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Review Article

Kinase components of the Ras-MAPK signaling cascade as potential targets for therapeutic intervention

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Abstract

The Ras-MAPK signaling cascade plays a role of central importance in cellular growth, proliferation and survival. Activation of this pathway by stimuli from hormones and growth factor receptors at the cell surface results in the transmission of signals to various transcription factors within the nucleus that mediate these processes. The downstream effector of Ras is Raf, a serine/threonine kinase. Upon activation by Ras, Raf phosphorylates the serine residues of MEK1 and MEK2, which in turn phosphorylate the MAPK family proteins ERK1 and ERK2. Activated ERK1 and ERK2 translocate to the nucleus, stimulating growth and proliferative processes by activation of various substrates. Aberrant signaling within this pathway has been associated with the formation of human tumors, making the kinase components of the Ras-MAPK signaling cascade attractive targets for pharmaceutical intervention. This review describes a variety of agents that have been identified as inhibitors of the kinase components of this signaling pathway – natural products, synthetic compounds, as well as antisense oligonucleotides. Preclinical studies have demonstrated that certain inhibitors are well tolerated in mammals, and can effectively inhibit the growth of selected human tumor cell lines. Several Raf and MEK kinase inhibitors have been advanced to clinical trials, where preliminary evidence of efficacy has been noted. Studies have been initiated to evaluate these agents in combination with cytotoxic drugs. The work described in this review suggests that this area of research has been fruitful, providing several potential anticancer agents currently under investigation.

Introduction

Protein kinases play a vital role as key regulators of a variety of critical cell functions. These enzymes function by catalyzing the transfer of a phosphate group from ATP to amino acid residues of substrate proteins. Sequencing of the human genome has provided evidence of the presence of more than 500 kinases. Aberrations of kinase function via mutation, hyperactivity or dysregulation are associated with the onset of certain diseases. In particular, tumorigenesis has been linked to the aberrant function of protein kinases that play key roles in cell proliferation, migration and invasion. Thus, kinases have become of particular interest as potential targets for anticancer agents.

One ubiquitous signaling pathway found in all eukaryotic organisms is the Ras-MAPK (mitogen-activated protein kinase) module, which is comprised of the Ras/Raf/MEK (mitogen-activated protein/extracellular signal-related protein kinase [MAP/ERK] kinase)/MAPK signaling cascade. This key pathway is involved in transmitting signals from growth factors and hormones from the extracellular compartment to the cytosol, and to transcription factors in the nucleus (1-3). Alteration of the Ras-MAPK pathway is associated with the formation of certain human tumors. This review will provide a brief description of the Ras-MAPK pathway components and the transformations leading to oncogenesis, followed by a detailed discussion of agents that target the kinases within this signaling cascade.

Components of the Ras-MAPK signaling cascade

Ras

The Ras/Raf/MEK/MAPK signaling cascade is activated by GTP loading of Ras, which occurs in response to stimuli from cell-surface receptors (1-3). Ras is a member of a large family of small (21 kDa) GTPases that act as molecular switches in the regulation of cell growth, differentiation, survival and apoptosis (1-4). Ras was initially defined as a component of oncogenic murine retroviruses (5), and it became the focus of intensive research in the early 1980s when the connection between mutant forms of Ras and human cancer was established (5-7). Ras mutations that lead to oncogenic activation occur primarily in two hotspots: Gly12 and Gln61 (8). The mutational changes at these residues found in human cancers impede the GTP hydrolysis activity of Ras, thus causing Ras to remain in the GTP-bound or "on" conformation (1, 2, 4, 8). With this finding, a better appreciation of the molecular basis of aberrant signaling associated with approximately 30% of all human cancers was obtained (1, 2).

There are three Ras isoforms associated with human cancer: H-Ras, N-Ras and K-Ras. Of these cancers, 95% are associated with K-Ras mutations. Ras proteins are closely related, having 85% amino acid identity with a 20-amino-acid variable region at the carboxy terminus (9). A cysteine residue occurs in all Ras proteins after the variable region, and this is the site of post-translational modification of Ras by addition of a farnesyl isoprenoid lipid (10). All Ras proteins are further modified by proteolysis of the three carboxy-terminal amino acids and subsequent methylation of the new carboxy terminus (1, 2, 10). These modifications stabilize Ras interaction with the inner cell membrane, where it must reside to form a multiprotein complex with Raf, MEK, MAPK and scaffold proteins to activate signaling in the Ras-MAPK module (11, 12).

As the consequences of mutated Ras function were elucidated, this attracted sufficient attention from numerous pharmaceutical laboratories to initiate discovery and development programs for small-molecule inhibitors of this signaling protein. The goal of these programs was to bring forward inhibitors of aberrant Ras signaling with minimal associated toxicities. Numerous Ras inhibitors have been described, and they impair Ras-MAPK signaling by blocking proper post-translational modification of Ras by protein farnesyltransferase, thereby preventing membane localization (1, 2, 4, 12). These Ras inhibitors have shown good preclinical efficacy, and are currently being evaluated in clinical trials (1, 2, 4, 12). The sequential downstream kinase effectors of Ras, namely Raf and MEK, are also viewed as equally attractive targets for the pharmacological intervention of cancer.

Raf

Raf proteins are fairly homologous (~60%). These 66-84-kDa serine/threonine kinases include A, B and C isoforms (C-Raf = Raf1, c-Raf), which coexist in many cell types and activate MEK (13-15). Three conserved regions occur in Raf proteins: CR1, which is at the *N*-terminus and contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD); CR2, which contains a serine/threonine-rich region; and CR3, which contains the catalytic kinase domain (13-15).

GTP-loaded Ras recruits Raf to the inner cell membrane. This is crucial for Raf activation, although activation is a complex process not yet fully understood. For example, there are at least 13 regulatory phosphorylation sites on C-Raf (16-24). Not only GTP-loaded Ras, but also various kinases (*e.g.*, C-TAK1 [transforming growth factor-β-activated kinase 1], PAK [p21-activated kinase], PKC [protein kinase C], PKA [protein kinase A], Src), phosphatases (PP1, PP2A), adapter proteins (14-3-3) and scaffold proteins (KSR, or kinase suppressor of Ras) are implicated in full Raf activation (16-27). The three Raf isoforms differ in their ability to interact with Ras isoforms, to activate MEK and to transform rodent fibroblasts *in vitro* (28). The B-Raf isoform in all cases is the most active, followed by C-Raf and then A-Raf (28).

A-, B- and C-Raf knockout mice have been described (29-31). B-Raf-deficient embryos die at mid-gestation due to apoptotic cell death in endothelial cells leading to vascular hemorrhage (30). C-Raf deficiency causes mid-gestational death due to more diffuse apoptotic tissue effects (31). A-Raf-deficient mice are born alive, but show neurological and intestinal defects (29). These divergent phenotypes show that Raf isoforms serve distinct functions in different tissues. These studies have shown that individual B-Raf and C-Raf survival functions cannot be performed by other Raf isoforms (28, 32). They also demonstrated that normal levels of MAPK activation occur in C-Raf-deficient mouse cells, indicating that the antiapoptotic function of C-Raf is not mediated by the MAPK cascade (28, 31).

