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Targeting the Mitogen-Activated Protein Kinase RAS-RAF Signaling Pathway in Cancer Therapy

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Abstract

Introduction—The mitogen-activated protein kinase (MAPK) pathway comprises several key signaling components and phosphorylation events that play important role in tumorigenesis. These activated kinases transmit extracellular signals that regulate cell growth, differentiation, proliferation, apoptosis and migration functions. Alteration of the RAS-RAF-MEK-ERK-MAPK (RAS-MAPK) pathway has frequently been reported in human cancer as a result of abnormal activation of receptor tyrosine kinases or gain-of-function mutations mainly in the *RAS* or *RAF* genes. Accordingly, these pathways are considered a potential therapeutic target for cancer treatment. Recently, several small-molecule inhibitors targeting this pathway have been developed and are currently being tested in clinical trials.

Areas covered—This paper focuses on the biological role of the RAS-MAPK pathway, the consequence of its disregulation, and the development of small-molecule inhibitors. The rationale for targeting the RAS-MAPK pathway will be reviewed here along with a discussion of the application and the results of various inhibitory molecules as anticancer agents in clinical trials.

Expert opinion—The RAS-MAPK pathway mediates cellular responses to growth signals and is often deregulated in human cancer. Activating mutations in the *RAS* and *BRAF* genes have been frequently identified in a wide range of cancers. Inhibitors of MEK and particularly of RAF kinases, have been effective in clinical trials with manageable side effects. *RAS* and *BRAF* genes need to be analyzed for mutations as markers of response to treatments and to avoid paradoxical effects. Further characterization of the RAS-MAPK molecular mechanisms regulation in malignant cells or underlying the acquired resistance to RAF inhibitors will facilitate development of novel combination therapies.

Keywords

mitogen-activated protein kinase (MAPK); extracellular signal-regulated kinase (ERK); MAP kinase kinase (MEK); RAS; RAF; inhibitors; targeted therapies

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1.0 Introduction

The Mitogen-activated protein kinase (MAPK) pathway encompasses different signalling cascades of which the Ras-Raf-Mek-extracellular signal-regulated kinase 1 and 2 (ERK1/2) is one of the most dysregulated in human cancer. This pathway regulates multiple critical cellular functions including proliferation, growth and senescence (1). The Ras is an important component of the large family of GTPases. The *ras* genes are transforming oncogenes that have initially been recognized as murine sarcoma viruses by Jennifer Harvey (Harvey-Ras [H-RAS], and Werner Kirsten (Kirsten-Ras [K-Ras]) in 1960s (2, 3). The association of activated and transforming *RAS* genes in human cancer was concurrently reported by several authors in 1982 (4–6). Subsequent studies led to the identification of a third human *RAS* gene, designated *NRAS* in human neuroblastoma cells (Neuroblastoma-Ras [N-Ras]). The three human *RAS* genes encode four highly related 188 to 189 amino acid proteins, designated as *H-RAS, N-RAS* and *K-RAS* (*K-RAS4A* and *K-RAS4B*). Ras proteins function as binary molecular switches that control intracellular signaling pathways involved in fundamental cellular processes such as cell polarity, proliferation, differentiation, adhesion, migration, and apoptosis. Ras and ras-related proteins are often dysregulated in cancers by activating mutations of Ras isoforms or its effectors in nearly one-third of all human cancers (7). Ras activates several pathways, including the RAF-MEK-ERK/MAPK cascade, which transmits signals downstream and results in the transcription of genes involved in controlling several cellular mechanisms. The Ras family members are anchored to the cytoplasmic side of the plasma membrane by carboxyl-terminal farnesylation. This localization places the Ras in close proximity to adaptors, the growth factor receptor bound protein 2 (Grb2) and the nucleotide exchange factor son of sevenless (SOS), to mitigate the exchange of nucleotide guanosine diphosphate (GDP) bound to Ras with guanosine triphosphate (GTP) in the cytosol (8). This exchange activates Ras conformationally, allowing its interaction with a number of downstream effectors. Accordingly, Ras communicates external cellular signals to the nucleus, and its altered activation leads to inappropriate cellular activities including enhanced cell growth, differentiation and survival and ultimately to cancer (1,8,9). The RAS-RAF-MEK-ERK pathway is activated by several known growth factors and cytokines that act through receptor tyrosine kinase signals and by activating mutations mainly in the *RAS* and *RAF* genes (1–3).

Aberrations in the RAS-MAPK complex are implicated in several human cancers, render them an attractive therapeutic targets (10–13). Several drugs including humanized monoclonal antibodies (*e.g.* EGFR inhibitors), and small molecule inhibitors (*i.e.* RAS, RAF and MEK inhibitors) are currently being tested in clinical trials.

2.0 RAS-MAPK Pathway Functions

Ras (H-, K-, N-isotypes) (guanine nucleotide-binding protein), is a single GTPase molecule related in structure to the G_{α} subunit of heterotrimeric G proteins. G proteins act as molecular switches and timers that cycle from inactive GDP-bound to active GTP-bound states (14). In normal quiescent cells, Ras is bound to GDP and is inactive (“off” state), while upon extracellular stimuli, Ras bind to GTP (“on” state), which has an extra phosphate group than GDP. This extra phosphate holds the two switch regions in a “loaded-spring” configuration (switch I includes Threonine-35, switch II Glycine-60). Upon the release of this phosphate, the switch regions relax leading to conformational modifications and return to the inactivate state. Therefore, the activation and the inactivation of Ras and several other small G proteins are controlled by a cycling switching between the active GTP-bound and inactive GDP-bound forms (15).

The cyclic process of GDP/GTP is facilitated by guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs). The Ras intrinsic GTPase activity, hydrolyze the GTP into GDP. However, this process is inefficient and requires additional GAPs for binding, stabilizing, and accelerating the Ras catalytic activity. This is achieved by additional catalytic residues, "arginine fingers", where a H_2O molecule is positioned for nucleophilic attack on the gamma-phosphate of GTP, leading to the release of the inorganic phosphate molecule with a subsequent binding of Ras to GDP (15).

GEFs catalyze a "push and pull" process that unhinges the GDP from Ras by positioning close to the P-loop and the magnesium cation binding site to block the interaction with the gamma phosphate anion. Acidic (negative) residues in switch II "pull" a lysine in the P-loop away from the GDP which "pushes" switch I away from the guanine. The contacts holding GDP in place break, leading to its release in cytoplasm. Because intracellular GTP is abundant relative to GDP, it predominantly re-enters the nucleotide binding pocket of Ras and reloads the spring. Thus, the GEFs and GAPs balance underlie and facilitate Ras activation and inactivation, respectively (16).

