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Selective Raf Inhibition in Cancer Therapy

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Abstract

Over the past 5 years, the Raf kinase family has emerged as a promising target for protein-directed cancer therapy development. The goal of this review is to first provide a concise summary of the data validating Raf proteins as high-interest therapeutic targets. We then outline the mode of action of Raf kinases, emphasizing how Raf activities and protein interactions suggest specific approaches to inhibiting Raf. We then summarize the set of drugs, antisense reagents, and antibodies available or in development for therapeutically targeting Raf or Raf-related proteins, as well as current strategies combining these and other therapeutic agents. Finally, we discuss recent results from systems biology analyses that have the potential to increasingly guide the intelligent selection of combination therapies involving Raf-targeting agents and other therapeutics.

Keywords

C-Raf; B-Raf; Ras; EGFR; MEK; kinase inhibitor; antisense inhibitor; combination therapy; systems biology; signal transduction; cancer

1. Introduction

Cancer arises from the abnormally increased expression and/or mutation-based activation of oncogenes, or the abnormally decreased expression and/or activity of tumor suppressor genes. Protein-targeted cancer therapies follow two primary strategies. One strategy is to directly target the oncogene or tumor suppressor gene that is functioning aberrantly, and hence is the primary lesion inducing a cancer. A second strategy is to target a protein that is an essential component of the oncogenic pathway, although it is not itself mutated or misexpressed. By each criterion, the Raf protein family (A-Raf, B-Raf, and c-Raf) has emerged in the past several years as an extremely promising target for protein-directed therapies.

1.1 Raf as a therapeutic target

The Raf proteins are central components of the mitogen-activated protein kinase (MAPK) pathway that regulates cell proliferation (**Figure 1**). The core pathway, first elucidated in the early 1990s, is now appreciated as one of the most common sources of oncogenic lesions in cancer. Overexpression or mutation of members of the epidermal growth factor (EGFR) protein family is a driving factor for numerous cancers, including pancreatic ([1,2]; reviewed in [3]), lung (adenocarcinoma and non-small cell lung cancer (NSCLC) [4]), head and neck squamous cell cancer [5], colorectal [6], glioblastoma [7], and (for EGFR2/HER2/NEU/ERBB2) breast cancer [8,9] **Table 1**. Increased expression and/or mutation-based activation of EGFR

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hyperactivates its downstream effector, Ras. Separately, Ras proteins are mutated, resulting in constitutive activation, in a high percentage of pancreatic, colon, and papillary thyroid cancers, and are also found in other cancers such as NSCLC and others ([10], **Table 1**). These changes in EGFR and Ras lead to a greatly enhanced level of Ras-dependent Raf activation, which in turn communicates signals downstream to MEK1/2 and the MAPKs ERK1 and ERK2. Although Ras has other important direct effectors in tumor promotion, including phosphoinositol-3-kinase (PI3K) and RalGDS ([11,12], and reviewed in [13]), the Raf > MEK > ERK signaling axis is essential for oncogenesis, based on validation in many systems [14]. Hence, elimination of Raf function is predicted to be an effective treatment for the many cancers initiating with EGFR and Ras lesions.

More recently, mutations increasing the catalytic activity of the Raf proteins themselves have been identified in an increasing number of human tumors **Table 1, Table 2**. The first V600E catalytically activating mutations were identified in B-Raf, in melanomas, in 2002 [15,16]. A recent database release annotating the incidence of somatic mutations in cancer indicated that activating B-Raf mutations (>85% involving V600E) were found in as many as 8% of human cancers, with the greatest association with cancers of the skin and thyroid [17]. V600E B-Raf and Ras mutations are almost mutually exclusive, implying that this B-Raf mutation fully captures the most important pro-oncogenic function of catalytically activated Ras. In contrast, a very limited number of cases of activated c-Raf have been reported, and no mutations in A-Raf, in spite of extensive scrutiny [18–20]. Although the reason B-Raf is so much more commonly mutated than c-Raf and A-Raf is not definitively established, several studies addressing the mechanism of kinase activation for the Raf family point to differences between family members that allow B-Raf to be activated by a single mutation, while c- and A-Raf require multiple mutational events [21,22].

Besides activation of Raf signaling in tumors, a number of studies implicate the activation of the Ras-Raf-MEK-ERK signaling pathway as a critical step in vasculogenesis and angiogenesis [23–25]. Such activation is induced by growth factors such as VEGFR2, FGFR2, EDG1 and Tie2, as well as by adhesion proteins such as the integrins $\alpha_v ß_3$ and $\alpha_v ß_5$ (e.g. [26–30]). Thus, inhibition of activation of Ras-ERK pathway could also represent a legitimate target for modulation tumor angiogenesis and vascularization.

Over the past two decades, drugs (small molecules, antisense, and antibodies) have been designed to target proteins at each point on the EGFR > Ras > Raf > MEK > ERK cascade. Some of these agents (particularly those targeting the kinase activity of EGFR) are promising in the clinic; others, such as farnesyltransferase inhibitors (FTIs) designed to target Ras, have not satisfied initial hopes. Over the next five years, the efficacy of Raf- and MEK- targeted agents should be clearly established.

2. Raf-targeting strategies: Issues

In considering the relationship of Raf to the EGFR > Ras > Raf > MEK > ERK signaling cascades, there are a number of alternative methods by which Raf activity can be targeted. 1) An antisense or short hairpin RNA (shRNA) approach can be used to knockdown the Raf mRNA, depressing the steady state level of the protein. 2) Raf levels can also be depressed by selectively reducing Raf transcription, or by destabilizing Raf at the protein level. 3) The kinase activity of Raf can be directly targeted with a catalytic inhibitor. 4) The interaction of Raf with essential partner proteins such as its activator (Ras) or its effector (MEK) can be inhibited. As detailed below (**Section 5**), each of these strategies has been explored. In thinking about the relevant merits of each approach and therapeutic applicability of these agents in regard to the specific biology of Raf, there are several key points to consider:

2.1 Raf paralogs and domains

The conserved domain structure of the oncogenic Raf paralogs B-Raf and c-Raf-1 is shown in **Figure 2**. Important structural elements include the Ras-binding domain (RBD), an ~80 amino acid module that is essential for Ras binding, and a flanking zinc finger-containing cysteine-rich domain (CRD), which binds Ras and phosphatidylserine. As Ras activation of Raf involves translocation and tethering of Raf at the plasma membrane, the RBD/CRD interactions are essential for transmission of signals from upstream in the EGFR>Ras signal cascade. Both RBD and CRD are contained within a larger region (CR1, conserved region 1), which marks an area of high homology within the Raf paralog group. CR2 and CR3 define two other highly conserved regions. CR2 encompasses an important inhibitory phosphorylation site that maintains Raf in an inactive conformation, and influences its localization. CR3 contains the Raf kinase domain, which phosphorylates MEK2/1; it is N-terminally flanked by a number of phosphorylation sites that are targeted by kinases to activate A- and c-Raf, and are either constitutively phosphorylated or mutated in B-Raf (contributing to the more ready activation of this protein).

The Raf proteins undergo dynamic conformational changes and interact with different protein partners as they transition through inactive, partial, and fully activated states [31,32](**Figure 3**). During this progression, the Raf proteins are constantly complexed with specific protein chaperones (Hsp90, 14-3-3) that stabilize the protein from degradation and help regulate Raf conformation, thus influencing the accessibility of binding sites for regulatory Raf-interacting proteins. These proteins, including the kinases Pak, Akt, and Src, and the phosphatase PP2A, govern the ability of Raf to bind Ras, localize to the plasma membrane, and activate its kinase activity. Finally, the protein KSR binds simultaneously to Raf, MEK, and ERK at the plasma membrane, regulating the rate and localization of EGFR > Ras > Raf > MEK > ERK signal transmission by providing a scaffold that brings multiple pathway constituents into proximity.

Translating these mechanistic observations into considerations for clinical practice, if a tumor arises from an oncogenic lesion in a single Raf family protein (typically B-Raf), an inhibitory strategy targeted specifically to that kinase (i.e., a specific antisense or shRNA) is appropriate. If a tumor instead involves a lesion upstream of Raf, a therapeutic agent that incapacitates or removes multiple Raf family members would logically be preferable. Such agents would include inhibitors of Raf kinase activity, Raf protein interaction inhibitors, and protein destabilizing drugs. It is also important to consider the possibility that eliminating the function of all Raf proteins may not have an “additive” effect, but reduce efficacy in some cases because of differing activities of different Raf family members. For example, increased expression of c-Raf-1 was associated with decreased survival, while increased expression of B-Raf was implicated in improved survival of ovarian cancer patients [33]. Although the molecular basis for these findings is not clear, this study suggests a critical role for c-Raf-1 in promoting ovarian cancer cell growth, and a potentially opposing effect for B-Raf.