The antiapoptotic function of C-Raf may be mediated by antagonism of apoptosis-stimulated kinase-1 (ASK-1). There is evidence to suggest that C-Raf impedes ASK-1 function via a protein–protein interaction that is not associated with C-Raf kinase activity (32). Raf can also impede apoptosis in a kinase-dependent manner. For example, Raf/MEK/MAPK signaling activates Rsk1 (ribosomal S6 kinase 1), which in turn phosphorylates and inactivates BAD, a proapoptotic protein (33). Additionally, C-Raf can be localized to the mitochondria by a Bcl-2-mediated process, where it can inactivate proapoptotic proteins by phosphorylation (34). Raf antiapoptotic effects are complex and will require further study to clarify. However, this characteristic of Raf enhances its appeal as a pharmaceutical target since a hallmark of cancer cells is resistance to apoptosis, which, at least in part, is likely attributable to improper Raf activation (35).

As with Ras, oncogenic forms of Raf have been found to be components of transforming murine retroviruses (36). Oncogenic Raf in murine retroviruses results from *N*-terminal deletions that remove the regulatory sequences that control Raf kinase activity (36). Most

recently, a systematic human genome-wide screening effort to detect alterations in genes that control cell proliferation, differentiation and death found activating B-Raf mutations in 66% of malignant melanomas (37). Additionally, B-Raf mutations were observed at lower frequencies in a wide range of other cancers, including colorectal, lung, breast and ovarian cancers (37).

The significance of B-Raf mutations in colorectal tumors was extended in a subsequent study showing that mutations in *either* B-Raf *or* K-Ras (not both) were detected in a sample of colorectal tumors examined at all stages of development, including premalignant lesions (38). The frequency of B-Raf mutations in these colorectal tumors was 10%, whereas the K-Ras mutation frequency was 51%. The cumulative K-Ras/B-Raf mutation frequency in colorectal cancer is therefore 61% (38). In the case of melanoma, an examination of N-Ras mutation (the Ras isoform mutated in melanoma) together with B-Raf has shown a cumulative mutation frequency of 81% (39). These statistics, when combined with the 2002 incidence of colorectal cancer (148,000) and melanoma (54,000) in the U.S. (American Cancer Society), make Raf a compelling pharmaceutical target.

MEK

MEK1 and MEK2 are ubiquitously expressed 43-46-kDa kinases activated by Raf phosphorylation of two serine residues (*e.g.*, Ser217–Ser221 of MEK1). MEK1 and MEK2 are members of a larger family of dual-specificity kinases (MEK1-7) that phosphorylate threonine and tyrosine residues within the TXY motif of various MAP kinases (40). MEK1 and MEK2 are encoded by distinct genes, but they have high homology (80%) within the *C*-terminal catalytic kinase domain and most of the *N*-terminal regulatory domain (40, 41). At the *N*-terminus of MEK1 and MEK2 there are 30 amino acids of divergent sequence that may direct differential interactions with both activators and substrates (40, 41). The only known substrates for MEK1/MEK2 are MAPK1 and MAPK2, which they phosphorylate on Thr202/183 and Tyr204/185, respectively.

MEK1-deficient mice have been described, and inactivation of MEK1 leads to embryonic lethality due to decreased placental vascularization during embryogenesis (42). MEK1 deficiency is not compensated for by MEK2 (42). In contrast, MEK2-deficient mice are viable and fertile, with no morphological alterations (43). These data demonstrate that MEK2 is not necessary for the normal development of mouse embryos, indicating that the loss of MEK2 can be compensated for by MEK1.

Oncogenic forms of MEK1 or MEK2 have not been described in retroviruses or human cancers. However, a MEK1 where Ser218 and Ser222 are both mutated to Asp is capable of causing oncogenic transformation of various rodent fibroblast cell lines (44).

MAPK

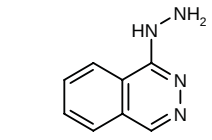
The MAPK components of the Ras-MAPK module are also known as ERK1 and ERK2. These MAPK isoforms (also designated p44 MAPK and p42 MAPK) are highly homologous (> 80%), ubiquitously expressed 44-42-kDa serine/threonine kinases that are members of a larger gene family that includes ERK1, 2, 3, 5 and 7, JNK1-3 and p38α, β, γ and δ. Experimental data indicate that ERK1 and ERK2 are functionally equivalent (11, 42, 45). ERKs are activated by MEK phosphorylation of their TEY sequence; dual phosphorylation is required for activation, and in the case of ERK2 results in a > 1,000-fold increase in activity (41). Downstream substrates of ERK1/2 include cytoskeletal proteins, kinases, phosphatases and transcription factors (11, 41). The pleiotropic effects of MAPK activation on cell growth and differentiation are undoubtedly mediated through this diverse array of effectors (11, 41).

No constitutively active MAP kinases are known, despite attempts at their genetic selection and site-directed mutagenesis. This failure suggests that cells cannot tolerate the continuous activation of MAP kinases. Among the kinase components of the Ras-MAPK signaling pathway, only the ERK2 atomic structure has been solved (46).

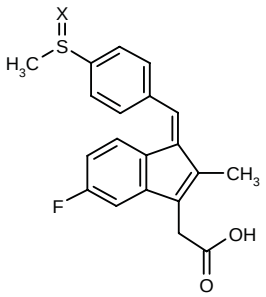
The ERK1/ERK2 components of the Ras-MAPK module are the most abundant (approx. 10^6 molecules per cell). MEK is also relatively abundant in most cell types (approx. 3.5 x 10^5 molecules per cell), whereas Raf and Ras molecules are less abundant (approx. 2 x 10^4 per cell) (47). All MAPK molecules can become fully activated in cells where only 10-50% of Ras molecules are GTP-bound (47). The predicted sensitivity of the Raf/MEK1/MAPK signaling cascade to inhibitors is: Raf > MEK1 > MAPK (48). This sensitivity profile results from the distributive (nonprocessive) mechanism of both Raf and MEK1, in which the rate of MEK1 activation depends on the concentration of Raf squared, and similarly, the rate of MAPK activation is dependent on the concentration of MEK1 squared (48). To date, potent inhibitors of Raf and MEK, but not ERK, have been reported (35).

Targeting the kinases of the Ras-MAPK signaling pathway

Kinase inhibitors of the Ras-MAPK signaling pathway are of keen interest because they have the potential to reverse MAPK activation that originates not only from mutant Ras and mutant B-Raf, but also MAPK activation that stems from deregulated growth factor receptors upstream of the Ras-MAPK pathway. Examples of such deregulated growth factor receptors include the protein tyrosine kinase receptors EGFR (epidermal growth factor receptor) and HER-2/neu. EGFR and HER-2/neu are, by mutation or overexpression, associated with mammary, ovarian and non-small cell lung cancers (49).



[**1**], Hydralazine



[**2**], X = O, Sulindac
[**3**], X = O_2, Sulindac sulfone
[**4**], X = lone pair, Sulindac sulfide

While the focus of this review will be on compounds specifically designed to inhibit the kinase components of the Ras-MAPK signaling pathway, it should be noted that certain synthetic compounds designed for different therapeutic uses have demonstrated an effect on this pathway. For example, the antihypertensive agent hydralazine [**1**] inhibited PMA (phorbol myristate acetate)-stimulated ERK phosphorylation in Jurkat cells and PHA (phytohemagglutinin)-stimulated T-cells at 5 μM (50). While no activity was observed against the JNK (c-Jun *N*-terminal kinase) and p38 signaling pathways, the specific target of inhibition by this agent was not established. It was proposed that hydralazine inhibited Ras, Raf or MEK, thereby decreasing DNA methyltransferase expression and inducing T-cell autoreactivity.

