ERK1/2 inhibitors: new weapons to inhibit the RAS-regulated RAF-MEK1/2-ERK1/2 pathway

Andrew M. Kidger, James Sipthorp and Simon J. Cook

Signalling Programme, The Babraham Institute, Babraham Research Campus, Cambridge, CB22 3AT, England.

Correspondence

andrew.kidger@babraham.ac.uk

simon.cook@babraham.ac.uk

Abstract

The RAS-regulated RAF-MEK1/2-ERK1/2 signalling pathway is de-regulated in a variety of cancers due to mutations in receptor tyrosine kinases (RTKs), negative regulators of RAS (such as NF1) and core pathway components themselves (RAS, BRAF, CRAF, MEK1 or MEK2). This has driven the development of a variety of pharmaceutical agents to inhibit RAF-MEK1/2-ERK1/2 signalling in cancer and both RAF and MEK inhibitors are now approved and used in the clinic. There is now much interest in targeting at the level of ERK1/2 for a variety of reasons. First, since the pathway is linear from RAF-to-MEK-to-ERK then ERK1/2 are validated as targets *per se*. Second, innate resistance to RAF or MEK inhibitors involves relief of negative feedback and pathway re-activation with all signalling going through ERK1/2, validating the use of ERK inhibitors with RAF or MEK inhibitors as an up-front combination. Third, long-term acquired resistance to RAF or MEK inhibitors involves a variety of mechanisms (KRAS or BRAF amplification, MEK mutation, etc.) which re-instate ERK activity, validating the use of ERK inhibitors to forestall acquired resistance to RAF or MEK inhibitors. The first potent highly selective ERK1/2 inhibitors have now been developed and are entering clinical trials. They have one of three discrete mechanisms of action – catalytic, 'dual mechanism' or covalent – which could have profound consequences for how cells respond and adapt. In this review we describe the validation of ERK1/2 as anti-cancer drug targets, consider the mechanism of action of new ERK1/2 inhibitors and how this may impact on their efficacy, anticipate factors that will determine how tumour cells respond and adapt to ERK1/2 inhibitors and consider ERK1/2 inhibitor drug combinations.

Keywords

BRAF, Cancer, ERK, Inhibitors, MEK, RAS

Abbreviations

Akt, oncogene in the transforming retrovirus, AKT8; AP-1, activator protein 1; ARAF, v-raf murine sarcoma 3611 viral oncogene homologue; ASK1, Apoptosis signal-regulating kinase 1; BCL2, B-cell lymphoma 2; BCL-X_L, B-cell lymphoma extra large; BRAF, v-raf murine sarcoma viral oncogene homologue B1;BTK, Bruton's tyrosine kinase; CD, common docking domain; CDK, cyclin-dependent kinase; C/EBP-β, CCAAT/enhancer-binding protein *beta; COSMIC*, Catalogue Of Somatic Mutations In Cancer; CRAF, v-raf-1 murine leukaemia viral oncogene homologue 1; DRS, D-recruitment site; DUSP, dual-specificity phosphatase; EGF, Epidermal growth factor; EGFR, epidermal growth factor receptor; elK1, ELK1, ETS-like gene 1; ERK, Extracellular signal-regulated kinase; ERKi, ERK inhibitor (see also MEKi, RAFi, etc); ETS, E26 transformation-specific; E2F, E2 transcription factor; FRS, F-recruitment site; HER2, human epidermal growth factor receptor 2; HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homologue; GAP, GTPase activating protein; IC_{50}, concentration causing 50% inhibition; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue; LE, ligand efficiency; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MCL1, myeloid cell leukaemia 1; MEK, MAPk or ERK kinase; MKK, MAP kinase kinase; MKP, MAP Kinase phosphatase; MOA, mechanism of action; MST2, Mammalian STE20-like protein kinase 2; MTD, maximum tolerated dose; MTOR, mechanistic target of rapamycin; NF1, neurofibromin gene product, mutated in type 1 neurofibromatosis; NRAS, neuroblastoma RAS viral (v-ras) oncogene homologue; PAINS, pan-assay interference compound; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PPI, protein-protein interaction; PROTAC, proteolysis targeting chimera; RAL, resorcyclic acid lactone; RB, retinoblastoma; RSK, ribosomal protein S6 kinase; RTK, receptor tyrosine kinase; SAR, structure-activity relationship; SOS, son-of-sevenless; SPRY, Sprouty; TCO, *trans*-cyclooctene; VEGFR, vascular endothelial growth factor receptor.

Contents

2. The ERK1/2 signalling pathway
3. Validation of the ERK1/2 pathway and ERK1/2 as targets in cancer
4. Experiences of RAF and MEK1/2 inhibitors
5. Small molecule inhibitors of ERK1/2 activity
    a. Reversible, ATP-competitive ERK1/2 inhibitors
    b. Covalent ERK1/2 inhibitors
    c. Allosteric ERK1/2 inhibitors
6. Prospects for ERK1/2 inhibitors as monotherapy and in combination
7. Conclusions

1. Introduction

The RAS-regulated RAF-MEK1/2-ERK1/2 signalling pathway is activated by a range of extracellular stimuli including growth and differentiation factors, cytokines, hormones and neuropeptides acting through their cognate receptors (receptor tyrosine kinases, cytokine receptors, G-protein coupled receptors, etc). This pathway is perhaps best known for its prominent role in controlling gene expression (Nabet et al., 2015; Yang, Sharrocks, & Whitmarsh, 2013). Activation of the terminal kinases in the cascade, ERK1 and ERK2, results in their accumulation in the nucleus where they phosphorylate various sequence-specific transcription factors to stimulate or repress gene expression. In this way activation of the ERK1/2 pathway can promote a range of cellular responses; notably, ERK1/2 signalling promotes cell cycle progression and cell division but can also drive cell cycle exit, differentiation and irreversible cell senescence (Meloche & Pouysségur, 2007). These opposing cell fates are made possible by pathway properties and cellular context; for example, the magnitude and duration of ERK1/2 activation, which is determined by feedback controls, can dictate whether a cell proliferates or undergoes cell cycle arrest (Blüthgen & Legewie, 2008) whilst the consequences of ERK1/2 signalling may also be determined by the activation of coincident parallel signalling pathways (Mendoza, Er, & Blenis, 2011). ERK1/2 signalling is by no means confined to the nucleus and ERK1/2 can also target substrates outside the nucleus to control metabolism and cell survival (Cook, Stuart, Gilley, & Sale, 2017). Indeed, many aspects of cell physiology are regulated by ERK1/2 signalling, from the earliest cell lineage choices during development to decisions to die or survive.

