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Invited Review

Allosteric MEK1/2 inhibitors including cobimetanib and trametinib in the treatment of cutaneous melanomas

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ABSTRACT

The Ras-Raf-MEK-ERK (Map kinase) cellular pathway is a highly conserved eukaryotic signaling module that transduces extracellular signals from growth factors and cytokines into intracellular regulatory events that are involved in cell growth and proliferation or the contrary pathway of cell differentiation. Dysregulation of this pathway occurs in more than one-third of all malignancies, a process that has fostered the development of targeted Map kinase pathway inhibitors. Cutaneous melanomas, which arise from skin melanocytes, are the most aggressive form of skin cancer. Mutations that activate the Map kinase pathway occur in more than 90% of these melanomas. This has led to the development of the combination of dabrafenib and trametinib or vemurafenib and cobimetanib for the treatment of *BRAF V600E* mutant melanomas. Dabrafenib and vemurafenib target *V600E/K BRAF* mutants while trametinib and cobimetanib target MEK1/2. The latter two agents bind to MEK1/2 at a site that is adjacent to, but separate from, the ATP-binding site and are therefore classified as type III allosteric protein kinase inhibitors. These agents form a hydrogen bond with a conserved β3-lysine and they make numerous hydrophobic contacts with residues within the αC-helix, the β5 strand, and within the activation segment, regions of the protein kinase domain that exhibit greater diversity than those found within the ATP-binding site. One advantage of such allosteric inhibitors is that they do not have to compete with millimolar concentrations of cellular ATP, which most FDA-approved small molecule competitive inhibitors such as imatinib must do. Owing to the wide spread activation of this pathway in numerous neoplasms, trametinib and cobimetinib are being studied in combination with other targeted and cytotoxic drugs in a variety of clinical situations. Except for *BRAF* and *NRAS* mutations, there are no other biomarkers correlated with treatment responses following MEK1/2 inhibition and the discovery of such biomarkers would represent an important therapeutic breakthrough.

Contents

1. The Ras-Raf-MEK-ERK (MAP kinase) signaling pathway 21
2. Overview of melanomas 21
   2.1. Clinical characteristics 21
   2.2. Pathogenesis of melanomas 22
   2.3. Targeted treatment of melanomas 22
3. Properties of MEK1 and MEK2 24
   3.1. Architecture and primary structures 24
   3.2. The secondary and tertiary structures of MEK1/2 and the K/E/D/D motif 25
   3.3. Hydrophobic spines from active and inactive protein kinases 26
4. Binding of cobimetinib and trametinib to MEK1/2 26
5. Epilogue 29

Abbreviations: AS, activation segment; CS or C-spine, catalytic spine; CL, catalytic loop; EGFR, epidermal growth factor receptor; MAP kinase, mitogen-activated protein kinase; MEK1/2, MEK1 and MEK2; PI3 kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; RS or R-spine, regulatory spine; Sh1, shell residue 1.

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5.1. Classification of targeted small molecule protein kinase inhibitors 29
5.2. MEK1/2 inhibitors in clinical trials 29
Conflict of interest 30
Acknowledgment 30
References 30

1. The Ras-Raf-MEK-ERK (MAP kinase) signaling pathway

Protein kinases play vital roles in nearly every aspect of cellular and molecular biology [1]. They regulate, *inter alia,* apoptosis, cell growth and division, cell migration, the immune response, metabolism, nervous system function, and transcription. Regulatory protein phosphorylation involves the action of both protein kinases and phosphoprotein phosphatases thus making phosphorylation-dephosphorylation an overall reversible process. Dysregulation of protein kinase signaling modules occurs in many diseases including cancer and inflammatory disorders. Protein kinases catalyze the following reaction:

$$MgATP^{1-} + protein\text{–}O:H \rightarrow protein\text{–}O:PO_3^{2-} + MgADP + H^+$$

Note that the phosphorylium moiety (PO_3^{2-}), and not the phosphate group (OPO_3^{2-}), is transferred from ATP to the protein substrate. Based upon the identity of the phosphorylated —OH group, these enzymes are classified as protein-serine/threonine kinases or protein-tyrosine kinases [2]. A small group of dual-specificity kinases such as MEK1/2 catalyzes the phosphorylation of both tyrosine and threonine in their target proteins (ERK1/2). The dual-specificity enzymes are members of the protein-serine/threonine kinase family.

The Ras-Raf-MEK-ERK cellular module is a highly conserved eukaryotic pathway that transduces extracellular signals from growth factors and cytokines into intracellular regulatory events [3–5]. This classical mitogen-activated protein kinase (MAP kinase) cascade is activated by a variety of transmembrane receptors including receptor protein-tyrosine kinases, G protein-coupled receptors, and cytokine receptors. Activated receptor protein-tyrosine kinases become phosphorylated at tyrosine residues within and outside of the activation segment and these phosphorylation sites recruit various adapter proteins and guanine nucleotide exchange factors (GEFs) such as SOS (from *Drosophila* son of sevenless). The exchange factors mediate the conversion of inactive Ras-GDP to active Ras-GTP at the inner leaflet of the plasma membrane [6,7]. H-Ras. K-Ras, and N-Ras are the products of three genes and each has a molecular weight of about 21 kDa. These proteins function as a switch; the conversion of Ras-GDP to Ras-GTP turns the switch on while Ras-GTPase activity stimulated by NF1 (neurofibromin-1) turns the switch off. To activate the MAP kinase pathway, Ras-GTP promotes the formation of active homo- or hetero-dimers of A-Raf, B-Raf, and C-Raf by a multistage process (Raf corresponds to rapidly accelerated fibrosarcoma). These three Ras effectors are protein-serine/threonine protein kinases that catalyze the phosphorylation and activation of MEK1 and MEK2 where MEK corresponds to MAP/ERK kinase.

MEK1/2 are classified as dual specificity protein kinases because they catalyze the phosphorylation of ERK1/2 at tyrosine before threonine in the sequence Thr-Glu-Tyr (TEY) that occurs within the ERK activation segments (ERK corresponds to extracellular signal-regulated protein kinase) [4,5]. ERK1 and ERK2 have very broad substrate specificity and can catalyze the phosphorylation of hundreds of proteins. In contrast, A/B/C-Raf and MEK1/2 have very narrow substrate specificity. In fact, the only known substrates of the Raf enzymes are MEK1/2 and the only known substrates of MEK1 and MEK2 are ERK1/2. To further illustrate their fastidious substrate specificity, MEK1/2 are unable to catalyze the phosphorylation of denatured ERK1/2 nor do they mediate the phosphorylation of peptides with sequences corresponding to the activation segment of ERK1/2. However, MEK1/2 are able to catalyze the phosphorylation of myelin basic protein, which is an adventitious substrate. Note that all of the activators of the classical MAP kinase pathway involve MEK1/2, which thus represents a regulatory bottleneck in the initiation and maintenance of important cellular responses (Fig. 1). The kinase suppressor of Ras1 (KSR1) is an impaired protein kinase (but not kinase dead) that functions as a scaffold to assemble Raf, MEK, and ERK to increase signaling effectiveness (not shown) [4]; since it increases signaling efficiency, the term suppressor is a misnomer.

ERK1/2 catalyze the phosphorylation of Ser69 of pro-apoptotic BIM, leading to its interaction with TRIM2 and its subsequent ubiquitylation and proteosomal destruction [8]. Similarly, phosphorylation of the pro-apoptotic MCL-1 leads to its ubiquitylation and proteosomal destruction. RSK (ribosomal protein 6 kinase) catalyzes the phosphorylation of many proteins that are involved in cellular proliferation and survival. MNK (MAP kinase interacting serine/threonine protein kinase) is involved in anti-apoptotic signaling. Moreover, ERK1/2 catalyze the phosphorylation of MSK (also known as ribosomal protein S6 kinase α5) at residues Ser360, Thr581, and Thr700. Activated MSK catalyzes the phosphorylation of several transcription factors that participate in mitogenic responses. FOS, ETS, and MYC are such transcription factors and substrates of ERK1/2 that participate in cellular proliferation and survival.