2.2 Non-canonical activities of Raf proteins

Although the best validated activities of Raf involve the interactions with Ras and MEK that dominate this discussion, it is important to bear in mind that a growing number of studies have identified MEK-independent roles for Raf (See **Figure 1**). Some of this work has demonstrated biological activity of kinase-dead forms of Raf, or for Raf in cells treated with MEK inhibitors (e.g. [34,35]): this has led to the proposal that Raf may have a separate function as a scaffolding protein (see discussion in [34,36]). If so, and if such a function contributes to Raf activity in cancer, Raf kinase inhibitors may not block all relevant Raf activities. Other work has nominated additional proteins as Raf targets, including cell cycle regulators (e.g., Rb [37], Cdc25 [38,39]), apoptosis modulators (e.g., BAD/BCL proteins [40–43], ASK1 [35,44], MST2 [45]), translation regulators (e.g., eEF-1A, [46]), and components of the cytoskeleton (e.g.,

vimentin [47], ROK-α [48], and keratins [49]). Raf interactions with some of these targets (such as BCL2 and ASK-1) occur at the outer mitochondrial membrane rather than the plasma membrane [34], and likely involve significantly different interactions with other cellular partner proteins than those involved in the canonical Raf signaling pathway. Further, although Raf clearly contributes to the pro-proliferation activity of the EGFR > Ras > Raf > MEK > ERK signaling pathway, Raf activity in the non-canonical pathways may either promote or oppose the process of carcinogenesis.

At present, the significance of these interactions in vivo (as opposed to in cell culture experiments) needs to be investigated. Further MEK-independent biomarkers for Raf inhibition in these other processes need to be developed, to better inform the use of Raf inhibitors in therapy. These biomarkers might reasonably arise from some of the recently identified Raf targets noted above, such as MST2, eEF-1A, vimentin, or others, assuming appropriate phospho-specific antibodies could be developed.

2.3 Limited knowledge of Raf structure

An additional issue that influences drug development and evaluation efforts involving Raf is the persistently limited structural information available for Raf. The solution of the B-Raf kinase domain in 2004 provided valuable insights into the mechanism by which activating mutations in the P loop disrupt the inactive conformation of the kinase [50]. This study also revealed the binding contacts between one Raf kinase inhibitor, BAY43-9006, and the interfacial cleft of the B-Raf kinase domain, demonstrating interaction between the compound and Raf-family conserved residues of the ATP-binding pocket [50]. NMR-based studies have yielded some information about the c-Raf RBD [51], and the CRD [52,53]. However, no structure of full length Raf either alone or in complex with Ras or MEK is available. As noted above, Raf normally exists in complex with a number of other proteins, including chaperones that are necessary to stabilize the protein from degradation [32]. This characteristic offers some therapeutic options discussed below, such as targeting the chaperones to destabilize Raf. However, it also suggests the lack of structural information will persist, limiting the potential of structure-based modeling for targeted drug design primarily to the Raf kinase domain, and to the isolated RBD/CRD domains.

3. Therapeutic approaches to target Raf

As noted above, reagents that have been developed to inhibit Raf include antisense oligonucleotides, small molecule kinase inhibitors, a Raf-Ras protein interaction inhibitor, and compounds that destabilize Raf proteins by targeting chaperone proteins critical for Raf stability. These are summarized in **Table 3 and Figure 4**. Critical clinical trials involving Raf-targeting agents are summarized in **Table 4**.

3.1 Antisense

Initially, the most attractive Raf family target was considered to be c-Raf. Two c-Raf-directed antisense oligonucleotides have advanced to clinical testing: ISIS 5132 and LErafAON. COM4, an shRNA targeting B-Raf, has shown encouraging characteristics in preclinical experiments.

3.1.1. ISIS 5132—ISIS 5132 (CGP 6984, ISIS Pharmaceuticals Inc, Carlsbad, CA) was designed to hybridize to the 3’ untranslated region of the c-Raf mRNA [54]. ISIS 5132 is the 19-sodium salt of a 20-base oligonucleotide (5'-TCCCGCCTGTGACATGCATT-3') with 19 internucleotide phosphorothioate linkages. In cell culture, ISIS 5132 inhibits cell proliferation with an IC50 of about 100 nM [55]. Studies of ISIS 5132 in breast and lung cancer mouse xenograft models revealed significant tumor growth inhibition [54]. Combined treatment of

ISIS 5132 and paclitaxel or carboplatin was strongly anti-proliferative in ovarian cancer cell lines with mutated p53 [56,57]. Additional xenograft combination studies with ISIS 5132 and cisplatin, adriamycin, tamoxifen and mitomycin C produced additive and super-additive effect in the majority of cancer models [58]. Liposomal formulations protect phosphorothioate oligonucleotides from rapid in vivo degradation, and improve their uptake by tumor cells and intracellular activity [59]. ISIS 5132 and its liposomal formulation Le-5132 produced strong radiosensitization effect in human laryngeal squamous cell carcinoma xenografts treated with ionizing radiation [60].

Based on these data, ISIS 5132 AON was evaluated in a series of Phase I clinical trials in patients with advanced cancer [61–64]. In the first study, 31 patients received ISIS 5132 as 2-hour i.v. infusions three times a week for 3 consecutive weeks at doses ranging from 0.5 to 6.0 mg/kg. The clinical toxicities were minor and included fever and fatigue. Significant decrease in c-Raf-1 expression was identified at doses >/=2.5 mg/kg. Two patients experienced prolonged stable disease for more than seven months, which was accompanied by reduction of c-Raf-1 expression in peripheral blood mononuclear cells (PBMCs) [63,64], providing a useful biomarker for response. In the next study, ISIS 5132 was administered to 34 patients as a continuous i.v. infusion for 21 days every 4 weeks. The study used a dose escalation protocol until 5 mg/kg/day or dose related toxicities were reached. The toxicities observed with doses up to 4mg/kg/day were modest, with the most common side effects fever (up to grade 3), and mild fatigue. Four of 34 patients with pancreatic cancer had stable disease up to 10 months and one woman with ovarian cancer had a significant partial response with 97% reduction in the tumor marker CA-125 for 11 months [62].

To determine the maximum tolerated dose (MTD) for ISIS 5132 in patients with advanced malignances, 22 patients were subjected to a dose escalation Phase I trial in a weekly 24-h i.v. infusion. The ISIS 5132 MTD was defined as 24 mg/kg/week. Serious adverse effects were documented in two patients treated with 30 mg/kg/week dose after the first dose. These side effects included acute hemolytic anemia and acute renal failure. No reduction of c-Raf-1 expression and no major responses in patients were observed [61]. Of three Phase II trials, involving patients with colorectal, recurrent ovarian, and hormone-refractory prostate cancer, no significant response was noted [65–67]. This agent was dropped from further clinical development.

3.1.2. LErafAON—LErafAON (NeoPharm, Inc) is Liposome Entrapped derivative of a 15-mer antisense oligodeoxyribonucleotide (5'-GTGCTCCATTGATGC-3') directed toward the translation initiation site of c-Raf-1 (rafAON) [68]. LErafAON toxicity, pharmacokinetics and antitumor activity were evaluated in CD2F1 mice, in New Zealand white (NZW) rabbits and in cynomolgous monkeys. CD2F1 mice received a total of 12 i.v. injections of LErafAON at a dose from 5.0 to 35.0 mg/kg administered over 17 days. Weight loss did not exceed 14% in any of the groups of mice, and all groups demonstrated weight gain after day 21 of treatment or within 5 days after treatment cessation. No hematological abnormalities, no liver dysfunction beyond mild hepatitis induced by the liposome control, and no gross or microscopic pathology were noted in any of the groups of mice treated with LErafAON. Treatment of NZW rabbits with up to 39 mg/kg of LErafAON liposomes similarly revealed no drug-related toxicities. In monkeys, LErafAON administration at doses up to 56 mg/kg, produced transient changes in complement profile, mild chronic inflammation in liver and kidney, as well as some morphological changes were observed in spleen, liver, and kidneys, attributable to the liposome formulation.

In initial preclinical pharmacokinetic and xenograft analysis of LErafAON, intravenously administered LErafAON (30 mg/kg) in nu/nu mice with a hormone-refractory PC-3 prostate cancer xenograft was detected in plasma for up to 48 h. LErafAON preferentially accumulated

in the liver, spleen, kidneys heart and lungs in these animals, and was also detected in PC-3 tumor tissue. In monkeys, intact LErafAON was detected in plasma 24 h after i.v. injection. Both in mice and monkeys, LErafAON drastically reduced c-Raf-1 protein expression in normal and tumor tissues, and in mice caused PC-3 tumor growth arrest. LErafAON (25 mg/kg/day) and ionizing radiation (3.8 Gy/day) led to a dramatic and steady decline in PC-3 xenograft tumor volume [68]. In the same xenograft model, LErafAON produced strong combination effect when co-administered with cisplatin, epirubicin or mitoxantrone. In ASPC-1 and Colo 357 pancreatic carcinoma cell xenografts, co-administration of LErafAON with docetaxel or gemcitabine significantly increased tumor growth inhibition than application all compounds individually [69]. c-Raf-1 protein expression was demonstrated to be markedly decreased in mice treated with docetaxel and LErafAON. In an independent preclinical study [70] LErafAON chemosensitized tumor cells towards doxorubicin and paclitaxel in prostate (PC-3), NSCLC (A549) and breast (MDA-MB231) mouse xenografts. Together, these results warranted clinical studies of LErafAON in combination with chemotherapy and/or ionizing radiation treatment for hormone-resistant prostate and pancreatic cancer.

