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Raf: A Strategic Target for Therapeutic Development Against Cancer

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ABSTRACT

The mitogen-activated protein kinase (MAPK) signaling pathway plays a critical role in transmitting proliferative signals generated by cell surface receptors and cytoplasmic signaling elements to the nucleus. Several important signaling elements of the MAPK pathway, particularly Ras and Raf, are encoded by oncogenes, and as such, their structures and functions can be modified, rendering them constitutively active. Because the MAPK pathway is dysregulated in a notable proportion of human malignancies, many of its aberrant and critical components represent strategic targets for therapeutic development against cancer. Raf, which is an essential serine/threonine kinase constituent of the MAPK pathway and a downstream effector of the central signal transduction mediator Ras, is activated in a wide range of human malignancies by aberrant signaling upstream of the protein (eg, growth factor receptors and mutant Ras) and activating mutations of the protein itself, both of which confer a proliferative advantage. Three isoforms of Raf have been identified, and therapeutics targeting Raf, including small-molecule inhibitors and antisense oligodeoxyribonucleotides (ASON), are undergoing clinical evaluation. The outcomes of these investigations may have far-reaching implications in the management of many types of human cancer. This review outlines the structure and diverse functions of Raf, the rationale for targeting Raf as a therapeutic strategy against cancer, and the present status of various therapeutic approaches including ASONs and small molecules, particularly sorafenib (BAY 43-9006).

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INTRODUCTION

The *ras* family of oncogenes and encoded proteins has been evaluated as a putative target for anticancer therapeutic development. These efforts have resulted in new insights into Ras-mediated cell signaling as it relates to human cancer. Ras plays a central role in an intricate array of signal transduction pathways, characterized by cross talk, feedback loops, and multicomponent signaling complexes.[1-3] One strategy to overcome the challenges inherent in developing therapeutics against signaling elements situated in redundant pathways is to target elements downstream of convergence points of critical signaling modules. This reasoning has led, in part, to interest in Raf kinase, which is one of several downstream effectors of Ras, as a target for therapeutic development against cancer.

The Raf serine/threonine kinases are the principal effectors of Ras in the mitogen-activated protein kinase (MAPK) pathway (Fig 1). Raf activation occurs immediately downstream of membrane and cytoplasmic receptors that relay mitogenic signals.[4] Although principally activated by Ras, Raf may also be activated by Ras-independent elements and, in turn, propagates signals through diverse effectors that mediate proliferation, angiogenesis, metastases, and survival.[5] Raf may be activated by signaling upstream or constitutively. Constitutive activation of Raf and Ras are indistinguishable in their potential to induce malignant transformation.[6-8] Activating *raf* mutations have been identified in melanoma, thyroid, colon, and other cancers (Table 1).[9-43] Furthermore, the disappointing clinical results of farnesyltransferase (FTase) inhibitors (FTIs) that were developed based on a flawed premise that they would effectively target malignancies with a high incidence of Ras mutation has led to scrutiny of signaling elements downstream of Ras, such

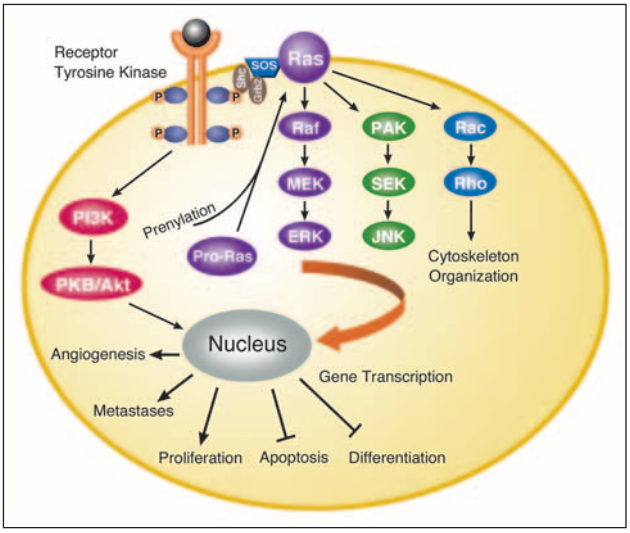

Fig 1. Ras-mediated signal transduction pathways. Abbreviations: PKB/Akt, protein kinase B; ERK, extracellular signal–regulated kinase; Grb2, growth factor receptor binding protein; JNK, c-JUN amino-terminal kinase; MEK, mitogen-activated protein kinase kinase; P, phosphate; PAK, p21-activated kinase kinase or JNK kinase; PI3K, phosphatidylinositol 3-kinase; Shc, Src homology domain-cytosol; SEK, stress-activated protein kinase; and SOS, son-of-sevenless exchange factor.

as Raf, as therapeutic targets.[44] This review highlights relevant information about the biology of Raf and novel strategies directed at exploiting this knowledge to more effectively treat malignant diseases.

SIGNALING THROUGH THE MAPK PATHWAY

The molecular mechanisms and signaling pathways that regulate cell proliferation and survival are receiving considerable attention as potential targets for anticancer strategies.[45,46] Recently, there has been a notable increase in efforts directed at targeting the MAPK pathway, which integrates a wide array of proliferative signals initiated by receptor tyrosine kinases (RTKs) and G protein–coupled receptors.[47,48] The network of signals emanating from the MAPK pathway are transmitted by proteins that serve as chemical switches, cycling between phosphorylated (activated) and dephosphorylated (inactivated) states.[49] These on and off switches are regulated by kinases and phosphatases, respectively. Activated signaling elements, in turn, phosphorylate amino acid residues on downstream signaling proteins in a cascade-like and expansive manner.[50] Not only does the centrality of the MAPK pathway render its components important targets for therapeutic development, but many genes that encode for its critical signaling elements undergo mutations, constitutively activating downstream signaling elements and conferring the potential for transformation and autonomous growth.[50-52] In addition to efforts directed at Ras, therapeutic strategies directed at the MAPK pathway are targeting the cascade of downstream effector proteins including Raf, MAPK kinase (MAPKK; also called MEK or extracellular signal–regulated kinase [ERK] kinase), and ERK. The Raf/MEK/ERK module (Fig 1) of the MAPK pathway, which is immediately downstream of Ras, may be less redundant and innately resistant to therapeutic manipulations compared with Ras (as discussed in the next section).

The Unfilled Promise of Targeting Ras

The Raf/MEK/ERK module of the MAPK pathway has been the focus of considerable attention because therapeutic efforts directed at Ras, which is situated at the apex of the MAPK pathway, have been disappointing.[44] Ras belongs to a superfamily of guanine nucleotide triphosphatases (GTPases) that transmit proliferative and survival signals to the MAPK, phosphatidylinositol 3-kinase (PI3K), and other pathways (Figs 1 and 2). Three *ras* proto-oncogenes encode four 21-kd proteins, called $p21^{ras}$ or Ras (H-Ras, N-Ras, K-Ras4A, and K-Ras4B, resulting from two alternatively spliced *K-Ras* gene products), that are localized to the inner surface of the cell membrane.[44] Of the three *ras* genes, *K-ras* mutations are most commonly found in solid malignancies, whereas *N-ras* mutations are encountered less often, and *H-ras* mutations are rarely encountered.[53,54] Ras isoforms impart distinct biologic effects as a result of the potential of these proteins to differentially activitate critical effectors.[55]

After synthesis as inactive cytosolic propeptides, Ras undergoes a series of post-translational modifications at its carboxyl terminus that increase its hydrophobicity.[56,57] These modifications render Ras functional and capable of localizing to the lipid-rich inner surface of the cell membrane. The first and most critical modification, farnesylation, which is principally catalyzed by protein FTase, adds a 15-carbon hydrobobic farnesyl isoprenyl tail to the carboxyl terminus of Ras. It is in the cell membrane where Ras cycles between inactive guanosine diphosphate–bound and active guanosine triphosphate (GTP) –bound states, thereby activating a series of effector kinases that phosphorylate a cascade of signaling proteins.[58] Ras mutants exhibit slightly less intrinsic GTPase activity than wild-type Ras; however, the principal consequence of the mutated proteins is a marked decrease in interactions between Ras and its GTPase activator protein.[59] Instead of reverting to its inactive guanosine diphosphate–bound state, the modified conformation of mutant Ras favors its active GTP-bound state, which has a higher propensity to activate downstream effectors even in the absence of growth factor stimulation, conferring a proliferative advantage to tumors.