The ERK1/2 signalling pathway has attracted particular attention because it is deregulated in a wide variety of cancers due to activating mutations in growth factor receptors and core pathway components, most notably RAS and BRAF (Montagut & Settleman, 2009). This has driven the development of a variety of pharmaceutical agents to inhibit RAF-MEK1/2-ERK1/2 signalling in cancer and both RAF and MEK

inhibitors (RAFi, MEKi) are now approved and used in the clinic (Caunt, Sale, Smith, & Cook, 2015; Holderfield, Deuker, McCormick, & McMahon, 2014). There is now much interest in targeting the pathway at the level of the terminal kinases, ERK1 and ERK2 (ERK1/2). ERK1/2 inhibitors (ERKi) will likely be used in combination to target tumour cells that are refractory to RAFi or MEKi monotherapy or to overcome or forestall acquired resistance to RAFi or MEKi. The first potent and highly selective ERKi have now been developed and are entering clinical trials. In this article we review the validation of ERK1/2 as anti-cancer drug targets, consider the mechanism of action of new ERKis and how this may impact on their efficacy, consider ERKi drug combinations and anticipate factors that will determine how tumour cells respond and adapt to ERKis.

2. The ERK1/2 signalling pathway.

The core ERK1/2 signalling pathway is a three-tier hierarchical protein kinase cascade (Figure 1). ERK1/2 exemplify a family of mitogen-activated protein kinases (MAPKs) which are activated by MAPK Kinases (MAPKKs) which are in turn activated MAPKKKs (Cargnello & Roux, 2011). Like most protein kinases ERK1/2 are synthesised in cells as inactivate zymogens. Activation requires phosphorylation of critical threonine and tyrosine residues in the T-E-Y motif found in the kinase activation loop; T-E-Y phosphorylation has the effect of rearranging the active site to allow the correct alignment of ATP and substrate for catalysis. Phosphorylation of the ERK1/2 T-E-Y motif is catalysed by the dual-specificity protein kinases MEK1 or MEK2 (the MAPKKs), which phosphorylate first the tyrosine and then the threonine in a processive manner (Aoki, Yamada, Kunida, Yasuda, & Matsuda, 2011). MEK1/2 are themselves activated by activation loop phosphorylation at conserved serine residues; this is catalysed by one of several different MAPKKKs including the three RAF protein kinases (ARAF, BRAF and CRAF). Growth factors activate the ERK1/2 pathway by first activating the RAS proteins, small monomeric GTPases that are tethered on the

inner leaflet of the plasma membrane and act as binary switches (Karnoub & Weinberg, 2008). Binding of growth factors to their receptors results in the recruitment of guanine nucleotide exchange factors such as Son-of-Sevenless (SOS) to the plasma membrane where they promote the dissociation of GDP from RAS proteins; the large molar excess of GTP over GDP in cells ensures that GDP is replaced by GTP which switches RAS into its active conformation. RAS-GTP then binds to RAF proteins forming active homo or heterodimers at the plasma membrane, which then phosphorylate MEK1/2, which in turn activate ERK1/2. Whilst the core pathway is frequently represented as a simple linear RAF-MEK1/2-ERK1/2 cascade, efficient signal transmission is dependent on various scaffold proteins, which bring the component kinases together and also serve to insulate the pathway from cross talk by other kinases (Good, Zalatan, & Lim, 2011). In addition to scaffolds, signalling fidelity is made possible by distinct kinase-substrate interactions. For example, ERK1/2 are proline-directed protein kinases, phosphorylating substrates at S/T-P motifs (Ünal, Uhlitz, & Blüthgen, 2017); such motifs are found in thousands of proteins that are not ERK1/2 substrates. Rather, ERK1/2 are directed to phosphorylate *bona fide* substrates by the presence of discrete kinase docking domains that are distinct from the S/T-P phosphoacceptor site (Bardwell, 2006; Cargnello & Roux, 2011).

All reported mammalian cells express two canonical ERK proteins, ERK1 and ERK2, encoded by distinct genes. ERK1 and ERK2 are 84% identical at the amino acid level; in the case of the human proteins ERK1 exhibits a 17 amino acid N-terminal extension and 2 extra amino acids at the C-terminus, making it larger than ERK2. Gene disruption of *erk1 or erk2* in mice has revealed striking phenotypic differences; *erk1-/-* mice live and reproduce normally whereas *erk2* disruption causes early embryonic lethality (reviewed in (Buscà, Pouysségur, & Lenormand, 2016). Numerous studies have sought to define functional differences between ERK1 and ERK2 that might account for these phenotypic differences. Indeed, any such differences would be a very important consideration in the design or search for ERK inhibitors. However,

very few rigorous studies have reported function differences between ERK1 and ERK2 and the emerging consensus is that the two enzymes are functionally redundant, with differences in mouse knockout phenotype reflecting differences in the abundance of ERK1 and ERK2. Studies of the mouse *erk1* and *erk2* proximal promoters suggest that the difference in expression is in part due to the *erk2* promoter being stronger than the *erk1* promoter (Buscà et al., 2015). Finally, mice lacking *erk2* live and reproduce normally when *erk1* is ubiquitously expressed from the chicken β-actin promoter (Frémin, Saba-El-Leil, Lévesque, Ang, & Meloche, 2015). These mice exhibited apparently normal ERK signalling suggesting that the differences in *erk1* and *erk2* knockout phenotype simply reflect the different contributions each isoform makes to global ERK activity (Buscà et al., 2016).

MEK1/2-catalysed phosphorylation results in a significant fraction of ERK1/2 entering the nucleus (Lidke et al., 2010) where it can bind to and phosphorylate transcriptional regulators to control gene transcription; indeed, this is arguably the classic paradigm for ERK1/2 signalling (Figure 1). ERK1/2 phosphorylate a variety of sequence-specific transcription factors, including many members of ETS and AP-1 families (O'Donnell, Odrowaz, & Sharrocks, 2012). In many cases these phosphorylation events promote transcription, through activation of transcriptional activation domains (TADs) or stabilisation of the relevant transcription factor; in other cases ERK1/2 phosphorylation represses transcription. As a result the ERK1/2 signaling is a major pathway by which growth factors, cytokines and hormones control gene expression (Schulze, Lehmann, Jefferies, McMahon, & Downward, 2001). However, ERK1/2 signalling is not confined to the nucleus and ERK1/2 can also target substrates outside the nucleus to control metabolism, mitochondrial fission and cell survival (Balmanno & Cook, 2008; Cook et al., 2017). In this way the ERK1/2 pathway serves as a master regulator of cell fate. Indeed, ERK1/2 signalling is essential for both human and mouse embryonic stem cell maintenance and self-renewal and the

maintenance of ESC genome stability (H. Chen et al., 2015), highlighting a critical role for ERK1/2 signalling in the earliest cell fate decisions.