Initial Phase I clinical evaluation of LErafAON formulated in cationic liposomes was conducted in 22 patients with advanced solid tumors, using a dose escalation protocol. Patients received LErafAON as weekly i.v. infusion at doses from 1 to 6 mg/kg/week for up to 8 weeks [71]. Treatment-related adverse effects up to grade four were seen in all dose cohorts, including flushing, dyspnea, hypoxia, rigors, back pain and hypotension. A dose-limiting thrombocytopenia was observed at 6 mg/kg/week. Pretreatment with acetaminophen, H1- and H2-agonists and steroids reduced the severity of these reactions. Two of five patients tested had clear reduction of c-Raf-1 mRNA in PBMC cells, but no objective response in tumors was observed. Overall, dose-independent adverse effects severely limited clinical evaluation of LErafAON: a modified liposomal formulation may significantly improve tolerability and efficacy [71,72].

After preclinical studies suggesting action of LErafAON in sensitizing transformed cells to radiation [73,74], a Phase I study of LErafAON in combination with palliative radiation therapy was performed [72]. Over 2 weeks, 17 patients with advanced solid tumors were treated with LErafAON in a dose escalation protocol, with i.v. daily infusions ranging from 1.75 mg/kg/week to 7mg/kg/week, and daily radiation at 300 cGy. Sixteen out of seventeen patients experienced significant adverse effects (reaching grades 3 and 4 in five patients) that included dyspnea, fatigue, fever, and hypertension. These were related to the liposomal formulation rather than the oligonucleotide, and were not dose dependent. Premedication with corticosteroids and antihistamines helped to alleviate some of these toxicities. Of twelve patients available for treatment evaluation 4 had partial response, and another 4 had a stable disease. Both the RNA and protein levels of c-Raf-1 were inhibited in 4 of 5 patients with partial response or stable disease. The study authors concluded that LErafAON was tolerated at 2mg/kg dose administered twice weekly with premedication and did not enhance radiation toxicity. The results of the trial lead to development of a modified liposomal formulation that is currently undergoing clinical evaluation.

3.1.3. Small interfering RNAs (siRNAs) against B-RafV600E and C-Raf—Recently, siRNAs to B-RafV600E have been tested for their ability to inhibit the proliferation and invasiveness of malignant melanoma cells in cell based assays, and in melanoma mouse xenograft models (118). These siRNAs inhibited the proliferation and angiogenic capacity of tumor cells without inducing apoptosis. Perhaps significantly, depletion of wild type B-Raf or C-Raf in melanoma cell lines lacking a B-RafV600E mutation produced no anti-tumorigenic effect, emphasizing the specific importance of B-RafV600E induced signaling for melanoma tumor development [75]. Efficacy of B-RafV600E-targeting siRNA has been demonstrated in B-RAF mutant papillary thyroid cancer (PTC) cells [76]. Together with observation of

effective anti-tumor activity of C-Raf siRNAs in prostate [77] and breast cancer cell models in vitro and in vivo [78] these results offer a new avenue for development of mRNA-targeted therapeutics.

3.2 Kinase inhibitors

At present, 7 independent Raf kinase inhibitors have been preclinically or clinically evaluated (**Table 3**). These include sorafenib, PLX4032, ZM336372, AZ628, Raf265, AAL881, and LBT613. In general, in evaluating the action of these Raf-targeted kinase inhibitors, it is very important to consider some of the efficacy of these compounds may arise from activity against non-Raf-kinases. In one extensive study, it was shown that sorafenib blocks the activity of over 60 kinases at clinically relevant concentrations [79]. Broadly speaking, all uses of such compounds represent an unselected "combination therapy" approach. At present, it is an open question whether increasing or decreasing the specificity of Raf kinase inhibitors would increase therapeutic value. While combinatorial inhibition of multiple oncogenic kinases may increase potency, the downside of this approach may be that low potency of blockade of essential kinases contributes to therapy-related toxicity. Hence, while antisense and siRNA-based approaches unequivocally seek to act "on-target", with kinase inhibitors, it is an open question as to restricting or exploiting "off-target" activities of the compounds is a better strategy.

3.2.1. Sorafenib—The bi-aryl urea sorafenib (Nexavar®, BAY-43-9006) is an orally bioavailable compound originally developed by Bayer HealthCare and Onyx Pharmaceuticals as C-Raf kinase inhibitor [80]. In biochemical assays, sorafenib is a potent inhibitor of pre-activated wild type c-Raf-1 and B-Raf, as well as oncogenically activated B-Raf kinases (IC50 values 6, 22 and 38 nM, respectively) and effectively reduces downstream phosphorylation of MEK and ERK kinases [81]. Crystal structures of wild type and V600E B-Raf kinase domains in complex with sorafenib revealed that the inhibitor held the activation segment in an inactive conformation, preventing ATP binding and subsequent kinase reaction [50]. Cell based assays have showed sorafenib potently inhibits anchorage-dependent and -independent growth in many human cancer cell lines [82].

Although the antiproliferative effect of sorafenib can be partially explained through its activity towards Raf kinases, like many kinase-directed inhibitors [79,83], sorafenib has additional off-target activities. Sorafenib is also a potent inhibitor of VEGFR1, 2 and 3, Flt-3, p38, and c-Kit kinases, with IC50 values in in vitro biochemical assays in each case <70 nM [81]. Sorafenib has been evaluated in numerous mouse xenograft models representing a broad spectrum of solid cancer tumors with Ras or Raf oncogenic mutations, including colon, breast, ovarian, pancreatic, thyroid, NSCLC and melanoma [75,81,84]. Despite potent sorafenib-induced MEK and ERK inhibition in many of these animal trials, the broad anti-tumor activity demonstrated by sorafenib is likely based in part on its anti-angiogenesis activity, i.e. inhibition of VEGFR kinases. The inhibition of angiogenesis is probably the most crucial activity of sorafenib in A549, NCI-H460 and Colo-205 xenografts and in a Renca murine renal cancer model, where no Nexavar-associated reduction in ERK phosphorylation was detected [81,85]. However, studies in K1735 murine melanoma model revealed a primary effect of sorafenib on inhibition of angiogenesis by modulation of endothelial cell proliferation through blocking Ras-Raf-MEK-MAPK signaling [25]. This phenomenon was accompanied by inhibition of p-ERK in endothelial but not tumor cells, as well as induction of hypoxia, reduction in vascularity, altered vessel characteristics and morphology. As noted above, activation of Ras signaling in endothelial cells could be triggered by multiple proangiogenic receptors including VEGFR2, FGFR2, Tie2, integrins ($\alpha_v ß_3$ and $\alpha_v ß_5$), and EDG1 (e.g. [28–30]). It is not yet clear whether only VEGFR2, Raf or both kinases are targeted by sorafenib to achieve the inhibition of angiogenesis in these cells.

Sorafenib induces apoptosis in a broad spectrum of cancer cell lines [86]. The mechanism of apoptosis induction in sensitive cell lines by sorafenib is largely independent of Raf activity in caspase activation and BAD dephosphorylation, but rather involves nuclear translocation of AIF (apoptosis inducing factor) [87]. It is not yet clear whether this represent Raf-directed or off-target activities of sorafenib. In hematopoietic cells sorafenib induces apoptosis by inhibiting translation of the Bcl-2 family member Mcl-1 [88]. This involved suppression of eIF4E phosphorylation through a MEK/ERK-independent mechanism. A similar mechanism of apoptosis induction by sorafenib was demonstrated in preclinical studies of the compound in hepatocellular carcinoma (HCC) models [89]. Whether sorafenib directly inhibits the activity of MNK1 or alternative eIF4E kinases remains to be elucidated.

The combination of anti-proliferative, anti-angiogenic and pro-apoptotic activities of sorafenib provided the basis for extensive clinical evaluation of the compound. Results of a series of phase I dose escalation studies of sorafenib used as a single agent in patients with advanced refractory solid tumors are summarized by [90]. Sorafenib was generally well tolerated, with skin rush, diarrhea and hypertension the major adverse effects [91], and MTD established as 400 mg twice daily. In these phase I studies, an unusual number of patients achieved partial responses and stable diseases in renal cell carcinoma (RCC) and HCC. In 2004, the FDA granted sorafenib/Nexavar® fast track status in development for RCC. In a subsequent phase III randomized double-blind placebo-control clinical trial, involving 903 patients with advanced clear-cell RCC [92] the median progression-free survival in patients treated with sorafenib was 5.5 months versus 2.8 in placebo group. This resulted in FDA approval for RCC in December, 2005. A more recent phase II study of sorafenib as oral mono-agent treatment administered at the MTD in patients with advanced HCC [93] demonstrated that 33% of the patients exhibited stable disease for at least 16 weeks, with manageable adverse reactions. In February 2007, a phase III clinical trial of sorafenib in patients with primary advanced HCC (SHARP) was halted based on early analysis indicating that the trial met its primary endpoint in superior overall survival of patients treated with Nexavar versus placebo, with no difference in adverse events [94]. These data are being submitted for FDA and European Union regulatory authority approval in 2007.