The considerable attention paid to targeting Ras as a therapeutic strategy is based on the high incidence of activating *ras* mutations in human malignancies, including

Table 1. *raf* Alterations and Mutations and Human Cancers

Raf Isoform	Predominant Genetic Alterations	Type of Malignancy	Reference	Frequency of Raf Mutation (%)*	Frequency of Ras Mutation (%)
B-Raf	Point (missense) mutations	Malignant melanoma	Davies et al[9] and Pollock and Meltzer[17]	55-68	16
		Anaplastic thyroid carcinoma	Nikiforova et al[15]	83	—†
		Papillary thyroid carcinoma	Kimura et al[16] and Cohen et al[18]	35.8-69	25
		Cholangiocarcinoma	Tannapfel et al[21]	0-21	56
		Colorectal carcinoma	Rajagopalan et al[14]	4-16	36
		Esophageal carcinoma, Barrett's	Sommerer et al[12]	15	—†
		Acute myeloid leukemia	Lee et al[22]	4	23
		Head and neck carcinoma, squamous	Cohen et al[19] and Weber et al[23]	3-4.8	23
		Lung carcinoma, non–small-cell	Brose et al[24]	2-3	22
		Gastric carcinoma	Lee et al[25]	2	—†
		Ovarian carcinoma, low-grade/high-grade	Singer et al[20]	63/0	23
		Mucinous ovarian carcinoma	Gemignani et al[26]	0	50
		Non-Hodgkins lymphoma	Lee et al[27]	2	—†
C-Raf	Gene rearrangements; point mutations; truncated amino-terminal regulatory domain	Renal cell carcinoma	Oka et al[28]	55	10
		Medullary thyroid carcinoma	Carson et al[29]	—†	—†
		Breast carcinoma	Callans et al[13] and McFarlin and Gould[30]	—†	2
		Lung carcinoma, non–small-cell	Kerkhoff et al[31]	—†	22
		Lung carcinoma, small-cell	Graziano et al[32]	> 90	< 2
		Head and neck carcinoma, squamous	Patel et al[33] and Riva et al[34]	—†	23
		Soft tissue and bone sarcomas	Ikeda et al[35] and Mitsunobu et al[36]	—†	—†
		CNS: glioma, glioblastoma, ependymoma	LaRocca et al,[37] Fukui et al,[38] and Korshunov et al[39]	—†	—†
		Hepatocellular carcinoma	Ting et al,[40] Jenke et al,[41] and Beer et al[42]	—†	31
		Pancreatic carcinoma	Berger et al[43]	—†	78
		Non-Hodgkins lymphomas, T-cell	Storm and Rapp[10]	—†	—†

*Frequency values based on a review of the literature, largely encompassing small and moderately sized studies that generally surveyed tumor biopsy samples in a retrospective manner.
†The precise frequency of the specific genetic alteration is not known. References discuss phenomena but relate to small numbers of patients.

approximately 22% of non–small-cell lung, 50% of colorectal, and 90% of pancreatic cancers.[53,60,61] Of the strategies directed at Ras, targeting FTase has received the most attention, but the FTIs are not Ras specific, and a bonafide Ras-specific therapeutic agent has not yet been evaluated in clinical trials.[62,63] Fortunately, because *K-ras* mutations constitute most *ras* mutations in the aforementioned malignancies, in which the therapeutic expectations of FTIs were among the highest, the failure of this strategy should not be surprising because geranylgeranyl transferase I can alternatively prenylate *K-ras*, rendering it functional even when FTase is completely inhibited.[64,65] Although the FTIs have shown notable antitumor activity in patients with advanced breast cancer and some hematologic malignancies, the low *ras* mutation rates in these cancers suggest that farnesylation of other critical proteins is being inhibited.[66]

DOWNSTREAM OF RAS: RAF AND OTHER RAS EFFECTORS

Localization of GTP-bound Ras to the inner surface of the cell membrane activates several downstream effectors, most notably the serine/threonine kinase Raf, which is the first signaling element in the MAPK pathway.[2,67,68] As shown in Figure 1, other downstream effectors of Ras include the PI3K cell survival pathway, the small GTP-binding proteins Rac and Rho, and the stress-activated protein kinase pathway (also referred to as the c-jun N-terminal kinase [JNK] pathway).[69-71] In addition, in response to cellular stress and cytokine stimulation mediated through Ras, the dual-specificity $p38^{MAPK}$ kinases (MKK3 and MKK6) and the JNK kinases (MKK4 and MKK7) phosphorylate $p38^{MAPK}$ and JNK, respectively.[72-76]

GTP-bound Ras interacts directly with Raf and mobilizes the inactive protein from the cytoplasm (Figs 1 and 2).

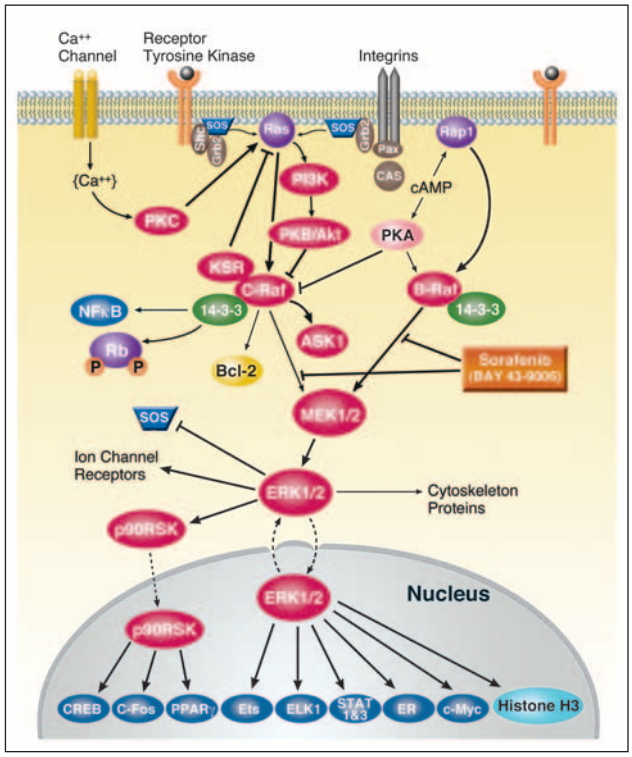

Fig 2. Raf is stimulated by diverse mitogenic stimuli and, in turn, activates multiple effectors. Abbreviations: ASK1, apoptosis signal-regulated kinase; CREB, cyclic adenosine monophosphate response element B; c-Fos, c-Myc, Ets, and ELK1, transcription factors; NF-κB, nuclear factor-kappa B; p90RSK, 90-kd ribosomal S6 kinase; PPARγ, peroxisome proliferator activated receptor gamma; Rb, retinoblastoma protein; Shc, Src homology domain-cytosol; STAT, signal transducer and activator of transcription.

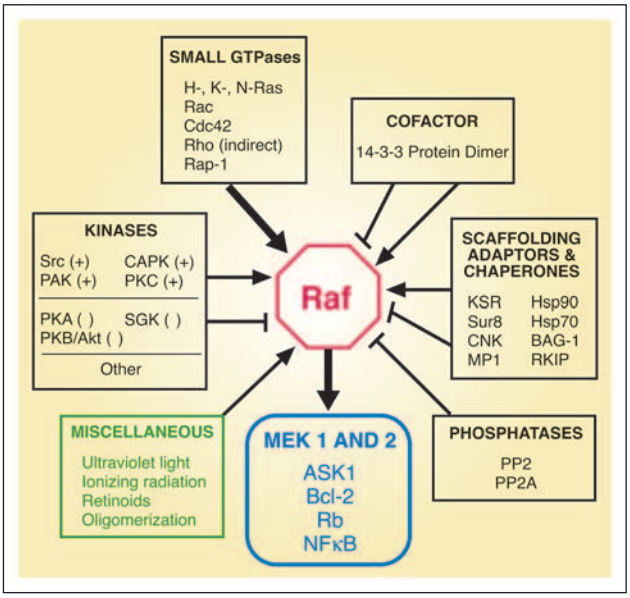

Fig 3. The activating and inhibitory stimuli converging on Raf and its principal downstream effectors. Abbreviations: ASK1, apoptosis signal-regulated kinase; BAG1, Bcl2- associated athanogene; CAPK, ceramide-activated protein kinase; cdc42, cyclin-dependent kinase; Hsp, heat shock proteins; KSR, kinase suppressor of Ras; MP1, MEK partner-1; NF-κB, nuclear factor-kappa B; PAK, p21-activated kinase; PKC, protein kinase C; PKB/Akt, protein kinase B; PP1 and PP2A, protein phosphatases; Rap1, repressor activator protein 1; Rb, retinoblastoma protein; RKIP, Raf kinase–inhibitor protein; SGK, steroid glucocorticoid kinase; Src, soluble nonreceptor tyrosine kinase; (+), activator; (−), inhibitor.

Once the Ras-Raf complex is translocated to the cell membrane, Ras activates the serine/threonine kinase function of Raf through an association between its Ras-binding domain (RBD) in the amino-terminal regulatory region and Ras-GTP. This is followed by a series of Ras-dependent phosphorylation events and conformational changes, which will be described later in this review.[77-84] The regulatory mechanisms of various Raf isoforms differ in that A-Raf and C-Raf require additional phosphorylation reactions for activity, whereas B-Raf has a much higher level of basal kinase activity.[85]

Raf is also activated by Ras-independent activators, including the soluble non-RTK Src and Janus kinase 1, which are involved in cytokine signaling.[86] Other Ras-independent activators of Raf include interferon beta, protein kinase C (PKC) alpha, antiapoptotic proteins (eg, Bcl-2), scaffolding proteins (eg, ceramide-activated protein kinase), ultraviolet light, ionizing radiation, retinoids, erythropoietin, and dimerization between Raf isoforms[86-94] (Fig 3). In addition, several *Raf* mutations confer constitutive activity to Raf irrespective of signaling activity upstream.[9,11,12] The multifactoral mechanisms of Raf activation imply that therapeutic strategies that depend on the abrogation of any single element of these pathways may not result in sufficiently robust tumor growth–inhibitory activity. Furthermore, the kinase activity of Raf is inhibited by its interactions with cholesterol-rich lipid rafts in the cell membrane and phosphorylation by protein kinases A (PKA) and B (PKB/Akt), as shown in Figure 2.[95-98] In essence, the activation status of Raf depends on the integration of both activating and inhibitory stimuli, the net result of which determines the downstream messages.

THE RAF FAMILY OF GENES AND PROTEINS

The *raf* family of genes was first identified as oncogenes in retroviruses that are the causative vectors of tumors in mice and chicken.[99,100] The first *raf* gene to be identified, *v-raf*, the transforming gene of the mouse sarcoma virus 3611, induces fibrosarcomas and erythroleukemia in newborn mice, and *C-raf* (also called *raf-1*) is its proto-oncogene homolog.[101,102] *A-raf* was found next by screening a mouse spleen cDNA library at low stringency with a *v-raf* probe. Next, *v-Rmil* was identified as the transforming gene in the avian retrovirus Mill Hill No. 2 (MH2) from a spontaneous ovarian tumor and found to be homologous to *v-raf*.[99] *C-Rmil* corresponded to a third mammalian *raf* gene, *B-raf*, which was also shown to be an oncogene.[103,104] However, initial attempts to identify activated versions of *raf* in human cancers failed to demonstrate unique DNA rearrangements in

any specific tumor type, which, in retrospect, can be attributed to the lack of requisite sensitivity of early assays to detect single point mutations. Furthermore, most early efforts were directed at *C-raf*, rather than at the more oncogenic *B-raf*.