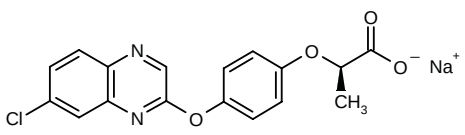
It has been shown that nonsteroidal antiinflammatory drugs (NSAIDs) such as sulindac [**2**] can delay or inhibit tumor growth (51, 52). Sulindac itself does not inhibit tumor growth, but two major metabolites are formed which have inhibitory effects on inflammation and cell proliferation. Both of these metabolites, sulindac sulfone [**3**] and sulindac sulfide [**4**], induce cells to undergo apoptosis (53), though by different mechanisms (for example, sulindac sulfide is a far more potent cyclooxygenase [COX] inhibitor than the sulfone). Sulindac sulfide has been shown to inhibit the interaction between Ras protein and Raf kinase at 10-50 μM (54).

In addition, certain experimental anticancer agents appear to exert their effects at least in part by activity against targets within the Ras-MAPK signaling pathway. A compound that has recently entered phase I clinical trials as an antitumor agent, XK-469 [**5**], is under extensive investigation to determine its mechanism of action (55-57). While multiple pathways have been implicated, one study demonstrated that this agent inhibited the activation of MEK in U-937 cells (58). A cytotoxic and antimetastatic ruthenium complex known as NAMI-A [**6**] has been shown to inhibit PMA-stimulated ERK1/2 phosphorylation in ECV304 cells (a transformed immortal endothelial cell line) at 100 μM (59). Further cellular studies with this agent indicated that it induced an apoptotic response at least in part by the inhibition of MEK/ERK signaling (60).

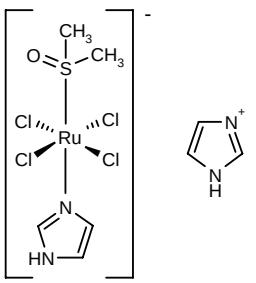
The two kinases of the Ras-MAPK pathway for which potent inhibitors have been described are Raf and MEK kinase. These inhibitors are comprised of natural products, synthetic inhibitors and antisense oligonucleotides.

Natural product inhibitors of Raf

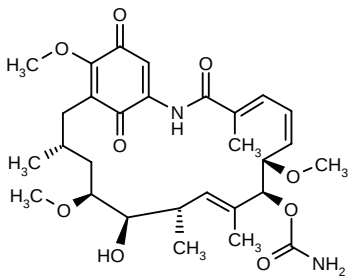
While a number of natural products exert an effect on Raf-mediated signaling, the majority of these exert their effect not by binding to Raf, but by binding to proteins that associate with Raf. Certain natural products lower the cellular concentrations of Raf by destabilizing its interaction with heat shock protein 90 (HSP90). These include geldanamycin [**7**] (61), radicicol [**8**] and its oxime derivatives



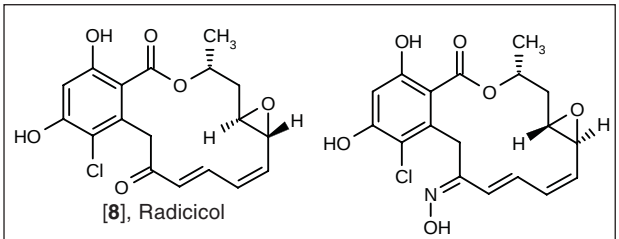
[**5**], XK-469



[**6**], NAMI-A

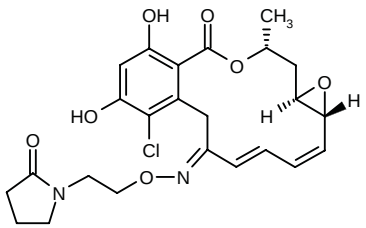


[**7**], Geldanamycin

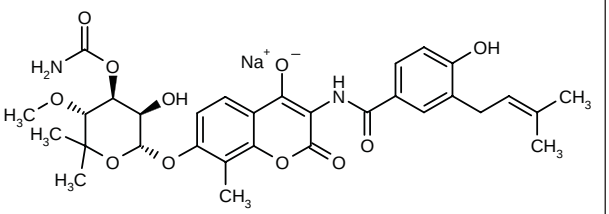


[**8**], Radicicol

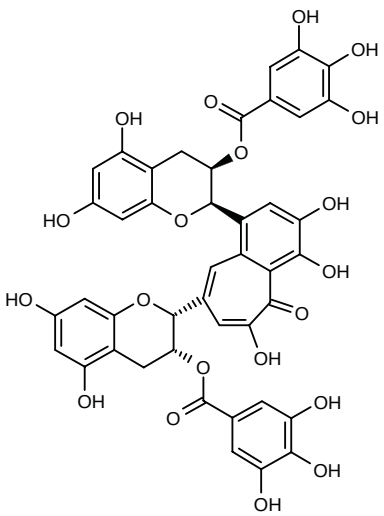
[**9**], KF-25706



[**10**], KF-58333



[**11**], Novobiocin



[**12**], Theaflavin-3,3'-digallate

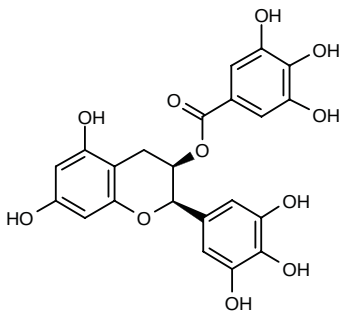
KF-25706 [**9**] and KF-58333 [**10**] (62, 63), and the DNA gyrase inhibitor novobiocin [**11**] (64). Although certain natural product derivatives might be of interest due to their antitumor activity *in vivo* (65), they are in fact non-specific inhibitors since HSP90 binds to a number of proteins and receptors (62).

A constituent of black tea, theaflavin-3,3'-digallate (TfdiG) [**12**], has been shown to rapidly degrade Raf-1 (< 15% remaining at 120 min) at 20 μM in 30.7b Ras 12 cells (66). While the precise mechanism of action was not delineated, it was speculated that TfdiG possibly altered the plasma membrane in such a way as to lead to vacuole formation and entrapment of Raf-1. In the same study (66), it was shown that the green tea substituent (–)-epigallocatechin-3-gallate (EGCG) [**13**] decreased the association between Raf-1 and MEK1 at 20 μM, possibly by binding to a proline-rich sequence on MEK1.

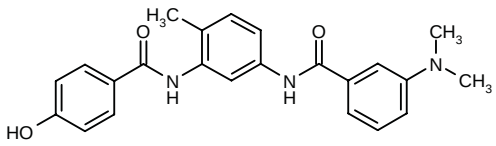
With the possible exception of EGCG, none of the natural products described in this section directly binds to Raf kinase, thereby inhibiting the phosphorylation of MEK1. In contrast, several synthetic inhibitors have been identified as Raf kinase inhibitors.