The Ras binding domain is found in many effectors and invariably binds to one of the switch regions. Activated Ras-GTP has a high affinity for numerous downstream effectors and other small GTPases such as arfaptin or second messenger systems such as adenylyl cyclase as well.

Typically, ligand binding to receptor tyrosine kinases induces dimerization of the receptor and autophosphorylation of specific tyrosine residues in the C-terminal region. This generates binding sites for adaptor proteins *e.g.* growth factor receptor-bound protein 2 (GRB2), that recruit the GEF Sos at the plasma membrane, and in turn activates the membrane-bound Ras by catalyzing the GDP to GTP. In its GTP-bound conformation, Ras combines with Raf and mobilizes the inactive protein from the cytoplasm recruiting the Raf kinases (ARAF, BRAF and CRAF) to the plasma membrane (17, 18). Once the Ras-Raf complex is translocated to the cell membrane, Ras activates the serine/threonine kinase function of Raf isoforms. Upon activation of Ras, Raf acts as a MAP kinase kinase kinase (MAPKKK) to activate MEK1 and MEK2, which, in turn, catalyze the activation of the effector ERK1 and ERK2 kinases, and their translocation into the nucleus. Once activated, ERK1/ERK2 broadly phosphorylate several nuclear and cytoplasmic effector genes involved in diverse cellular responses such as cell proliferation, survival, differentiation, motility, and angiogenesis (19–21). Although RAF can also be activated by RAS-independent activators, (22) considerable experimental evidence indicates that the RAF-MEK-ERK cascade is a major mediator of Ras-induced oncogenesis. Recent data have clearly shown that Ras can activate other downstream signaling pathways including phosphatidylinositol 3-kinase (PI3K) and Rac and Rho proteins, associated with the regulation of the cytoskeleton and invasiveness of tumor cells. Through RAS, other signals may be activated such as p38 MAPK, and the stress-activated protein kinase pathway, c-Jun N-terminal [JNK] pathway (Figure 1) (23–25).

3.0 Deregulation of RAS-MAPK Pathway in Human Cancer

3.1) Ras activation

Activating point mutations of the RAS family genes (*H-RAS, K-RAS, and N-RAS*) are not uncommon and comprise up to 30% of all human cancers (7). A summary of the frequencies of *RAS* mutations in tumours of various sites is shown in Table 1. It has been reported that 90% of pancreatic adenocarcinomas harbour a *RAS* mutation and the majority of these mutations involve the codon 12 of the *K-RAS* gene in the majority of cases. These findings link mutation in the *K-RAS* proto-oncogene to the development of pancreatic cancer. *K-*

RAS mutations are also a frequent event in colorectal, lung, and biliary tract carcinogenesis, while mutations at the *H-RAS* and *N-RAS* gene have rarely been detected. Conversely, melanomas present a quite high percentage of *N-RAS* mutations while salivary gland tumours present a majority of *H-RAS* mutations (Table 1). Of note, thyroid tumours based on their histotype origins may present different types of *RAS* alterations with *K-RAS* mutations mainly associated with papillary thyroid carcinoma (PTC) and *N-RAS* with follicular thyroid carcinomas, follicular variant PTCs, and poorly differentiated thyroid carcinomas (26–28); whereas medullary thyroid carcinomas present rare mutations of *RAS* genes (29). Ras has also been shown to be a key downstream effector in a number of activated receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR) (30). Moreover, ERK activation can induce over-expression of EGFR ligands, promoting an autocrine growth loop critical for tumor growth (1). It is, therefore, conceivable to target these genes for new therapeutic strategies in cancer bearing these alterations.

The most prevalent mutations in *Ras* genes occur at residue G12 in the P-loop and the catalytic residue Q61, which are commonly in tumors associated with *Ras* mutations (Table 1). This mutation leads to clycin to valine substitution at *residue 12* that renders the GTPase domain of Ras insensitive to inactivation by GAP and autonomous activation (“on state”). However*, residue 61* is responsible for stabilizing the transition state for GTP hydrolysis and because enzyme catalysis in general is achieved by lowering the energy barrier between substrate and product, this mutation reduces the rate of intrinsic Ras GTP hydrolysis to physiologically low levels (31, 32).

3.2) Raf activation

The RAF family members, (A-Raf, B-Raf, and C-Raf or Raf-1), are highly conserved serine/threonine kinases of the MAPK [RAS-RAF-MEK-ERK] pathway (Figure 2). The RAF proteins activate the MAPK pathway (33) where inappropriate and/or persistent activation leads to abnormal differentiation, proliferation and apoptosis, and cancer development. *BRAF* is frequently mutated in a variety of human tumors, especially in malignant melanoma, thyroid and colon carcinomas (34) (Table 2). The incidence of activating *RAF* gene mutations in cancer is presented in Table 2. Among different *BRAF* mutations, a single-base missense substitution (T to A at nucleotide 1,799) that substitute valine for glutamic acid at codon 600 (V600E) of the kinase domain (exon 15) is the most prevalent in melanomas and papillary thyroid carcinomas (35, 36). The mutant V600E BRAF protein results in impaired kinase activity, which induces hyperactivity of the MAPK pathway in a RAF1(CRAF)-dependent manner promoting the development of these tumors (37). Activation results from conformational change in the protein structure caused by glutamic acid acting as a phosphomimetic between the Thr598 and Ser601 phosphorylation sites that disrupts the interaction between the P-loop and the activation segment of the BRAF protein (38). *In vitro* and/or *in vivo* studies have demonstrated the oncogenic potential for *BRAF* V600E mutation in different settings.

Marked activation of the MAPK pathway can also suppress cellular growth in a wide variety of normal and cancer cells by inducing cellular senescence (39, 40). This mechanism of senescence is usually modulated by cyclin dependent kinase inhibitors (*e.g.* p27Kip1), and it is adopted by normal cells to overcome oncogene activation (40, 41). In contrast, moderate levels of MAPK pathway activation could induce abnormal cellular functions (42). These different effects were found to cause transformation and immortalization of mouse melanocytes, increased *in vitro* colony formation, and elevated Erk1/2 activities (43). It has also been shown that *BRAF* V600E activating mutation initially promotes nevi development but further tumor progression is inhibited by marked activation of the MAPK. In this setting, subsequent genetic abnormalities such as loss of p16INK4a, or elevation in Akt3 activity, are required for the senescent melanocytic cells to reenter the cell cycle. Recent studies

show that Akt3 and mutant V600E BRAF cooperate to promote early melanoma development; specifically Akt3 is shown to phosphorylate V600E B-Raf to lower its activity as well as that of the downstream MAP kinase pathway promoting cell proliferation (44).