The mammalian *raf* family consists of the following three genes: *A-raf*, *B-raf*, and *C-raf*, which are located on chromosomes Xp11, 7q32, and 3p25, respectively. The *raf* proto-oncogenes encode three 68- to 74-kd cytosolic proteins, termed A-Raf, B-Raf, and C-Raf (Raf-1), which share highly conserved amino-terminal regulatory regions and catalytic domains at the carboxyl terminus (Fig 4).[10] As serine/threonine kinases, Raf proteins phosphorylate serine and threonine residues on essential modulatory proteins downstream of Ras. Each Raf species has a distinct expression profile in tissues, which suggests that individual Raf isoforms perform clearly defined functions.[4] C-Raf is ubiquitously expressed in most tissues. Both A- and B-Raf have more restricted expression profiles than C-Raf, with A-Raf overexpressed in urogenital tissues (eg, kidney, ovary, prostate, and epididymis) and B-Raf overexpressed in neural, testicular, splenic, and hematopoietic tissues.[105] Unlike *A-raf* and *C-raf*, *B-raf* undergoes differential splicing in exons 8b and 10a, and its spliced variants are translated into 10 B-Raf isoforms.[106,107] Both A-Raf and C-Raf undergo localization to the mitochondria, which supports the notion that Raf regulates apoptosis, but the specific proportions of Raf isoforms that are localized to the mitochondria are not known.[108-112] This localization may be a result of isoform-specific lipid- or protein-binding partners, which recruit Raf to distinct membrane rafts.

From a functional standpoint, although all Raf proteins are serine/threonine kinases and capable of activating the MAPK cascade, they have distinct downstream phosphorylation targets and play unique roles in signaling.[113] Their distinct roles are supported by *Raf* knockout studies, in which mice lacking each of the three Raf proteins have disparate phenotypes.[113,114] *B-raf* knockouts die in utero by day 12, usually as a result of massive internal hemorrhage, whereas *A-raf* and *C-raf* knockouts die postpartum with extensive intestinal distension (*A-raf* knockout) or failure of lung maturation (*C-raf* knockout). Additional support for the diverse functionality of Raf family members is provided by the disparate responses of B-Raf and C-Raf to identical stimuli, as well as the distinct messages that each isoform relays downstream to Rap1, which is a small GTPase that functions as both an activator and repressor of Raf.[115] For example, Rap1-mediated stimulation of B-Raf by cyclic adenosine monophosphate (cAMP) phosphorylates ERK, whereas stimulation of C-Raf inhibits ERK phosphorylation.[115]

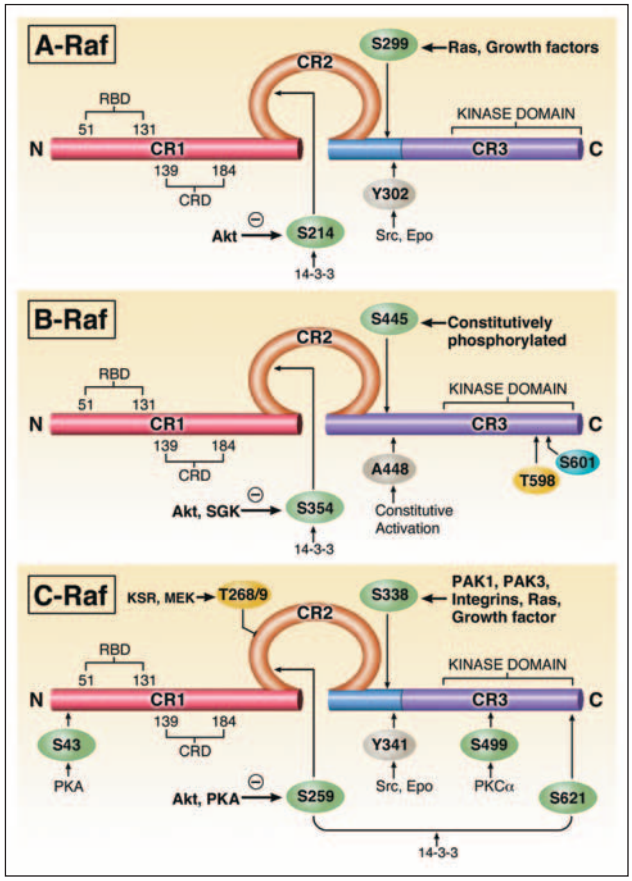

Fig 4. Schema of the domain structure of A-Raf, B-Raf, and C-Raf. The amino acid phosphorylation sites (S, serine; T, threonine; Y, tyrosine) and phosphorylating stimuli regulating the Raf kinases are shown. Abbreviations: C, N, carboxyl and amino terminus; RBD, Ras-binding domain; CRD, cysteine-rich domain; Epo, erythropoietin; 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. (Reproduced from Dowsett et al: Short-term changes in Ki-67 during neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival. Clin Cancer Res 11:951S-958S, 2005)

The Structure of Raf

The structure of Raf consists of the following: (1) an amino terminus that contains the regulatory domain; (2) an activation loop; and (3) a carboxyl terminus that contains the kinase domain[116-118] (Fig 4). All Raf kinases are composed of three conserved regions, CR1 (adjacent to the amino terminus), CR2, and CR3 (adjacent to the carboxyl terminus). The regulation of Raf kinase activity is a complex process involving phosphorylation of the regulatory and catalytic domains of the protein and both inter- and intramolecular interactions. The initial process of Raf activation involves the interaction of active GTP-bound Ras with the RBD of Raf and the adjacent zinc-binding cysteine-rich domain (CRD) of CR1, facilitating recruitment of Raf to the cell membrane for activation.[78] The role of CR2, which is rich in serine and threonine residues, is less well defined; however, the phosphorylation of moieities within CR2 and various protein-protein interactions involving CR2 also affect Raf localization and activation.[89,119-121] Deletions of the amino-terminal regulatory domains CR1 and CR2, similar to *v*-Raf, are found in

several types of human cancers with activating *Raf* mutations, suggesting that these domains negatively regulate Raf function. CR3, the catalytic domain of Raf, is also subject to regulation by phosphorylation.

Regulation of Raf Kinase Activity

General. The overlapping functional aspects of the three Raf isoforms have been elucidated by studies involving Raf knockout mice. In C-Raf knockouts, B-Raf can compensate for the loss of C-Raf in activating MEK in the MAPK pathway, but C-Raf knockouts are much more susceptible to apoptotic stimuli, despite the presence of A-Raf and B-Raf.[122] With regard to differences in signaling between the Raf isoforms, A-Raf is a weaker activator of MEK than B-Raf or C-Raf. Furthermore, A-Raf can activate MEK1 only, whereas C-Raf activates both MEK1 and MEK2.[123-125] As shown in Figure 3, Raf kinases are activated by Ras, other small GTPase regulatory proteins, and scaffolding proteins, and the magnitude and quality of downstream signaling are dependent on the integration of activating events and protein interactions.

C-Raf exists in the cytoplasm as a 300- to 500-kd protein complex. The complex consists of C-Raf, heat shock protein 90 (Hsp90), and the dimeric protein cofactor 14-3-3. 14-3-3 binds to two specific phosphoserine residues of C-Raf, which masks its kinase domain and inactivates the protein. The binding of Ras to C-Raf displaces the 14-3-3 dimer, rendering C-Raf accessible to dephosphorylation by protein phosphatase 2A.[126] This action enables subsequent activation of C-Raf by mitogenic stimuli. The stability and function of C-Raf are also regulated by the phosphorylation status of C-Raf itself, the binding of C-Raf to Ras, and interactions between C-Raf and cellular lipids.

Activation by Ras and other small GTPases. The initial event in the activation of Raf is its recruitment to the inner surface of the cell membrane by the small GTPase Ras. The effector domain of GTP-bound Ras binds to C-Raf through the RBD and CRD in the CR1. Although binding to both sites is required for Raf activation, the most critical interaction is between Ras-GTP and the RBD.[127] All Ras isoforms are capable of interacting with Raf, but K-Ras is the most potent activator, whereas N-Ras is much more efficient than H-Ras.[128] The interaction between Ras and C-Raf alone is insufficient to activate C-Raf, but it serves to translocate C-Raf to the cell membrane where it can be activated.

The activation of B-Raf by Ras has been less well studied; however, the interacting amino acids in the Ras-Raf interface are identical for B-Raf and C-Raf.[129,130] The association of Ras with B-Raf also translocates B-Raf to the cell membrane where it is activated.[124] Interestingly, a membrane-free complex of B-Raf and 14-3-3 can be activated in vitro by recombinant Ras. This is in stark contrast to A-Raf and C-Raf, which must undergo a series of phosphorylation reactions on serine and tyrosine residues in the cell membrane and cannot be activated by Ras alone.[124,130] Of the Raf isoforms, B-Raf is activated first, and on stimulation by Ras, it heterodimerizes with C-Raf, the significance of which is not known.[94]

Both B-Raf and C-Raf can bind to other small GTPases, most notably Rap1.[115,131,132] The effector domains of Rap1 and Ras are nearly identical, but activation of these proteins produces vastly different downstream effects. Furthermore, Rap1 mediates distinct effects after binding to various Raf isoforms. The B-Raf–Rap1 complex activates B-Raf, whereas the C-Raf–Rap1 interaction does not activate C-Raf and, in fact, may be inhibitory.[115,132,133] This occurs because Rap1 binds to the CRD of C-Raf with higher affinity than Ras and excludes Ras from binding.