Synthetic inhibitors of Raf

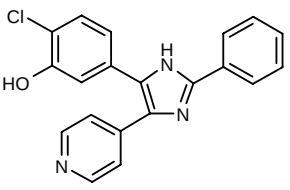
Researchers at AstraZeneca (67) identified ZM-336372 [**14**] as a potent inhibitor of C-Raf (IC_{50} = 70 nM). Variation of the ATP concentration caused a change in the IC_{50}, indicating that this compound was ATP-competitive. ZM-336372 displayed good selectivity for C-Raf, being 10-fold more active against C-Raf than B-Raf. In addition, ZM-336372 did not inhibit 17 of 19 other kinases at 50 μM. The two kinases against which this agent had some activity were the stress-activated protein kinases SAPK2a/p38α and SAPK2b/p38β2 (IC_{50} = 2 μM). Surprisingly, this compound failed to inhibit activation of the downstream kinases MEK1 and ERK2 (p42 MAPK) in Swiss 3T3 cells. Further experiments revealed that ZM-336372 induced the activation of C-Raf in Swiss 3T3 cells, causing an increase of several hundred-fold in C-Raf activity. Similar activation of C-Raf was observed in other cell lines, including monkey COS-1 cells and human 293 cells. C-Raf activation by ZM-336372 did not induce MEK1 and MAPK activation in Swiss 3T3 cells, but removal of this inhibitor did trigger the activation of these



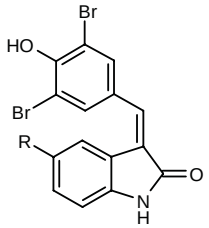
[**13**], (–)-Epigallocatechin-3-gallate



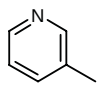
[**14**], ZM-336372



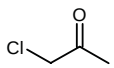
[**15**], L-779450



[**17**], R =



[**18**], R =



[**16**], GW-5074, R=I

kinases. Thus, ZM-336372 appears to paradoxically cause Raf-1 hyperactivation and counterbalance its effect as an inhibitor.

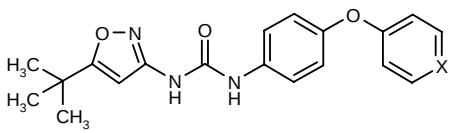
A research group at Merck & Co. reported L-779450 [**15**] to be a potent and selective, synthetic, small-molecule inhibitor of C-Raf kinase (68). This ATP-competitive compound displayed an IC_{50} of 1.4 nM against activated recombinant C-Raf. When tested against a panel of 11 additional kinases, L-779450 was highly selective (> 200-fold), with the exception of p38 MAP kinase. The potent inhibition of p38 (IC_{50} = 47 nM) was not unexpected, given the structural similarity of L-779450 to SB-203580, a potent p38 inhibitor (69). It was demonstrated that the inhibition of C-Raf by L-779450 in human tumor cells led to inhibition of the MAP kinase cascade, blocking the downstream *fos* promoter element (IC_{50} = 0.7 μM). L-779450 inhibited the growth of Ras-transformed rodent fibroblasts and the human tumor cell lines A549, PSN-1 and HCT 116 at concentrations ranging from 0.3 to 3.0 μM. Due to the low cellular potency and poor pharmacokinetics of this compound, *in vivo* efficacy was not observed.

A benzylidene oxindole, GW-5074 [**16**], has been described to be a potent C-Raf kinase inhibitor by a group at GlaxoSmithKline (70, 71). Solution-phase parallel synthetic methods, as well as discrete compound synthesis, were utilized to provide over 2,000 benzylidene oxindole analogues. The SAR of this series were thoroughly explored, with several trends being observed. The optimal benzylidene ring was a 4-phenol with two flanking substituents that enhanced its acidity. In fact, a strong correlation was shown for phenol acidity and potency of C-Raf inhibition. Of the possible positions that could be substituted on the oxindole ring, the C-5 position proved ideal for the attachment of a variety of substituents that enhanced potency and/or its drug-like properties. Planar rings fused to the C-4 and C-5 positions also significantly enhanced potency. It was observed that the double bonds of these compounds typically existed as a mixture of *E*- and *Z*-isomers (as observed by NMR). In water, compounds with a pKa < 7 rapidly equilibrated to a 1:1 mixture of isomers.

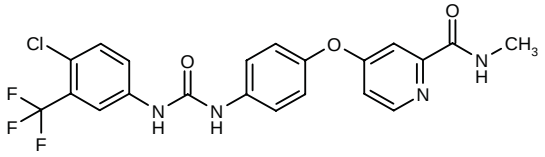
GW-5074 was shown to be highly selective for C-Raf kinase (71), with ≥ 100-fold selectivity for Raf kinase versus a panel of other kinases, including CDK1 (cyclin-dependent kinase 1), CDK2, c-Src, ERK2, MEK, p38 MAPK, Tie2, VEGFR2 (vascular endothelial growth factor receptor 2) and c-Fms. A cell-based assay demonstrated that GW-5074 and several analogues inhibited EGF-stimulated activation of MAP kinase. GW-5074 at 5 μM caused 90% inhibition in this assay. A serum-free survival assay was utilized to determine the cellular activities of GW-5074 and selected analogues against RasA1 (a K-Ras-transformed rat fibroblast line), DLD-1, HCT 116 and A549 (70). With IC_{50} values of 0.58-2.56 μM and adequate pharmacokinetic profiles when dosed subcutaneously (s.c.) or intravenously (i.v.), respectively, compounds **17** and **18** were further evaluated *in vivo*. Tumor growth inhibition of Ras/p53 A1 and SW620 was observed on treatment with **17** and **18,** respectively.

Researchers at Bayer and Onyx Pharmaceuticals have collaborated to discover heterocyclic ureas and substituted diphenyl ureas as potent C-Raf kinase inhibitors. Compound **19** was described as a second-generation lead (IC_{50} = 540 nM) following extensive modification of a commercially available 3-thienyl urea (72, 73). Further modification led to the somewhat more potent **20** (IC_{50} = 230 nM), which was the first compound to demonstrate *in vivo* activity on oral (p.o.) dosing (74).

The importance of the urea NH groups for optimal kinase-inhibitory activity was established by showing that *N*-methylation, or the formation of a cyclic urea derivative, caused a dramatic loss in activity (73). Substitution of NH with a methylene group provided a similar loss of activity. Further improvements in activity were discovered by exploration of substituted diphenylureas (73, 75). Following exhaustive substitution on both phenyl residues, as well as on the distal pyridyloxy moiety, Bay-43-9006 [**21**] was discovered as a potent lead candidate (IC_{50} = 12 nM). Bay-43-9006 exhibited selectivity for



[**19**], X = CH
[**20**], X = N



[**21**], Bay-43-9006

C-Raf kinase, showing essentially no activity against MEK1, ERK1, HIR (human insulin receptor) and EGFR.

As would be expected for a Raf kinase inhibitor, this compound inhibited phosphorylation of downstream kinases in cells. Thus, Bay-43-9006 inhibited ERK phosphorylation in HCT 116 and NIH/3T3 cells as determined by Western blot analysis, and additionally inhibited MEK1 activation in B-Raf: ER 3T3 cells (76). Anchorage-dependent and -independent proliferation of HCT 116 cells was inhibited by Bay-43-9006 with IC_{50} values of 4.8 μM and 5.1 μM, respectively. Anchorage-independent cell growth of MIA PaCa-2 cells was inhibited with an IC_{50} of 7.8 μM. *In vivo* antitumor activity was demonstrated against several human tumors, including HCT 116, MIA PaCa-2, NCI-H460 and SK-OV-3, implanted subcutaneously in nude mice (77, 78). Dose-dependent tumor inhibition was observed at 10, 30 and 100 mg/kg/day p.o. in all of these models, with the best response being observed against SK-OV-3. Additionally, Bay-43-9006 was active against advanced-stage HCT 116 tumors.