BRAF codon 600 (V600E) mutations also play important roles in thyroid tumorigenesis although additional signals may be needed for thyroid follicular cells to acquire full metastatic capabilities (36, 45). This mutation has been reported in ~45% of papillary thyroid carcinomas (PTC) and in ~25% of undifferentiated thyroid cancers (35, 36, 46). Of interest, diverse types of *BRAF* mutations have been reported in the follicular variant of PTC (28). Recently, high *BRAF* mutations were reported in pleomorphic xanthoastrocytomas and gangliogliomas (47), colon carcinomas and, in other tumor types (Table 2). The frequency of mutational changes of *ARAF* and *CRAF* in human cancers is low (Table 2) except for a truncated form of CRAF (11).

4.0 Anticancer agents targeting the RAS-MAPK pathway

Strategic focus on the development of novel biologically-based treatment has gained remarkable momentum. Target-based therapies are widely considered to be the future of cancer treatment and much attention has been focused on developing inhibitors of the RAS-RAF-MEK-ERK/MAPK signaling pathway and its upstream activators. In this context, several MEK1/2 and RAF inhibitors have been tested clinically or are currently undergoing clinical trial evaluation (Table 3 and 4). RAS inhibition did not achieve the expected results in clinical trials probably due to the fact that these inhibitors are not able to hit specific target proteins. Inhibition of RAS remains an interesting target although challenging.

4.1) MEK inhibitors

Blocking MAPK via small-molecule inhibitors has become a biologically viable model for targeted cancer therapeutics. PD 098059, the first MEK inhibitor described, was identified through screening a compound library of inhibitors by assessing the phosphorylation of an ERK target protein in the presence of both MEK1 and ERK. The compound inhibited MEK with an IC50 ~10 nmol/L but had no inhibitory effects when tested against a panel of other serine/threonine kinases (48). Another second MEK inhibitor, U0126, was also identified by screening a library of molecules using an assay designed to identify antagonist for activator protein-1–driven (c-Fos and c-Jun) transcription without blocking the glucocorticoid response elements (49). U0126 inhibited MEK1 and MEK2 with IC50 ~5 to 7 nmol/L but had little effect on other kinases and the inhibition was noncompetitive with respect to the MEK substrates ATP and ERK (49). Both PD 09859 and U0126 have shown *in vitro* antiproliferative effects on transformed cell lines. Although U0126 and PD 098059 have been extremely useful in animal studies of MAPK signaling, they have not been targeted for clinical development because of poor pharmacologic properties.

CI-1040 (PD 184352), a potent MEK inhibitor (IC50 of 17 nM on purified MEK1) that progressed to clinical testing, was identified during the screening of the PD 098059 (50). CI -1040 selectively inhibited MEK1 in a noncompetitive manner with respect to ATP. The drug also inhibited MEK in cell-based assays and human colon cancer xenograft cell growth in a noncompetitive mechanism, suggesting an allosteric inhibitory effect (51). CI-1040 binds to specific hydrophobic pocket of the MEK1 and MEK2 that has low homology for other kinases, supporting the high degree of specificity. A phase I study of orally administered CI-1040 in 77 patients with advanced cancers showed that this drug was well tolerated at doses resulting in a median 73% inhibition of phospho-ERK1/2 expression in tumor biopsies. Approximately 60% of patients experienced adverse effects, mostly grade 1 or 2, with no patient having drug-related grade 4 events. The most common toxicities included diarrhea, asthenia, rash, nausea, and vomiting. Interestingly, a single patient with

pancreatic cancer achieved a partial response with significant symptom improvement that lasted 12 months. Moreover, 19 patients with different cancers had stabilization of the disease lasting from 4 to 17 months (50). A phase II study was initiated in patients with advanced pancreatic, breast, colon and non-small cell lung cancers (52). Unfortunately, none of the patients achieved a complete, partial response, and only 8 patients achieved stabilization of disease (median of 4.4 months). The poor antitumor activity, low bioavailability and solubility of this drug precluded further clinical studies.

Two additional MEK inhibitors have been developed and have advanced to clinical testing. PD 0325901 is a second generation analogue of CI-1040 with an IC50 of 1 nmol/L that is more potent and soluble than CI-1040 *in vivo*, with a single oral dose providing >50% inhibition at 24 h. The anticancer activity of this drug has also been demonstrated in a variety of human tumor xenografts bearing *BRAF* mutations (53). The clinical activity of PD 0325901 was first evaluated in a phase I–II dose-escalating study of 35 patients with advanced solid tumors with doses ≥ 2 mg BID that efficiently suppressed ERK1/2 phosphorylation (average of 84%) and Ki67 expression in tumor biopsies and resulted in two partial responses and eight stable disease that lasted 3–7 months of 27 patients. An extension of a phase I study documented, 3 partial responses and 24 stable diseases (22 melanoma and 2 non-small cell lung cancer) in 66 patients (54). However, PD 0325901 was associated with more toxicity than CI-1040, including acute neurotoxicity and blurred vision and retinal vein occlusion in patients receiving more than 15 mg BID leading to the termination of the clinical trial.

AZD6244 (ARRY-142886, Selumetinib) is an oral potent second-generation MEK1/2 inhibitor with similar structure to the CI-1040, but with improved pharmacologic properties (55). Similar to other MEK inhibitors, AZD6244 is ATP non-competitive with no significant inhibitory effects when tested against a broad range of serine/threonine kinases. Preclinical evaluation has shown anticancer activity in a variety of human tumor xenograft models with remarkable target inhibition (55–58). AZD6244 antitumor activity was found to correlate with suppression of ERK activation, in a phase I clinical trial, the pharmacokinetics and pharmacodynamics of AZD6244 in 57 patients with advanced cancer showed that the 50% maximal effective dose (100 mg BID) was well tolerated with cutaneous rash being the most frequent and dose-limiting toxicity. Most other adverse events were of grade 1 or 2. In this trial seven patients developed transient and reversible blurred vision, nine patients showed disease stabilization lasting for at least 5 months and a strong reduction in ERK1/2 phosphorylation (mean inhibition of 79%) in tumor biopsies (59). Preliminary results from four randomized phase II clinical trials of AZD6244 have recently been reported. A first trial compared AZD6244 to the alkylating agent temozolomide in advanced melanoma patients. AZD6244 showed a potent antitumor activity, but there was no significant difference in progression-free survival between the two treatment arms (60). In a second study, the efficacy of AZD6244 was compared with the antimetabolite pemetrexed as second- or third-line treatment of patients with non-small cell lung cancer. The study showed evidence for single agent antitumor activity, but failed to demonstrate a difference in primary disease progression endpoint (61). In a third study, AZD6244 versus capecitabine in patients with metastatic colorectal cancer, who had failed prior irinotecan and/or oxaliplatin treatments, showed no difference between the two treatments in the number of patients with disease progression (62). Another Phase II study of Selumetinib in 28 patients (thirty-nine percent of whom had received one prior systemic therapy) with metastatic biliary cancers, had a confirmed objective response in three patients (12%). Another 17 patients (68%), experienced stable disease (SD), 14 of whom (56%) experienced prolonged SD (> 16 weeks) (63). Finally, the results of a phase II study of AZD6244 in patients with advanced or metastatic hepatocellular carcinoma, showed lack of radiographic improvement that led to the termination of the trial (64).