Raf and Ras mutations are rare events in HCC and RCC, suggesting either anti-angiogenesis via targeting VEGFR may be the major therapeutic activity of the drug in these tumors, or that sorafenib is interrupting proliferative signaling arising upstream of Ras. Further complicating assessment of sorafenib's mode of action, a recent phase II randomized discontinuation study of sorafenib as a monoagent at MTD in advanced malignant melanoma (a type of tumor well-documented as depending on activating mutations in either B-Raf or N-Ras, [95], and with a high percentage of patients with these mutations in the treated cohort), failed to show benefits in overall patient survival [96]. It is possible that the inefficacy of sorafenib in these patients arises from feedback, alternative induction of c-Raf phosphorylation, as has been documented in melanoma cell lines after treatment with sorafenib [97]: however, this remains speculative. Ongoing trials of sorafenib as mono- or combination agent are discussed below (**Section 5**).

Building from suggestive pre-clinical studies (see section 2.2), a combination treatment using sorafenib and IFNα-2b was evaluated as a first and second line treatment for metastatic renal cell carcinoma in a phase II clinical trial [98,99]. The regimen consisted of subcutaneous treatment with 10×10^6 units of IFNα-2b three times weekly, and 400 mg of sorafenib bid. Both studies indicated patients undergoing combination treatment showed better response than those receiving drugs as monoagents: 19% of objective confirmed response and almost 50% unconfirmed partial response or stable disease as best response in one study [98] and 33% of partial response in the second [99]. However, the adverse effects associated with IFNα-2b treatment (commonly fatigue, anorexia, anemia, diarrhea, nausea, rigors/chills, leukopenia,

fever, and transaminase elevation) have limited further development of this combination treatment.

3.2.2. PLX4032—PLX4032 is an orally bioavailable kinase inhibitor currently under development by Plexxicon and Roche. PLX4032 potently inhibits mutant V600E and wild type B-Raf kinases, with significant selectivity for the mutant allele (IC50 of 31 and 100 nM, respectively). PLX4032 inhibits cell proliferation with a submicromolar IC50 in thyroid carcinoma and melanoma cell lines with mutant B-Raf [100]. PLX4032 synergizes strongly with taxol, vinblastine and oxaliplatin compounds in inhibiting the proliferation of B-RafV600E transformed colon and melanoma cell lines. In mouse xenograft experiments in colorectal and melanoma models, PLX4032 reduced tumor size and slowed the progression of tumor growth for a significant time after the completion of treatment, without body weight loss. PLX4032 has entered phase I clinical trial to evaluate safety, maximum tolerated dose, and pharmacokinetics in patients with refractory solid tumors (Identifier: NCT00405587, http://ClinicalTrials.gov); for melanoma patients, a confirmed V600E BRaf mutation is a criteria for enrollment.

3.2.3. ZM336372—Developed by AstraZeneca, ZM336372 was the first described small molecule that inhibited activation of c-Raf-1 and B-Raf in in vitro biochemical assays (IC50 of 10 and 100 nM, respectively) [101]. Some inhibitory activity against other kinases, including notably p38, was observed. Unexpectedly, this compound produced paradoxical activation of MAPK signaling cascade in vivo in cell-based assays, leading the authors to suggest that Raf suppressed its own activation by a kinase-dependent feedback loop. However, other Raf-kinase-directed agents do not activate ERK, suggesting this may represent some off-target activity of the compound. Although inhibition of ERK signaling normally is associated with tumor cell death, it has been recently appreciated that ERK activation induces apoptosis in certain types of human cancers. For this reason, ZM336372 has attracted new interest as a potential therapeutic agent for treatment of pheochromocytomas, hepatocellular carcinomas, Merkel cell carcinomas, and neuroendocrine tumors, in which ERK activation causes tumor cell death [102–104].

3.2.4. AZ628—The quinazilinone AZ628 is a new pan-Raf kinase inhibitor from AstraZeneca. AZ628 reduces activities of preactivated B-Raf, B-RafV600E, and c-Raf-1 in in vitro kinase assays, with IC50 values of 105, 34 and 29 nM, respectively [105]. Specificity profliling indicates that AZ628 also inhibits activation of number of tyrosine protein kinases including VEGFR2, DDR2, Lyn, Flt1, FMS and others. AZ628 inhibits anchorage-dependent and -independent growth, causes cell cycle arrest, and induces apoptosis in colon and melanoma cell lines harboring B-RafV600E mutation. The profile of AZ628 cross-reactivity suggests that similar to sorafenib, AZ628 may be antiangiogenic based on inhibition of VEGFR2. Preclinical evaluation is in progress.

3.2.5. Raf265—Raf265/CHIR-265 is an orally bioavailable substituted benzazole compound codeveloped by Chiron and Novartis. Raf265 inhibits activity of all wild type Raf kinases as well as B-Raf oncogenic mutant kinases (in vitro IC50 values 3–60 nM) [106], and effectively inhibits proliferation and survival of cancer cell lines with activated MAPK signaling pathway [107]. The compound also potently inhibits VEGFR2 and several other tyrosine kinases, providing the basis for a putative antiangiogenesis activity. Currently, Raf265 is under evaluation in phase I clinical trial in patients with metastatic melanoma (Identifier NCT00304525, http://ClinicalTrials.gov).

3.2.6. AAL881—The isoquinolone AAL881 is an orally administered small molecule ATP-mimetic inhibitor, under development by Novartis. AAL881 shows significant potency in

inhibition of Raf protein kinases, with selectivity for V600E mutated B-Raf protein (IC50 220 nM) over wild type B-Raf (940 nM) or C-Raf (430 nM) [108]. AAL881 also effectively inhibits VEGFR2 and several others protein kinases. AAL881 demonstrated a strong antiproliferative effect in thyroid carcinoma [109] and multiple glioblastoma cell lines [108] in cell based assays and in sub-cutaneous and intracranial glioblastoma xenograft models. AAL881 inhibits proliferation of thyroid carcinoma cell lines both in cells with wild type or B-V600E Raf, either confirming the general importance of B-Raf inhibition in thyroid malignancies, or suggesting off-target activities of the compound are important. The recent observation that siRNA directed to B-Raf inhibits these tumors supports the first interpretations [109]. Moreover, glioblastoma xenograft studies indicated superior tumor growth inhibition in animals treated with AAL881 versus a selective VEGFR2 inhibitor [108]. AAL881 may represent a future therapeutic option for treatment thyroid and brain cancers and is currently under preclinical development.

3.2.7. LBT613—LBT613 is a pan-Raf kinase inhibitor that belongs to the same class of compounds as AAL881, and is currently under preclinical development by Novartis. The compound is ~10 fold more potent than AAL881, and may prove to be efficacious in treatment thyroid cancers [110].

3.3. Alternative Raf-targeting strategies

In contrast to targeting Raf kinase activity directly, some drugs have been developed to impair Ras-dependent Raf activation by blocking the Ras-Raf interaction, or to reduce overall Raf expression, by removing essential Raf chaperones.

3.3.1 MCP110—MCP110, an aryl amide [111] under development by NexusPharma, is so far the only small molecule compound that inhibits Ras and Raf protein interaction [112]. An analog of a compound originally selected by yeast two-hybrid high throughput screening based on its ability to disrupt Ras-Raf interactions, MCP110 limited anchorage-dependent and –independent growth in multiple cell lines where MAPK pathway was activated by oncogenic mutations in K-, N-, and H-Ras or by the receptor tyrosine kinases like EGFR or PDGFR (IC50s 10–15 μM). In contrast, MCP110 is inactive in cells transformed with constitutively active (Ras-independent) c-Raf-1 kinase domain (Raf22W) or with constitutively active MEK1, and in untransformed fibroblasts [113], supporting a specific action at the point of Ras-Raf interaction. MCP110 also inhibits multiple phenotypes associated with malignant transformation including cell cycle progression, invasion and survival [113,114], and inhibits Ras-dependent activation of MEK and ERK [112]. MCP110 has demonstrated low toxicity and dose-dependent tumor growth inhibition in SW620 colon carcinoma and LXFA629 mouse xenografts. Moreover, MCP110 produced clear synergistic effect with MAPK pathway inhibitors including sorafenib, and with the microtubule-targeting agents paclitaxel, docetaxel and vincristine [113]. MCP110 remains in preclinical development.

3.3.2 HSP90-targeting compounds—HSP90 is a molecular “cancer” chaperone that maintains the stability and function of a number of proteins that regulate signaling, cell cycle progression, and other growth properties of cancer cells (reviewed in [115]). HSP90 stabilizes c- and A-Raf, is required for the activity of V600E mutated B-Raf, and also supports the stability and/or activity of > 50 other proteins including AKT, HER2, MET, estrogen and androgen hormone receptors [115,116]. In concept, chemical inhibition of HSP90 would simultaneously blockade multiple pathways necessary for cancer cell growth, and limit opportunities for cancer cells to develop resistance [117]. The benzoquinone compound geldanamycin (GA) has been pursued as an anti-tumor agent [118] based on its ability to inhibit HSP90: 17-allylamino-17-demethoxy-geldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin 17-DMAG are two less toxic analogs of geldanamycin that are currently undergoing clinical evaluation in a series of phase I/II

clinical trials for advanced pediatric and adult tumors as well as renal cell carcinoma and in hormone refractory prostate cancer [119–121]. Other agents designed based on consideration of HSP90 structure are currently in development (e.g. [122]).