The Rho family of small GTPases, consisting of Rho, Rac, and cyclin-dependent kinase (Cdc) 42, regulate cytoskeletal organization during the cell cycle and also mediate Ras-induced activation of Raf, especially C-Raf.[134-136] These GTPases do not directly bind to Raf but, instead, signal by activating downstream kinases. Rho signals by activating the serine/threonine protein kinases N1 and N2 and Rho-associated kinase 1, whereas Rac and Cdc42 signal through p21-activated kinase (PAK).[134-136]

Phosphorylation. Raf is principally activated by phosphorylation of specific amino acid residues as shown for each isoform in Figure 4. From an evolutionary standpoint, the Raf activation sites are highly conserved from yeast to humans. Several amino acids in Raf, particularly serine (S) 259 and S621, which bind 14-3-3 and maintain C-Raf in a closed auto-inhibited conformation, are phosphorylated in the basal state.[137] On stimulation, Ras-GTP displaces 14-3-3 from S259, and C-Raf is translocated to the cell membrane, where it can be dephosphorylated at S259 by protein phosphatase 2A or other phosphatases.[126] S259 also represents the site of inhibitory phosphorylation by PKB/Akt, PKA, and serum glucocorticoid-inducible kinase.[121,138,139] Phosphorylation at S621 seems to have greater significance because mutations at this site inactivate Raf's kinase activity. Hence, a balance of phosphorylation and dephosphorylation is required to prime Raf in the basal state before stimulation by Ras or mitogens.[137]

Raf is also phosphorylated at other serine and threonine residues, the most important of which are S338 and tyrosine (Y) 341, which are situated adjacent to the C-Raf kinase domain.[140] Phosphorylation of these residues relieves the inhibitory effects of the regulatory domain on the kinase domain.[141] S338, which is the evolutionarily conserved PAK phosphorylation site that resides on the amino-terminal side of the kinase domain, is critical for Raf activation.[134-136,140,142] This site is also phosphorylated in response to stimulation by growth factors, integrins, Ras, PAK1, and PAK3.[78,136,143] The homologous site on B-Raf, S445, is constitutively phosphorylated, accounting for the higher basal activity of B-Raf. Ras presumably phosphorylates this site by activating PI3K. Activated mutants of Rac

and Cdc42 are also capable of inducing phosphorylation of S338 by activating PAK. Y341 is phosphorylated by the Src family of non-RTKs, Janus kinase, and erythropoietin.[85,92] The substitution of this tyrosine residue by aspartate in B-Raf may explain why B-Raf is fully inducible by Ras, whereas A-Raf and C-Raf require both Ras and Src for full activation.[124] However, Ras-mediated recruitment of C-Raf to the cell membrane and Src activation are not the only steps involved in the activation of C-Raf.

A-Raf, which is structurally similar to C-Raf, is activated in a similar manner; however, the pertinent structural and activational aspects of B-Raf differ from those of A-Raf and C-Raf. Although the structural domains and phosphorylation sites of Raf proteins differ, the greater degree of phosphorylated amino acids in B-Raf confers a 15- to 20-fold higher level of kinase activity in the basal state than either A-Raf or C-Raf, and B-Raf is therefore a much more robust activator of ERK phosphorylation.[85,144,145] The differential splicing of *B-raf* may also account for the distinct kinase activity of the protein. In addition, the structures of several B-Raf mutants mimic the conformational changes unique to phosphorylated wild-type B-Raf, which may explain the ability of B-Raf mutants to activate ERK in the absence of stimulation.

Other interactions. In addition to phosphorylation events, the activation status of C-Raf is regulated by protein-protein and protein-lipid interactions. As shown in Figure 2, C-Raf interacts with a diverse array of scaffolding proteins (kinase suppressor of Ras and MEK partner-1), adaptor proteins (Bcl-2–associated athanogene-1), chaperone proteins (Hsp90 and Hsp70), substrates (retinoblastoma protein [Rb]), lipids (phosphatidic acid, cholesterol-rich caveolae, and cytosolic lipid rafts), and cellular constituents, many of which, in turn, modulate its kinase activity.[95]

Activation of Downstream Effectors by Raf

Activated Raf principally propagates signaling by phosphorylating the two dual-specificity MAPKKs, MEK1 and MEK2 (also referred to as MKK1 and MKK2; Figs 1 and 2).[75] The Raf isoforms are the best characterized MEK1 and MEK2 activators, and all Raf isoforms activate MEK1, whereas only B-Raf and C-Raf activate MEK2. MEK1 and MEK2 contain a proline-rich sequence that enables recognition and activation by Raf.[125,146-153] This sequence, which is not present in other MAPKKs, may explain why Raf preferentially activates MEK1 and MEK2, whereas the dual-specificity $p38^{MAPK}$ kinases (MKK3 and MKK6) and JNK kinases (MKK4 and MKK7) phosphorylate $p38^{MAPK}$ and JNK, respectively. Although both A-Raf and C-Raf are capable of activating other signaling elements independent of MAPK pathway activation, such as nuclear factor-kappa B (NF-κB), Rb, and Bcl-2, MEK1 and MEK2 are the only known substrates for B-Raf.[154-157] A consistent theme in studies on MEK/ERK activation by Raf is that B-Raf is far more potent at activating downstream kinases than either A-Raf or C-Raf. Several lines of evidence also indicate that B-Raf has a much higher affinity for its substrate than the other Raf isoforms and is 50-fold more potent at phosphorylating MEK1 and MEK2 than either A-Raf or C-Raf.[125,158]

The respective downstream substrates of MEK1 and MEK2 are ERK1 ($p44^{MAPK}$) and ERK2 ($p42^{MAPK}$), which are translocated to the nucleus where they ultimately induce an array of cytoplasmic and nuclear regulatory proteins.[50,159-162] Effectors include the nuclear transcription factors Elk-1, Fos, Jun, AP-1, and Myc, which regulate genes encoding proteins that play key roles in proliferation, angiogenesis, metastasis, and resistance to anticancer therapeutics.[51] As a result, cell cycle regulators, such as cyclins D1 and E and Cdc activator 25 phosphatase, are positively regulated,[163,164] whereas $p27^{kip-1}$ and other inhibitors of cyclin-dependent kinases (cdk) are negatively regulated.[51] These actions favor progression through cell cycle checkpoints, aberrant growth, dedifferentiation, and cell survival.

C-Raf activates other cellular effectors, but the extent of the interdependence of these actions on MEK1 and MEK2 is not clear. For example, C-Raf activation regulates cytoskeleton formation by modulating the polymerization status of vimentin.[165] Cell survival signaling is also regulated by C-Raf, which induces phosphorylation of Iκ B in the NF-κB–Iκ B complex. This action releases activated NF-κB, which is then translocated to the nucleus where it mediates transcription of antiapoptotic factors.[155,166] Other antiapoptotic effects of C-Raf are mediated by a mitochondrial pool of the protein, which, on stimulation, localizes to the mitochondrial membrane where the protein interacts with and phosphorylates Bcl-2, Bcl-2–associated athanogene, and other pro-apoptotic regulators, abrogating their pro-apoptotic effects.[157,167] The antiapoptotic effects of C-Raf are also mediated through the ankyrin-repeat protein Tvl-1 and apoptosis signal-regulated kinase-1.[166,168,169] In addition, C-Raf phosphorylates Rb, p53, Cdc25, and other cell cycle regulatory proteins in metaphase.[156,170,171] Lastly, C-Raf induces transcription of the multidrug resistance gene *mdr-1,* and its activation has been associated with multidrug resistance.[172] In summary, Raf mediates essential cellular processes that signal proliferation, survival, and drug resistance.

RAF MUTATIONS IN HUMAN CANCER

General

Constitutively active mutant Raf proteins are predominately a result of point (missense) mutations, deletions, amplification, and rearrangements of *raf.*[173-176] Such genetic alterations have been identified in malignant melanoma, hematopoietic cancers, and cancers of the thyroid, breast, kidney, liver, larynx, biliary tract, and other organs, as shown in

Table 1.[173-176] Although initial efforts at identifying *raf* mutations in human cancer focused on *C-raf*, the advent of high-throughput gene sequencing led to the identification of activating *B-raf* mutations as the predominant genetic aberrations.[11,122,145,177]

B-raf *Mutations*

Recently, a sequence screen of 923 cancer samples for genes mutated in human cancers identified somatic mutations in a notable proportion of tumor samples.[9] Somatic *B-raf* mutations were demonstrated in 60% to 70% of malignant melanomas and in moderate to high rates in carcinomas of the colon, ovary, and thyroid (papillary), implicating activating oncogenic *B-raf* mutations as critical promoters of these malignancies.[9,14-17] Furthermore, somatic *B-raf* mutations were found, albeit at lower rates, in glioma, sarcoma, non-Hodgkin's lymphoma, acute myeloid leukemia, and carcinomas of the breast, lung, and liver. Interestingly, *C-raf* mutations were not identified in a series of 545 cancer samples, including melanomas and carcinomas of the colon, ovary, and lung.[9]