Based on their high potency, oral bioavailability, and preclinical evidence of anticancer activity, PD 0325901 and AZD6244 were the first candidates MEK inhibitors for treatment of human cancers.

GDC-0973/XL518 (Exelixis/Genentech) is an oral active inhibitor of MEK1/2 (IC50 of <1 nM). Cell-based studies using this compound demonstrated an inhibition of ERK1/2 phosphorylation at subnanomolar concentrations, and antiproliferative effects in multiple tumor cell lines harboring *K-RAS* or *BRAF* mutations. Animal model studies have shown that a single dose of GDC-0973 inhibits phosphorylation of ERK1/2 in tumors for up to 48 hours strongly inhibiting tumor growth in human xenograft models (65). Remarkably, GDC-0973 has minimal side effects on the central nervous system. A phase I dose-escalating study of GDC-0973 was initiated in patients with solid tumors. Preliminary results of 13 patients' trial indicate that GDC-0973 is well tolerated with no drug-related severe adverse events being reported (66). A patient with non-small cell lung cancer had stabilization of disease for 7 months. Another phase I trial of GDC-0973 in combination with the PI3K inhibitor GDC-0941 of 30 patients showed decreases in RECIST measurable target lesions in 5 patients, 2 melanoma, 1 prostate cancer, 2 NSCLC and three patients had prolonged stable disease (> 6 months) (67). Another phase Ib, dose-escalation study of GDC-0973 in combination with PLX4032/RO5185426 in patients with BRAFV600E-positive metastatic melanoma, is ongoing.

GSK1120212 (GlaxoSmithKline) is an orally bioavailable, allosteric and selective inhibitor of MEK1/2 enzymes. Cell-based assays and xenografts mouse models showed an important antitumor activity of this drug (68, 69). A phase I study of GSK1120212 patients with solid tumors and lymphoma and the preliminary evaluation of 6 patients treated at four dose levels, was well tolerated with no dose-limiting toxicity reported (70). Two other phase I/II studies of GSK1120212 have started to enroll patients with relapsed or refractory leukemias, and in combination with everolimus in patients with solid tumors. Randomized Phase III study comparing single agent GSK1120212 to chemotherapy (either dacarbazine or paclitaxel) in subjects with stage IIIc or stage IV cutaneous melanoma, is being conducted on patients with *BRAF* mutation-positive tumor.

RDEA119 (BAY 869766, Ardea Biosciences/Bayer) is an oral bioavailable, allosteric *in vitro* inhibitor of MEK1 (IC50 of 19 nM) and MEK2 (IC50 of 47 nM) in a non-ATP competitive manner. *In vitro* assays showed that RDEA119 inhibits ERK1/2 phosphorylation and cell proliferation (IC50 from 2.5 to 16 nM) in human cancer cell lines (71). *In vivo*, RDEA119 exhibits potent antitumor activity in xenograft models of melanoma and colon carcinoma. Interestingly, this compound has low central nervous system penetration and it is currently being evaluated as single agent in a phase I study in advanced cancer patients and in a phase I/II study in combination with the multikinase Raf inhibitor sorafenib.

4.1.1 Other MEK1/2 Inhibitors—Other MEK1/2 inhibitors are currently being evaluated in phase I clinical trials of advanced cancer patients. These are AZD8330 (Array BioPharma/AstraZeneca), RO5126766 and RO4987655 (Hoffmann La Roche), TAK-733 (Millenium Pharmaceuticals) and AS703026 (EMD Serono), XL518 (Genentech/Exelixis). Other novel MEK1/2 inhibitors such as RO4927350, RO5068760 and PD318088 have recently been reported on preclinical models (72).

4.2) RAF inhibitors

Efforts to develop antisense inhibitors of Raf expression and activity are being made (Table 4). ISIS-5132, a 20base phosphorothioate DNA oligonucleotide, blocks mainly the c-Raf-1 protein expression and showed antitumor activity in preclinical xenograph models and early

clinical trials. Further trials, however, have been halted due to the lack of clinical responses (73). A similar approach, using a liposome encapsulated antisense c-raf-1 oligonucleotide LErafAON, showed antitumor activity in xenograft models (74) but in phase I clinical trials as a single agent and in combination with radiation or chemotherapy resulted in modest results (75, 76).

To date, the most successful anti-Raf inhibitor has been Sorafenib (tosylate salt of BAY 43-9006; Nexavar), an orally administered compound used for treatment of advanced renal cell carcinoma (RCC). Sorafenib is a bi-aryl urea compound that was originally developed as an inhibitor of Raf-1 (77). Subsequent studies revealed that sorafenib is also a potent inhibitor of both wild-type and mutant B-Raf V600E kinases *in vitro*, and has a potent activity for other protein kinases, such as, the proangiogenic tyrosine kinase receptors and VEGFR-2, VEGFR-3, FGFR-1, PDGFR-b, Flt-3, and c-Kit (77). Sorafenib may also inhibit angiogenesis-related kinases as well as other non-Raf kinases as evidenced through antitumor activity independent of *RAS* or *BRAF* mutation status in different studies. However, trials of sorafenib have not provided evidence to conclude that inhibition of Raf has clinical value. Furthermore, although preclinical data showed that continued expression of mutant Braf is critical for melanoma growth and tumorigenicity, phase II clinical trials with sorafenib (400 mg BID) showed only modest or no antitumor activity (78). Phase II trial of sorafenib and chemotherapy showed early response in melanoma irrespective of the *BRAF* mutational status. Moreover, a phase III trial of sorafenib in combination with carboplatin and paclitaxel failed to achieve improvement in overall survival (79). These results may lend credence to the concept that the primary mechanism of activity of sorafenib in solid cancer is likely anti-angiogenic and not RAF related. It's worth noting, however, that phase II/III clinical trials of sorafenib in patients with advanced hepatocellular carcinoma have shown promising results (80). Clinical trials using sorafenib in combination with other agents for the treatments of advanced or metastatic breast and thyroid cancers are ongoing (81, 82).

