (small molecular weight compounds: C<sub>29</sub>H<sub>27</sub>ClN<sub>2</sub>O<sub>3</sub>, M<sub>r</sub> 487) and its derivatives can also target the ERK pathway at the level of Raf. The mechanism of action has been speculated to involve MCP1 physically binding to Raf-1 and blocking its activation by Ras. *In vitro*, this effectively prevents MEK1 and ERK activation, and as a

result has several downstream effects. These include morphologic reversion of Ras-transformed cells, decrease in matrix metalloproteinase activity, and inhibition of the invasiveness of cells with N-Ras activation, decreased cyclin D levels and enrichment of cells in G<sub>1</sub> phase. And, unlike the farnesyltransferase inhibitors, MCP1 and its derivatives reverse all Ras-transformed phenotypes, including K-Ras-activated tumors which constitute the majority of Ras-mediated human cancers. Currently MCP1 is in preclinical development where preliminary results are encouraging (58).

## Summary

Over the last decade, significant advances in our understanding of cell signaling have strongly implicated the ERK pathway in many aspects of tumorigenesis including proliferation, differentiation, invasion, angiogenesis, and apoptosis. Inappropriate activation of the ERK pathway frequently occurs in cancer due to increased exposure to growth factors, up-regulated or mutated receptor tyrosine kinases, Ras or B-Raf mutations.

The Raf kinase inhibitors are promising anticancer agents because they may be effective not only in tumors with constitutively active or aberrant Ras or Raf signaling, but also in those with deregulated signal transduction due to overexpression or overactivation of upstream growth factors or their receptors (6). Specifically, BAY 43-9006, which has entered phase III trials in RCC, has shown efficacy and minimal toxicity both as a single agent and in combination with standard chemotherapies in many tumor sites.

The mechanism of action of BAY 43-9006 represents a fundamental change in our approach to the design of targeted therapies. Unlike conventional therapies, where specificity is important, there may be a significant advantage to less precise inhibitors—perhaps based on the fact that cell signaling is not a linear process but rather involves a complex interplay of several pathways. Responses seen with BAY 43-9006 could therefore be explained by concomitant inhibition of other key cellular pathways, such as VEGF; especially given the preliminary negative results seen with the pure MEK inhibitor CI-1040. However, the exact effect of pure Raf kinase or MEK inhibitors in terms of suppression of ERK signaling and clinical outcome in cancer patients is not yet known. Combining inhibitors of the ERK pathway with inhibitors of other pathways such as phosphoinositide-3-kinase or EGFR may be a reasonable therapeutic approach, as long as toxicities are nonoverlapping. Similarly, combinations with chemotherapy and/or radiation therapy may also be particularly effective, and may for example prevent repopulation of cells between cycles of treatment.

At present, it is unclear how best to use these novel agents. At higher doses, as indicated by the IC<sub>50</sub> values, BAY 43-9006, may inhibit more pathways relevant to tumorigenesis, but ultimately dose would be limited by toxicity. It is also unknown what proportion of BAY 43-9006 is protein-bound and what is free and able to interact

with relevant targets. These factors would certainly have implications for drug dose, duration, and schedule, especially when BAY 43-9006 is used in conjunction with other agents.

Another important issue with this and other classes of molecular targeted agents is the choice of appropriate trial end-points to evaluate drug efficacy. Whereas conventional drug evaluation defines efficacy based on tumor response rate, such an approach may not be applicable to targeted agents. For instance, the signal transduction inhibitors such as the EGFR-targeting compounds, when given as single agents, have primarily produced objective tumor responses in small proportions of patients and disease stabilization in more substantial proportions of patients. This phenomenon may be due to the evaluation of these agents in patients with more advanced and thus more refractory diseases. As well, it likely reflects the cytostatic nature of these agents, such that their antitumor activity may not be properly captured by conventional efficacy criteria. Furthermore, cytorreduction or tumor growth inhibition observed with many biological agents effective in mouse tumor models do not translate to tumor shrinkages in human clinical trials. These caveats have led to the search for surrogate markers of drug activity in peripheral blood mononuclear cells, but since drugs such as BAY 43-9006 can have multiple downstream effects, knowing which target to specifically measure may pose a further challenge.

As with other biologically targeted therapies, there remain several key unanswered questions relating to patient selection. In the setting of lung cancer for example, Lynch et al. (59) have recently identified somatic mutations in the tyrosine kinase domain of the EGFR, which increased the sensitivity to treatment with gefitinib (Iressa), another novel targeted agent. These EGFR mutations were found more commonly in women, in Japanese patients, in patients with adenocarcinomas and those without a prior smoking history (60). In the case of the Raf kinase and MEK inhibitors, it is not yet known whether there is a specific subset of patients who will be more likely to respond to treatment. Existing data suggest that these agents are primarily cytostatic; however, there may be a subset of tumors where oncogenic mutations have made the cancer more dependent on the ERK signaling pathway, and as a result, more susceptible to treatment with single-agent Raf kinase or MEK inhibitors. Otherwise, use of these agents in combination with other anticancer treatments such as chemotherapy, radiation, or molecular agents may be a better approach. Interestingly, a recent paper by Wan et al. (18), proposes that BAY 43-9006 preferentially interacts with an inactive conformation of B-Raf, suggesting that activating oncogenic mutants, specifically of B-Raf, may actually be less sensitive to the inhibitor than wild-type. Consistent with this hypothesis, the V600E B-Raf mutant is 2-fold less sensitive to BAY 43-9006 than wild-type B-Raf as reflected by the IC<sub>50</sub>s of 38 and 22 nmol/L, respectively (18). Clearly, further research in this area is needed to delineate which patients, and specifically, which mutational phenotypes are the most likely to benefit from these novel therapies.

Over the last decade, advances in our understanding of molecular genetics and tumor biology have led to the identification of several key molecular pathways implicated in the pathogenesis and progress of human cancers. Novel biological agents which target various aspects of these pathways are now in different stages of preclinical and clinical development. These agents are exciting because they may be superior to current systemic cytotoxic chemotherapies which are limited both by their nonspecific mechanisms of action and unwanted toxicities. As we gain more experience with molecular targeted agents, their therapeutic potential will undoubtedly increase greatly and ultimately translate into improved overall patient outcomes.

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