Sequence analysis of *B-raf* in human cancer has identified more than 30 single-site missense mutations, principally encoding amino acids in the kinase domain of B-Raf, whereas the constitutive activity and transforming potential of C-Raf result from loss of the auto-inhibitory amino-terminal region, as well as gene rearrangements.[6,175,176] Most *B-raf* mutations are caused by thymidine-to-adenine transversions at nucleotide position 1796 in exon 11 or 15, which encode a valine-to-glutamic acid substitution at amino acid 599 (^{V599E}B-Raf) in the activation segment (kinase domain) of the protein. Interestingly, structural changes in the activation segment as a result of the insertion of an acidic residue close to a site of regulated phosphorylation mimic phosphorylated B-Raf.[9] *^{V599E}B-Raf* possesses the hallmarks of a conventional oncogene because the kinase activity of its encoded protein is greatly elevated; it constitutively stimulates ERK in vivo in the absence of Ras activation; and it transforms NIH3T3 cells.[144] Furthermore, the basal kinase activity of ^{V599E}B-Raf is 12.5-fold higher than that of wild-type B-Raf, and its responsiveness to stimulation by oncogenic H-Ras is diminished. Furthermore, the transforming capacity of ^{V599E}B-Raf in NIH3T3 cells is 667-fold more efficient than that of wild-type B-Raf, whereas the equivalent mutation introduced into C-Raf (V492E) confers 10-fold lower kinase activity and transforming capacity.[85]

The discovery of *B-raf* mutations in 60% to 70% of malignant melanomas is surprising because early studies attributed the hyperactivation of the Raf/MEK/ERK module of the MAPK pathway in melanoma to an abundance of autocrine and paracrine growth factors. Interestingly, *B-raf* mutations are not found in uveal melanoma, which differs from cutaneous melanoma in that abnormalities of chromosome 6 are found only in uveal melanoma, suggesting that there are distinct pathways for melanoma formation.[178,179] Further studies evaluating the function of ^{V599E}B-Raf in benign and dysplastic nevi may yield important information about the type and timing of events required for tumorigenesis. Interestingly, the ^{V599E}B-Raf allele is found in as many as 80% of benign nevi, suggesting a role for oncogenic *B-raf* in nevus formation and melanoma initiation.[180] However, there is no direct evidence that benign nevi harboring ^{V599E}B-Raf progress to malignancy, and most cases may actually represent terminally differentiated lesions analogous to nondysplastic colorectal aberrant crypt foci that harbor *K-ras* mutations in the absence of *adenomatous polyposis coli* (*APC*) mutations. *APC* mutations are generally considered to be of low malignant potential, whereas *K-ras* mutations that arise after *APC* mutations promote colorectal tumor progression.[181,182] Further studies are also needed to determine whether the prevalence of *B-raf* mutations in melanoma relates to the site of the primary tumor, sun exposure, and radiation damage. Similar findings have been noted in the setting of papillary thyroid carcinoma, in which up to 69% of tumors harbor ^{V599E}B-Raf, whereas benign thyroid tumors and both follicular and medullary thyroid carcinomas do not.[18,19] It is notable that *B-raf* mutations are common in melanoma and thyroid cancers and that both melanocyte and thymocyte growth is positively regulated by cAMP. Interestingly, B-Raf is thought to be the key Raf isoform that transduces cAMP-dependent growth signals in both cell types, which may account for their vulnerability to transformation by activating mutations of this kinase.[183,184]

Analysis of other much less common oncogenic B-Raf mutants, most of which cluster adjacent to valine 599 or in the G loop ATP-binding region, suggest that the mutated proteins stimulate kinase activity in a manner similar to ^{V599E}B-Raf.[9] Nevertheless, it is intriguing that several of these mutations involve highly conserved or invariant residues in the catalytic domain, which are required by other kinases for optimal activity. This raises the question of how these mutants promote tumorigenesis.[8,9,185] It should also be noted that *B-raf* mutations outside the kinase domain have been identified, and other mutations will likely be identified as the gene is sequenced in other types of malignancies.[11]

Mutations of *B-raf* and *ras* are essentially mutually exclusive, implying that these genes belong to the oncogenic signaling pathway. Fewer than 1% of cancers with *B-raf* mutations have simultaneous *ras* mutations, and of the 1% that have mutations of both genes, the *B-raf* mutations are almost never ^{V599E}B-Raf.[8,9,14] In colorectal carcinoma, both genes are mutated at high frequencies in the same types of premalignant lesions and at the same stages in the transition from adenoma to carcinoma.[8,14] A strong association exists between mismatch repair deficiency and the presence of the mutant ^{V599E}B-Raf protein in colorectal carcinoma, which

may be a result of the underlying DNA repair defect.[14] Further reflecting the redundancy of the MAPK pathway, a high fraction of papillary thyroid cancers harbor either *V599E B-Raf*, mutant *K-ras*, or mutant *RET*.[9,20] Harboring more than one mutation is quite rare, although a moderate fraction of low-grade ovarian tumors harbor either *V599E B-Raf* or mutant *K-ras*.[9,20] This finding may represent a unique paradigm of human tumorigenesis through mutations of these signaling proteins that lie in tandem.[8,9] However, concomitant *ras* mutations have been identified in cancers that harbor uncommon *B-raf* mutations in the G loop region, suggesting that there may be differences in molecular pathways used by distinct mutant B-Raf proteins.[9]

C-raf *Mutations*

In contrast to *B-raf* mutations, no underlying genetic mechanisms predominate in human cancers that harbor *C-raf* mutations. Several types of genetic alterations, particularly gene rearrangements, have been demonstrated in human cancers sampled from patients with non–small-cell lung carcinoma and T-cell lymphoma harboring *C-raf* mutations.[10] In addition, constitutively active C-Raf has been associated with site-specific *C-raf* mutations, and a structurally aberrant C-Raf protein that is truncated in its amino-terminal regulatory domain has been identified in tumor samples from patients with carcinomas (kidney, lung [small cell], liver, and pancreas), sarcomas (soft tissue and bone), and CNS malignancies (glioma, glioblastoma, and ependymoma).[6,186,187] However, neither specific genetic nor structural aberrations have been identified in a sizeable proportion of human cancers in which C-Raf is activated in the absence of upstream Ras activation.[6,175,177]

THERAPEUTIC STRATEGIES TARGETING RAF

Given the high proportion of cancers with constitutively activated Raf, Ras mutations, or growth factor hyperactivity, which result in increased signaling through Raf, Raf is an ideal target for therapeutic development. Although there have been many attempts to develop therapeutics against Raf, most efforts have been directed at C-Raf rather than B-Raf. To decrease Raf production and inhibit its activation, antisense oligonucleotides (ASONs), small-molecule kinase inhibitors, and dominant interfering DNA constructs are being developed. In addition, other therapeutics that indirectly target Raf include inhibitors of chaperone proteins (eg, geldanamycin analogs), which destabilize Raf, and histone deacetylase inhibitors, which reduces *raf* expression.[188]

ISIS 5132 (CGP 69846A): AN ASON INHIBITOR OF C-RAF

The specificity of nucleotide base pairing provides the rationale for using ASONs as therapeutics against Raf.[189,190] This approach relies on the intracellular uptake of short synthetic ASONs that are complementary to Raf mRNA by mechanisms that have not been clearly elucidated. The ASON then hybridizes with its cognate mRNA, leading to RNAase H–mediated degradation of the complex. Alternatively, the ASON can sterically inhibit translation, which reduces synthesis of the encoded protein.

ISIS 5132 (CGP 69846A; ISIS Pharmaceuticals Inc, Carlsbad, CA) is a 20-base phosphorothioate ASON designed to hybridize to the 3′ untranslated sequence of *C-raf*.[191] Binding induces degradation of the C-Raf mRNA, which, in turn, decreases synthesis of C-Raf in a concentration-dependent manner.[192] The 50% inhibitory concentration (IC_{50}) value for both tumor proliferation and C-Raf expression is approximately 100 nmol/L.[192] Furthermore, treatment of mice bearing human lung and breast cancer xenografts produces impressive decrements in C-Raf, as well as antitumor activity.[191] In other models, ISIS 5132 decreases C-Raf expression and enhances sensitivity to both cytotoxics and radiation.[193] The phosphorothioate backbone of ISIS 5132 was engineered to confer resistance to digestive nucleases, which is manifested by plasma half-life values ranging from 30 to 85 minutes and extensive tissue distribution in mice.[194-196]

The feasibility of administering ISIS 5132 was explored in patients with advanced solid neoplasms on the following schedules: (1) 21-day continuous intravenous (IV) infusion (CIVI) every 28 days; (2) 2-hour IV infusion thrice weekly for 3 weeks every 28 days; and (3) 24-hour IV infusion weekly for 3 weeks every 28 days.[197-199] The principal toxicities were fever and malaise. Thrombocytopenia and anemia, which were typically moderate in severity, brief, and not cumulative, were also noted. Transient prolongation of the activated partial thromboplastin time and activation of the alternate complement pathway, which have been attributed to the phosphorothioate backbone of ISIS 5132, occurred in a dose-dependent manner. Dose-dependent elevations of the compliment component C3a, but not Bb or C5a, were noted. Although maximum tolerated doses were not clearly defined in the first two studies, plasma concentrations of intact ISIS 5132 achieved at the highest doses (6 and 4 mg/kg/d) exceeded IC_{50} values derived in vitro and were known to activate the alternate complement pathway in monkeys.[198] In the third study, an unacceptably high incidence of intolerable toxicities, particularly Coombs hemolytic anemia and acute renal insufficiency, was noted in patients treated at doses greater than 24 mg/kg/wk. The toxicities of ISIS 5132 were similar to those of other ASONs and, therefore, should not be interpreted as being related to target inhibition. Although several patients experienced protracted periods of stable disease, major tumor regression did not occur. *C-raf* mRNA levels in peripheral-blood mononuclear cells were consistently suppressed in patients receiving ISIS 5132 as a 2-hour IV

infusion thrice weekly for 3 weeks, but suppression of *C-raf* mRNA was not detected on the schedule of 24-hour CIVI weekly for 3 weeks every 28 days and not evaluated in the study of ISIS 5132 as a 21-day CIVI.