Although the Raf isoforms are the primary initiators of the MAP kinase cascade, MEKK1/2/3, MLK1/2/3/4, and COT (also known as cancer Osaka thyroid kinase or MAP3K8) are additional ERK1/2 MAP3Ks that participate in specialized cell type and stimulation specific responses (Fig. 1) [8]. Ras-GTP has several downstream effector pathways including the MAP kinase, the phosphatidyl inositol 3-kinase (PI3 kinase), and the Ral-GDS modules [5,9,10]. PKB is a downstream effector of PI3 kinase. This raises the possibility of combining targeted inhibitors of the MAP kinase pathway at the level of Raf, MEK1/2, or both along with inhibition of PI3 kinase pathway at the level of PI3 kinase, PKB, or both in the treatment of various neoplasms.

2. Overview of melanomas

2.1. Clinical characteristics

Cutaneous melanomas, which arises from skin melanocytes, are the most aggressive form of skin cancer [11]. Although its incidence is far below that of squamous cell and basal cell skin carcinomas, melanomas account for about 75% of all skin-cancer related deaths. Siegel et al. estimate that 76,000 new cases of melanoma will be diagnosed in the United States in 2016 and 10,000 people will die of the disease [12]. In the absence of metastasis or spread, about 85% of patients with melanomas are cured by surgical excision [11]. The only cytotoxic chemotherapeutic agent approved by the FDA for the treatment of advanced, or metastatic, melanomas is dacarbazine, which is a DNA alkylating agent. However, the response rate is only 5–10%. Newer and more effective therapies are being developed including the use of (i) agents targeting the MAP kinase pathway and (ii) immune checkpoint inhibitors [8,13].

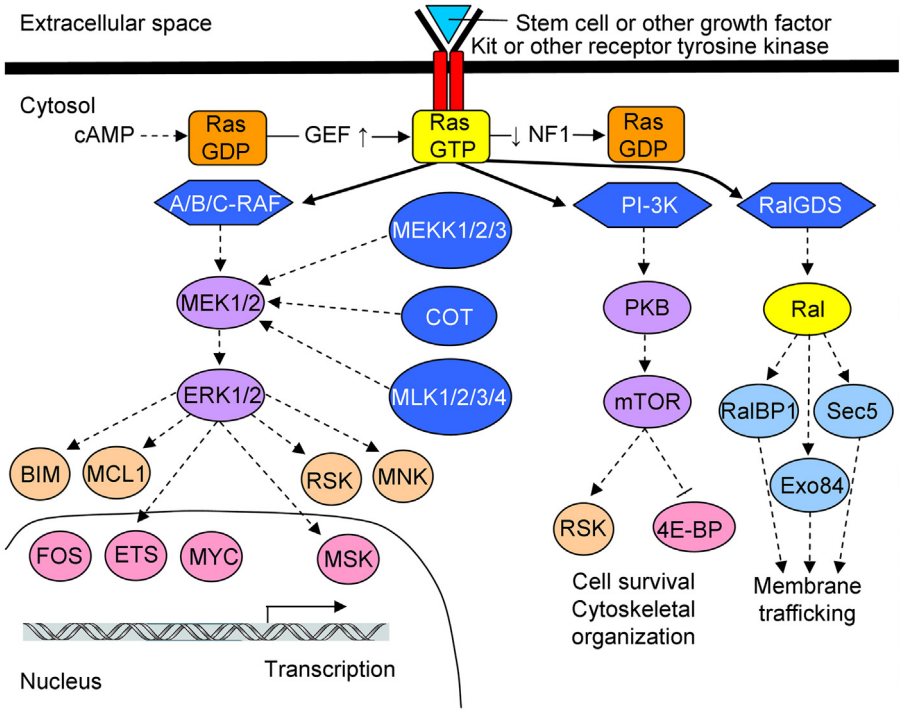

Fig. 1. Ras effector modules including the classical MAP kinase Ras-Raf-MEK-ERK pathway. GEF action increases the level of Ras-GTP and NF1 activity decreases the level of Ras-GTP. The dashed lines indicate that several steps may be involved.

2.2. Pathogenesis of melanomas

Mutations that activate the Ras-Raf-MEK-ERK pathway occur in more than 90% of cutaneous melanomas [14,15]. The Cancer Network consortium described the landscape of the genetic alterations of cutaneous melanomas in 331 patients based upon DNA, RNA, and protein analysis [14]. They reported the incidence of mutations in *BRAF* (52%), *NRAS* (28%), and *NF1* (14%); they referred to the remainder as Triple-WT (wild type). The *BRAF* and *NRAS* gain-of-function and the *NF1* loss-of-function mutations activate the MAP kinase pathway. These investigators found potential driver events in their Triple-WT melanomas including *KIT* amplifications/mutations and co-amplified *PDGFRA* and *VEGFR2*. They found no significant correlation of this genomic classification with the clinical outcome. However, they reported that samples enriched for immune gene expression accompanied by lymphocytic infiltration and high expression of the LCK T-cell marker were associated with increased patient survival. The PI3 kinase pathway is also downstream of Ras (Fig. 1) and it represents a potential target in melanomas. Similarly, aberrations in the cyclin-dependent kinase 4 and one of its inhibitors (INK4A) are implicated in the pathogenesis of melanomas.

2.3. Targeted treatment of melanomas

Although *H/K/NRAS* mutations occur in perhaps 30% of all cancers, attempts to target these proteins have been unsuccessful. However, recent advances have occurred as described by Ostrem and Shokat [16]. Although mutations in *KRAS* are the most common *RAS* mutation in solid malignancies, the most common *RAS* mutation in melanoma occurs in *NRAS*. The discovery that activating mutations of *BRAF* occur in the majority of melanomas [17] prompted the search for B-Raf inhibitors [18]. Several clinical trials of sorafenib were initiated in patients with metastatic melanomas. Sorafenib is a multikinase inhibitor with actions against B-Raf (wild type and V600E mutants), Kit, Flt3, and VEGFR1/2/3 that is currently approved for the treatment of liver, renal cell, and differentiated thyroid carcinomas (www.brimr.org/PKI/PKIs.htm). Eisen et al. reported that this drug produced clinical responses in fewer than 5% of patients with advanced melanomas [19]. In contrast, Chapman et al. reported on a clinical trial that pitted vemurafenib (Fig. 2A, also known as PLX4032) against dacarbazine in

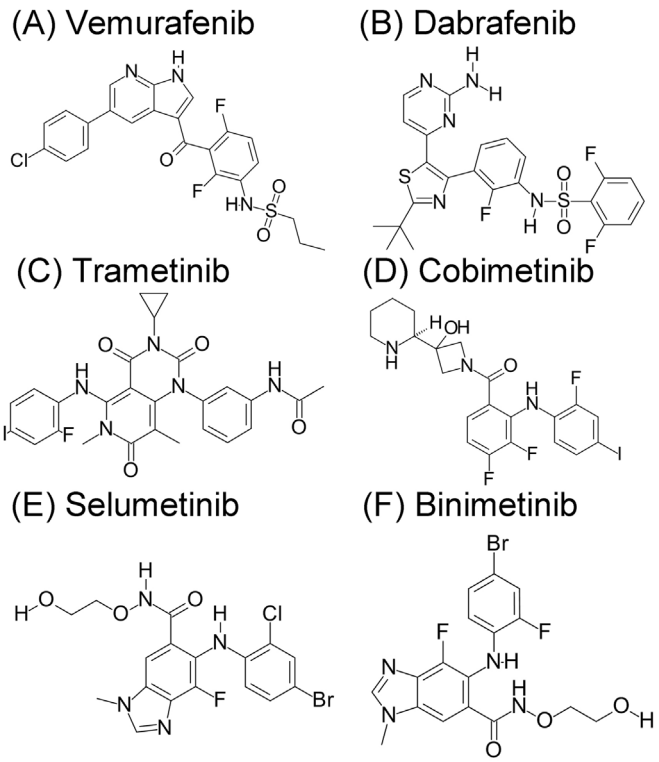

Fig. 2. Structures of selected B-Raf (A and B) and MEK1/2 (C–F) inhibitors.

a total of 675 randomized melanoma patients with the *BRAF V600E* mutation [20]. They reported that the response rates for vemurafenib were 48% while those for dacarbazine were 5%. Common adverse events included arthralgia, fatigue, fever, nausea, diarrhea, and rash along with the development of keratoacanthomas and well-differentiated squamous cell skin carcinomas. The latter two tumors are easily identified, simple to excise, and are generally not metastatic. However, the development of such neoplasms is an undesired outcome. Rash, fatigue, and diarrhea are side effects that occur with nearly all small molecule protein kinase inhibitors [21]. Vemurafenib was approved by the FDA for the treatment of *BRAF V600E*-positive melanomas in 2011.