Given the role of ERK1/2 signalling in control of cell fates it comes as no surprise that the pathway is regulated by a complex array of homeostatic negative feedback controls that fine-tune pathway output. These can broadly be divided into those that are rapid and direct, operating on the minutes timescale and proceed through pre-existing components (shown in red in Figure 1), and those that are slower in onset, depend on new protein synthesis and operate on the hours timescale (shown in green in Figure 1). Rapid direct feedback mechanisms involve direct inhibitory phosphorylation of upstream pathway components by ERK1/2 or by ERK1/2-dependent protein kinases such as RSK. Perhaps the best examples of this are the ERK1/2 catalysed phosphorylation of CRAF (Dougherty et al., 2005), BRAF (Ritt, Monson, Specht, & Morrison, 2010) and MEK1 (Eblen et al., 2004), which inhibits phosphorylation of MEK by the RAF proteins and the phosphorylation of ERK1/2 by MEK1. The net effect is that ERK1/2 activation propagates a negative feedback loop that limits its own activation, whereas loss of ERK activity (for example, by treatment with a RAF or MEK inhibitor) inhibits these feedback loops, thereby activating RAF, MEK and ERK1/2 and allowing the pathway to adapt to perturbations (Sturm et al., 2010). Slower feedback loops that confer regulation in the longer term involve the *de novo* expression of the dual-specificity phosphatases (DUSPs, also known as MAP kinase phosphatases or MKPs) and the Sprouty (SPRY) proteins. The DUSPs inactivate ERK1/2 by dephosphorylating the pT-E-pY motif, providing a temporally delayed ERK-driven feedback loop that is dependent upon ERK-dependent gene expression and so 'reports' on delivery of the ERK1/2 signal to nuclear targets (Kidger & Keyse, 2016). In turn, different DUSPs function in different locations allowing spatial regulation of ERK1/2 output; for example, DUSP5 resides within the nucleus whilst DUSP6 is found in the cytoplasm. SPRY proteins inhibit ERK signalling at the level of

RTKs, SOS and by interfering with the RAF catalytic domain (Masoumi-Moghaddam, Amini, & Morris, 2014).

3. Validation of the ERK1/2 pathway and ERK1/2 as targets in cancer

RAS proteins are mutated at high frequency in human cancer (Bos, 1989). 20-30% of all human cancers have RAS mutations whilst in some diseases the incidence is especially high; for example 80-90% of pancreatic ductal adenocarcinomas have activating mutations in KRAS (Bos, 1989; Karnoub & Weinberg, 2008). These mutations (typically at codons 12, 13 or 61) render the RAS proteins resistant to the activity of GTPase-activating proteins (RAS-GAPs) (Martin et al., 1990; Trahey & McCormick, 1987), which normally terminate RAS signalling by accelerating their otherwise modest GTPase activity. As a result mutant RAS proteins accumulate in their active GTP-bound state in the absence of an inductive signal, thereby driving inappropriate cell proliferation and survival. The discovery of the RAF-MEK-ERK1/2 signalling cascade as the first effector pathway of the RAS proteins (Crews, Alessandrini, & Erikson, 1992; Howe et al., 1992; Karnoub & Weinberg, 2008; Leevers & Marshall, 1992; Warne, Vician, & Downward, 1993) immediately prompted interest from the pharmaceutical sector and quickly led to the discovery of PD98059, an allosteric inhibitor of MEK1/2, as the first selective pathway inhibitor (Dudley, Pang, Decker, Bridges, & Saltiel, 1995). Other MEK inhibitors quickly followed (Caunt et al., 2015) and the availability of these probes quickly led to the recognition that ERK1/2 signalling was a major pathway by which growth factors and RAS drive cell proliferation (Meloche & Pouysségur, 2007).

Despite these early studies there was some delay before mutant forms of RAF, MEK or ERK proteins were identified in human cancer. Prior to this a variety of studies demonstrated that engineered mutant forms of RAF or MEK proteins that were constitutively active could confer malignant transformation upon tissue culture cells whereas inactive dominant interfering forms of RAF, MEK or ERK could inhibit RAS-

dependent transformation. The subsequent demonstration of activating mutations in BRAF (notably $BRAF^{V600E}$) in human cancer (Davies et al., 2002), especially in melanoma but also thyroid, colorectal and more recently hairy cell leukaemia (Tiacci et al., 2011), was a landmark and provided critical validation that core components of the ERK1/2 pathway were subject to disease-driving mutations in human cancer. More recently, activating oncogenic mutations in MEK1 or MEK2 have been described in melanoma, colorectal cancer and lung cancer (Caunt et al., 2015). Although the incidence of activating mutations in MEK1 or MEK2 is much lower than that of RAS or BRAF they may define a subset of lung adenocarcinoma (Arcila et al., 2015) and provide a critical validation that the RAF-MEK-ERK1/2 pathway is a common cancer-driving pathway. In addition, emergent mutations in MEK1 or MEK2 are also seen in tumours that have acquired resistance to BRAF or MEK inhibitors (see below).

Mutations in ERK1 or ERK2 have been reported on the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). In addition, the $ERK2^{E322K}$ mutant has been observed at significant frequency in cervical and head and neck squamous cell carcinomas (Arvind et al., 2005; Lawrence et al., 2014; Ojesina et al., 2014). This mutant maps to the D-recruitment site or common docking domain and may exhibit more sustained activity due to defects in DUSP binding. However, it is apparent that primary disease driving mutations in ERK1 or ERK2 tend to be rarer than BRAF or even MEK1/2 mutations. Despite this, ERK1/2 remain very well validated targets themselves for a variety of critical reasons. First, the pathway topology suggests that signalling proceeds in a linear fashion, namely RAF->MEK1/2->ERK1/2, and the overwhelming body of evidence supports this contention. ERK1 and ERK2 exhibit >80% sequence identity (Boulton et al., 1991; Buscà et al., 2016) and are the only known substrates for the dual threonine/tyrosine kinase activity of MEK1/2. For example, despite the kinase domain ERK5 being highly related to ERK1 and ERK2 (66% sequence identity) and including the same T-E-Y motif within its activation loop, MEK1 fails to bind to ERK5 (Zhou, Bao, & Dixon, 1995) and fails to activate ERK5 in