HDAC6-induced deacetylation regulates HSP90 chaperone activity [123–125]. The broad-spectrum histone deacetylase (HDAC) inhibitors SAHA/vorinostat (Merck) and NVP-LAQ824 (Novartis) induce acetylation of HSP90, promoting the destabilization and degradation of HSP90-associated proteins including c-Raf-1 in multiple myeloma and in leukemia cell lines [119,126]. Currently SAHA is undergoing 36 clinical trials as a monoagent or in combination with other chemotherapeutic agents: in October 2006, SAHA won FDA approval for treatment of cutaneous T-cell lymphoma. These agents, and more specific HDAC6-targeting agents in development (e.g. tubacin [127]) have not yet specifically been involved for efficacy in Raf-involved cancers.

4. Therapeutic approaches to Raf near-neighbor targets

A large suite of therapeutic agents have been developed that target points upstream and downstream of Raf in the EGFR > Ras > Raf > MEK signaling cascade. Although in depth discussion of these is beyond the scope of this review (see excellent recent work by Roberts and Der, [14]), consideration of results with these reagents is valuable in view of future applications of Raf-targeted therapeutics.

4.1 MEK

With the early appreciation of the importance of Ras mutations in cancer, initial drug developments sought to inhibit Ras function, most notably through the use of farnesyl transferase inhibitors (FTIs). These efforts were generally unsuccessful, and are not currently in clinical use. However, a number of agents are now in clinical and pre-clinical development for inhibition of the important Raf effector MEK. MEK kinase inhibitors that have advanced to phase II clinical trials include CI-1040, AZD6244/ARRY142886, and PD0325901 (see for example [128–131]). Although CI-1040 did not meet pre-specified criteria for advancement as a single agent therapy, some positive results were obtained, and the results with other MEK inhibitors are pending. Significantly, activating mutations in B-Raf and, to a lesser extent, in Ras are sensitizing to the effect of MEK inhibitors [131], suggesting particular efficacy of these compounds in B-Raf mutant tumors. Currently, a phase I clinical trial with MEK Inhibitor PD-325901 to treat advanced breast cancer, colon cancer, and melanoma is in progress, sponsored by Pfizer. AZD6244/ARRY142886 has entered a phase II clinical trial for advanced or metastatic pancreatic cancer, sponsored by Astra-Zeneca (www.ClinicalTrials.gov). Both trials are focused on safety and objective response rates.

4.2 EGFR

Two small molecule EGFR kinase inhibitors, erlotinib (Tarceva®) and gefitinib (Iressa®), are currently in use in the clinic. Gefitinib has been approved as a second line therapy for NSCLC, although a placebo-controlled phase III trial indicated no survival benefit. Erlotinib has been approved both for pancreatic cancer and NSCLC, and has shown survival benefits. Both compounds are currently in phase II and III trials for additional cancer types. Additional EGFR-family-targeted small molecule kinase inhibitors acurrently under clinical evaluation include vandetanib and lapatinib, which have advanced to phase III trials for NSCLC, breast cancer, and other cancers. A major issue in treatment with these agents is the identification of responding versus non-responding patients. In one study of 60 NSCLC patients, K-Ras mutations were prevalent in non-responders to erlotinib and gefitinib [132]. In a TRIBUTE randomized clinical trial, 21% of patients treated with cytotoxic chemotherapy and erlotinib with tumors characterized by mutant K-Ras showed poorer survival [133]. These data indicate

that, not surprisingly, downstream constitutive activation of the EGFR-Ras-Raf-MAPK axis is associated with worse survival and resistance to treatment strategies aimed to inhibit the upstream growth factor receptors.

Antibodies have also been used to target EGFR-family receptors [134]. The antitumor effects of therapeutic antibodies are exerted through a number of mechanisms, including perturbing receptor signaling, inducing receptor recycling followed by lysosomal degradation, and antibody-dependent cell-mediated cytotoxicity. EGFR-targeting antibodies that have been approved as drugs include cetuximab (Erbitux®) and panitumumab (Vectibix®) for EGFR1; additional monoclonals targeting EGFR and family members are currently in clinical trials.

The effect of EGFR family-inhibiting antibodies used as monotherapies is modest. Overall, about 10% of patients show partial responses to monotherapy regardless of cancer type. As with EGFR-targeting small molecule inhibitors, a downstream mutation in K-Ras that maintains the activity of the EGFR-Ras-MAPK signaling axis in spite of EGFR inhibition is commonly associated with treatment resistance [135–137]: mutant K-Ras typically rescues the apoptosis caused by antibody-induced EGFR-blockade [138]. Among responders, Moroni et al. [139] found increased EGFR gene copy number in 8 of 9 colon cancer patients who had a response to treatment with cetuximab or panitumumab. By contrast, only 1 of 20 non-responders had an increased EGFR copy number. Lièvre et al. [140] similarly correlated EGFR gene amplification with response to cetuximab plus chemotherapy, and K-Ras mutations with failure to respond. In some metastatic cancer cells, autocrine production of EGFR ligands such as epiregulin and amphiregulin leads to a sustained high level of EGFR signaling activity without the need for increased cell surface expression of the receptor [141]. Progression free survival (PFS) after cetuximab treatment was twice as long as for patients with high versus low levels of the mRNAs for epiregulin and amphiregulin ($p<0.05$ in each case) [135].

5. Expert opinion

5.1 Current state of the art

As of 2007, pathway-validated agents exist to target multiple steps in the EGFR > Ras > Raf > MEK > ERK signaling cascade. As illustrated by the examples above, these agents show some promise and clinical efficacy, but to date only in relatively limited patient populations. There are a number of high priority issues going forward. First, it is necessary to understand how best to integrate the new reagents into existing standard therapies. Second, among the existing agents targeting the Raf-centered pathway, specific combinations may result in greater potency in eliminating overall pathway function, yielding therapeutic benefit. Third, it is important to develop biomarkers or other strategies to identify which patients are most likely to benefit from Raf-targeted therapies.

Currently, Bayer/Onyx is sponsoring phase III randomized placebo-controlled combination trials of sorafenib with paclitaxel and carboplatin in chemotherapy-naïve patients with advanced malignant melanoma, as well as phase II randomized placebo-controlled combination trial with dacarbazine for the same indication. In addition, more than 30 additional clinical trials with sorafenib as mono-agent or in combination with various chemotherapeutic agents are ongoing sponsored by Bayer/Onyx or NCI CTEP program (see also **Table 4**).

There are accumulating precedents for the idea that combinations of specific targeted agents offer higher efficacy against their targets. For example, combinatorial blockade of the EGFR family receptors with EGFR and HER2 [142,143] or IGF-R1 [144] antagonists, or with an EGFR-targeting antibody and a tyrosine kinase inhibitor [145], induces additive or synergistic activity against xenograft tumors in mice, and such studies are being advanced to the clinic. For Raf therapeutics, it would be of interest to assess the combination of two kinase inhibitors,

or a kinase inhibitor and antisense approach. The criteria for selection of patients for trials with Raf-directed therapeutics remain poorly defined at present. As noted above, in some studies, efficacy of Raf-targeted kinase inhibitors does not correlate with B-Raf mutational status. This may reflect the fact that agents such as sorafenib derive significant therapeutic potency from off-target activities, or may reflect the complexity of cellular rescue pathways. For both patient selection and drug combination selection, one promising avenue to investigate is the exploitation of the rapidly growing informatics resources available to expand our understanding of Raf-centered signaling pathways.

As discussed above (sections 1.1, 2.2), targeting of Raf may also be useful in combination with agents inhibiting known "near neighbor" signaling pathways such as IFNα, or targeting non-tumor tissue (i.e., angiogenesis controls). For example, although IFNα has some desirable anti-tumor properties, the induction of EGFR by IFNα in some cells [146] causes c-Raf-1 activation and inhibits apoptosis. In this respect, a combination treatment of IFNαwith Raf or MEK1 [146] inhibitors, or even FTI inhibitors (e.g. R115777/tipifarnib), may be beneficial in epidermoid carcinomas and other cancers. Based on the observation that c-Raf can inhibit apoptosis based in part on upregulation of the protein translation machinery (e.g. [46]), combination of Raf-targeted agents with agents such as temsirolimus, that targets the mTOR protein and has been approved by FDA for treatment of advanced renal cell carcinoma, may be desirable for renal and other cancers. Finally, combination of Raf-targeting agents with others targeting the angiogenic machinery is also likely to be a productive strategy.

5.2 Systems biology approaches to augment the targeting of Raf-involved cancers: future prospects

Cancer therapy is in the early stages of being transformed by high throughput datasets describing the human genome, transcriptome, proteome, and other "omes". Researchers in model organisms including yeasts, *C. elegans*, and *D. melanogaster* now commonly perform experiments guided by very extensive curated bioinformatics resources that summarize the physical, genetic, and functional interactions between a protein of interest and a large set of other cellular proteins (e.g. FlyGrid; Wormbase; SGD). A key observation arising from such work is that genes selected based on their proximity to a target of interest (e.g. Ras) in an interaction network are enriched relative to an unbiased gene set for "sensitizers" to the consequences of mutating the target. For example, Zhong and Sternberg identified sensitizers to *let-60* (*C. elegans* Ras) mutations at a greatly increased rate from a set of genes in a *let-60*/Ras-centered network [147], with siRNAs targeting these genes enhancing physiologically relevant phenotypes associated with *let-60* mutations.