The antitumor activity of ISIS 5132 was evaluated in phase II studies in patients with advanced colorectal (15 patients, no prior treatment for metastatic disease), hormone-refractory prostate (16 patients, no prior chemotherapy), ovarian (22 patients, one to two prior systemic therapies), small-cell lung (four patients, one prior therapy), and non–small-cell lung (18 patients, no prior therapies) carcinomas.[200-203] Stable disease lasting 2.5 to 5.5 months was the best response in a sizeable proportion of patients, but there were no major tumor regressions. Nonetheless, these disappointing results should not diminish the potential importance of Raf as a therapeutic target because several alternative hypotheses, including the lack of validation of ASON technology as a platform that can confer robust anticancer activity and lack of documentation of *raf* mutational status in these clinical studies, may explain these results.

Small-Molecule Inhibitors of Raf Kinase

The identification of nearly 500 kinases that can be classified into at least 20 families based on structural homology and recent successes with kinase inhibitors have produced bountiful opportunities for small-molecule inhibitors of Raf kinase.[185] The elucidation of the crystalline structure of the ATP-binding domain of Raf has even further brightened these prospects.[204,205] Several classes of small molecules are currently being optimized from both mechanistic and pharmaceutical standpoints. In addition to blocking Raf kinase, small molecules directed at Raf also inhibit a wide range of other kinases by virtue of structural homology between the kinase families. Although it may be desirable for small-molecule therapeutics to impart inhibitory effects on multiple critical signaling pathways, these multifunctional aspects may also impart greater toxicity. Of the small-molecule Raf inhibitors in development, sorafenib (BAY 43-9006; Bayer Corporation Pharmaceutical Division, New Haven, CT; and Onyx Pharmaceuticals, Inc, Richmond, CA; Fig 5) is the furthest along.

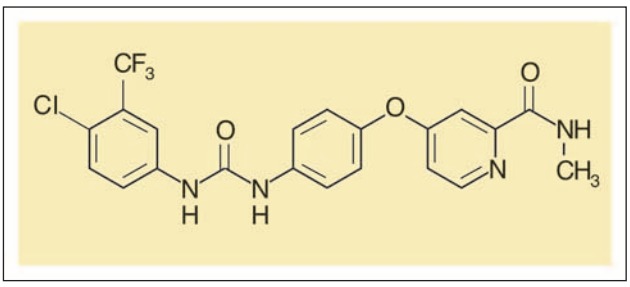

Fig 5. Chemical structure of sorafenib (BAY 43-9006).

Sorafenib (BAY 43-9006): Mechanism of Action and Preclinical Results

The bi-aryl urea sorafenib (4-{4-[3-(4-chloro-3-trifluoromethyl-phenyl)-ureido]-phenoxyl}-pyridine-2-carboxylic acidmethylamide-4-methylbenzene-sulfonate; BAY 43-9006) is the first molecule of its class to undergo clinical development. Originally identified by high-throughput screening of small molecules against C-Raf kinase, sorafenib was found to be a potent competitive inhibitor of ATP binding in the catalytic domains of C-Raf, wild-type B-Raf, and ^{V599E}B-Raf mutant. As shown in Table 2, the IC_{50} values of sorafenib against C-Raf, wild-type B-Raf, and the ^{V599E}B-Raf mutant in a biochemical assay are in the low nanomolar range, whereas sorafenib does not inhibit MEK1, ERK1, erbB1, or erbB2.[206] Sorafenib potently inhibited activation of the MAPK pathway and ERK phosphorylation in human cancer cell lines, irrespective of whether they harbored *K-ras* mutations, ^{V599E}*B-Raf*, or both.[207] Further characterization of sorafenib in biochemical assays showed potent inhibition of pertinent RTKs involved in tumor progression and angiogenesis, including human and murine vascular endothelial growth factor receptor (VEGFR) -2, VEGFR-3, platelet-derived growth factor receptor-beta (PDGFR-β), Flt-3, c-Kit, p38α, and fibroblast growth factor receptor-1 (Table 2). In contrast, erbB1, insulin-like growth factor-1, c-met, and erbB2 RTKs were not inhibited. The kinase activities of PKA, PKB, PKCα, PKCγ, cdk1/cyclin B, and pim-1 were also insensitive.[208] Furthermore, sorafenib inhibited various nonkinase

Table 2. Biochemical Kinase Selectivity Profile for Sorafenib (BAY 43-9006)[206]

Biochemical Assay	IC_{50} (nmol/L)
C-Raf	6
B-Raf, wild-type	22
B-Raf mutant, V599E	38
VEGFR2	90
mVEGFR-2	6
mVEGFR-3	12
mPDGFR-β	57
Flt-3	58
c-Kit	68
p38α	38
FGFR-1	580
EGFR, HER-2, ERK1, MEK1, IGFR-1, c-met, c-yes, PKB, PKA, cdk1/cyclin B, PKCα, PKCγ, pim-1	> 10,000

Abbreviations: cdk, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; FGFR-1, fibroblast growth factor receptor-1; HER-2, human epidermal growth factor receptor 2; IC_{50}, concentration of sorafenib that inhibits the kinase activity by 50%; IGFR, insulin-like growth factor receptor; mPDGFR, mouse platelet-derived growth factor receptor; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; m, murine; MEK1, mitogen-activated protein kinase kinase 1; VEGFR, vascular endothelial growth factor receptor.

targets, including adenosine A3, dopamine D1, and muscarine M3, albeit at much higher (micromolar) concentrations than kinase targets.[209] In cellular assays, sorafenib reduced basal phosphorylation of the MAPK pathway in a panel of human breast, melanoma, pancreatic, and colon cancer cell lines expressing either mutant K-Ras, mutant B-Raf, or wild-type Ras or Raf. Interestingly, several non–small-cell lung cancer cell lines expressing mutant *K-ras* were resistant, presumably because Raf-independent activation of MEK is operative in these cells.[208,210] In other cell-based assays, sorafenib inhibited phosphorylation of several pro-angiogenic RTKs, including both human VEGFR-2 and murine VEGFR-2, murine VEGFR-3, PDGFR-β, and Flt-3.[208,210] In nude mice bearing human xenografts derived from melanoma and colon, pancreas, breast, and lung carcinomas, sorafenib treatment resulted in a high level of tumor growth inhibition without appreciable toxicity.[206] Most early evaluations used the HCT116 human colon xenograft because its tumorigenicity depends on K-Ras activation. Extending the duration of sorafenib treatment attained protracted antitumor efficacy, even when treatment was initiated in settings of high tumor burden. Significant growth inhibition was noted after treatment of well-established human xenografts with *B-raf* mutations (HT-29, Colo205, and DLD-1 colon), *K-ras* mutations (NIH-H460 and A459 lung; MiaPaCa pancreas), and both *K-ras* and *B-raf* mutations (MDA-MB-231 breast) with sorafenib at doses of 7.5 to 60 mg/kg daily for 9 days. *B-raf*–mutated MDA-MB-231 breast cancer xenografts, which were reduced in size by 42%, on average, after only 9 days of treatment with 30 mg/kg of sorafenib, were the most sensitive.[208,210] Relevant activity against the human SKOV-3 ovarian xenograft that harbors wild-type Ras but overexpresses both erbB1 and erbB2 was also noted.

Concurrent with the demonstration that sorafenib is efficacious in a molecularly diverse range of human tumor xenografts, translational studies have demonstrated inhibition of the MAPK pathway after 5 days of sorafenib treatment of HT-29, DLD-1, HCT-116, and MDA-MB-231, but not Colo-205 xenografts. In the Colo-205 tumors, in which concurrent assessments of vascular effects were performed, tumor neovascularization was reduced dramatically. The cumulative results of these studies suggest that sorafenib inhibits tumor progression by blocking cellular proliferation that is dependent on activation of the MAPK pathway and/or inhibiting tumor angiogenesis through VEGFR-2, VEGFR-3, and/or PDGFR-β. Recent studies suggest C-Raf inhibition may also promote the death of endothelial cells as a result of their specific requirements for stimulation by VEGFR-2.[211] The results indicate that sorafenib is efficacious not only against human tumors with *ras* and/or *raf* mutations, but also against tumors that overexpress growth factor receptors that signal through Ras and the Raf/MEK/ERK module. However, it is important to note that the relative potency of sorafenib against various kinases, particularly VEGF, must be considered in assessing the value of sorafenib as a Raf kinase inhibitor, as well as in drawing conclusions about the value of Raf kinase as a molecular target against cancer.

Favorable cytotoxic effects were noted after treatment of a broad spectrum of human cancer cell lines and xenografts harboring both wild-type and mutated forms of *ras* or *raf* with sorafenib and either fluorouracil, paclitaxel, gemcitabine, gefitinib, vinorelbine, doxorubicin, irinotecan, or its active SN-38 metabolite.[212] Treatment of human tumor xenografts with sorafenib plus paclitaxel, irinotecan, gemcitabine, or cisplatin did not enhance the toxicity or diminish the activities of the therapeutics.

Pharmacokinetic studies in rodents and dogs have demonstrated that sorafenib clearance is much lower than normal liver plasma flow. Its low steady-state volume of distribution (approximately 0.7 to 0.93 L/kg) suggests that tissue affinity is low and plasma protein binding is high (mean free fraction, 1.2% [human] to 2.5% [mouse]). The pharmacokinetics in mice are dose proportional over a biologically relevant dosing range, and tissue concentrations are several fold higher than IC_{50} values in vitro.[206,208,213] At higher doses, drug exposure increases disproportionately, possibly because of saturation of gastrointestinal absorption. Autoradiographic studies have revealed homogeneous drug distribution to peripheral tissues and modest penetration across the blood-brain barrier. The mean terminal half-life ranges from 6 to 7 hours. In rodents, oral bioavailability is high (approximately 79%). Drug disposition is principally by CYP3A4 metabolism, followed by biliary and fecal excretion (approximately 90%). CYP1A, CYP2C9, CYP2C19, and CYP3A are not induced after incubating drug with microsomal extracts from human hepatocytes. However, in vitro metabolism studies in human systems indicate extensive metabolism by CYP3A, and early clinical data indicate that disposition is principally by hepatic metabolism and fecal excretion. Sorafenib is a modest inhibitor of CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, and the propensity for interactions between sorafenib and drugs that inhibit and induce P-450 systems exists.