Dabrafenib (Fig. 2B) was the second mutant-selective inhibitor to enter clinical trials vs. dacarbazine. Hauschild et al. reported that the overall response rate for dabrafenib was 50% and that for dacarbazine was 6% [22]. The former drug resulted in an improved progression-free survival of 5.1 months vs. 2.7 months for dacarbazine. The side effects are similar to those described for vemurafenib. Dabrafenib was approved by the FDA for the treatment of patients with metastatic melanomas with the *BRAF V660E* mutation in 2013 (www.brimr.org/PKI/PKIs.htm). About 20–25% of patients receiving vemurafenib or dabrafenib develop keratoacanthomas or well-differentiated squamous cell carcinomas. These hyperproliferative disorders are treated by surgical excision and these drugs may be resumed safely without dose adjustments. However, these drugs are ineffective in the treatment of patients without the *V600E BRAF* mutation. Moreover, these drugs produce a paradoxical activation of the MAP kinase pathway in the absence of a *V600E* mutation [3,5]. This paradoxical activation is caused by several mechanisms including mutations of *NRAS*, production of a truncated p61 B-Raf V600E, activation of the PI3 kinase pathway, and overexpression of either COT, B-Raf V600E, PDGFRβ, or the insulin-like growth factor-1 receptor [5].

Although nearly all patients with metastatic melanomas with the *BRAF V600E* mutation derive clinical benefit, median progression-free survival is approximately six months while 90% of patients develop resistance within one year [21]. The rapid development of resistance has prompted the exploration of other inhibitors of the MAP kinase pathway. One of the first of these was trametinib (Fig. 2C), which is a selective and potent inhibitor of MEK1 and MEK2. In a clinical trial involving 322 advanced melanoma patients with *BRAF V600E/K* mutations vs. dacarbazine or paclitaxel, Flaherty et al. reported that trametinib resulted in an improved overall response rate (22% vs. 8%) and progression-free survival (4.8 vs. 1.5 months) when compared with the groups receiving the cytotoxic treatments [23]. Rash, diarrhea, and peripheral edema were the chief trametinib adverse events, which were easily managed. Mild grade 1 or 2 ocular toxicity consisting of blurred vision occurred in 9% of the patients most likely as a result of the development of reversible serous retinopathy. In contrast to dabrafenib and vemurafenib therapy, these investigators did not observe any secondary skin neoplasms.

In a clinical trial involving 97 patients, Kim et al. observed significant clinical activity with trametinib (Fig. 2D) in B-raf-inhibitor-naive patients previously treated with chemotherapy, immunotherapy, or both [24]. However, they reported minimal clinical activity with trametinib as a second-line therapy in patients previously treated with a B-Raf inhibitor. They suggested that B-Raf-inhibitor resistance mechanisms likely confer resistance to MEK-inhibitor monotherapy. Accordingly, the FDA approved trametinib for the treatment of patients who have not been previously treated with targeted B-Raf inhibitors (www.brimr.org/PKI/PKIs.htm).

In a clinical study involving 247 patients with metastatic melanomas and *BRAF V600* mutations, Flaherty et al. compared trametinib and dabrafenib monotherapy with the combination of these two medicinals [25]. They found that the rate of complete or partial responses was 76% for the combination group compared with 54% for monotherapy groups. Furthermore, median progression-free survival was 9.4 months for the combination therapy group compared with 5.8 months for the monotherapy groups. Pyrexia (fever) was much more common in the combination group when compared with the monotherapy groups (71% vs. 26%). The incidence of cutaneous squamous cell carcinoma (7% vs. 19%) and hyperkeratosis (9% vs. 30%) was decreased in the combination therapy group when compared with the monotherapy group; however, these differences did not achieve statistical significance ($P = 0.09$). The protective effect of combination therapy may be due to the MEK inhibitor-induced blockade of the paradoxical activation of the MAP kinase pathway produced by dabrafenib. The combination of trametinib and dabrafenib was approved by the FDA for the treatment of *BRAF V600E/K* in 2014.

Larkin et al. reported on the results of a clinical trial involving 495 patients with previously untreated advanced *BRAF V600*-mutation positive melanomas receiving combination vemurafenib and cobimetinib or vemurafenib plus placebo (the control group) [26]. The rates of complete or partial responses (68% vs. 45%) and the lengths of median progression-free survival (9.9 vs. 6.2 months) were better in the combination group when compared with the control group. The incidence of cutaneous squamous cell carcinoma in the combination group was 2% compared with 11% in the control group while the incidence of keratoacanthomas was 1% in the combination group compared with 8% in the vemurafenib only group (the authors did not indicate whether or not these were statistically significant differences). This is an unusual case where a combination therapy exhibits fewer adverse effects than monotherapy [21]. This study and the study of Flaherty et al. [25] reported above indicate that the use of the combination of the B-Raf and MEK1/2 inhibitors is more efficacious than that of B-Raf or MEK1/2 monotherapy alone.

For the patients with advanced melanomas that possess a *BRAF V600* mutation (about 50% of all advanced melanoma patients), the National Comprehensive Cancer Network recommends (i) combination therapy with dabrafenib/trametinib, or vemurafenib/cobimetinib, or (ii) B-Raf inhibitor monotherapy with either vemurafenib or dabrafenib [27]; it is therapeutically advantageous that each of these four drugs is orally effective. Although FDA approved, this consortium no longer recommends trametinib monotherapy and they favor B-Raf/MEK1/2 combination therapy over the B-Raf inhibitor monotherapy.

Besides targeted therapies, the use of immune checkpoint inibitors has emerged in the efficacious treatment of melanomas; each of these therapies must be given parenterally [13]. Ipilimumab was the first of these agents; it is a monoclonal antibody that activates the immune system by targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4), which is a protein receptor that down regulates the immune response. Pembrolizumab is a humanized antibody that targets the programmed cell death-1 (PD-1) receptor, a process that also activates the immune system. Nivolumab is a human IgG4 anti-PD-1 monoclonal antibody that works as a checkpoint inhibitor by blocking a signal that prevents activated T cells from attacking the melanomas. All three of these immune checkpoint inhibitors are approved for the treatment of metastatic melanomas regardless of *BRAF* mutation status. For patients with documented *BRAF V600* mutations, selection between targeted therapy and immune checkpoint therapy is currently problematic owing to the lack of results from ongoing clinical trials comparing the two approaches. The studies documented in this section show that great strides have been made in the past five years in the treatment of metastatic melanomas and additional studies that are underway may add to the effectiveness of both targeted and immune checkpoint modalities.