As a result of these and other studies (79), Bay-43-9006 advanced to clinic trials, and is currently in phase II evaluation. Four phase I clinical trials were initiated with Bay-43-9006, with doses ranging from 50 mg once a week to 800 mg twice daily in patients with a variety of refractory solid tumors (80, 81). Preliminary evidence of efficacy (81) was noted, with 38 of 115 patients who received at least 200 mg/kg twice daily having disease stabilization for at least 12 weeks. A total of 28 patients remained on the study for more than 6 months, and 9 for more than a year. Partial responses and tumor shrinkage were obtained in 6 patients. Dose-limiting diarrhea was observed for patients receiving the highest doses of compound. Other side effects reported at higher doses include skin toxicity, nausea, hypertension, fatigue and hand-and-foot syndrome. With respect to the pharmacokinetic studies, while interpatient variability was high, the C_{max} and AUC increased at higher doses. Inhibition of ERK phosphorylation in peripheral blood monocytes of patients treated with Bay-43-9006 at 400 mg/kg twice daily has been demonstrated (82). Phase I trials have been initiated with Bay-43-9006 in combination with the cytotoxic agents carboplatin and paclitaxel (83), and gemcitabine (84).

Antisense oligonucleotides targeting Raf

ISIS-5132/CGP-69846A is an antisense agent targeting C-Raf, which is under joint development by Isis Pharmaceuticals and Novartis (85). This 20-mer phosphorothioate oligodeoxynucleotide (PS-ODN) was found to be the most potent of a series of PS-ODNs targeting the 3'-untranslated region of C-Raf mRNA. ISIS-5132/CGP-69846A was shown to significantly inhibit C-Raf mRNA levels at a concentration of 200 nM in the human tumor cell lines A549, SW480 and T24. When tested for its antiproliferative effects against A549 cells, this agent had an IC_{50} of 50-100 nM. Inhibition of A549 cell growth occurred in a concentration-dependent manner consistent with the levels of this PS-ODN required to inhibit C-Raf mRNA. ISIS-5132/CGP-69846A demonstrated *in vivo* activity against A549 xenografts at doses as low as 6 μg/kg/day i.v. Similar antitumor effects were seen against T24 and MDA-MB-231 xenografts at daily i.v. doses of ISIS-5132/CGP-69846A of 0.06-6 mg/kg.

Additional studies have shown ISIS-5132/CGP-69846A to be active against several ovarian cancer cell lines (86). Cellular proliferation studies carried out with ISIS-5132/CGP-69846A at 200 nM showed it to inhibit proliferation in 9 of 12 ovarian cell lines by ≥ 60%, in contrast to the < 30% inhibition by random ODN against selected cell lines. In SK-OV-3 cells, ISIS-5132/CGP-69846A was found to be specific for C-Raf versus A-Raf and ERK. In contrast, random control oligomers had no effect on C-Raf levels. The lead agent and ISIS-13650 (a second-generation antisense ODN) were both highly effective as antiproliferative agents against SK-OV-3 cells at 200 nM, inhibiting cell growth by > 80%. DNA analysis showed that ISIS-5132/CGP-69846A arrested cells in the S and G_2/M phases of the cell cycle, and enhanced apoptosis in a concentration-dependent manner. In a xenograft model, ISIS-5132/CGP-69846A significantly inhibited the growth of SK-OV-3 tumors at 10 and 25 mg/kg/day i.p.

ISIS-5132/CGP-69846A has been evaluated in several phase I clinical trials, administered 3 times per week at doses ranging from 0.5 mg/kg to 6 mg/kg by 2-h infusion (87, 88), by continuous infusion for 21 days at 0.5-5 mg/kg (89), or by 24-h infusion weekly up to 30 mg/kg/week (90). With the exception of the 24-h infusion study, the compound was well tolerated, with no severe toxicities observed. Anemia, fever and fatigue were the major side effects. In the study arm wherein patients received multiple 2-h infusions, significant reductions in C-Raf mRNA levels were observed by the third day in peripheral blood mononuclear cells (PBMCs) in 13 of 14 patients (87). Two patients experienced stable disease lasting for more than 7 months. Frequent short infusions of drug appear to be more effective than weekly 24-h bolus doses, presumably because of the short half-life of this agent (12). Clinical trials have more recently progressed to phase II to determine activity against metastatic colorectal cancer (91), hormone-refractory prostate cancer (92) and non-small cell lung cancer (NSCLC) (93). No objective responses have been observed in the single-agent studies, although some cases of transient stable disease were noted (91). A combination study has been initiated evaluating ISIS-5132/CGP-69846A with 5-FU and leucovorin (94), with disease stabilization being observed in 3 of 12 patients.

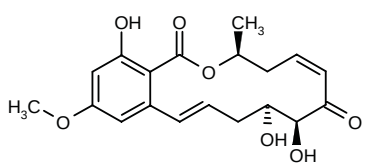
Researchers at NeoPharm have described a liposome-entrapped Raf antisense oligodeoxyribonucleotide (LErafAON) as a potential antitumor agent (95). The 15-mer antisense oligonucleotide (rafAON) was synthesized to target the translation initiation region of C-Raf mRNA, with phosphorothiate modifications limited to the terminal base at the 5'- and 3'-ends. A novel cationic liposome delivery system was designed to protect the highly

unstable (in plasma) rafAON, allowing it to become efficiently distributed to the tissues. In PC-3 tumor-bearing athymic BALB/c mice, intact rafAON was found in the plasma, liver, spleen, kidneys, heart, lungs and tumor tissue for up to 48 h following a dose of 30 mg/kg i.v. of LErafAON. Antitumor activity of LErafAON was correlated with a decrease in C-Raf levels in the tumor tissue. Multiple doses over 14 days (25 mg/kg i.v.) in athymic mice bearing human tumor PC-3 xenografts was sufficient to arrest tumor growth, while tumor regression was observed when this treatment was combined with irradiation (3.8 Gy/day). Significant chemosensitization has been observed when LErafAON was dosed i.v. in combination with several cytotoxic agents, with potent antitumor activity being observed against diverse tumor lines, such as human lung (A549), breast (MDA-MB-231), prostate (PC-3) and pancreatic (AsPC-1) tumors implanted in athymic mice (96-98).

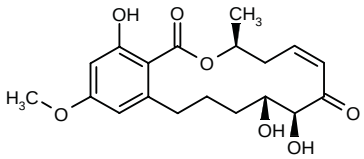
On the basis of these *in vivo* studies, as well as toxicology data demonstrating that multiple doses of this agent were well tolerated in mice, rabbits and monkeys (95), LErafAON was advanced to clinical trials. In a phase I study with LErafAON (99), 12 patients were enrolled to receive 1-6 mg/kg/week dosed intravenously. Acute infusion-related reactions were recorded in 10 patients (3 discontinued the treatment), while 2 of 6 patients receiving 4 mg/kg/week continued with stable disease for 16 or 24 weeks. Additional studies are under way with LErafAON delivered by intermittent bolus dosing (100) and in combination with radiotherapy (101).