cells (Gilley, March, & Cook, 2009). Indeed, more than 20 years on from their discovery there is no convincing evidence that MEK1 or MEK2 serve any function other than to activate ERK1/2, a contention supported by the similar early embryonic lethality and placental disruption observed in Mek1-/- mice (Giroux et al., 1999) and Erk2-/- mice (Saba- El- Leil et al., 2003). Much the same can be argued for the RAF proteins. Wild type RAF proteins act as RAS-dependent homo- or heterodimers (Freeman, Ritt, & Morrison, 2013) and can activate their dimer partners *in trans* whilst the common $BRAF^{V600E}$ mutant is constitutively active and acts as a monomer; however, in both cases the only known and universally accepted catalytic function of activated RAF proteins is to phosphorylate and activate MEK1 and MEK2. The significance of interactions between RAF proteins and components of other signalling pathways such as MST2 (Nguyen, Matallanas, Romano, Kholodenko, & Kolch, 2015) or ASK1 (J. Chen, Fujii, Zhang, Roberts, & Fu, 2001) in cancer is at present unclear. Below ERK1/2, signalling diverges to hundreds of ERK1/2 substrates and interacting proteins (Carlson et al., 2011; Courcelles et al., 2013; Kriegsheim et al., 2009; Kubiniok, Lavoie, Therrien, & Thibault, 2017) that each mediate a subset of the global effects of ERK1/2 signalling. Whilst some of these ERK1/2 substrates are kinases themselves, and therefore druggable, the effect of targeting these components is limited to a subset of ERK1/2-dependent events and prcesses and this inevitably limits efficacy. Indeed, ERK1/2 can be viewed as playing a pivotal distributive role in the pathway, receiving information from MEK1/2 and then passing it on to hundreds of targets. Thus, the core RAF-MEK1/2-ERK1/2 pathway can be viewed as a critical bottleneck with inhibition of ERK1/2 signalling achievable by targeting at the level of RAFs, MEK1/2 or ERK1/2. Second, innate resistance to clinically approved RAFi or MEKi arises through relief of negative feedback and ERK1/2 pathway re-activation, validating the use of ERKi with RAFi or MEKi as an up-front combination (see section 4 below). Third, long-term acquired resistance to RAFi or MEKi arises through a variety of mechanisms but the majority of these re-instate ERK1/2 activity validating the use of ERKi to forestall or

overcome acquired resistance to RAFi or MEKi (Caunt et al., 2015; A. S. Little, Smith, & Cook, 2013) (See below).

4. Experiences of RAF and MEK1/2 inhibitors

The first ERK1/2 pathway inhibitor to be clinically approved was the RAFi sorafenib, which is now used in the treatment of renal cell carcinoma, liver and thyroid cancer, though its efficacy is thought reflect its significant activity against several RTKs (Wilhelm et al., 2004). The real breakthrough in ERK1/2 pathway therapeutics came with the description of the BRAF inhibitor (BRAFi) vemurafenib, which emerged from a structure-based drug design process and exhibits selectivity for the mutationally activated forms of BRAF, especially the commonest $BRAF^{V600E}$ mutant, that is particularly abundant in melanoma (Joseph et al., 2010). Indeed, $BRAF^{V600E}$-positive melanoma cells are acutely addicted to their driving oncoprotein and ERK1/2 signalling, such that BRAFis like vemurafenib cause striking inhibition of melanoma cell proliferation and tumour regression in the clinic (Bollag et al., 2010). This led to the rapid clinical approval of vemurafenib, and subsequently dabrafenib, both of which have transformed the treatment of melanoma driven by $BRAF^{V600}$ mutants (Holderfield et al., 2014). The high incidence of $BRAF^{V600E}$ mutations in hairy cell leukaemia suggests that BRAFis may hold promise in this disease also (Tiacci et al., 2015). However, the striking efficacy of BRAFis is very much limited to $BRAF^{V600}$-mutant tumours where the mutant oncoprotein signals as a monomer. In cells with wild type BRAF, which signals as dimer, binding of BRAFi to one protomer elicits conformational changes in the dimer partner that result in its activation, driving MEK1/2 and ERK1/2 activation (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Holderfield et al., 2013; Poulikakos, Zhang, Bollag, Shokat, & Rosen, 2010). This paradoxical RAF activation requires RAS-dependent RAF dimerization and so is especially profound in cells with activating mutations in RAS, upstream components (RTKs) or negative regulators of RAS (NF1). A consequence of this paradoxical RAF activation is the development of

a range of secondary cutaneous lesions, including papillomas, squamous cell carcinomas and basal cell carcinomas, all of which limit the efficacy of BRAFi monotherapy to those cells harbouring $BRAF^{V600}$ mutants (Holderfield et al., 2014).

The very strong ERK1/2 pathway addiction observed in $BRAF^{V600E}$ melanoma has meant that MEKis are also effective, with the result that the MEKis trametinib and cobimetinib are both approved for the treatment of this disease (Caunt et al., 2015). However, because MEKis are not selective for mutant forms of MEK1/2 (as is the case for vemurafenib and $BRAF^{V600E}$) and MEK1 and MEK2 are more rarely mutated in cancer there is a less profound therapeutic index; as a result the efficacy of MEKi monotherapy is currently limited by toxicity in normal tissue. This toxicity is mitigated by combination with BRAFi. Indeed, the recognition that acquired resistance to BRAFi most frequently involves re-activation of MEK1/2-ERK1/2 signalling quickly led to the development of BRAFi+MEKi combination therapies (Long et al., 2014) and cobimetinib is actually approved as part of a BRAFi combination. At least three different BRAFi+MEKi combinations have been tested and in all cases provide improved response rates and improved progression free survival and overall survival with fewer side effects (Queirolo, Picasso, & Spagnolo, 2015). This increased therapeutic index reflects two unique aspects of the drug combination. First, the drugs synergise to cause a more profound and durable inhibition of ERK1/2 signalling in $BRAF^{V600E}$ tumour cells. Second, they are thought to antagonise each other in normal cells with wild type BRAF; this is because the MEKi counters the paradoxical activation of ERK1/2 by BRAFi, whilst the paradoxical effects of BRAFi counter ERK1/2 inhibition by MEKi (Caunt et al., 2015; Holderfield et al., 2014). As a result, the combination has now become the standard of care in patients with $BRAF^{V600}$-mutated advanced melanoma (Holderfield et al., 2014). That said, the experience with BRAFi or MEKi monotherapy and with BRAFi+MEKi combination therapy is that $BRAF^{V600}$-mutated tumours will eventually adapt and acquire resistance (Ahronian et al., 2015). In the vast majority of cases resistance involves reactivation of the ERK1/2 pathway through

a variety of different mechanisms (Ahronian et al., 2015; A. S. Little et al., 2013). These include: amplification of genes encoding mutant RAS proteins or $BRAF^{V600E}$ (Corcoran et al., 2010; Annette S. Little et al., 2011); the emergence of BRAF splice variants (Poulikakos et al., 2011); a switch to other RAF isoforms (Villanueva et al., 2010) or other MEK activators (Johannessen et al., 2010); or the emergence of MEK1/2 mutations (Ahronian et al., 2015; A. S. Little et al., 2013). In these cases there is a strong rationale for inhibiting at the level of ERK1/2 to forestall or overcome acquired resistance.