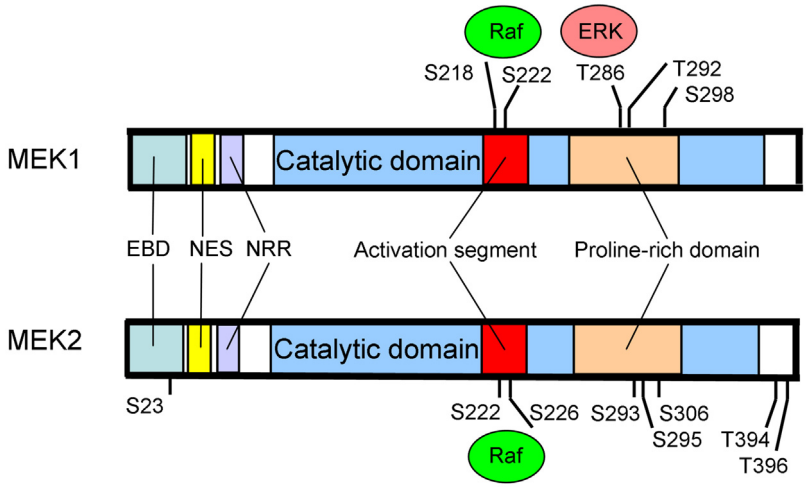

Fig. 3. Architecture of MEK1 and MEK2 depicting the location of the tri-functional N-terminal segment and the C-terminal protein kinase catalytic domain including its proline-rich component. Selected Raf and ERK serine (S) and threonine (T) phosphorylation sites as well as sites related to other protein kinases are indicated. EBD, ERK-binding domain; NES, nuclear export sequence; NRR, negative regulatory region.

3. Properties of MEK1 and MEK2

3.1. Architecture and primary structures

The dual specificity MEK protein kinases contain a trifunctional sequence consisting of about 70 amino acids that are N-terminal to the 270 residue protein kinase catalytic domain. The trifunctional sequence contains an ERK-binding domain (EBD), a nuclear export sequence (NES), and negative regulatory region (NRR) (Fig. 3) that we will see resides within the A-helix that is found near the amino-terminal lobe. The activation segment of MEK1/2 contains two serine residues that result in enzyme activation following phosphorylation as catalyzed by any of the Raf enzymes. Moreover, Thr292 within the proline-rich segment of MEK1, but not MEK2, is a site of phosphorylation catalyzed by ERK1/2 that results in the feedback inhibition of the pathway.

The protein kinase catalytic domains consist of about 250–300 amino acid residues. Hanks and Hunter examined the sequences of about five dozen protein kinases and divided the primary structures into one dozen domains (I-VIA,VIB-XI) [28]. Domain I of MEK1/2 is the Gly-rich loop (GRL) and contains a GxGxxG signature ($^{475/479}$GAGNGG$^{480/484}$). Generally the glycine-rich loop is the most flexible part of the protein kinase domain and the penultimate residue is a very hydrophobic residue such as phenylalanine. In the case of MEK1/2, this residue is glycine thereby increasing the flexibility of the G-rich loop even more. This loop connects the β1- and β2-strands and overlays the ADP/ATP-binding site (Fig. 4A). The great flexibility of the Gly-rich loop is related to the switches required for ATP binding and ADP release during each catalytic cycle. Domain II of MEK1/2 contains a conserved Ala-Xxx-Lys (ARK) sequence in the β3-strand and domain III contains a conserved glutamate in the αC-helix that forms a salt bridge with the conserved ARK-lysine in the active protein kinase conformation. Apparently there are no tertiary structures of active MEK1/2 in the public domain, but this salt bridge (K483-E501) in active B-Raf is depicted in Fig. 4B. Domain VIB of MEK1/2 contains a conserved HRD sequence, which forms part of the catalytic loop (HRDVKPSN), which begins with histidine and ends with asparagine (the catalytic

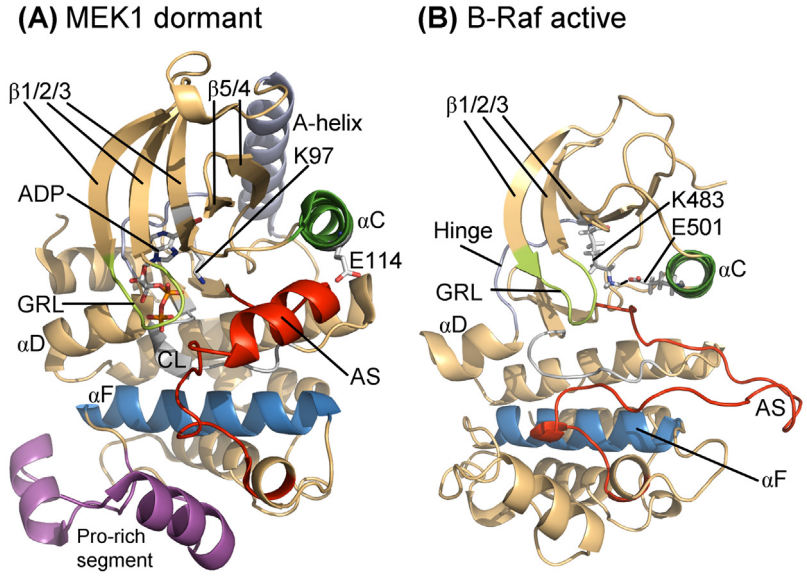

Fig. 4. Ribbon diagram structures of (A) dormant human MEK1 (PDB ID: 3EQI) and (B) active human B-Raf (PDB ID: 4MNE). AS, activation segment; CL, catalytic loop; GRL, glycine-rich loop. Figs. 4–7 were prepared using the PyMOL Molecular Graphics System Version 1.5.0.4 Schrödinger, LLC.

loop of PKA and related enzymes begins with tyrosine). Domain VII contains a DFG signature and domain VIII of MEK1 contains a ^{231}SPE^{233} while that of MEK2 contains a ^{235}APE^{237} sequence; together domain VII and VIII represent the beginning and end of the protein kinase activation segment. The end of the activation segment of most protein kinases consists of an APE signature, which is found in MEK2 but not MEK1. Domains IX–XI consist of the αF–αI helices. The tertiary structure of protein kinase A provided an invaluable framework for understanding the role of the 12 domains on the protein kinase catalytic mechanism, which is described next.

3.2. The secondary and tertiary structures of MEK1/2 and the K/E/D/D motif

The determination of the X-ray crystal structure of the catalytic subunit of cyclic AMP-dependent protein kinase (PKA) bound to a polypeptide antagonist by Knighton et al. has shed light on the fundamental properties of the protein kinase superfamily (PDB ID: 2CPK) [29,30]. All protein kinases including MEK1/2 and B-Raf have a small N-terminal lobe and large C-terminal lobe [31]. The N-terminal lobe contains five β-strands (β1–5) and a regulatory αC-helix; the large lobe contains four short β-strands (β6–β9) and seven conserved α-helices (αD–αI and αEF). A gap or cleft between the small and large lobes functions as the binding site for ATP/ADP. Of the hundreds of protein kinase structures that are known, all of them possess the original protein kinase fold described first for PKA [29,30].

Virtually all active protein kinases contain a K/E/D/D (Lys/Glu/Asp/Asp) entity that plays an essential role in protein kinase catalysis (Table 1) [1]. The lysine and glutamate function together within the amino-terminal lobe and the two aspartate residues function within the carboxyterminal lobe. Although both lobes participate in ATP/ADP binding, the N-terminal lobe plays a major role in this process. Based upon the structures of other protein kinases, we infer that K97/101 (the K of K/E/D/D) of the β3-strand of MEK1/2 holds the α- and β-phosphates of ATP in an active position. The carboxylate groups of E114/118 (the E of K/E/D/D) of the MEK1/2 αC-helix forms a salt bridge with the ε-amino groups of K97/101 and stabilize their interactions with the α- and β-phosphates. The existence of a salt bridge linking the β3-lysine and αC-glutamate is necessary for the formation of an active protein kinase conformation; this is designated as the "αC-in" structure as depicted for B-Raf in Fig. 4B. In contrast the lysine and glutamate of inactive enzymes fail to make contact; this is designated as the "αC-out" structure. The MEK1/2 A-helix, the primary structure of which occurs before the first β-strand of the small lobe, interacts with the N-terminal lobe between the αC-helix and β4-strand and stabilizes the inactive "αC-out" structure (Fig. 4A). For the entire protein kinase enzyme family, the αC-in configuration is necessary, but not sufficient, for catalytic activity.