The limited activity of sorafenib in tumors with *BRAF* mutation prompted the development of second-generation RAF inhibitors with greater selectivity for mutant *BRAF.* PLX4032/R7204 (Vemurafenib) and its analog PLX4720 have shown potent antiproliferative effects in several preclinical models specifically in cell lines harboring *BRAF* V600E mutations (83). In a recent phase 1 clinical trial of PLX4032, high (30 to 50 μM) steady state serum levels of the drug were tolerated with modest toxicity and intense inhibition of ERK signaling in tumors. The antitumor activity observed was 78% response rate by RECIST and tumor shrinkage in almost all patients (84). Notable toxicities included the development of squamous cell carcinomas and skin rash in approximately one third of patients. The average duration of response in phase I trial was approximately 9 months. A recent randomized phase III clinical trial comparing PLX4032 to dacarbazine as first-line therapy in patients with previously untreated *BRAF* V600E mutation positive melanoma patients showed that PLX4032 improved overall and progression-free survival in comparison to dacarbazine group. Common adverse events associated with PLX4032 were arthralgia, rash, fatigue, alopecia, keratoacanthoma or squamous-cell carcinoma, photosensitivity, nausea, and diarrhea (85). Despite the important impact made by these inhibitors for the treatment of melanoma patients bearing *BRAF V600E* mutations, early studies of these agents in patients with *BRAF V600E* colorectal carcinoma have thus far been disappointing (86). This data could be explained by the activation of different and/or redundant activating cellular mechanisms of the MAPK pathway in colorectal cancers.

It has been reported that PLX4032 inhibited ERK activation only in cancer cells with *BRAF* mutations, while in cells with wild-type *BRAF*, it induced ERK phosphorylation and activation (87). The underlying mechanism for this paradoxical effect is likely related to the

allosteric effect of the drug, which enforces the dimerization of endogenous BRAF with CRAF or ARAF (88–90). Within these dimers, only one active component is required for activation of MEK–ERK pathway. At non-saturating concentrations, the inhibitors activate rather than inhibit the pathway, particularly in the presence of *RAS* mutations or activated upstream receptor kinases (by Ras activation). It is also possible that the rapid development of skin tumors in patients treated with RAF inhibitors might be enhanced by such mechanism (91, 92). The paradoxical activation of ERK by PLX4032 in wild type BRAF can also be explained by the low RAS activity, and that dimer formation is not required for the activation. It's worth noting that such paradoxical activation of ERK signaling is not unique to PLX4032 since it is also observed with other ATP-competitive inhibitors of RAF and sorafenib. These results suggest that the clinical activity of PLX4032 will be restricted to tumors harboring activating mutations of *BRAF*.

GSK2118436 (another ATP competitive BRAF inhibitor), has recently shown a dramatic effect, with response rates of 70–80% as single agent in metastatic melanoma patients (84, 93, 94). A phase I/II clinical trial of patients with metastatic brain melanoma showed that ninety percent of the patients had reductions in size of metastases. The overall reductions ranged from 20 to 100% of brain metastases that were 3mm or larger in diameter before treatment. These drugs are currently being tested in other clinical trials in patients with other solid tumors with *BRAF* V600E mutations, such as thyroid carcinomas or colon cancers (95, 96). Potential risk during treatment of thyroid cancer patients with inhibitors that target mutants BRAF is the multifoci nature of PTCs and the heterogeneity of the *BRAF* V600E mutation in these lesions. Additional small molecule inhibitors of RAF are also in their early clinical testing. XL281 (BMS-908662) has shown modest biological activity and modulation of MAPK pathway activity in tumor tissue: clinical benefit (partial response or stable disease) was observed in 43% (13 of 30) of patients in the dose-escalation phase of a recent phase 1 study (97).

4.2.1 Other RAF Inhibitors—RAF265 is an oral highly selective inhibitor of RAF, including B-RAF and C-RAF and mutant B-RAFs. RAF265 has also an important anti-angiogenic activity through inhibition of vascular endothelial growth factor receptor 2 (VEGFR-2). Interestingly, growth inhibitory effects have been demonstrated in combination of RAF265 and mammalian target of rapamycin inhibitor RAD001 (everolimus) (98). In a BRAF mutant xenograft mouse model, RAF-265 induced dose dependent tumor regression and is currently being tested in Phase I clinical trials in patients with advanced malignant melanoma.

AZ628 is a selective RAF inhibitor of CRAF (Raf-1) as well as BRAF wild type and the V600E BRAF. Cytotoxic activity in many tumor cell lines, especially those with V600E *BRAF* mutations have been achieved using this agent. It has been demonstrated that CRAF overexpression is associated with both primary and acquired resistance to BRAF inhibition by AZ628 in *in vitro* melanoma model. Interestingly, AZ628 resistant melanoma cells are sensitive to HSP90 inhibitors, which promote CRAF degradation (99), suggesting that both drugs may overcome resistance in a subset of patients with CRAF overexpression.

SB-590885 is another selective smallmolecule inhibitor of the B-Raf kinase. It has been usedto overcome the dependence of MAPK signaling and tumor cellgrowth on B-Raf kinase activity. In melanoma and colorectal cancer cell lines with *B-Raf* V600E mutation, an effective inhibition of cell proliferation was achieved at concentrations that inhibited ERK phosphorylation. Both cytostatic and cytotoxicmechanisms of growth inhibition were observed based upon compound concentration and exposure time (100). The clinical safety and toxicity of this compound, however, requires further investigations.

GDC-0879 is a highly selective and orally available BRAF inhibitor that is effective in BRAF V600E containing cells in various *in vitro* and cell-based assays. For this compound, subnanomolar enzyme potency translated into very effective reduction of cellular viability of BRAF-mutant Malme3M cell lines (EC50 values were 0.75 μmol/L for GDC-0879) (101).

5.0 Expert Opinion & Discussion

Extensive preclinical data support an important role for the RAS-MAPK signaling pathway in cancer biology and its potential as a therapeutic target in human cancers. Molecular and pharmacologic studies have shown that MEK and ERK are required for the transforming activities of Ras and different oncogenes. More recently, mutationally activated Braf and Ras have been identified in several tumors suggesting a tight link between Braf activation and Ras function. The RAF–MEK–ERK cascade, therefore, constitutes a key node in the complex signalling network, with multiple converging signals affecting Raf activation.