Together with other studies supporting the idea of proximity-based sensitization (discussed in [148]), these data have a direct prediction for cancer therapeutics: to enhance the effectiveness of a protein-targeted drug, combination of such an agent with other agents targeting proximal proteins will be a productive strategy. Indeed, such approaches have in some cases been productively applied. For example, the PI-3K inhibitor PX-866 strongly potentiates the action of the EGFR inhibitor Iressa; these agents "vertically" target two distinct but connected points the EGFR > Ras > PI-3K signaling cascade, with drugs inhibiting multiple steps in a signaling cascade [149]. Synergistic effect has also been documented in glioblastoma cells treated with C-Raf or MEK kinase inhibitors (GW5074 and U0126), which synergize with ILKAS, an antisense oligonucleotide that inhibits the PI-3K-regulated ILK and AKT kinases [150]; in this case, two "horizontally" related Ras effectors are inhibited in parallel. The studies evaluating combination of Raf inhibitors with VEGF-, mTOR, and IFNa-targeting agents described above represent expansion of this strategy to include Raf "near neighbors". How might such a strategy be extended?

A growing number of resources support analysis of the Raf-proximal signaling network. For example, some research teams have used high throughput, protein-interaction based screening methods to identify candidate sets of proteins physically interacting with EGFR [151,152]. Functional data regarding genes interacting with Raf or its near neighbors in multiple organisms is available in central databases, based not only on high throughput data, but well-validated data curated from the scientific literature [153]: existing cancer-relevant databases include among others NetPath, BioGrid, DIP, BIND, KEGG, HPRD, CellCircuits, and NCBI GEO, as well as "expert systems" focused on pathway analysis (NetPath, Protein Lounge, Molecular Systems Biology, Biocarta, STKE). In addition, studies by the Ideker group and others have demonstrated the robustness of predictions of interaction networks based on comparison of interaction networks cross-species [154–158]. The Cytoscape and PathBLAST tools [155, 157] can be mastered with relatively limited effort by biologists and clinicians with minimal sophistication in use of computer programs. These programs allow the individual investigator to generate and query protein interaction maps focused on their gene of interest, exploiting extensive and constantly updating databases available on line.

A simplified 2007-current, Raf-centered network developed using these tools is shown in **Figure 5**. As this demonstrates, many different proteins have been identified as candidate Raf regulators or effectors, based on physical interactions with one or more members of the Raf protein family. Although these interactions are generally defined as "high confidence" based on detection in multiple experimental systems, supporting publications, or robust characteristics of the interaction in a single detection system, not 100% will be validated as functionally important. Nevertheless, taken as a group, these proteins provide a rich Raf "neighborhood" of proteins that might plausibly be targeted to sensitize cells to the effect of Raf therapies. While there is a significant gap between designing therapeutic strategies, and having the tools immediately available to translate the strategies to the clinic, some of the proteins thus linked to Raf have independently been of interest for development of small molecule therapeutics: agents to these proteins would be logical candidates to evaluate in combination with Raf inhibitors for synergistic effect.

Beyond studies of physical interactions, the EGFR > Ras > Raf >MEK > ERK signaling pathway has been heavily exploited for molecular modeling; as of 2005, over 30 mathematical models had analyzed dynamics of signal transmission (reviewed in [159]), and efforts to develop efficient predictive models continue. In parallel, interaction network studies are building a conceptual infrastructure to understand the relationship of disease-focused genes to the total network of cellular interactions [160,161], while other researchers seek to define the complete set of mutations associated with specific cancers (e.g. [162]), or to identify transcriptional profiles marking genes with significantly altered expression in specific cancers (reviewed in [163,164]). Together, these efforts will continue to enhance the context for thinking about Raf and desirable near-neighbor targets to ablate.

Finally, this concise review has focused specifically on some of the most important aspects of Raf kinase biology relevant to state-of-the-art therapies. For lengthier, in depth review of Raf structure and Raf pathway signaling, the interested reader is urged to consult a number of excellent recent reviews addressing these topics [14,36,159,165–169].

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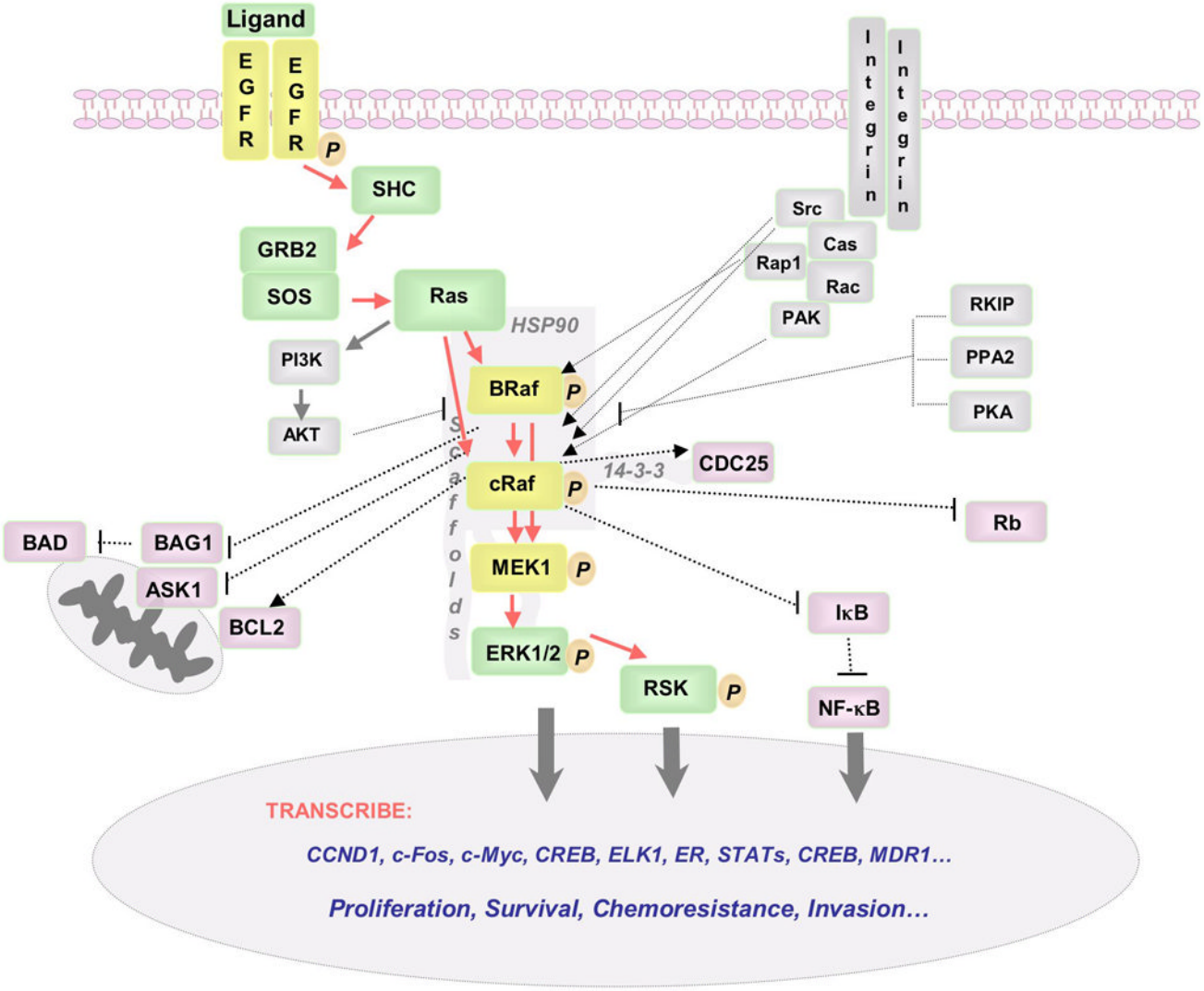

Figure 1. The core Raf signaling pathway

Components of the central activation cascade proceeding from ligand-bound EGFR through Raf to the nucleus are indicated in green or yellow boxes, connected by red arrows. Additional proteins regulating Raf are indicated in gray boxes. Additional Raf phosphorylated/regulated proteins beyond the central cascade are indicated in pink boxes: not all are shown (see text). After Ras stimulation, B-Raf heterodimerizes with c-Raf-1; mutationally activated B-Raf binds and activates C-Raf and MEK independent of Ras [170]. Therapeutics targeting B-Raf and c-Raf1 are the predominant topic of this review: agents targeting EGFR and MEK1 are also briefly discussed.