The large lobe plays a major role in protein/peptide substrate binding and participates in the catalytic cycle. Two Mg^{2+} ions participate in each catalytic cycle of a number of protein kinases [32] and are most likely required for the functioning of MEK1/2. By inference, MEK1/2 D208/212 (the DFG-D and the first D of K/E/D/D) binds to $Mg^{2+}(1)$, which in turn binds to the β- and γ-phosphates of ATP. In the functionally active enzyme, DFG-D points inward toward the active site. In the dormant form of many protein kinases, the DFG-D is directed outward from the active site (small molecule protein kinase inhibitors that bind to the DFG-D outward conformation are classified as type II inhibitors [33]). MEK1/2 N195/199 of the catalytic loop bind $Mg^{2+}(2)$, which in turn forms salt bridges with the α- and γ-phosphates of ATP. We infer that the activation segment of the MEK1/2 active conformation forms an open structure that allows protein/peptide binding; such an open structure is depicted for B-Raf in Fig. 4B. In the inactive or less active enzyme, the activation segment forms a closed and compact structure that obstructs protein/peptide substrate binding (Fig. 4A). The 6-amino exocyclic nitrogen of ATP typically forms a hydrogen bond with the carbonyl backbone residue of the first protein kinase hinge residue connecting the small and large lobes while the *N*1 nitrogen of the ATP adenine forms another hydrogen bond with the N—H group of the third hinge residue (not shown).

The activation segment is responsible for both protein-substrate binding and catalytic efficiency [34]. This segment in MEK1/2 contains two phosphorylatable serine residues. The beginning of the activation segment occurs near (i) the N-terminus of the αC-helix and (ii) the conserved catalytic loop HRD. The spatial contact of these three components is hydrophobic in nature. As is the case for most protein kinases [35], phosphorylation of one or more residues within the activation segment converts an inactive enzyme to an active one. Zheng and Guan reported that human MEK1 activation necessitates the phosphorylation of both S218 and S222 within the activation segment [36]. They reported that the expression of either the S218A or the S222A mutant in murine Swiss 3T3 cells abolishes EGF-stimulated MEK1 activation; this finding implies that both S218 and S222 must be phosphorylated in order to achieve MEK1 activation.

Alessi et al. reported that human C-Raf catalyzes the phosphorylation of the equivalent serine residues in rabbit MEK1 [37]. Thus, the phosphorylation and activation of MEK1/2 is mediated

Table 1
Important residues in human MEK1/2 and B-Raf.

	Comments	MEK1	MEK2	B-RAF
UniProtKB accession no.		Q02750	P36507	P15056
No. of residues		392	400	765
Protein kinase domain		68–361	72–367	457–717
Molecular Wt (kDa)		43.4	44.4	84.4
N-lobe				
Glycine-rich loop: GxGxxG	Anchors ATP β-phosphate	^{75}GAGNGG^{80}	^{79}GAGNGG^{84}	^{464}GSGSFG^{469}
β3-lysine (K of K/E/D/D)	Forms salt bridges with ATP α- and β-phosphates	K97	K101	K483
αC-glutamate (E of K/E/D/D)	Forms ion pair with β3-K	E114	E118	E501
Hinge residues between the N- and C-lobes	Connects N- and C-lobes	^{144}EHMDGGS^{150}	^{148}EHMDGGS^{154}	^{530}QWCEGSS^{536}
C-lobe				
Catalytic loop HRD (first D of K/E/D/D)	Catalytic base (abstracts proton)	D190	D194	D576
Catalytic loop Asn (N)	Chelates $Mg^{2+}(2)$	N195	N199	N581
Activation segment	Positions protein substrate	208–233	212–237	594–623
AS DFG (second D of K/E/D/D)	Chelates $Mg^{2+}(1)$	D208	D212	D594
AS phosphorylation sites	Stabilizes the AS after phosphorylation	S218, S222	S222, S226	T599, S602
End of activation segment		^{231}SPE^{233}	^{235}APE^{237}	^{621}APE^{623}

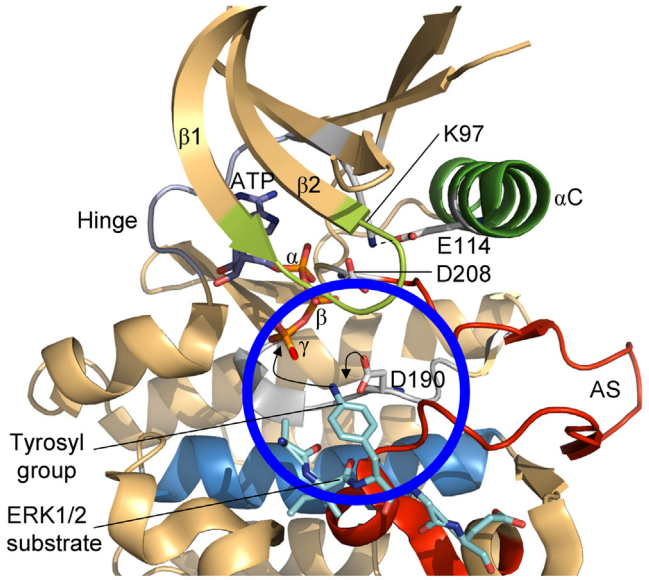

Fig. 5. Inferred mechanism of the MEK1/2 reaction involved in the first step in the activation of ERK1/2. The chemistry occurs within the blue circle.

by upstream Raf protein kinases. Similarly, the phosphorylation and activation of ERK1/2 is mediated by MEK1/2, also upstream protein kinases. This differs from the phosphorylation of many other protein kinases such as EGFR where phosphorylation and activation is catalyzed by a *trans* autophosphorylation with one EGFR protomer acting on a second EGFR [38]. Alessi et al. also reported that subsequent to the first phosphorylation *in vitro*, the second phosphorylation occurs promptly thereby precluding the accumulation of the mono-phosphorylated species [37]. These investigators also found that the protein phosphatase 2A catalyzed hydrolytic removal of one phosphate from doubly phosphorylated MEK1 leaves the enzyme fully activated. This finding indicates that double phosphorylation is required for activation, but the subsequent removal of a single phosphate leaves MEK1 in its activated state. The corresponding residues on MEK2 are S222 and S226. The MEK1/2 catalytic aspartate (HRD-D190/194), which is the first D of K/E/D/D, serves as a base that accepts a proton from the protein–OH group during the catalytic cycle (Fig. 5).

3.3. *Hydrophobic spines from active and inactive protein kinases*

Kornev et al. examined the structures of the active and inactive conformations of about two dozen protein kinases and they established the role of crucial residues by a local spatial pattern alignment algorithm [39,40]. Their investigation led to a classification of four hydrophobic residues that make up a regulatory or R-spine [39] and eight hydrophobic residues forming a catalytic or C-spine [40]. Each spine consists of amino acids found in both the N- and C-terminal lobes. The regulatory spine contains one residue from the αC-helix and another from the activation segment, both components of which are key structures in determining high activity and low activity states. The adenine base of ATP makes up a portion of the C-spine. The R-spine stabilizes the binding site for the peptide/protein substrate and the C-spine positions ATP within the active site thus promoting catalysis. Additionally, the correct alignment of each spine is required for the formation of an active protein kinase as described for the Bruton protein kinase, the Janus kinases, cyclin-dependent protein kinases as well as ERK1/2, EGFR, and Src [5,32,38,41–43].