In general, MEK inhibitors appear to be well tolerated with only rash, edema, and transient blurred vision as common side effects. Importantly, suppression for each compound in tumor tissue with plasma concentrations corresponding to those sufficient to inhibit MEK *in vitro* has been demonstrated. Despite the preclinical data, the objective response rate in these studies has been modest and could be attributed to several factors among which are: i) unknown concentration for tumor cytotoxicity, ii) the existence of alternative pathways that compensate for the effects of the MEK inhibitors iii) alterations of multiple signaling pathways in cancer, and iv) inhibition of only a single pathway may not be sufficient to promote apoptosis or cell growth arrest. Given the excellent safety records of these drugs, it is likely that combinations of cytotoxic agents and/or other targeted therapies will be tested in the future as evidence by preclinical data support the potential development of MEK inhibitor combinations. Targeted therapies are likely to benefit only a subset of patients as single agents. The inhibition of a single signal (*e.g.* RAS) is not able to block pathways downstream of RAS-RAF, which can be regulated by other signals. Some cellular pathways can be activated by both RAS-dependent and RAS-independent mechanisms. Preclinical studies, increased knowledge of the complexity of the Ras-MAPK pathways, and their intersection with other oncogenic pathways, provide the opportunity to design better therapeutic strategies. Combining different RAS-MAPK pathway inhibitors (*e.g.* RAS-, RAF-, MEK-, PI3K inhibitors), the use of different RAS-signaling inhibitors applied sequentially, or interfering with its upstream supportive signals (*e.g.* RTKs) might tailor drug combinations, which could have a significantly higher potential for killing tumor cells reducing the side effects on normal cells.

Raf-kinase inhibitors also remain an attractive therapeutic target, and additional preclinical and clinical studies are warranted in order to evaluate the clinical activity and benefit of these types of compounds. Further studies are also needed to better understand the specific biological mechanism of these inhibitors. In particular the identification of the “subpopulation” of patients that may likely benefit.

Efforts to identify predictive molecular markers or gene signatures of response are needed to advance tailored therapy. The identification of *EGFR* mutations that predict for response to EGFR inhibitors is a good example. *BRAF* V600E and *RAS* mutations may also represent viable biomarkers to predict clinical responsiveness to RAF and MEK inhibitors. Therefore, analysis of *BRAF* and *RAS* mutations should always be required in future therapeutic trials using these new classes of inhibitors. Particularly using BRAF inhibitors, which have been shown to enhance ERK phosphorylation and tumor growth in *BRAF* wild type cancer cells. Second line continue treatments with BRAF inhibitors may lead to resistance of cancer cells therefore proper combination therapies should be developed to overcome acquired and

adaptive resistance to these agents. A model of resistance to BRAF inhibitors developed by chronic treatment of BRAF V600E melanoma cells with the BRAF inhibitor SB-590885 has recently been described where resistance was attributed to the flexible switching among the three RAF isoforms, underscoring the ability of melanoma cells to adapt to pharmacological challenges. It has been suggested that the activation of other pathways in particular IGF-1R/PI3K signaling, underlie this resistance. Only combined treatment with IGF-1R/PI3K and MEK inhibitors induced death of BRAF inhibitor-resistant cells. An increased IGF-1R and pAKT level in a post-relapse human tumor sample was consistent with a role for IGF-1R/PI3K-dependent survival in the development of resistance to BRAF inhibitors (99). The future design of potential drug combination therapies and the follow-up of their outcome will undoubtedly be facilitated by gene profilings. As the clinical trials of these inhibitors progress, more efforts should be directed to further unravel the complex biology and genetics, and the crosstalk signals of the cancer cells. We contend that combinational therapeutic approaches would be the best approach to address cancer cells escaping drug inhibition and developing resistance.

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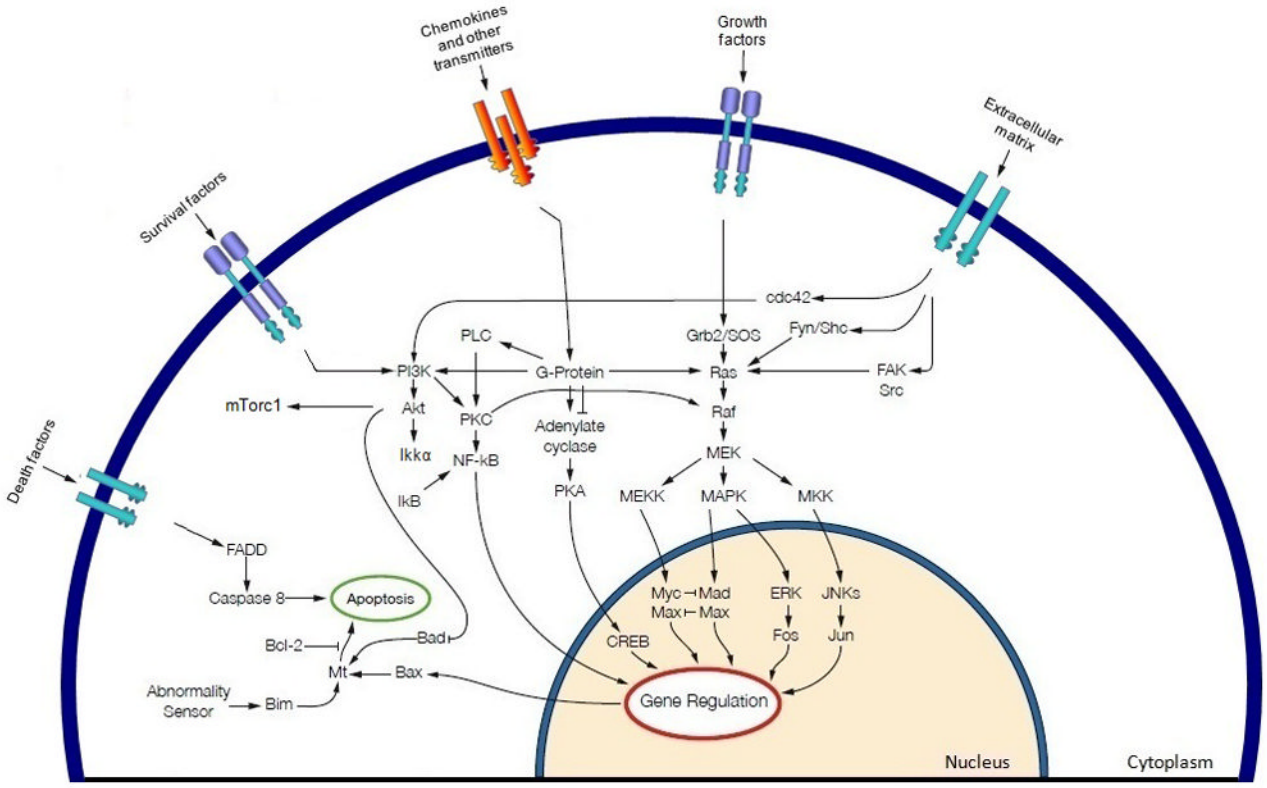

Figure 1.