In rodents and dogs, sorafenib is well tolerated. Principal toxicities include emesis, diarrhea, and transaminase elevations. Histopathologic studies have revealed dose-related degenerative changes in the liver, stomach, duodenum, pancreas, kidneys, heart, testes, and ovaries and regenerative changes in the liver, pancreas, duodenum, and kidneys. Hypocellularity and necrosis of hematopoietic and lymphoid tissues and unusual findings involving the teeth and growth plate of the femur have been noted.

Clinical Evaluations

Phase I studies. Phase I end points were evaluated in patients with advanced solid malignancies in studies of the

following daily oral schedules: (1) 7 days every 15 days; (2) 21 days every 28 days; (3) 28 days every 35 days; and (4) continuous treatment. The principal dose-related toxicities were diarrhea, vomiting, skin rash, fatigue, hypertension, and palmar-plantar erythrodysesthesia (hand-foot syndrome). Hand-foot syndrome was characterized by desquamation and discomfort of the digits, all of which were reversible. Clinically relevant elevations in serum amylase and lipase and both lymphopenia and anemia were uncommon. The incidences of intolerable toxicities, particularly diarrhea and hand-foot syndrome, were unacceptably high at sorafenib doses exceeding 400 mg twice daily on a continuous schedule, which was recommended for phase II trials. Tumor regression was noted on several schedules, particularly when doses exceeded 200 mg twice daily. One patient each with hepatocellular carcinoma and renal cell carcinoma (RCC) had partial responses, whereas tumor regressions of lesser magnitude occurred in patients with RCC and colorectal and ovarian carcinomas. Furthermore, approximately 50% of patients with colorectal, ovarian, hepatocellular, renal, and breast carcinomas had stable disease as their best response.[214] Pharmacokinetic studies revealed dose proportionality up to 600 mg twice daily and high interpatient variability. Steady-state was achieved by 7 days, and terminal half-life values ranged from 30 to 45 hours. ERK1/2 phosphorylation in $CD7^+$ peripheral-blood mononuclear cells was inhibited.[215,216]

Disease-directed studies. The principal paradigm adopted for disease-directed evaluations of sorafenib represents a radical departure from traditional phase II approaches. Although phase II studies are being performed in malignancies of high interest, the principal disease-directed evaluation strategy was a randomized discontinuation trial. This unorthodox approach was undertaken because the predominant clinical benefit of the agent, particularly in patients whose tumors were not screened for molecular aberrations known to increase the probability of responding, was projected to be increased progression-free survival (PFS), which was also the principal beneficial effect in preclinical studies. In addition, because sorafenib inhibits multiple kinases, the use of any empiric screening and/or enrichment strategy, as well as any particular malignancy, could produce false-negative results. In contrast to randomized phase II studies, which lack sufficient statistical power to discern small to moderate, albeit relevant, differences between treatments, the randomized discontinuation study is designed so that there is an initial process of natural enrichment of the study population with patients who may have experienced benefit to treatment before patients are randomly assigned to either continue or discontinue drug treatment.[217]

The randomized discontinuation study is felt to be ideal for sorafenib and agents whose main benefit is expected to be tumor growth delay, which is not readily detected in nonrandomized studies. At the end of an initial lead-in phase, in which all patients receive the study drug, patients who experience a relevant degree of tumor growth are removed from the study. This weeding out process enriches the study population with patients who will most likely benefit from further treatment, thereby increasing the probability that the randomization step will be more efficient at detecting tumor growth inhibition related to drug. In essence, the lead-in period may furnish data about the inherent potential of the agent to induce tumor regression and can suffice as multiple phase II studies, each of which can be sized in real time to provide a requisite level of statistical power. At the end of the lead in period, patients whose tumors have not progressed are randomly assigned to either continue or discontinue treatment, ideally in a double-blinded, placebo-controlled fashion. The natural selection or enrichment of the population before random assignment increases the efficiency of the trial, with as few as 20% of the standard number of randomly assigned patients. Nonetheless, a shortcoming of this approach relates to its inability to precisely quantify the magnitude of antitumor activity. However, if there is a clear difference in PFS between the randomly assigned arms, conclusions can still be generated about the general activity of the agent. Nevertheless, if the results meet a sufficient level of interest, resource-intensive phase III studies may ensue.

The randomized discontinuation study, as depicted in Figure 6, was designed to discern differences in PFS between patients treated with either sorafenib or placebo in the randomization period. The randomization stage was sized to discern PFS in patients with colorectal carcinoma, which frequently harbors *ras* mutations, although patients with many tumor types were enrolled. At the end of the 12-week period, in which all patients received sorafenib 400 mg twice daily, patients whose target lesions had increased in excess of 25% were taken off study. Because of concerns about randomly assigning patients who had potentially benefited from treatment, patients whose target lesions had regressed by greater than 25% were not randomly assigned and, instead, continued treatment until disease progression. Patients who experienced neither objective benefit of this magnitude nor disease progression were randomly assigned to either continue treatment with sorafenib or placebo. Because PFS was the primary end point in the randomization phase, placebo-treated patients who experienced progressive disease could be re-treated with sorafenib.

Patient accrual ended in January 2004, with 484 patients accrued at a rate of 36 patients per month at only five institutions in the United States and Europe. Of these, 408 patients were the focus of a recent report.[218] Tumor regression was noted in previously treated patients with advanced melanoma, sarcoma, RCC, and colorectal, thyroid, and pancreatic cancers. Most colorectal carcinoma patients treated with sorafenib developed disease progression before random assignment. RCC emerged as a central focus of the

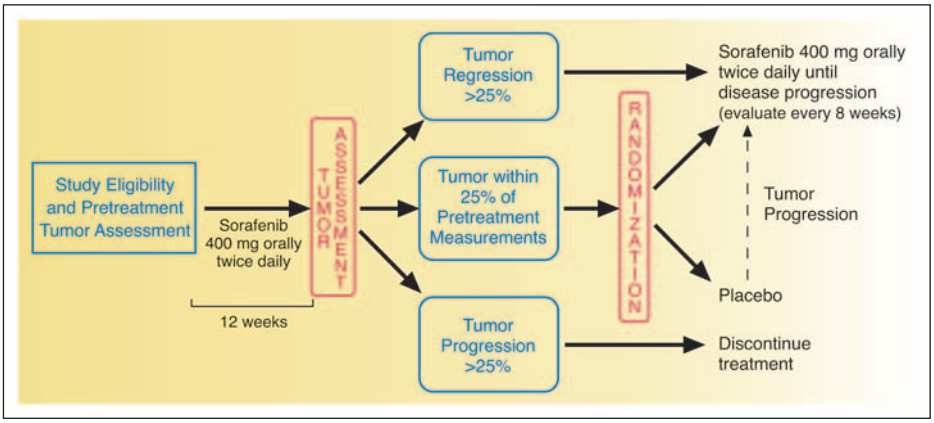

Fig 6. Schema of the randomized discontinuation trial with sorafenib (BAY 43-9006).

study, and the accrual targets for RCC were reset higher as the activity of sorafenib in RCC became increasingly evident. In the run-in phase, 202 patients with RCC were treated with sorafenib and were the focus of a recent report in May 2005.[219] At 12 weeks, 144 patients (71%) experienced tumor shrinkage or disease stabilization. Independently confirmed tumor shrinkage of $\geq$ 25% (partial response) was noted in eight (4%) of these patients. A total of 65 patients were entered onto the randomization phase, of whom 32 were treated with sorafenib and the rest received placebo. Sixteen patients (50%) in the sorafenib arm were progression free at 12 weeks postrandomization, compared with six patients (18%) in the blacebo arm (P = .0077). The median progression-free survival from randomization in the two arms was 24 weeks and 6 weeks, respectively (P = .0087). The unique design of this study served the broad mechanism of action of this promiscuous tyrosine kinase inhibitor, providing a sufficient level of flexibility to evaluate a wide range of end points and tumor types. The intriguing results in patients with RCC provided the basis for a phase III study, in which 800 patients with unresectable and/or measurable RCC who have received at least one prior systemic therapy are being randomly assigned to treatment with either sorafenib or placebo. The primary and secondary end points were overall survival and progression free survival, respectively. A total of 905 patients were randomly assigned, of whom 769 patients were the focus of a recent report.[220] Three hundred eighty-four patients were randomly assigned to the sorafenib arm, and the rest to the placebo arm. An independent response assessment was performed in 574 patients. Seven partial responses (2%) were noted in the sorafenib arm compared with none in the placebo arm. Disease stabilization was seen in 261 (78%) versus 186 (55%) patients in the sorafenib and placebo arms, respectively, and disease progression was noted in 29 (9%) versus 102 (30%) patients, respectively. The median progression-free survival for patients in the sorafenib arm was 24 weeks, compared with 12 weeks in the placebo arm (hazard ratio = 0.44; P = .000001). The substantial benefit due to sorafenib was apparent across all patient subgroups. Because of the magnitude of the benefit noted upon analysis, treatment unblinding was performed, and patients randomly assigned to placebo were allowed to cross over to sorafenib treatment.