Natural product inhibitors of MEK

By high-throughput screening of microbial broths, Roche researchers identified Ro-09-2210 [**22**] as a potent inhibitor of peripheral blood T-cell activation (102). This agent was also an effective inhibitor of IL-2 secretion, with IC_{50} values of 30-50 nM. Ro-09-2210 was shown to be a potent inhibitor of MEK1 kinase (IC_{50} = 59 nM), with some activity (4-10-fold higher IC_{50} values) against the other dual-specificity kinases MEK4, 6 and 7. No activity was observed at less than micromolar concentrations against a number of other purified kinases, including PKC, PKA, Phk (phosphorylase kinase), ZAP-70 (70-kDa zeta-associated protein), ERK, JNK, p38 MAPK, MAPKAP kinase 2



[**22**], Ro-09-2210



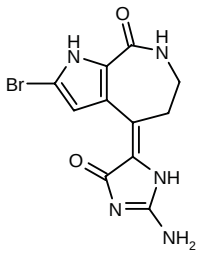
[**23**], L-783277

(mitogen-activated protein kinase-activated protein kinase 2) and p56 Lck.

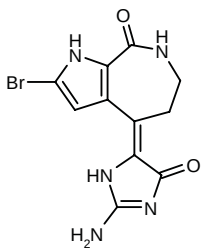
A resorcylic acid lactone, L-783277 [**23**], was discovered by Merck & Co. scientists to be a potent inhibitor of MEK kinase, with an IC_{50} of 4 nM (103). This compound was isolated from the organic extracts of a *Phoma* sp. (ATCC 74403), which came from the *Helvella acetabulum* fruitbody. When tested against a selected panel of additional kinases, L-783277 showed weak activity against Lck (IC_{50} = 750 nM), but no activity against Raf, PKC and PKA. In experiments to determine the mode of MEK inhibition, it was shown that the IC_{50} values became progressively higher as the ATP concentrations were increased, thereby indicating that the binding of L-783277 was competitive with ATP. Preincubation with MEK for 60 min lowered the IC_{50} from 4 to 0.4 nM. This was explained by adduct formation between MEK and L-783277, possibly by the reaction of the active site of MEK with the reactive unsaturated ketone of L-783277. Reduction of the unsaturated ketone moiety with $NaBH_4$ provided an isomeric mixture of alcohols with greatly diminished activity. It was demonstrated that enzyme preincubated with L-783277 abolished MEK activity, confirming that L-783277 was acting as an irreversible inhibitor. Similar irreversible inhibition was not observed with Lck kinase.

In cellular assays, L-783277 potently inhibited the growth of several human epithelial tumor lines in soft agar (IC_{50} = 100-200 nM), and reportedly inhibited MAP kinase phosphorylation in PSN-1 tumor cells with submicromolar IC_{50} values (103). L-783277 reportedly reduced tumor growth in nude mouse xenografts at 100 mg/kg.

In a collaborative effort among several academic groups and Wyeth researchers, certain aldisine alkaloids extracted from the Philippine sponge *Stylissa massa* were discovered to be potent inhibitors of MEK1 (104). Using a high-throughput Raf/MEK/MAPK cascade enzyme-linked immunosorbent assay (ELISA), 10*E*-hymenialdisine [**24**] and 10*Z*-hymenialdisine [**25**] were identified as potent inhibitors of MEK1 with IC_{50} values of 3 and 6 nM, respectively. Since 10*E*-hymenialdisine readily converts to 10*Z*-hymenialdisine in dimethyl sulfoxide (DMSO), a cosolvent used in the study, it is likely that little or none of the *E*-isomer was present when the IC_{50} was measured. While these compounds were inactive in a Raf to MEK1 assay, similar IC_{50} values were obtained in the MEK1 to MAPK assays, confirming these compounds to be MEK1 inhibitors. Both compounds inhibited K-Ras-mutant human colon tumor LoVo cell growth *in vitro* (IC_{50} = 586 and 710 nM, respectively, for the *Z*- and *E*-isomer),



[**24**], 10E-hymenialdisine



[**25**], 10Z-hymenialdeisine

while showing little activity against wild-type K-Ras Caco-2 cells (IC_{50} = 3867 and 7799 nM, respectively, for the *Z*- and *E*-isomer). Again, since the compounds were initially dissolved in DMSO for the cellular growth inhibition studies, the *E*-isomer data are likely derived from an isomeric mixture of hymenialdisines.

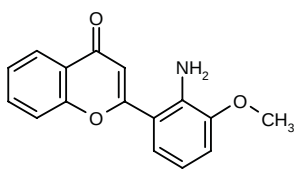
Synthetic inhibitors of MEK

Screening of a compound library at the former Parke-Davis (now Pfizer) led to the discovery of PD-098059 [**26**] as an inhibitor of MEK (105). PD-098059 inhibited the activity of basal MEK and a partially activated mutant MEK–glutathione *S*-transferase (GST) fusion construct (IC_{50}~ 10 μM). The ability of MEK to phosphorylate both the tyrosine and threonine residues of MAPK was inhibited. Kinetic analysis indicated that PD-098059 did not bind competitively with ATP or at the MAPK binding site. No inhibitory activity was seen against Raf kinase, cAMP-dependent kinase, PKC, v-Src, EGFR, IGFR (insulin-like growth factor receptor), PDGF (platelet-derived growth factor), PI3-kinase (phosphatidylinositol 3-kinase), JNK and p38 MAPK. When Swiss 3T3 cells were incubated with PD-098059, the PDGF-stimulated tyrosine phosphorylation of MAPK was inhibited at similar concentrations (IC_{50} ~ 10 μM), indicating that the compound readily crossed the cell membrane. In an experiment to determine the effect of PD-098059 on DNA synthesis, this agent was shown to inhibit thymidine incorporation into Swiss 3T3 cells with an IC_{50} of 7 μM.

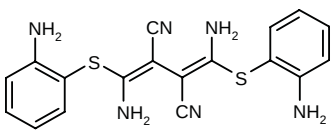
Further studies with PD-098059 indicated that it did not inhibit Raf-phosphorylated MEK1, although it had been shown to prevent the activation and phosphorylation of inactive MEK1 (106). Since it had been shown that PD-098059 did not bind at the active site, it was concluded that this agent bound to a site on unphosphorylated MEK1 that blocked access to activating enzymes, possibly displacing an allosteric effector. PD-098059 was selective for MEK1 versus MEK2 (IC_{50} = 50 μM), as well as a panel of 18 protein serine/threonine kinases (no inhibition was observed at 50 μM), in addition to the previously mentioned kinases. Subsequent studies have shown that PD-098059 inhibits MEK5 (107), as well as KSR1, an upstream activator of Raf-1 (IC_{50} = 1.5 μM), in HL-60 monocytes (108). PD-098059 has also been shown to inhibit cyclooxygenase type 1 (COX-1) and type 2 (COX-2) (109), indicating that the cellular effects of this agent might be due to the inhibition of several targets.