Schematic representation of the MAPK cascade activation and potential cross talk signals. Growth factors binding, and causing activation of, tyrosine kinase receptors, activating mutations of *BRAF* and *RAS*, and additional external cell membrane stimuli may cause persistent activation of the MAPK cascade in human cancers. Numerous effectors signals converge on RAF, activated RAF phosphorylates MEK in the cytoplasm, which in turn phosphorylates ERKs that translocates to the nucleus where they phosphorylate and regulate various nuclear and cytoplasmic substrates involved in diverse cellular responses, such as cell proliferation, survival, differentiation, motility, and angiogenesis. RAS may cross-talk with different signalling pathways, *e.g.* PI3K, to enhance tumorigenesis in cancer cells.

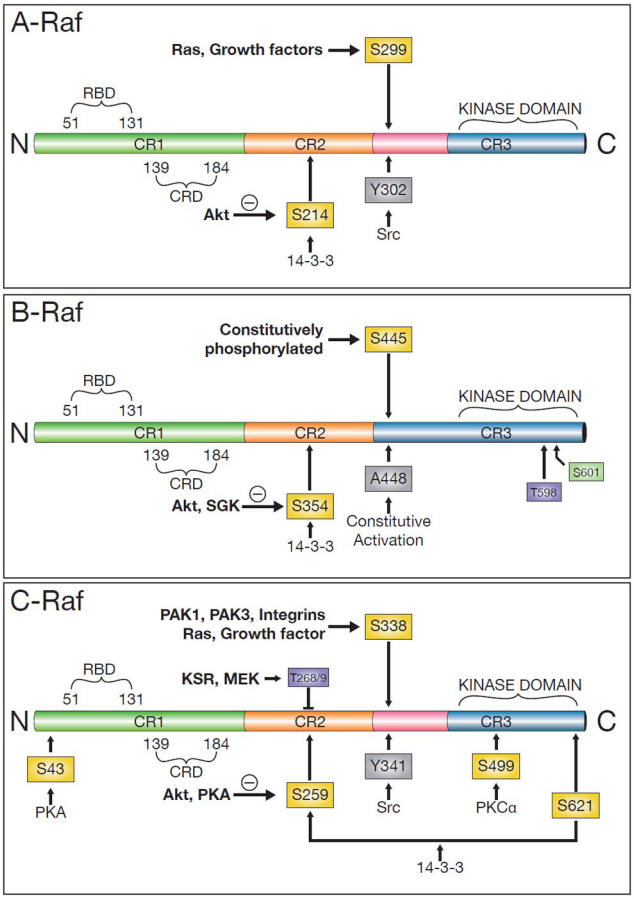

Figure 2.

Schema of the domain structure of human A-Raf, B-Raf, and C-Raf. The three mammalian RAF serine/threonine protein kinases, ranging from 70 to 100 kDa in size, mediate the transduction of proliferative and differentiative signals from cell surface receptors to the nucleus catalyzing the phosphorylation of hydroxyl groups on specific Ser and Thr residues. *A-Raf* gene (Location: Xp11.4-p11.2). This isoform is the weakest activator of MEK, and can only activate MEK1 but not MEK2. *B-Raf* gene (Location: 7q34); *BRAF* point mutations within the kinase domain of the protein occur in several tumor types including, melanomas, papillary thyroid and colorectal carcinomas. *C-Raf* gene (Location: 3p25); CRAF is the cellular homolog of viral raf gene (v-raf). The encoded protein is a MAP kinase kinase kinase (MAP3K), which functions downstream of the Ras family of membrane associated GTPases to which it binds directly. Among 3 RAF isoforms, BRAF displays the highest basal kinase activity.

S, serine; T, threonine; Y, tyrosine: amino acid phosphorylation sites regulating the Raf kinases. Abbreviations: C, carboxyl terminus; N, amino terminus; RBD, Ras-binding domain; CRD, cysteine-rich domain; KSR, kinase suppressor of Ras; MEK, mitogen-activated protein kinase kinase; PAK, p21-activated kinase; PKA, protein kinase A; PKCα, protein kinase C alpha; SGK, steroid glucocorticoid kinase; Src, soluble nonreceptor tyrosine kinase.

Table 1

Frequency of *RAS* (*K-RAS, N-RAS, H-RAS)* mutations in different types of tumors

Tumors	Frequency of *K-RAS* mutations	Total samples analyzed
pancreas	**57%**	5169
large intestine	**33%**	30793
biliary tract	**31%**	1555
small intestine	**20%**	316
lung	**17%**	15080
endometrium	**14%**	2143
ovary	**14%**	2956
gastrointestinal tract (site indeterminate)	8%	49
cervix	7%	637
prostate	7%	1126
soft tissue	7%	1070
peritoneum	6%	86
stomach	6%	2571
haematopoietic and lymphoid tissue	5%	5762
liver	5%	450
urinary tract	5%	868
breast	4%	544
eye	4%	90
genital tract	4%	25
oesophagus	4%	359
penis	4%	28
testis	4%	432
autonomic ganglia	3%	63
salivary gland	3%	170
skin	3%	1422
upper aerodigestive tract	3%	1554
thymus	2%	186
thyroid	2%	4710
bone	1%	231
central nervous system	1%	1032
kidney	1%	617

Tumors	Frequency of *N-RAS* mutations	Total samples analyzed
skin	**18%**	4759
NS	**17%**	282
haematopoietic and lymphoid tissue	**10%**	8548
thyroid	7%	4206
autonomic ganglia	6%	102
adrenal gland	5%	170
ovary	4%	133

Tumors	Frequency of *N-RAS* mutations	Total samples analyzed
soft tissue	4%	481
liver	3%	310
testis	3%	283
upper aerodigestive tract	3%	807
breast	2%	330
cervix	2%	132
large intestine	2%	1056
pancreas	2%	248
prostate	2%	530
stomach	2%	215
urinary tract	2%	655
biliary tract	1%	213
central nervous system	1%	995
endometrium	1%	314
eye	1%	103
lung	1%	2851

Tumors	Frequency of *H-RAS* mutations	Total samples analyzed
salivary gland	**15%**	161
urinary tract	**10%**	1499
cervix	9%	264
upper aerodigestive tract	9%	1083
penis	7%	28
prostate	6%	500
skin	6%	2100
soft tissue	5%	712
stomach	4%	384
testis	4%	130
thyroid	4%	3681
pituitary	3%	300
bone	2%	199
thymus	2%	46
adrenal gland	1%	135
breast	1%	542
endometrium	1%	291
oesophagus	1%	161

In bold all somatic mutations that are higher than 10% of frequency. (Sanger mutation database: http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Table 2

Frequency of *RAF (ARAF, BRAF, CRAF)* mutations in different types of tumors

Tumors	Frequency of *ARAF* mutations	Total samples analyzed
ovary	4%	27
large intestine	2%	157