RAFis are not appropriate for use as monotherapy in tumours driven by mutations in RAS or components upstream of RAS since they promote RAS-dependent paradoxical activation of RAF and so fail to inhibit, and may even promote ERK1/2 signalling. This is not an issue with MEKi and inhibition of MEK1/2 remains an attractive option in RAS-mutant or RAS-dependent tumours (Caunt et al., 2015). Most clinical trials with MEKi monotherapy in solid tumours have reported clear but modest response rates. A notable exception is the durable response rate to the MEKi selumetinib in paediatric neurofibromatosis type 1 (NF1), a disease associated with loss of RAS-GAP activity and increased activation of wild type RAS (Dombi et al., 2016). However, it is increasingly apparent that MEKis may require combination with other agents to fulfil their potential (Caunt et al., 2015) and one important combination option is dual intra-pathway inhibition through combination with a RAFi or ERKi. One major limitation of MEKi monotherapy, especially in tumours with mutant RAS, is the rapid rebound in pathway activation. The ERK1/2-dependent feedback loops that inhibit MEK1 and the RAF proteins are lost when cells are treated with MEKi with the result that RAF is reactivated. Since cells typically only utilise a small fraction of their total MEK1/2 and ERK1/2 this means there is considerable spare MEK1/2 that can be activated following loss of feedback and which will reinstate or sustain ERK1/2 signalling. This pathway reactivation following loss of feedback (or feedback relief) is much reduced in cells with $BRAF^{V600E}$ (Friday et al., 2008; Pratilas et al., 2009), which

signals as a monomer and although still subject to ERK1/2-dependent phosphorylation (Ritt et al., 2010) appears to be refractory to the inhibitory effects of this ERK1/2-depndent phosphorylation. However, ERK1/2 rebound is particularly robust in cells with mutant RAS and therefore limits the durability of pathway inhibition and clinical response.

In summary, the lessons learnt from BRAFi and MEKi are that durable ERK1/2 pathway shutdown can be achieved in the case of $BRAF^{V600}$-mutant melanoma and this can translate into striking clinical responses. However, these responses are usually short-lived (6-9 months) due to the emergence of acquired resistance which typically involves reinstating MEK1/2-ERK1/2 signalling. In cells with wild type BRAF, including those with RAS mutation, MEKi elicits only transient pathway inhibition that quickly (hours or a few days) rebounds, limiting primary efficacy. In both cases dual pathway inhibition is indicated, validating ERKi as a rational combination with BRAFi or MEKi.

5. Small molecule inhibitors of ERK1/2 activity

Protein kinase catalytic domains share a common structure characterised by the amino- and carboxy-terminal kinase lobes connected by a hinge region (Jianming Zhang, Yang, & Gray, 2009). The ATP binding pocket is located in a cleft between the two lobes adjacent to the hinge region, forming a highly druggable cavity which has been the focus of most kinase inhibitor development (Müller, Chaikuad, Gray, & Knapp, 2015). However, kinase inhibitors can vary based on the activation status of the kinase that is preferentially recognised (active or inactive), the mechanism of binding (reversible or covalent) and the binding site (ATP-binding pocket or allosteric) (Gross, Rahal, Stransky, Lengauer, & Hoeflich, 2015). Therefore, kinase inhibitors have been categorised based on their binding mode.

All kinases have a conserved Asp-Phe-Gly (DFG) motif at the N terminus of the activation loop, which is important for regulating kinase activity. When a kinase is

active the DFG phenylalanine sits in the hydrophobic pocket between the N- and C-lobes of the kinase, bringing the DFG aspartate into position to coordinate magnesium at the active site. Kinase inactivation can occur if the phenylalanine moves out of the hydrophobic pocket, disrupting the orientation of the aspartate and in some cases sterically blocking ATP binding (Jianming Zhang et al., 2009). Type I kinase inhibitors are ATP-competitive compounds that target the ATP-binding pocket when the activation loop is in an active, “DFG-in”, conformation and mimic the hydrogen bonding normally formed by the adenine ring of ATP. In contrast, type II kinase inhibitors recognise, or induce the formation of, an inactive, “DFG-out” conformation of the active site, thus trapping the kinase in its inactive conformation. Furthermore, this “DFG-out” conformation exposes an extended, diverse binding pocket consisting of an additional hydrophobic region directly adjacent to the ATP binding site (Jianming Zhang et al., 2009). Due to this increased structural diversity it has been postulated that type II inhibitors may have improved selectivity within the kinome, although the evidence for this is less clear (Zhao et al., 2014).

Instead of targeting the highly-conserved ATP-binding pocket, kinase inhibitors can be targeted to other less-conserved allosteric pockets on the target kinase. Allosteric kinase inhibitors can be classified as either type III inhibitors, which bind in a pocket adjacent to the ATP-binding site in a mechanism that does not compete with ATP binding, but may displace it, or type IV inhibitors, which bind to an allosteric site distinct from the substrate/ATP-binding sites (Wu, Clausen, & Nielsen, 2015). Other classes of kinase inhibitors include bivalent type V inhibitors that combine elements from type I–IV inhibitors (Lamba & Ghosh, 2012), and covalent inhibitors which are able to form irreversible covalent bonds to the kinase active site, typically by reacting with a nucleophilic cysteine residue (Bauer, 2015).

Within the last 15 years, many small molecule inhibitors of ERK1/2 activity have been described in the literature and some of these have progressed into clinical trials **(Table 1)**. Based on their mechanism of action, these ERKis can be classified into four

distinct groups, which might have significant consequences for how cells respond and adapt. The majority of the ERKis in development target ERK1/2's catalytic activity in a reversible, ATP-competitive manner. These reversible catalytic ERKis can be further stratified based on their ability to interfere with the activating phosphorylation of ERK1/2 at its pT-E-pY motif by MEK1/2. Catalytic ERKis solely possess the ability to inhibit ERK1/2 catalytic activity, whereas "dual mechanism" ERKis are characterised by their ability to antagonise T-E-Y phosphorylation and thus prevent the formation of the active conformation of ERK1/2, in addition to inhibiting ERK1/2 catalytic activity. Additionally, some covalent ERKis are in development; these inhibitors also target the ERK1/2 active site but are characterised by a prolonged duration of activity due to the formation of an irreversible covalent bond to the target site. Finally, a range of approaches have been published attempting to generate allosteric ERKis, which selectively modulate the ability of ERK1/2 to interact with its binding partners, and thus enabling more precise regulation of ERK1/2 activity or ERK1/2 biological effects.

(a) Reversible, ATP-competitive ERK1/2 inhibitors

In 2005 Astellas Pharmaceuticals published the first report of a small molecule ERKi. FR180204 **(1 – Table 1)** was identified from an in-house chemical library utilising a high-throughput kinase screen to identify compounds capable of inhibiting ERK1/2-catalysed phosphorylation of myelin basic protein (MBP) (Ohori et al., 2005). FR180204 inhibited the *in vitro* kinase activity of ERK1 and ERK2 with IC_{50} values of 0.51 µM and 0.33 µM respectively, and was shown to be very selective against ERK1/2 over a group of other kinases including MEK1, with the exception of the structurally-related MAPK p38α (IC_{50} 10 µM). The co-crystal structure of FR180204 bound to ERK2 revealed that the compound targeted the ATP-binding pocket and this evidence, together with Lineweaver–Burk analysis of the binding interaction, demonstrated that FR180204 acted as an ATP-competitive inhibitor of ERK1/2.