The classical protein kinase R-spine consists of a residue near the beginning of the β4-strand, a residue near the C-terminal end of the αC-helix (four residues C-terminal to the conserved αC-glutamate), the DFG-Phe of the activation segment, and the HRD-His of the catalytic loop [39,40]. The backbone of the N—H group of the HRD-His is anchored to the hydrophobic αF-helix by a hydrogen bond to an invariant aspartate carboxylate. Going from the bottom to the top of the spine, Meharena et al. labeled the R-spine residues RS0, RS1, RS2, RS3, and RS4 (Fig. 6A) [44]. The R-spine of dormant MEK2 is bent with RS3 displaced while that of active B-Raf is linear (Fig. 6B and C).

The C-spine of protein kinases consists of residues from both the N- and C-terminal lobes and it is completed by the ATP adenine (Fig. 6A) [40]. The two residues of the N-terminal lobe of protein kinase domains that interact with the adenine component of ATP include a conserved valine near the beginning of the β2-strand (CS7) and a conserved alanine from the invariant AxK of the β3-strand (CS8). Additionally, a β7-strand hydrophobic residue (CS6) interacts with the adenine base. The CS6 residue is flanked by two additional residues labeled CS4 and CS5 that make hydrophobic contact with the CS3 residue near the beginning of the αD-helix. These three residues (CS4/5/6) directly follow the asparagine residue of the catalytic loop (HRDxxxxN). Finally, CS3 and CS4 interact with the CS1 and CS2 residues within the αF-helix to complete the C-spine (Fig. 6) [40]. Importantly, the hydrophobic αF-helix anchors both the catalytic and regulatory spines. Furthermore, both spines play an important role in placing the protein kinase catalytic residues in an active conformation. When comparing the locations of the spinal residues, the greatest divergence in the structures between more active and less active protein kinase domains usually involves RS3.

Based upon site-directed mutagenesis methodologies, Meharena et al. identified three residues within the catalytic subunit of PKA that stabilize the regulatory spine, which they named Sh1, Sh2, and Sh3, where Sh refers to shell [44]. The Sh2 moiety represents the traditional protein kinase gatekeeper residue. The term gatekeeper signifies the role that this amino acid plays in regulating access to a back pocket [45], which is sometimes called the back cleft. The amino acids that constitute both spines were identified by their location in more active and less active protein kinases based upon their tertiary structures as determined by X-ray crystallography [29,30]. This contrasts with the identification of the APE, DFG, or HRD signatures, which were based upon their amino acid sequence [28]. Table 2 provides a listing of the spine and shell residues of human MEK1/2, human B-Raf, and murine PKA. Nearly all ATP-competitive small molecule protein kinase inhibitors interact with residues that make up the C-spine and sometimes the Sh1 residue within the αC–β4 loop [33]. This contrasts with the allosteric MEK1/2 inhibitors, which do not interact with the C-spine residues.

4. Binding of cobimetinib and trametinib to MEK1/2

Cobimetinib binds adjacent to, but not overlapping with, the ATP-binding site as determined by X-ray crystallography, a property which defines an allosteric type III protein kinase inhibitor [33]. Allostery is a concept developed by Monod et al. and refers to ligands binding to sites other than the active site [46]; in the case of protein kinases this site is the ATP-binding site. The methanone carbonyl group of the drug forms a hydrogen bond with β3-K97 and the 3-hydroxy azetidinyl group forms hydrogen bonds with (i) the γ-phosphoryl oxygen of ATP and (ii) N195 at the end of the catalytic loop. The azetidinyl N—H group also forms a hydrogen bond with the HRD-D190 (Fig. 7A). The drug makes hydrophobic contacts with L115 and L118 within the αC-helix, V127 (Sh1), I141 and M143 before the hinge along with D208, F209 (RS2), V211, and L215 within the activation segment. The drug makes van der Waals

Table 2
Spine and shell residues of human MEK1/2, B-Raf, and murine PKA.

	Symbol	MEK1	MEK2	B-RAF	PKA[a]
Regulatory spine					
β4-strand (N-lobe)	RS4	F129	F133	F516	L106
C-helix (N-lobe)	RS3	L118	L122	L505	L95
Activation loop F of DFG (C-lobe)	RS2	F209	F213	F595	F185
Catalytic loop His/Tyr (C-lobe)	RS1	H188	H192	H574	Y164
F-helix (C-lobe)	RS0	D245	D249	D638	D220
R-shell					
Two residues upstream from the gatekeeper	Sh3	I141	I145	I527	M118
Gatekeeper, end of β5-strand	Sh2	M143	M147	T529	M120
αC-β4 loop	Sh1	V127	V131	V511	V104
Catalytic spine					
β2-strand (N-lobe)	CS8	V82	V86	V471	V57
β3-AxK motif (N-lobe)	CS7	A95	A99	A481	A70
β7-strand (C-lobe)	CS6	L197	L201	F583	L173
β7-strand (C-lobe)	CS5	V198	V202	L584	I174
β7-strand (C-lobe)	CS4	I196	I200	I582	L172
D-helix (C-lobe)	CS3	L151	L155	L537	M128
F-helix (C-lobe)	CS2	S252	S256	V645	L227
F-helix (C-lobe)	CS1	M256	L260	L649	M231

[a] From Ref. [39,40,44].

contact with N78 within the G-rich loop as well as S212 and T226 within the activation segment.

X-ray crystal structures of trametinib bound to MEK1/2 and cobimetinib bound to MEK2 have not been reported. To obtain an idea on the possible interactions of these medicines with MEK1/2, the Schrödinger Glide Suite (2016-1 release) [47] was used to generate poses of these drugs bound to both targets. For trametinib binding to MEK1, the X-ray structure of cobimetinib bound to MEK1 was used as the starting point (PDB ID: 4AN2). The model indicates that the 2-pyrido[4,3-*d*] pyrimidine carbonyl group of trametinib forms a hydrogen bond with β3-K97 and the 7-pyrido[4,3-*d*] pyrimidine carbonyl group forms hydrogen bonds with the S212 N—H and O—H groups of MEK1 (Fig. 7B). Additionally, the acetamide carbonyl group forms two hydrogen bonds with the side chain of HRD-R189. The drug makes numerous hydrophobic contacts with MEK1 including residues L115 and L118 within the αC-helix, V127 (Sh1), I141 and M143 before the hinge along with F209 (RS2), V211, L215, I216, M219, and A220 within the activation segment. These hydrophobic contacts closely resemble those described above for cobimetinib. Trametinib also makes van der Waals contact with HRD-H188 and DFG-D208.

The Glide model of cobimetinib binding to MEK2, using 1S9I with bound 5-{3,4-difluoro-2-[(2-fluoro-4-iodophenyl)amino] phenyl}-*n*-(2-morpholin-4-ylethyl)-1,3,4-oxadiazol-2-amine as the starting point, reveals that the methanone carbonyl group of the drug forms a hydrogen bond with β3-K101. Moreover, the azetidinyl 3-hydroxy group forms two hydrogen bonds with the γ-phosphoryl oxygen of ATP and the azetidinyl N—H forms a hydrogen bond with the HRD-D194 (Fig. 7C). The drug makes hydrophobic contacts with L119 adjacent to αC-E118, V131 (Sh1), I145 before the hinge, C211 immediately before the activation segment along with DFG-F213, V217 (Sh1), L219, and M223 of the activation segment. Cobimetanib makes van der Waals contacts with N82 of the G-rich loop, I103 in the αC-helix, DFG-D212, and S216 within the activation segment. The pattern of hydrogen bonding seen here mimics that observed with the drug binding to MEK1, but the patterns of hydrophobic and van der Walls interactions differ somewhat.