Tumors	Frequency of *BRAF* mutations	Total samples analyzed
NS	**57%**	547
skin[^]	**39%**	7731
thyroid	**38%**	19675
genital tract	**12%**	33
large intestine	**12%**	36546
biliary tract	**11%**	242
eye	**11%**	493
ovary	**11%**	1898
gastrointestinal tract	6%	31
meninges	5%	19
small intestine	5%	101
central nervous system	4%	1312
endometrium	4%	825
prostate	4%	1130
bone	3%	147
breast	3%	424
testis	3%	235
adrenal gland	2%	154
haematopoietic and lymphoid tissue	2%	1235
lung	2%	3539
oesophagus	2%	170
pancreas	2%	503
soft tissue	2%	1172
upper aerodigestive tract	2%	723
autonomic ganglia	1%	149
cervix	1%	451
liver	1%	145
pituitary	1%	115
salivary gland	1%	90
stomach	1%	955
urinary tract	1%	345

Tumors	Frequency of *CRAF* mutations	Total samples analyzed
liver	**100%** *	1 *
ovary	4%	26
lung	1%	225

NS, nervous system;

^ *BRAF V600E* mutation has been demonstrated in a higher percentage of melanomas.

* This percentage of mutations deserves further investigations; only 1 case of this type of cancer has been analyzed and demonstrated to be positive for *CRAF* mutations. In bold all somatic mutations that are superior to 10% of frequency. A high percentage of BRAF V600E mutations has recently been reported at American Society of Clinical Oncology 2011 in 47 consecutive patients with hairy cell leukemia (also published by Tiacci E. et al. on NEJM 2011 "*BRAF* Mutations in Hairy-Cell Leukemia").(Sanger mutation database: http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Table 3

MEK inhibitors

Compound	Tumor	Primary Target(s)	Potential side effects
CI-1040 (Pfizer Inc.)	Breast cancer, Lung cancer, Pancreatic cancer, Colorectal cancer, Breast Neoplasm, Non small cell lung cancer, Pancreatic Cancers	MEK	Diarrhea, Asthenia, Rush, Nausea, Vomiting
PD-0325901 (Pfizer Inc.)	Carcinoma, Non small cell lung cancer, Melanoma, Breast cancer	MEK	Diarrhea, Nausea, Fatigue, Rash, Reversible visual disturbances, Vomiting
ARRY-438162 (Array BioPharma)	Metastatic Biliary Cancer, and Metastatic Colorectal Cancer	MEK	Recruiting Phase
AZD6244 (AstraZeneca)	Breast cancer, Colon cancer, Lung cancer, Kidney cancer, Metastatic colorectal cancer, Advanced/metastatic Melanoma, Pancreatic cancer, Non small cell lung cancer, Hepatocellular carcinoma	MEK	Papulopustular rash, Xerosis, Fissures, Pruritus, Telangiectasias, Hyperpigmentation, Dermatitis, Alopecia, Angular chelitis, Paronychea, Fatigue
RDEA119/BAY 86-9766 (Bayer/Ardea Biosciences)	Advanced cancer, cancer, Thyroid cancer Pancreatic	MEK	Low toxicity, further studies are needed
GSK1120212 (GlaxoSmithKline)	Solid tumors, Melanoma, Non small cell lung cancer, Pancreatic cancer	MEK, C-Raf, B-Raf, V600E, BRAF wt	Fatigue, Diarrhea
TAK-733 (Millennium Pharmaceuticals)	Advanced Non-hematologic malignancies, Metastatic melanoma	MEK	Recruiting
GDC-0973/XL581 (Genentech/Exelixis)	Metastatic Melanoma, Solid cancers	MEK	High toxicity, Diarrhea, Fatigue, Rash, Nausea, Vomiting
AZD8330/ARRY-424704 (AstraZeneca)	Advanced malignancies	MEK	Ongoing trial
RO5126766 (Hoffmann-La Roche)	Metastatic or advanced solid tumors	MEK	Recruiting
RO4987655 (Hoffmann-La Roche)	Advanced and/or metastatic solid tumors	MEK	Recruiting
RO5068760 (Hoffmann-La Roche)	Melanoma, Colorectal cancer	MEK	Preclinical Phase Studies
AS703026 (EMD Serono)	Acute Myeloid Leukemia, and Hematological Malignancies	MEK	Recruiting

Table 4

RAF inhibitors

Compound (Pharmaceutical Company)	Tumors	Primary Target(s)	Potential side effects
Bay 43-9006 * Sorafenib (Bayer)	Squamous cell carcinoma, Non small cell lung, Leukemia, Hepatocellular carcinoma, Kidney cancer, Pancreatic cancer, Bladder cancer, Lung cancer, Urothelial cancer, Neuroendocrine tumors, Thyroid cancer, Renal cell carcinoma	C-Raf B-Raf B-Raf V600E	Fatigue, Anorexia, Diarrhoea, Nausea, Skin reaction, Alopecia, Rash, Stomatitis, Elevated bilirubin, Pancreatitis
LErafAON (NeoPharm, Inc)	Different advanced malignancies	C-Raf	Back Pain, Chills Fatigue, Hypertension, Fever, Flushing, Dyspnea
ISIS 5132 (Isis Pharmaceuticals)	Ovarian cancer, Breast cancer	C-Raf	Minimal side effects
PLX4720 (Plexxikon/Roche)	Melanoma	B-Raf V600E	Minimal side effects, further studies are needed
PLX4032 (Plexxikon/Roche)	Malignant melanoma, Colorectal carcinoma	B-Raf B-Raf V600E	Cutaneous side effects, Arthralgia, Fatigue
Raf-265 (Novartis Oncology)	Metastatic Melanoma	Raf	Recruiting phase
XL281 (Exelixis)	Non small cell lung cancer, Colorectal cancer, Melanoma, Papillary thyroid cancer	Raf	Recruiting phase
GDC-0879 (Genentech)	Melanoma	Raf	Preclinical Phase Studies
GSK2118436 (GlaxoSmithKline)	Melanoma	B-Raf V600E	Nausea, Fatigue, Fever, Rash, SCC, Headache

WT, wild type,

* sorafenib is a multityrosine kinase inhibitors, which inhibits additional targets such as, VEGFR2, drugs target additional markers. SCC, squamous cell carcinoma. Another novel multikinase inhibitor is BAY 73-4506 (Regorafenib), which potently inhibits endothelial cell kinases in biochemical and cellular kinase phosphorylation assays. Furthermore, regorafenib inhibits additional angiogenic kinases such as VEGFR1/3, platelet-derived growth factor receptor-β and FGFR 1 and the mutant oncogenic kinases KIT, RET and BRAF. This compound exhibited potent dose-dependent tumor growth inhibition in various preclinical human xenograft models in mice, with tumor shrinkages observed in breast MDA-MB-231 and renal carcinoma models.