The first nanomolar potency inhibitors of ERK1/2 were a pyrrole-based series from Vertex Pharmaceuticals which were developed through structure-guided optimisation (Aronov et al., 2007). This work led to the publication of VTX-11e **(5 – Table 1)** a highly selective, type I kinase inhibitor with potent cellular activity against ERK1/2 (Aronov et al., 2009). Further development of this compound series lead to the generation of BVD-523 **(8 – Table 1)** (ulixertinib - Biomed Valley Discoveries), a reversible, ATP-competitive small-molecule ERK1/2 kinase inhibitor which has generated promising results in Phase 1 clinical trials (ClinicalTrials.gov Identifier: NCT01781429) (Germann et al., 2015; B. T. Li et al., 2017).

Following the development of VTX-11e/BVD-523 a range of other reversible, ATP-competitive ERKi have been published including SCH772984 (**6** - Merck) (Morris et al., 2013), an unnamed tetrahydropyrrolo-diazepenones series (**10** - Novartis) (Bagdanoff, Jain, Han, Poon, et al., 2015; Bagdanoff, Jain, Han, Zhu, et al., 2015), GDC-0994 (**7** – Genentech) (Blake et al., 2016) and an unnamed pyrrolopyrazinone-based series (**16** - AstraZeneca) (Ward et al., 2017). In addition to BVD-523, GDC-0994 and MK-8353/SCH900353 (a derivative of SCH772984 selected for clinical development), undisclosed compounds from Eli Lily (LY3214996) and Novartis (LTT462) have reached phase I clinical trials.

Of these compounds perhaps the most unique is SCH772984, as this compound was shown to effectively inhibit both ERK1/2 catalytic activity as well as its phosphorylation by MEK1/2 on its activating pT-E-pY motif (Morris et al., 2013). This dual mechanism of action demonstrated by SCH772984 clearly contrasted with that of the previously disclosed ERKi VTX-11e, which in cell-based assays was only able to inhibit ERK1/2 catalytic activity (measured by the phosphorylation of the ERK1/2 substrate p90RSK), and not the phosphorylation of ERK1/2 itself. Interestingly, when the effects of SCH772984 treatment were examined over a 36-hour time-course in A375 cells the pathway rebound, following feedback relief, was able to overcome the inhibition of ERK1/2 phosphorylation, but not the inhibition of p90RSK phosphorylation,

which was maintained. This indicates that SCH772984 is more effective at inhibiting ERK1/2 catalytic activity than the phosphorylation of ERK1/2 by MEK1/2 (Morris et al., 2013).

SCH772984 is structurally distinct from ERKis **5**, **8**, **10** and **7**, all of which feature *N*-acyl β-amino alcohols**,** being formed of a hinge binding pyridine-indazole with an extended piperazinephenyl-pyrimidine decoration (Figure 2A). Furthermore, the dual mechanism of action of SCH772984 suggested that it was able to either bind inactive ERK1/2 to prevent the phosphorylation and activation of the kinase, or facilitate dephosphorylation of the active kinase. This mechanism of action and its unique structure led to the initial theory that SCH772984 displayed properties of both type I and type II kinase inhibitors (Chaikuad et al., 2014; Morris et al., 2013). If so this would contrast with other previously reported reversible, ATP-competitive ERK inhibitors; for example, co-crystal structures have indicated that VTX-11e exclusively exhibits a type I binding mode (Figure 2B) (Aronov et al., 2009; Chaikuad et al., 2014; Ohori et al., 2005). The prevalence of type I inhibitors, also seen within the GDC-0994 crystal structure (Fig 2C), is thought to be due to residues in the catalytic domain of ERK1/2 stabilising the “DFG-in” conformation (Hari, Merritt, & Maly, 2013), thereby minimising the availability of a “DFG-out” state for type II kinase inhibitor development. However, detailed structural analysis of SCH772984 bound ERK1 or ERK2 subsequently revealed that the inhibitor did not extend into the type-II binding pocket; rather the compound induced a novel binding pocket located between the phosphate binding loop (P-loop) and αC helix making a *π*-stacking interaction with Y64 (Figure 2A) (Chaikuad et al., 2014). This binding pocket was not present in unphosphorylated, inactive or phosphorylated, active forms of the kinase. To generate this binding pocket SCH772984 induces significant structural distortion of the P-loop, where Y36 is flipped into the ATP pocket above the pyrrolidine ring of SCH772984. This distortion creates the binding pocket between the P-loop and helix αC, occupied by the pyrimidine ring of SCH772984, whilst not affecting other key elements, leaving the DFG motif in an

“in” conformation (Chaikuad et al., 2014). Multiple kinases can form folded P-loop conformations and this binding mode correlates with a high degree of selectivity (Müller et al., 2015).

During the development of SCH772984, researchers at Merck identified initial hit compounds that were able to bind to both unphosphorylated, inactive and phosphorylated, active ERK2. Therefore, as well as a conventional, active ERK2 enzyme assay, they developed a coupled ERK2 assay where unphosphorylated ERK2 was pre-incubated with the test compound, then the ability of ERK2 to phosphorylate a polypeptide substrate was measured following the addition of active MEK1. This coupled assay encompasses the processes of phosphorylation and activation of ERK2 (and attendant conformational changes) and its ability to phosphorylate a substrate; it can therefore capture the inhibition of both inactive and activated ERK2 (Deng et al., 2014; Zhu et al., 2015).

SCH772984 demonstrated high ERK1/2 potency, kinome selectivity and *in vivo* efficacy but its high molecular weight and poor ligand efficiency (LE) could present problems later in development, therefore more drug-like properties were sought through a novel series. Interestingly, when developing a further compound series with lower molecular weight and higher LE, researchers at Merck selected a lead compound which apparently did not retain the ability to inhibit the phosphorylation of ERK1/2 (Lim et al., 2016). To what extent this choice suggested that the dual mechanism of action, and specifically the inhibition of T-E-Y phosphorylation, was not sufficiently valued is unclear. It will be interesting to see what mechanism of action is ultimately selected as this programme develops.

Recently, when developing a novel pyrrolopyrazinone-based series of reversible, ATP-competitive ERKi researchers at AstraZeneca also determined the ability of their compounds to prevent the activation of ERK1/2, in this case by measuring the levels of phosphorylated ERK1/2 following 2 hour treatment of A375 melanoma cells (Ward et al., 2017). This study revealed that relatively minor changes

in the structure of the compound, such as switching the chirality of a single methyl group, could have significant impacts on the ability to inhibit ERK1/2 phosphorylation, yet retain a similar ability to inhibit ERK1/2 catalytic activity. This indicates that dual-mechanism and catalytic ERKis are unlikely to be distinct classes, but rather two extremes of a spectrum of compounds with the ability to prevent ERK1/2 phosphorylation. Structure-activity relationships indicated that the interactions between the compound and the glycine-rich loop of ERK1/2 might influence the ability of the compound to inhibit ERK1/2 phosphorylation. However, further studies are required to identify the key requirements for this binding mode, and the mechanism by which it prevents ERK1/2 phosphorylation. The lead compound from this study, compound 35 **(16)**, is a potent, selective ERKi with an *in vitro* IC_{50} of <0.3 nM, IC_{50}'s of 12/62 nM for inhibition of p-ERK1/2 and p-p90RSK respectively in A375 cells and efficacy in tumour xenograft models (Ward et al., 2017). This data suggests that compound 35 possesses a dual mechanism, however the co-crystal structures generated for this compound series appear to show a binding mode more similar to that of the catalytic ERKi VTX-11e than the dual mechanism inhibitor SCH772984. Therefore, it would be interesting to determine whether this compound is able to induce robust, durable inhibition of p-ERK in RAS mutant cell lines that are more liable to feedback relief and pathway rebound.