There was considerable interest in the melanoma patients who participated in the randomized discontinuation study based on the high incidence of *B-raf* mutations in melanoma.[218] In the June 2004 report that focused on the first 20 patients enrolled, five patients developed cutaneous toxicity of grade 3 severity, and two patients developed hypertension that required intervention.[221] Of 19 patients whose disease had been evaluated, 15 patients developed progressive disease before or at the planned 12-week assessment, whereas one patient had a partial response, and three patients had stable disease. Although a complete survey of *B-raf* mutations was not available for the June 2004 report, the negligible antitumor activity in a malignancy with a 60% to 70% incidence of constitutive *B-raf* mutations implies that sorafenib alone on the dose schedule evaluated lacks sufficient activity at inhibiting B-Raf kinase. Phase II studies are also ongoing in advanced hepatocellular and non–small-cell lung carcinomas and other malignancies.

Combination studies. The feasibility of administering sorafenib with various other agents is being evaluated in early clinical evaluations. Flaherty et al,[222] who conducted a phase I study of sorafenib plus carboplatin and paclitaxel and then focused on the activity of the regimen in patients with melanoma who were enrolled at the maximum-tolerated dose, have provided some of the most intriguing results. Both untreated and previously treated patients with progressive growth of solid neoplasms before study enrollment were treated with fixed doses of carboplatin area under the curve 6 and paclitaxel 225 mg/m^2 IV over 3 hours on day 1 followed by sorafenib at doses of 100, 200, or 400 mg twice daily from days 2 to 19 of a 3-week course. All dose iterations were well tolerated, and toxicity rates did not exceed those expected with carboplatin and paclitaxel in the absence of sorafenib. Sixty-seven patients with melanoma,

most of whom were enrolled onto the phase II stage, have been treated. Of the 35 assessable patients with melanoma at the time of the report, 14 patients (40%) had partial responses, all of which lasted for at least 6 months, and 15 patients (43%) had stable disease as the best response. The median PFS time was estimated at 8.4 months. Of 25 patients whose mutational status was assessed, 15 (60%) had ^{V599E}*B-Raf*, but the propensity to respond did not depend on *B-Raf* mutational status. Five (33%) of 15 patients with ^{V599E}*B-Raf* and six (60%) of 10 patients with wild-type B-Raf had partial responses. These results are impressive in light of the negligible activity of sorafenib as a single agent in patients with melanoma, implying that sorafenib may operate principally by inhibiting VEGF RTKs, perhaps by enhancing the penetration of cytotoxics into tumors and/or decreasing intratumoral pressure. A phase III study evaluating the paclitaxel-carboplatin regimen with and without sorafenib is planned.

In a phase I study of sorafenib and gemcitabine, the maximum-tolerated doses were gemcitabine 1,000 mg/m^2 IV weekly for 7 of every 8 weeks, followed by weekly for 3 of every 4 weeks and sorafenib 400 mg twice daily.[223] Of 19 previously treated patients in the phase I study, two patients with ovarian carcinoma had partial responses; whereas one (4.3%) of 23 previously untreated subjects treated in an expanded stage at the recommended dose had a partial response.[223] No pharmacokinetic interactions were evident. Similarly, regimens consisting of sorafenib plus doxorubicin, irinotecan, or oxaliplatin were well tolerated, and pharmacokinetic interactions were not apparent.[224-226] In a phase I study of sorafenib and doxorubicin, the principal toxicities were neutropenia and hand-foot syndrome, which did not preclude administering each agent at relevant doses (sorafenib 400 mg twice daily and doxorubicin 60 mg/m^2 IV every 3 weeks).[222] One patient with mesothelioma had a partial response, and 17 patients (52%) had stable disease. Of note, four patients with hepatocellular carcinoma experienced stable disease lasting at least 12 months. Relevant doses of sorafenib (400 mg twice daily) and oxaliplatin (130 mg/m^2 every 3 weeks) were well tolerated.[227] Two patients with gastric carcinoma had partial responses lasting 14 and 21 weeks, and eight (42%) of 19 assessable patients had stable disease. Pharmacokinetic interactions were not evident. The activity of these agents in patients with colorectal carcinoma and the feasibility of administering sorafenib with other therapeutics are being studied.

Other Phrarmacologic Inhibitors of Raf Kinase

Besides sorefanib, other small-molecule competitive inhibitors of the ATP-binding site of Raf proteins have been developed. L-779450 (Merck Pharmaceuticals Inc, Nutley, NJ), a competitive inhibitor of the ATP-binding site of C-Raf, has demonstrated activity in the nanomolar range against C-Raf–overexpressing human tumors in vitro and is much more effective at inhibiting the kinase activity of C-Raf and A-Raf than B-Raf.[228,229] Likewise, the phenol substituted oxindole derivative SB203580 (GlaxoSmithKline Pharmaceuticals, Philadelphia, PA) inhibits C-Raf kinase in the low nanomolar range, but micromolar concentrations are required to inhibit ERK phosphorylation in cell culture.[230,231] Interestingly, SB203580 may paradoxically activate C-Raf through an autocrine feedback loop as a result of MEK inhibition.[232] Although C-Raf is a weak kinase, the significance of this observation is not known.

The tumor-inhibitory and cytotoxic effects of naturally occurring ansamycin antibiotics, particularly geldanamycin analogs that bind to Hsp90 and destabilize HSP90-dependent proteins, produce impressive decrements in C-Raf expression.[233] However, the effects of these agents on C-Raf may be nonspecific because Hsp90 plays a critical role in stabilizing and conferring functionality to a wide array of important cellular proteins such as v-Src, EGFR, HER-2/*neu*, cdk4, Akt2, and mutated p53, all of which are inhibited by the abrogation of Hsp90.[233] Thus, the growth inhibitory and pro-apoptotic effects of geldanamycin analogs, such as 17-allylamino-17-demethoxygeldanamycin, may not be a result of direct effects on C-Raf alone, considering the pleiotropic effect of these compounds.[234] Radicicol, a novel macrocyclic antibiotic isolated from the fungus *Monosporium bonorden* and potent inhibitor of Hsp90, has exhibited impressive tumor growth–inhibitory activity against a wide range of human tumor cell lines and xenografts, and both its antitumor and pharmaceutical properties are being optimized.[233] Radicicol may, in part, inhibit tumor growth by destabilizing and depleting C-Raf.[235] The *O*-carbamoylmethyloxime derivatives may be superior to radicicol from both mechanistic and pharmaceutical perspectives.[235]

Dominant Interfering DNA Constructs

Dominant interfering DNA constructs that specifically target tumor cells with anti-*raf* genes have been described. One such method, which involves coupling a cationic lipid-based nanoparticle to an $\alpha v\beta 3$ integrin ligand, seems to deliver genes to newly developing blood vessels of tumors that arise in mice after injection of melanoma cells. Hypothetically, by coupling a cDNA-encoding kinase-inactive C-Raf to the nanoparticle, this dominant negative version of C-Raf is delivered to the neovasculature of developing tumors and induces apoptosis and tumor regression. Paradoxically, the inhibition of C-Raf may induce tumor regression not by affecting MEK/ERK activation, but by inhibiting the MEK kinase–independent role of C-Raf in promoting tumor survival.[236] It is certainly possible that other therapeutics directed against C-Raf act in a similar fashion.

CONCLUSION

It is hopeful that therapeutics designed based on understanding the primary molecular defects governing malignant cell proliferation will be more efficacious and less toxic than nonspecific cytotoxics. It is clear that aberrant forms of Raf are the principal drivers of many types of cancer. Furthermore, the potential therapeutic benefit conferred by therapeutics targeting Raf may not be limited to malignancies with mutant Raf because some cancers with wild-type Raf associated with growth factor receptor hyperactivity and/or Ras mutations are extraordinarily sensitive to Raf inhibition.

The knowledge that some tumors have driving target aberrations coupled with gene-sequencing data has provided the means to establish proof of principle about the validity of targets and/or targeted therapeutics. For Raf, rational patient enrichment strategies based on the presence of specific aberrations of Raf, Ras, and growth factor receptors can be formulated from the outset and dynamically optimized in the course of development. This is not to say that therapeutics targeting Raf will ultimately be restricted to niche indications because molecular aberrations are shared by many cancers. Instead, after proof of principle in cancers that are solely driven by a target aberration, such as melanoma and pancreatic carcinoma in the case of Raf and Ras, respectively, studies could then be conducted in tumors in which the target contributes to, but may not be the sole driver of, tumor growth. Although tumor regression rates may be negligible in these settings, the principal therapeutic effects of target inhibition in tumors with multiple contributory molecular aberrations may be best appreciated in randomized trials designed to detect differences in overall survival, PFS, and other end points that reflect tumor growth inhibition.

Although the early results with sorafenib have been encouraging, emerging clinical data do not irrefutably validate Raf as its relevant target. Instead, the intriguing activity of sorafenib in RCC, its lack of robust single-agent activity in melanoma, and its ability to enhance the activity of chemotherapeutics may reflect its greater potency at inhibiting VEGFR or other, as of yet unidentified, RTKs. To this end, the lack of robust activity with ASONs targeting Raf should not negate the importance of Raf as a therapeutic target because mutations of *Raf* and *Ras* were not assessed in studies of these agents and there are many unanswered questions about the validity of antisense strategies as therapeutic platforms. Although knowing the precise mechanism of sorafenib's antitumor activity may make little difference with regard to its ultimate utility, such information may be used to optimize the therapeutic indices of the next generation of therapeutics targeting Raf. In concert with clinical evaluations, *Ras/Raf* gene sequencing studies and assessments of relevant biologic markers may facilitate these efforts. Most importantly, the role of Raf in driving tumor proliferation must be further understood. Issues pertaining to the structural and functional basis of Raf and Ras mutations and their interrelationships and roles in tumorigenesis, proliferation, and cell survival must be addressed to develop more effective therapeutics against Raf and related targets.

Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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