While searching for antiinflammatory agents with a novel mechanism of action, researchers at the former DuPont Merck (now Bristol-Myers Squibb) identified U-0126 [**27**] as a compound that functionally antagonized AP-1 transcriptional activity (110). Mechanistic studies demonstrated that this agent was a potent inhibitor of MEK1 and MEK2 (IC_{50} = 72 and 58 nM, respectively). Little or no activity was observed when tested against PKC, Abl, Raf, MEKK (mitogen-activated protein/ERK kinase kinase), ERK, JNK, MKK-3 (MAPK kinase-3), MKK-4/SEK (SAPK/ERK kinase), MKK-6, CDK2 and CDK4. Like PD-098059, U-0126 was not a competitive inhibitor with ATP or ERK, although U-0126 was a more potent inhibitor (IC_{50} = 70 nM vs. 10 μM for PD-098059) of constitutively active mutant MEK1 (ΔN3-S218E/S222D). Both PD-098059 and U-0126 were significantly less active against wild-type activated MEK1 (IC_{50} > 100 μM and 530 nM, respectively). This suggests that subtle conformational differences in these enzymes affect binding to the activated forms. Equilibrium binding experiments indicated that PD-098059 and U-0126 bound at a common site of ΔN3-S218E/S222D MEK1. Subsequent reports have indicated that U-0126 inhibits MEK activation at much lower concentrations than those required to suppress MEK activity (111, 112). It appears that U-0126 exerts its biological effects in a similar manner to PD-098059, even displaying activity against MEK5 (107, 113) and KSR (108). Unlike PD-098059, however, U-0126 does not display activity against COX-1 or COX-2 (111).

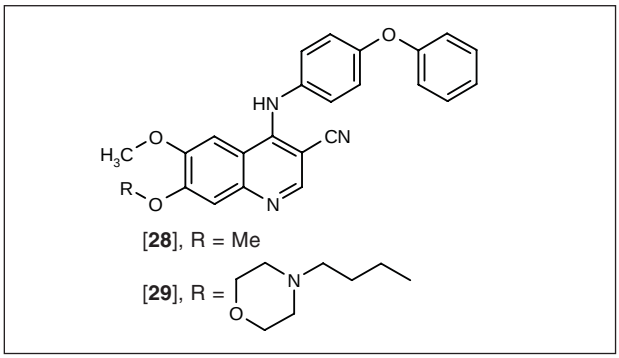
Analogues of U-0126 have been synthesized and evaluated *in vitro* in an effort to explore the SAR of this agent (114). It was first necessary to establish that the predominant *Z,Z*-isomer was the most active form of the compound. Separation of the *Z,Z-, Z,E*- and *E,E*-isomers was achieved by HPLC. Biological evaluation of the separated isomeric forms of U-0126 demonstrated that the *Z,Z*-isomer was indeed the most potent, followed by the *Z,E*- and *E,E*-forms. It was concluded that any activity observed for the *Z,E*- and *E,E*-isomers was most likely



[**26**], PD-098059



[**27**], U-0126



due to the presence of small amounts of *Z,Z*-isomer formed in solution. When dissolved in DMSO, or treated with dilute acid, cyclization to mono- or bisthiazolines or pyrroles occurred. Similarly, when dosed in rats, cyclization products were detected in the plasma. These cyclized compounds were less active than the parent U-0126, as well as cyclized analogues that were further modified by methylation or acylation. The SAR of the phenyl ring substituents were also explored. Electron-donating substituents provided the best activity, while electron-withdrawing groups dramatically lowered activity. All the changes made to U-0126 resulted in less active compounds as measured by MEK inhibition or AP-1 suppression.

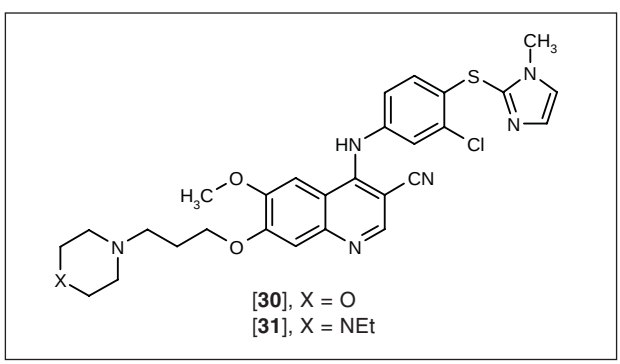
A series of 4-anilino-6,7-dialkoxyquinoline-3-carbonitriles has been reported as potent MEK1 inhibitors by researchers at Wyeth (115, 116). In the initial report (115), methoxy substituents were placed at the C-5 through C-8 positions of a 4-(phenoxyanilino)quinoline-3-carbonitrile core to determine the SAR, with enzyme activity being determined in a Raf/MEK/ERK ELISA assay, and a direct MEK/ERK ELISA assay. Placement of the methoxy group at the C-8 position abolished all activity, while optimal potency was observed with a compound [**28**] possessing methoxy substituents at C-6 and C-7 (IC_{50} = 9 nM, coupled assay). Compounds with morpholine appended via a C-7 alkoxy group proved to be even more potent against MEK, and showed enhanced activity against human colon tumor cell lines. It was also shown that the phenoxy substituent of the C-4 aniline group provided the best activity at the *para*-position, with significantly decreased activity at the *meta*- and *ortho*-positions. Compound **29** was the most potent overall (IC_{50} = 2.4 nM, coupled enzyme assay; IC_{50} = 360, 190 and 380 nM, respectively, against COLO 205, LoVo and SW620 cell lines). Unlike PD-098059 and U-0126, **29** was shown to be competitive with ATP. While this compound was not very cytotoxic at concentrations below 5 μM, the cellular activity was 50-100-fold weaker than that observed for enzyme activity. This was concluded to be due to poor cellular uptake, or due to the possibility that specific inhibition of the MEK/ERK pathway could be compensated for by alternative growth signals (115). Further exploration of the aniline substituents demonstrated that the *para*-ether linker could be replaced with methylene or sulfur to provide compounds of equivalent activity, while removal of this linker atom or replacement by ethyl decreased activity (116). Replacement of the phenyl group with heteroaryl groups generally provided compounds with slightly diminished activity (2-10-fold). Smaller alkyl substituents or cycloalkyl groups in place of the phenyl group were significantly less active. These compounds were shown to have little or no effect versus other kinases, including EGFR, ECK (epithelial cell kinase), KDR (kinase insert domain receptor), CDK2, CDK4 and Akt.

Another series of 4-anilinoquinoline-3-carbonitriles have been reported (117-119) to be highly potent MEK1 inhibitors with exceptional inhibitory activity against certain human tumor cell lines. The most potent of these analogues, **30**, had an IC_{50} of 2 nM in the Raf/MEK/MAPK cascade assay, and inhibited LoVo cell proliferation with an IC_{50} of 5 nM (119). This compound additionally showed potent antiproliferative activity against BxPC-3 cells (IC_{50} = 29 nM), LnCAP cells (IC_{50} = 40 nM) and HT-1197 cells (IC_{50} = 50 nM), but little activity against Caco-2 cells (IC_{50} > 1000 nM).

While a number of C-4 aniline substituents were explored (117), only the 4-(1-methyl-1*H*-imidazol-2-ylsulfanyl)aniline-substituted compounds possessed highly potent activity in both the enzyme (IC_{50} < 30 nM) and LoVo cell assays (IC_{50} < 45 nM). A 3-chloro or 3-bromo substituent on the aniline equally provided the best activity, while the corresponding hydrogen- or methyl-substituted compounds were less active. Of all the other substituted anilines tested, only 3-chloro-4-(thiazol-2-ylsulfanyl)aniline provided almost comparable (LoVo cell IC_{50} = 56 nM) antiproliferative activity (119).