To generate a dual mechanism profile compounds are likely to induce or stabilize a conformational state in ERK1/2 that prevents the binding of, or activation by, MEK, in addition to the ATP-competitive inhibition of ERK1/2 catalytic activity. However, with the exceptions of VTX-11e, SCH772984 and Compound 35 very little biological data has been published for most reversible, ATP-competitive ERKis, therefore it is not possible to determine how many of these inhibitors are solely catalytic, or possess a dual mechanism of action. However, based on the precedent of MEKis, we speculate that these two mechanisms of action (dual mechanism versus purely catalytic) could have significant consequences for how cells respond and adapt

following compound treatment. In the context of MEKi, compounds that are able to block the phosphorylation of MEK1/2 by RAF, in addition to inhibiting the catalytic activity of MEK1/2, have been shown to delay pathway rebound following the collapse of negative feedback (Hatzivassiliou et al., 2013; Ishii et al., 2013). These MEKi, which include trametinib, CH5126766 and GDC-0623, have been termed "feedback buster MEKis" due to their ability to mitigate some of the consequences of feedback relief and induce a more durable inhibition of ERK1/2 phosphorylation and cell proliferation in RAS mutant cell lines (Caunt et al., 2015; Hatzivassiliou et al., 2013; Lito et al., 2014). Consequently, it is possible to hypothesise that ERKis would also follow this paradigm with dual mechanism ERKis delaying pathway rebound relative to solely catalytic inhibitors due to their ability to prevent the phosphorylation and activation of ERK1/2 by MEK1/2. This is to some extent supported by the durable inhibition of p90RSK phosphorylation observed in the context of negative feedback relief and pathway rebound following SCH772984 treatment of A375 melanoma cells (Morris et al., 2013). However, this conclusion is clouded by the fact that A375 cells harbour $BRAF^{V600E}$, which is known to exhibit weaker pathway rebound due to feedback relief following ERK1/2 pathway inhibition, as $BRAF^{V600E}$ is active as a monomer and insensitive to ERK1/2 inhibitory phosphorylation (Pratilas et al., 2009). Furthermore, no studies to date have compared the ability of catalytic and dual mechanism ERKi to maintain durable inhibition of ERK1/2 activity, therefore it is possible that targeting ERK1/2 will result in prolonged pathway inhibition no matter which mechanism of inhibitor is used.

Catalytic ERKi treatment has been shown to result in the accumulation of p-ERK (Goetz, Ghandi, Treacy, Wagle, & Garraway, 2014; Morris et al., 2013), and the phosphorylation of ERK1/2 is known to induce its release from its cytoplasmic anchor MEK1/2 and enable its subsequent translocation to the nucleus (Plotnikov, Zehorai, Procaccia, & Seger, 2011). Together this evidence indicates that catalytic ERKi treatment might induce the nuclear accumulation of phosphorylated, but inhibited

ERK1/2, whereas dual-mechanism ERKi would prevent the nuclear translocation of inhibitor bound ERK1/2 (Figure 3). This “primed-state” following catalytic ERKi treatment may facilitate accelerated nuclear ERK1/2 activity and ERK1/2-dependent gene expression when pathway rebound overcomes compound efficacy, and could potentially result in cells recovering more rapidly from treatment with catalytic ERKi compared to dual-mechanism ERKi.

Alternatively, this nuclear p-ERK1/2 pool induced by catalytic ERKi treatment could potentially play a role in maintaining the non-catalytic functions of ERK1/2. Although it is clear that the vast majority of the biological functions of ERK1/2 are performed via phosphorylation events, evidence for non-catalytic functions of ERK1/2 is accumulating. Direct interaction with ERK2 has been shown to activate a range of proteins including topoisomerase II (Shapiro et al., 1999), poly(ADP-ribose) polymerase (PARP) 1 (Cohen-Armon et al., 2007) and DUSP6 (MAPK phosphatase 3, MKP-3) (Camps et al., 1998), in a manner-independent of phosphotransfer activity. ERK2 is also able to act as a transcriptional repressor of interferon-γ responsive genes by directly binding DNA in the promoter regions of these genes and preventing the binding of the transcription factor C/EBP-β (Hu et al., 2009). Furthermore, ERK1/2 have been suggested to contribute to regulation of cell-cycle entry in a kinase-independent manner. Upon nuclear translocation ERK1/2 was able to interact with lamin A and displace the retinoblastoma (RB) protein, enabling RB phosphorylation by cyclin-dependent kinases and the release of E2F transcription factors to promote cell cycle entry (Rodríguez et al., 2010).

Interestingly, with the exception of the activation of its negative feedback regulator DUSP6, the majority of the known non-catalytic functions of ERK1/2 occur in the nucleus. This raises the possibility that these processes are regulated by the nuclear translocation of ERK1/2 for which T-E-Y phosphorylation is a major driver (Lidke et al., 2010), although not the sole determinant (Caunt & McArdle, 2010). Therefore, the inability of solely catalytic ERKis to prevent the nuclear accumulation of

ERK1/2 could be permitting a range of non-catalytic functions of ERK1/2 to continue. These non-catalytic functions could potentially contribute to reduced efficacy of catalytic ERKis relative to dual mechanism ERKis or RAF/MEK inhibitors which target upstream of ERK1/2 and prevent its phosphorylation and nuclear import.

Overall, current evidence would imply that it could be advantageous to develop dual mechanism inhibitors to target ERK1/2. However, due to the very limited mechanistic data published on the current ATP-competitive ERKis, the majority of this evidence relies on knowledge gained from studies of ERK1/2 signalling rather than ERK1/2 inhibition or knowledge inferred or extrapolated from the mechanism of action of MEKis. Further studies are required to investigate the different mechanisms of action of current ERKis in the appropriate signalling and disease context to utilise these compounds successfully or to design compounds that are more effective.