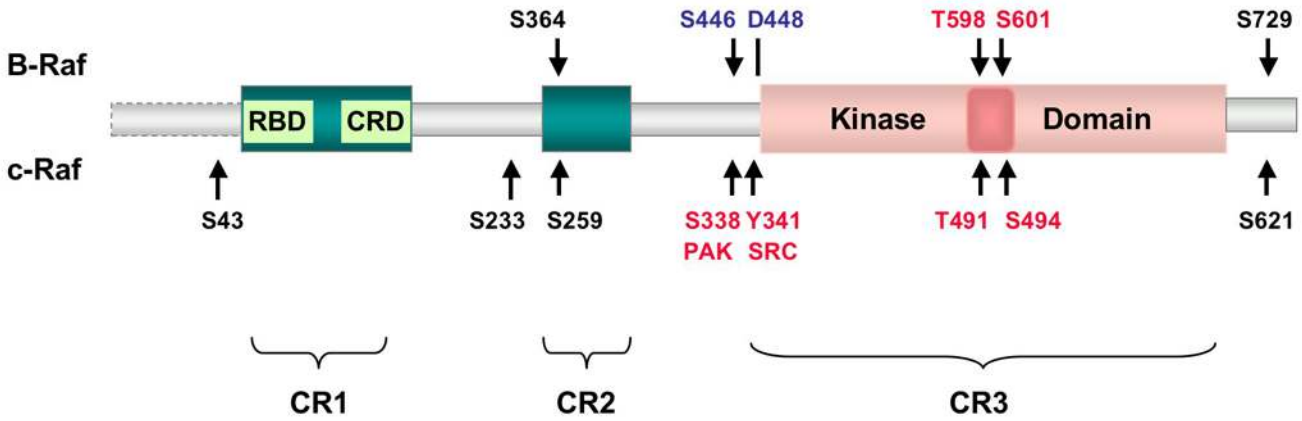

Figure 2. Domain structure and key phosphorylation sites for B-Raf and c-Raf-1

This is a simplified depiction of the regulatory phosphorylation sites governing activity of the Raf kinases. Darker red region within the kinase domain indicates the catalytic loop. Key activating phosphorylations on c-Raf-1 (shown in red) include S338, Y341, T491 and S494. Akt confers an inhibitory phosphorylation on S259, which is dephosphorylated during c-Raf-1 activation. Note that on B-Raf that S446 (equivalent to S338 of C-Raf) is constitutively phosphorylated, while D448 (positionally equivalent to Y341 on c-Raf-1) is a phospho-mimic. These differences are thought to contribute to the greater ease of mutationally activating B-Raf1 through a single V600E mutation. Because of its relatively minimal contribution to cancer, A-Raf is not shown; the structure and regulation of A-Raf are similar to those of c-Raf. Darker red region within the kinase domain indicates the catalytic loop. Additional phosphorylation sites indicated represent basal/constitutive phosphorylation sites that contribute to Raf interactions. See detailed discussions of regulatory phosphorylation of Raf in [32,165,169]

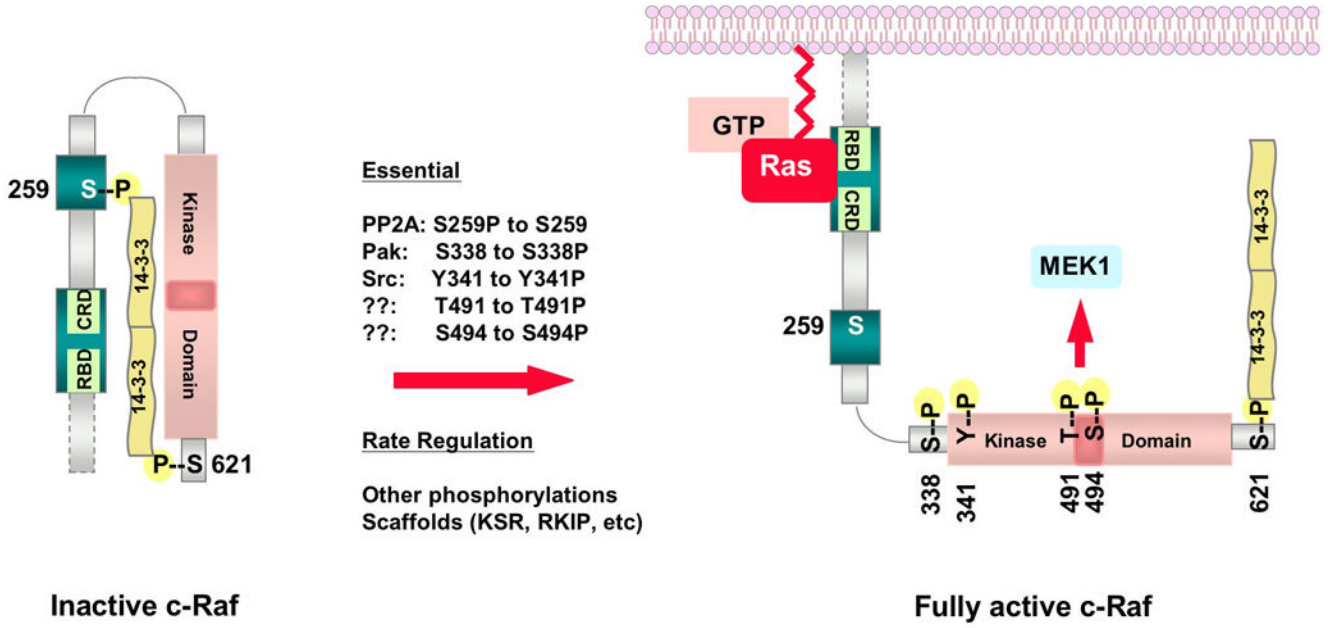

Figure 3. Comparison of inactive and active Raf kinase

Inactive Raf is locked in a closed conformation anchored by binding of 14-3-3 to N- and C-terminal phosphorylated residues on Raf. Sequential dephosphorylations by PP2A, followed by activating phosphorylations, lead to opening of the Raf conformation, interaction with Ras and recruitment to the membrane, and ability to bind and phosphorylate substrates such as MEK1. Additional Raf-interacting proteins that support these processes (e.g., the chaperone HSP90; the scaffold KSR) are not shown.

Figure 4. Chemical structures of Raf-targeting agents

See text and Table 3 for additional details. Structures for AAL881 and LBT613 are currently unavailable. Structure provided for Raf265 represents patent example.

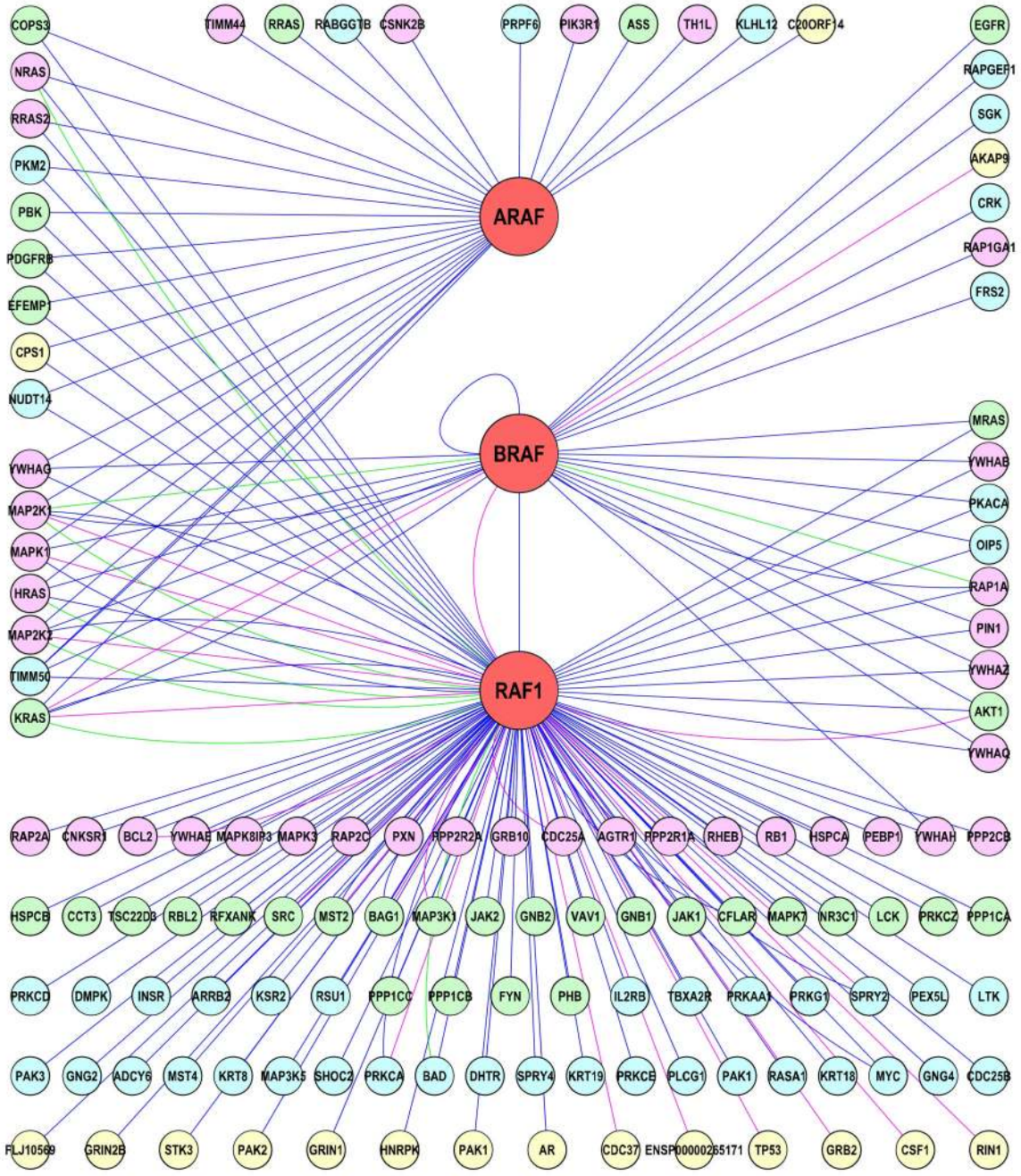

Figure 5. The Raf interaction neighborhood

The program STRING (‘Search Tool for the Retrieval of Interacting Genes/Proteins’, http://string.embl.de/) collects known physical interactions from the following databases: BIND, DIP, MINT, BioGRID, and HPRD. Each interaction is annotated by STRING with a benchmarked numerical confidence score. In addition, STRING can retrieve indirect protein associations (e.g., genetic and functional interactions) from pathway databases (e.g., http://pid.nci.nih.gov), and by text mining the scientific literature. C-Raf-1, A-Raf, and B-Raf interactions were collected with a cut-off score of 0.4 (medium confidence) for experimental data only; 0.9 (highest confidence) for text mining; and 0.98 for pathway mining. Data were imported in the Cytoscape software (www.cytoscape.org). In addition, BIND, DIP, MINT,