The SAR of the substituents at the C-6 and C-7 positions were thoroughly explored (118). Alkoxy, amino and amide-linked substituents at the C-6 position, in combination with C-7 alkoxy groups substituted with basic amines, provided the greatest potency in enzyme and cellular assays. A piperazine substituted analogue [**31**] showed statistically significant activity when dosed i.p. at 30 mg twice daily against LoVo xenografts (119) in nude mice (T/C = 44% at day 21).

In order to determine the origin of the potent cellular activity of **30** versus other compounds such as **29** (which possesses equivalent activity against MEK1), their ability to inhibit phosphorylated MEK1, MAPK and selected

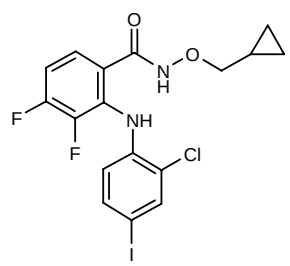


kinases was measured in LoVo cells (119). While **29** selectively inhibited phosphorylation of MAPK with an IC_{50} of 380 nM, **30** inhibited the phosphorylation of MEK1 and MAPK with IC_{50} values of 8 and 0.01 nM, respectively. Neither compound showed effects on phosphorylation levels of Akt or EGFR, nor was there an effect on overall cell phosphotyrosine levels. Compound **30** was not particularly active against Raf kinase (IC_{50} = 850 nM), suggesting that the inhibition of MEK phosphorylation is mainly due to a kinase upstream of Raf. Thus, the exceptional cellular activity of **30** appears to be due to activity against an additional target to MEK1, the identity of which is currently under investigation.

A potent MEK inhibitor, PD-184352 [**32**] was discovered by researchers at the former Parke-Davis (now Pfizer) (120). A screening effort led to the discovery of a series of compounds with inhibitory activity towards MEK kinase, which were ultimately optimized to provide PD-184352 (IC_{50} = 17 nM). This compound was shown to be noncompetitive with respect to ATP and the MAPK site on MEK, thereby identifying it as an allosteric inhibitor of MEK. The selectivity of this compound was high, with essentially no activity at 10 μM against a large panel of kinases (111, 120). However, PD-184352 has been reported to downregulate the activation of MKK5/ERK5, possibly by a negative feedback mechanism resulting from MEK inhibition (113).

Exposure of serum-starved colon 26 murine carcinoma cells (stimulated with PDGF) to concentrations of PD-184352 as low as 100 nM completely suppressed MAPK phosphorylation (120). It was also shown that growth-inhibitory effects correlated with the phospho-MAPK levels of different cell lines. Thus, certain colon cell lines (colon 26, HT-29 and COLO 205), which had higher phospho-MAPK levels than breast (MDA-MB-468 and MCF7), prostate (PC-3) and lung (A549) cell lines, were the most sensitive in cell proliferation assays run in soft agar (IC_{50} = 120-180 nM). Inhibition of cellular growth was shown to be due to the ability of PD-184352 to prevent cell cycle progression, inducing a block in the G_1 phase. In addition to blocking cell growth, PD-184532 significantly affected cell morphology and invasiveness.

In vivo activity was demonstrated with PD-184352 in several models (120). Nude mice implanted subcutaneously with colon 26, HT-29 and SK-OV-3 tumors were treated orally with 48-200 mg/kg every 12 h. Significant tumor growth inhibition was observed at all doses. Furthermore, substantial reduction of phospho-MAPK levels was observed for the cells of all tumors when evaluated *ex vivo* within 1 h of p.o. dosing. Consistent with the results observed in the cellular proliferation studies, PD-184352 was inactive even when dosed at 300 mg/kg against P388 murine leukemia cells (which possess very low levels of phospho-MAPK) implanted intraperitoneally. No toxicity was observed at this dose. Subsequently, it was shown that PD-184352 was highly active against pancreatic tumor xenografts BxPC-3 and MIA PaCa-2 when dosed orally at 200-300 mg/kg t.i.d., although it was inactive against Panc-1 (121).



[**32**], PD-184352

On the basis of the *in vivo* activity and a lack of toxicity noted in these studies, PD-184352 (subsequently known as CI-1040), was brought into development and subsequently to phase I clinical studies. Initial reports (122, 123) indicated that the compound was well tolerated at doses up to 1600 mg once daily or 800 mg b.i.d. or t.i.d. Best exposure was achieved with multiple dosing, particularly when administered together with food. Side effects included fatigue, rash and diarrhea. Target therapeutic plasma levels were achieved, and stable disease (at least 12 weeks) was observed in about 30% of all patients (123). One patient with pancreatic cancer achieved a partial response for more than 6 months. However, in a subsequent phase II report (124), patients with advanced NSCLC, breast, colon or pancreatic cancers showed no objective responses when treated with CI-1040. Of 67 patients, only 4 achieved a clinical response. It has been reported (125) that clinical studies with CI-1040 have been discontinued.

Summary and outlook

Recently, researchers within the pharmaceutical industry have become especially adept at developing potent protein kinase inhibitors with anticancer activity. For example, STI-571 (Gleevec®, imatinib), an Abl kinase inhibitor, has proven effective in the treatment of chronic myelogenous leukemia (CML) (126). Recently approved kinase inhibitors with activity against solid tumors include the HER-2/neu inhibitor (127) trastuzumab (Herceptin®) and the EGFR inhibitor (128) ZD-1839 (Iressa®). Numerous other kinase inhibitors targeting HER-2/neu and EGFR, as well as other kinases (including, for example, mTOR [mammalian target of rapamycin], PKC, KDR and CDK inhibitors), are currently undergoing clinical trials (127).

Because of the central importance (12, 129) of the Ras-MAPK signaling pathway with regard to cell proliferation, transformation and invasion, inhibitors of the kinase components of this pathway would appear to have great potential as anticancer agents (130, 131). Despite the central nature of this pathway, selective inhibitors of Raf and MEK appear to cause no ill effects in normal cells. Certainly, preclinical data from the research efforts described in this review have confirmed that such

compounds have potential for anticancer activity, and are well tolerated in laboratory animals. A potential issue for a selective kinase inhibitor is resistance. Because of the degree of cross-talk between the various signaling pathways, it is possible that some tumor cells might be able to overcome inhibition of this pathway (131). Thus, it is likely that these kinase inhibitors will be more effective in combination with agents acting by other mechanisms. Preclinical studies suggest that the combination of inhibitors of the Ras-MAPK signaling module with cytotoxic agents (96-98) or irradiation (95) can be particularly effective against human tumor xenografts.

To date, small-molecule inhibitors of Raf and MEK kinase, as well as antisense agents targeting C-Raf mRNA, have reached phase II and phase III clinical trials, showing signs of efficacy in these early studies. Several combination studies with these compounds and a variety of cytotoxic agents are under way. The outcome of clinical trials on kinase inhibitors of the Ras-MAPK signaling pathway is of great interest to both the basic and clinical research communities. Any positive outcome would be a compelling example of translating basic scientific understanding into a successful human cancer therapy.

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