The induced-fit pose [48] of trametinib binding to MEK2, using 1S9I as the initial structure, reveals that its 2-pyrido[4,3-*d*] pyrimidine carbonyl group forms a hydrogen bond with β3-K101 and the 7- pyrido[4,3-*d*] pyrimidine carbonyl group forms hydrogen bonds with the V215 and S216 N—H groups within the activation segment (Fig. 7D). Furthermore, the acetamide carbonyl group forms a hydrogen bond with the N—H group of S226 within the activation segment. Trametinib makes numerous hydrophobic contacts with MEK2 including I103 within the β3-loop, L119 and L122 within the αC-helix, V131 (Sh1), I145 and M147 before the hinge, C211 before the activation segment plus F213, V215, L219, I220, M223, A214, and N225 within the activation segment. The drug also makes van der Waals contact with N82 within the G-rich loop and DFG-D212. The hydrogen-binding pattern of trametinib to MEK2 closely resembles that of its binding to MEK1, but the hydrophobic and van der Waals interactions differ marginally.

Most FDA-approved protein kinase antagonists are steady-state competitive inhibitors with respect to ATP and bind within the ATP-binding site [33]. Such medicinals often interact with catalytic spine residues CS6/7/8 and Sh1. Cobimetinib and trametinib are type III allosteric inhibitors of MEK1/2 based upon their binding adjacent to the ATP-binding site [33]; these drugs are steady-state non-competitive inhibitors with respect to both ATP or a protein substrate. Such inhibitors are unable to interact with catalytic spine residues. The present examples indicate that they interact with residues within the G-rich loop, the β3-lysine, residues within the αC-helix, the Sh1 residue within the αC–β4 (back) loop, the β5-strand, and several residues within the activation segment. Because the region adjacent to the ATP-binding site displays greater diversity than the ATP-binding site per se, type III allosteric effectors are expected to have greater target specificity with fewer off-target effects [49]. Moreover, these drugs bind to their targets without competing with millimolar concentrations of ATP, a property which is an additional therapeutic advantage that classical ATP-competitive inhibitors lack. As a consequence, type III inhibitors do not necessarily have to possess low nanomolar affinity to be effective. However, the affinities of trametinib and cobimetinib for MEK1/2 are less than 5 nM.

Of mechanistic importance, trametinib and cobimetinib stabilize the inactive conformation of MEK1/2 in which the αC-helix is displaced; this stabilization results in the misalignment of the K/E/D/D catalytic residues and enzyme inhibition. Moreover, the activation segment in the drug-enzyme complexes occurs in its closed and less active conformation that occludes the peptide/protein binding site. Moreover, both drugs promote the dissociation of Raf-MEK1/2 complexes [8,50,51]. Trametinib blocks MEK1 and MEK2 phosphorylation as catalyzed by Raf, while cobimetinib does not antagonize this reaction. Trametinib forms

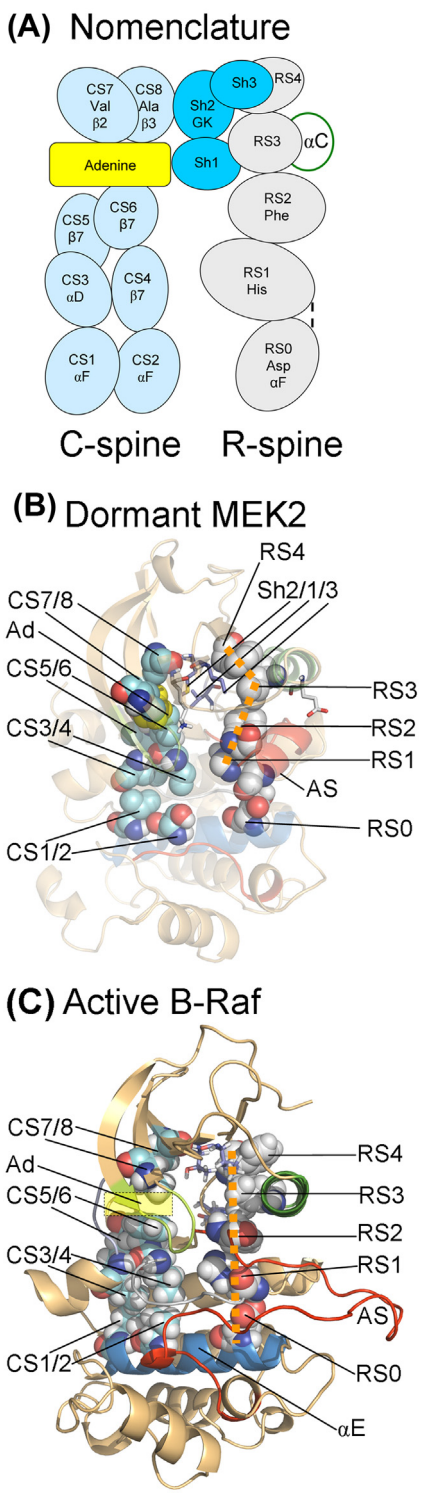

Fig. 6. Catalytic and regulatory spine structures. (A) Catalytic spine (CS), regulatory spine (RS), and shell (Sh) residue labels as observed from the classical frontal view of protein kinases. (B) Spines of dormant human MEK2 (PDB ID: 1S9I). Orange dashes indicate the non-linearity of the R-spine. (C) Spines of active human B-Raf (PDB ID: 4MNE). Orange dashes indicate the linearity of the R-spine. Ad, adenine; AS, activation segment.

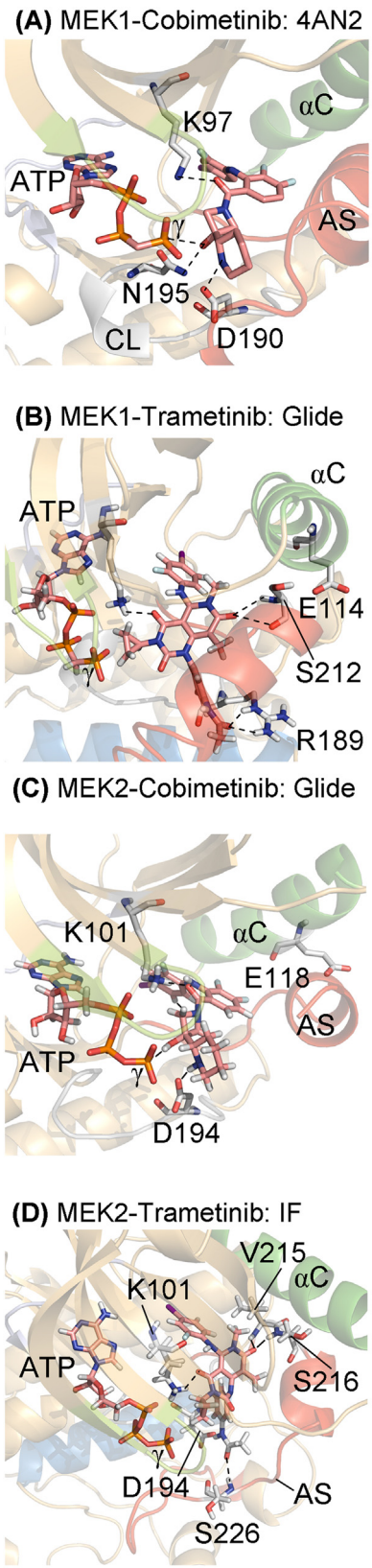

Fig. 7. Binding of cobimetinib (A) and trametinib (B) to human MEK1. Binding of cobimetinib (C) and trametinib (D) to human MEK2. Dashed lines indicate polar bonds. The Schrödinger program Glide [47] (Version 2016-1) was used to prepare B and C and the Schrödinger program Induced Fit (IF) [48] (Version 2016-1) was used to prepare D. AS, activation segment; CL, catalytic loop.