BioGRID, HPRD and Intact databases were also searched using the BioNetBuilder plugin for Cytoscape and additional search tools. All data were imported in Cytoscape and merged. Although there are numerous interactions among the group of Raf family-interacting proteins, only direct interactions with Raf proteins are shown here (a full version is available on request). Nodes (circles; indicating discrete proteins) were color-coded according to confidence level of the interaction with Raf as follows: pink, >0.9; green, >0.7; blue, > 0.4; yellow, not rated (e.g. the database providing the information lacked sufficient annotation for assignment). In addition, the edges (lines) are color-coded as follows: blue, direct protein-protein interaction; red, pathway maps; green, text mining.

Table 1

Frequency of genetic lesions involving Raf, Ras, and EGFR proteins in common human cancers.

Cancer Type	EGFR	Ras family	Raf family
NSCLC	17	10–50	3
SCLC	0	0	***
Colorectal	3	40	9
Pancreatic	*	78	0
Hepatocellular	11	0	**
Prostate	0	7	10
Breast	4	2	**
Ovarian	*	22–75	31–36
Head and Neck	*	6	3
Thyroid	3	0	10–54
Melanoma	0	16–29	45–68
Glioblastoma	30–40	2	3–6
Sarcoma	**	0	*
Acute Myeloid Leukemia	0	17	2–4
Gastric	5	3	2–12

Numbers shown represent the frequency of activating mutations in EGFR, the Ras family (K-, H-, and N-Ras) and the Raf family (A-, B-, and C-Raf), in clinically significant human cancers.

* gene amplification observed rather than point mutation

** elevated level of protein expression seen

*** chromosomal rearrangement resulting in aberrant expression of protein. Note, for many tumors, mutational activation involves either Raf or Ras, but almost never both. Further, additional members of the large EGFR family (particularly EGFR2/HER2/ErbB2/neu) are mutated or amplified in an additional large number of tumors.

Table 2

Observed occurrence of mutations in specific Raf isoforms in different classes of tumors.

Raf Isoform	Raf Mutation or other change	Mutation Frequency (%)	Cancer Type	Reference
A-Raf	PM (exon 10, introns 9,13), G*	*n.d.*	Colorectal carcinoma	[20]
B-Raf	PM (exon 15)	11.5	Colon carcinoma	[20]
	PM (exon 11,)	9	Colorectal carcinoma	[171]
	PM (exon 11, 15)	2–12	Gastric carcinoma	[172,173]
	PM (exon 11, 15)	3	NSCLC	[15]
	PM (exon 15)	36–55	Papillary thyroid carcinoma	[109,174–177]
	PM (exon 15)	10–14	Anaplastic thyroid carcinoma	[109,178]
	PM (exon 15)	45	Primary melanoma	[76]
	PM (exon 11,15)	55–68	Malignant melanoma	[16,179,180]
	PM (exon 15)	22	Cholangiocarcinoma	[181]
	PM (exon 15)	3	Head and neck, squamous carcinoma	[182]
	PM (exon 15)	11	Barrett's carcinoma	[183]
	PM (exon 15)	31–36	Ovarian carcinoma, serous borderline, low-grade	[184,185]
	PM (exon 15)	0	Ovarian carcinoma, mucinous borderline	[184,185]
	PM (exon 15)	*n.d.*	Struma ovarii	[186]
	PM (exon 15)	3.2–5	Glioblastoma	[187,188]
	PM (exon 11, 15)	2.1–4.4	Acute myeloid leukemia	[189,190]
	PM (exon 11, 15)	2.4	Non-Hodgkin's lymphoma	[191]
	PM (exon 15)	21	Childhood acute lymphoblastic leukemia	[192]
c-Raf-1	PM (intron 9), G*	*n.d.*	Colorectal carcinoma	[20]
	PM (exon 12), G*	*n.d.*	Acute myeloid leukemia	[18]
	increased activation	*n.d.*	Pancreatic carcinoma	[193]
	increased expression	*n.d.*	Breast cancer	[194]
	gene amplification	*n.d.*	NSCLC	[195]
	chromosomal rearrangement	90	SCLC	[196]
	increased expression	50	Hepatocellular carcinoma	[197]
	increased expression	*n.d.*	Ependymoma	[198]
	gene amplification	*n.d.*	Glioblastoma	[187,199]
	4 bp deletion (exon 17)	*n.d.*	Squamous cell carcinoma	[200,201]
	gene amplification	*n.d.*	Osteosarcoma	[202]
	increased expression	*n.d.*	Mantle cell lymphoma	[203]

For B-Raf, the significant majority of mutations represent V600E. G*, mutation is observed in the germline rather than somatically. n.d., although the mutation indicated has been observed, the statistically significant estimation of frequency was not determined.

Table 3

Agents directly or indirectly targeting Raf kinases

Inhibitor Class	Agent	Chemical Class	Primary Target(s)	Additional Targets	References
Antisense olligonucleotide	ISIS 5132	phosphorothioate oligonucleotide	c-Raf	*n.a.*	[66,204]
	LErafAON	liposome-entrapped oligonucleotide	c-Raf	*n.a*	[71]
siRNA	(various)	siRNA	B-Raf	*n.a*	[205]
Raf kinase inhibitor	BAY-43-9006 (sorafenib, Nexavar)	Diphenyl urea	c-Raf, B-Raf, B-RafV600E	VEGFR1, VEGFR2, FLT-3, p38, c-Kit, PDGFR, RET	[80] [81][84]
	PLX4032	Pyrrolo [2,3-B] Pyridine	B-Raf, B-RafV600E	?	[100]
	ZM336372	Benzamide		?	[101]
	AZ628	Quinazilinone	c-Raf, B-Raf, B-RafV600E	DDR2, EphA2, EphB2, Lyn, Flt2, FMS, VEGFR2	[105]
	Raf265	Substituted benzazole	Raf*	VEGFR2	[106]
	AAL881	Isoquinoline	c-Raf, B-Raf, B-RafV600E	?	[108]
	LBT613	Isoquinoline	c-Raf, B-Raf, B-RafV600E	VEGFR2	[110,206]
Ras-Raf PPI inhibitor	MCP110	Aryl Amide	Ras-Raf interaction	GPCR?	[112,113]
HSP90 inhibitor	17-AAG	Benzoquinone	HSP90	(many HSP90-stabilized proteins)	[119,120]
	17-DMAG	Benzoquinone	HSP90		[207]
HDAC inhibitor	SAHA	Phenyl-octanediamide	HDAC6	(all HDACs)	[208]
	NVP-LAQ824	Cinnamic acid hydroxamate	HDAC6		[209,210]

n.a., agent is specifically targeted to complementary Raf mRNA. ?, information not availaible on additional targets.

* specific preference for specific Raf family members not known. ?, additional targets not known.

Table 4

Clinical trials with Raf-targeting agents.

Drug	Cancer Type	Phase	Result	References
sorafenib	HCC	phase III, placebo control	Positive OS (10.7 vs 7.9 months)	[94]
	RCC (2^{nd} line)	phase III, placebo control	10% PR, improved PFS (5.5 vs 2.8 months)	[92,211]
	melanoma	randomized phase II, dacarbazine sorafenib	Improved PFS (21.1 versus 11.7 weeks)	[212]
	NSCLC	phase II, single agent	30/51 patients with SD, with PFS of 23.7 months	[213]
	Head and neck, squamous cell	phase II, single agent	2/38 patients PR, PFS 4 months	[214]
	Metastatic thyroid, iodine refractory	phase II, single agent	PR in 5/15, SD in 3/15	[215]
	soft tissue sarcomas	phase II, single agent	PR in 2/37 leiomyosarcomas, and in 3/23 angiosarcomas	[216]
PLX4032 RAF-265	melanoma, solid tumors	Phase I ongoing	not available	—

OS, overall survival; PFS, progression free survival; PR, partial response; SD, stable disease. For Nexavar, these data represent a selection from a large number of trials now in progress, for use as single agent and combination therapy.