Table 3
Properties of selected small molecule MAP kinase pathway inhibitors approved and in clinical trials.[a]

Name, code, trade name®	Targets	PubChem CID	Formula	MW (Da)	D/A[b]	cLogP[c]	FDA-approved indications
Vemurafenib, PLX-4032, Zelboraf®	B-Raf V600E	42611257	$C_{23}H_{18}ClF_2N_3O_3S$	489.9	2/7	5.1	B-Raf mutant melanoma (2011)
Dabrafenib, GSK2118436, Tafinlar®	B-Raf V600E/K	44462760	$C_{23}H_{20}F_3N_5O_2S_2$	519.6	2/11	4.3	B-Raf mutant melanoma (2013)
Trametinib, GSK1120212, Mekinist®	MEK1/2	11707110	$C_{26}H_{23}FIN_5O_4$	615.4	2/6	3.4	B-Raf V600E/K mutant melanoma (2013) as monotherapy and in combination with dabrafenib (2014)
Cobimetinib, GDC-0973, Cotellic®	MEK1/2	16222096	$C_{21}H_{21}F_3IN_3O_2$	531.3	3/7	3.9	B-Raf V600E/K mutant melanoma together with vemurafenib (2015)
Selumetinib, AZD6244	MEK1/2	10127622	$C_{17}H_{15}BrClFN_4O_3$	457.7	3/6	3.6	Not approved
Bimetinib, MEK162	MEK1/2	10288191	$C_{17}H_{15}BrF_2N_4O_3$	441.2	3/7	3.1	Not approved

[a] clinicaltrials.gov.
[b] No. of hydrogen bond donors/acceptors.
[c] Calculated log of the partition coefficient as determined by MedChem Designer® v.1.0.1.15.

strong hydrogen bonds with the MEK1/2 S212/216 phosphorylation sites whereas cobimetinib does not, which may explain the ability of the former to block MEK1/2 phosphorylation.

5. Epilogue

5.1. Classification of targeted small molecule protein kinase inhibitors

The mode of binding of each medicinal agent with its protein kinase target is unique. Nonetheless, it is advantageous to classify these binding modalities in order to relate such interactions with the drug development process. We have previously classified targeted protein kinase antagonists into six possible types based upon the structures of the drug-kinase complexes [33] following the lead of previous authors [52–55]. Type I drugs bind to the active structure of the enzyme with (i) an open activation segment, (ii) the αC-helix and the DGF-Asp directed inward, and (iii) a linear R-spine. The type I½ drugs bind to a less active structure with a closed activation segment, a non-linear R-spine, or both, but with the DGF-Asp directed inward [33]. We subdivided this group into type I½A drugs, which extend into the back cleft, and type I½B drugs, which do not extend into the back cleft [45]. Initial information indicates that the type A drugs are characterized by an extended residence time while the type B drugs are not [33].

The type II drugs interact with enzyme forms with the DFG-Asp directed away from the active site, which corresponds to a dormant structure [56]. The αC-RS3 residue is shifted away from the RS2 and RS4 residues [33]. The type IIA drugs occur in the front cleft, the gate area, and they extend into the back pocket while the type IIB drugs occur within the front cleft and gate area, but they do not extend into the back pocket. Both type III and IV inhibitors are allosteric antagonists. FDA-approved type III inhibitors including trametinib and cobimetinib bind adjacent to the ATP-binding site. In contrast, the type IV inhibitors bind outside of the ATP-binding site, but not adjacent to it. For example, GNF-2 is a non-FDA approved BCR-Abl antagonist that binds to the myristate-binding site between the αE and αF helices within the carboxyterminal lobe and is thus a type IV inhibitor [57]. Three drugs classified as type IV inhibitors that have been approved by the FDA include everolimus, sirolimus, and temsirolimus. These medicinals form a complex with FK-binding protein-12 (FKBP-12)and the complex then blocks the action of the mammalian target of rapamycin (mTOR) complex-1 (mTORC1) [9]. mTOR is a protein-serine/threonine kinase that is related to the family of PI3 kinase-related kinases (PIKKs). The type V drugs span two different protein kinase regions and the type VI inhibitors bind covalently to the target protein [33]. About half of the FDA-approved drugs bind to the active conformation of their target protein kinase and are thus type I inhibitors [33,41].

In addition to the type III medicinals that target MEK1/2, several other type III agents that target other protein kinases are in development [49]. For example, several potential type III antagonists are directed against protein-serine threonine kinases including PKB, CDKs, LIMK, PKCζ, PDK1, RIP1, PAK, IRE1, WNK, and CHK1. Other likely type III antagonists are directed against protein-tyrosine kinases including Abl, FAK, ITK, and IGF-1R. Furthermore, allosteric inhibitors of PI3 kinase, mTOR, and IKK have been described. MK-2206 (also known as LY294002) is a PKB inhibitor, which is not FDA approved, but is in more than four dozen clinical trials (www.clinicaltrials.gov). It is being studied as a monotherapy in acute myeloid leukemia, endometrial and stomach cancers, and lymphomas. It is also being studied as part of combination regimens for the treatment of chronic lymphocytic leukemias, non-small cell lung cancers, melanomas, and breast, kidney, and pancreatic carcinomas. See ref. [49] for a review of the status of allosteric protein kinase inhibitors.

5.2. MEK1/2 inhibitors in clinical trials

Owing to the activation of the MAP kinase pathway in about one-third of all malignancies, trametinib and cobimetinib are in clinical trials for the treatment of a variety of malignancies (www.clinicaltrials.gov). For example, trametinib (also known as GSK-1120212 and JTP-74057 [58]) is being evaluated as a monotherapy for the treatment of multiple myeloma and prostate cancers. It is also being evaluated in combination with targeted agents or cytotoxic agents in the treatment of acute myeloid leukemias, breast, cervical, colorectal, and follicular thyroid cancers as well as gastrointestinal stromal tumors, melanomas, and non-small cell lung cancers. Moreover, cobimetinib (also known as GDC-0973 and XL518 [59]) is being evaluated for the treatment of acute myeloid leukemias, melanomas, and non-Hodgkin lymphomas as well as breast, colorectal, and non-small cell lung carcinomas. Because *KRAS*-mutant tumors including those of non-small cell lung cancer represent an important clinical need, there is considerable interest in using MEK1/2 inhibitors such as trametinib as part of drug combination therapies in the treatment of disorders with these mutations.

Selumetinib (Fig. 2E) and binimetinib (Fig. 2F) are two other MEK1/2 inhibitors that are in clinical trials. For example, selumetinib (also known as AZD6244 and ARRY-142886 [60]) is being tried

as a monotherapy for the treatment of acute myeloid leukemias, diffuse large cell lymphomas, and gliomas (www.clinicaltrials.gov). The drug is also being evaluated as part of combination regimens for the treatment of breast, colorectal, pancreatic, stomach, and thyroid cancers as well as melanomas. Binimetinib (also known as MEK162 and ARRY- 438162 [61]) is under evaluation as part of combination regimens for the treatment of acute myeloid leukemias, gliomas, melanomas, and gastrointestinal stromal tumors as well as *KRAS* mutant non-small cell lung cancer and multiple myeloma along with biliary, colorectal, and ovarian cancers. Table 3 provides additional information about drugs targeting the MAP kinase pathway.

Additional clinical trials with these four targeted drugs in combination with cytotoxic agents, monoclonal antibodies, and small molecule agents targeting the MAP kinase and other signaling modules including that of PI3 kinase are either being planned or are underway. For example, studies combining trametinib with dabrafenib, erlotinib, pazopanib, everolimus, GSK2141795 (a PKB inhibitor), GSK2256098 (a focal adhesion protein kinase inhibitor), navitoclax (a BCL-2 inhibitor), BKM120 (a PI3 kinase inhibitor), 5-fluorouracil (a cytotoxic agent), or radiation therapy are underway [21]. A half dozen other MEK inhibitors are in various stages of drug development [49]. All of these antagonists possess a narrow spectrum of clinical activity being most effective in *BRAF*-mutant and *NRAS*-mutant melanomas [21]. However, there have been suggestions of activity in hepatic biliary and serous ovarian cancers. Except for *BRAF* and *NRAS* mutations, there are no other biomarkers correlated with treatment responses following MEK1/2 inhibition and the discovery of such biomarkers would represent an important therapeutic breakthrough.

Conflict of interest

The author is unaware of any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this review.

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