(b) Covalent ERK inhibitors

In recent years there has been an increased interest in developing targeted covalent inhibitors for many kinases. This has been driven by the successes of the EGFR/HER2 inhibitors afatinib and neratinib (D. Li et al., 2008; Park et al., 2016) the BTK inhibitor ibrutinib (Claro et al., 2015) and the $EGFR^{T790M}$–specific inhibitor osimertinib (Khozin et al., 2017). Targeted covalent inhibitors act through a two-step mechanism. First, they bind to their target protein through a reversible interaction, which presents an electrophilic moiety within the inhibitor in the correct vector towards a nucleophilic residue within the target protein (typically a cysteine). In the second step this nucleophilic residue then reacts with the inhibitor to form a covalent interaction, which can be either reversible or irreversible depending on the nature of the bond formed (Baillie, 2016; Chaikuad, Koch, Laufer, & Knapp, 2017). This method of inhibition is proposed to facilitate prolonged inhibition of the target protein and high potency, as the covalent interaction results in either a very slow off-rate for the inhibitor or a permanently inhibited target protein, consequently *de novo* synthesis of the target is

required to re-establish activity. However, a negative consequence of the inherent reactivity of covalent inhibitors is that they may exhibit off-target effects by reacting with cysteines and other reactive residues within the proteome.

An analysis of the kinome, performed by Liu et al., estimated that 200 kinases possess a cysteine either in or proximal to the ATP binding site, including both ERK1 and ERK2 **(Liu et al., 2013)**. Cys166 of ERK1/2 is a non-catalytic residue located in the back of the ATP binding pocket, one residue prior to the conserved DFG motif. The ability of this cysteine residue to form a covalent adduct with an inhibitor was demonstrated with the resorcyclic acid lactone (RAL) natural product 5*Z*-7-oxozeaenol (also known as FR148083, **4** - Table 1) **(Ohori et al., 2007)**. A crystal structure showed it covalently linked to Cys166 of ERK2 through the *cis*-enone functionality of the RAL to form a covalent adduct. However, 5*Z*-7-oxozeaenol and structurally related RAL analogues were found to be promiscuous, forming covalent adducts with a number of kinases that possess a cysteine within their catalytic sites, including MEK1, MKK7, MKK4 and VEGFR1/2 **(Ohori et al., 2007; Schirmer, Kennedy, Murli, Reid, & Santi, 2006)**

Building on the discovery that ERK1/2 is susceptible to covalent inhibition, AstraZeneca utilised an approach to develop covalent inhibitors through repurposing of existing scaffolds and a structure-guided screen. This identified a number of compounds that showed good biochemical and cellular activity against ERK2 **(Ward et al., 2015)**. This compound series binds in the ATP-pocket and forms a covalent bond with Cys-166 (Figure 2D). The lead, compound 13 (**11** – Table 1), showed an *in vitro* IC_{50} of 6.9 nM against ERK2 activity and an IC_{50} of 53 nM in an A375 cell proliferation assay. However, it should be noted that IC_{50} values for covalent inhibitors are dependent on the timing of the assay as longer incubation times will lead to increased protein-inhibitor adducts.

This molecular series has since been used as a template for the synthesis of chemical probes to manipulate and study ERK1/2 function. Lebraud *et al.* appended a *trans*-cyclooctene (TCO) group to the core pharmacophore of compound 13, allowing for functionalisation of the inhibitor, and as a result its target protein, through biorthogonal chemistry **(Lebraud, Wright, East, et al., 2016)**. Such TCO-derivatives have been used for a number of purposes including in-gel activity based protein profiling, whereby the selectivity of the inhibitor can be assessed by reacting the TCO tag with a fluorophore, and identification of protein-inhibitor adducts using an SDS PAGE gel **(Lebraud, Wright, East, et al., 2016)**. Further uses of these TCO-derivatives include guiding an E3 ubiquitin ligase to accelerate the degradation of ERK1/2 in a proteolysis targeting chimera (PROTAC) approach **(Lebraud, Wright, Johnson, & Heightman, 2016)**, and imaging of endogenous inhibitor-bound ERK1/2 using confocal microscopy to track localisation of ERK1/2 within cells **(Sipthorp et al., 2017)**.

Although covalent inhibitors have the potential to be an effective tool to inhibit ERK1/2 signalling they have yet to achieve any success in the clinic. The only Phase I clinical trial to date utilising a covalent ERKi was initiated by Celgene in 2014, however, this study was terminated because "the maximum tolerated dose (MTD) did not offer a sufficiently encouraging profile to proceed with the additional dosing schedule or the cohort expansion phase" (NCT02313012). This possibly reflects the reactivity of covalent inhibitors and their propensity for off-target effects. As such further studies are required to develop selective, covalent ERK1/2 inhibitors with efficacy in the clinic.

(c) Allosteric ERK1/2 inhibitors

In addition to targeting the catalytic site of ERK1/2, efforts have been made to develop allosteric ERKis, which bind at sites distinct from the catalytic cleft and interfere with the ability of ERK1/2 to interact with its binding partners. The disruption of protein-

protein interactions (PPIs) is considered challenging due to the extensive and sometimes relatively featureless interfaces involved; however the identification of interaction hot-spots led to the appreciation that blocking the entire interface is not required to successfully block PPIs (Whitty & Kumaravel, 2006; Yap, Worlikar, MacKerell, Shapiro, & Fletcher, 2011). This knowledge, coupled with the diverse structural nature of allosteric binding pockets makes them promising alternatives to catalytic kinase inhibitors, with potentially high selectivity and the ability to target tumours which evolve drug-resistance to conventional catalytic kinase inhibitors (Wu et al., 2015). Furthermore, ERK1/2 are known to have more than 200 substrates and/or binding partners and contain two major substrate binding sites, the D-recruitment site (DRS) and the F-recruitment site (FRS); therefore allosteric inhibitors targeted at one site may enable modulation of the activity of select ERK1/2 substrates (Yap et al., 2011).

Shapiro and colleagues reported the first putative small molecule, allosteric ERKi in 2005 (Hancock et al., 2005). This study utilised computer-aided drug design to perform an *in silico* screen for compounds which could target a polar cleft between the acidic common docking (CD) domain and the ED (Glu-Asp) site of the DRS observed in the crystal structure of unphosphorylated ERK2. From this screen, 80 potential compounds were selected for testing in biological assays, of which several were able to inhibit ERK1/2-mediated phosphorylation of the D-domain containing substrates p90RSK and the transcription factor ELK-1. Furthermore, these compounds inhibited the proliferation of several cancer cell lines. Lead compound 76 (**2** – Table 1) was shown to interact with ERK1/2 in a manner which did not compromise its phosphorylation or catalytic activity, supporting its predicted binding mode to the DRS of ERK1/2 (Hancock et al., 2005). Structure-activity relationship (SAR) studies have investigated the crucial structural features of compound 76 responsible for its biological activity and further optimised the potency of this compound series (Q. Li et al., 2009; Boston et al., 2011; Jung et al., 2013). However, it should be noted that these allosteric

inhibitors possess functionality that would not be desirable for a medicinal chemistry program. For example, compound 76 has a core structure of an ene-rhodanine, a reported pan-assay interference compound (PAINS) motif (Baell & Walters, 2014). Therefore, when using these compounds suitable controls should be put in place to ensure that any effects seen are due to the desired on-target mechanism of action (MOA).

The activation of ERK1/2 by phosphorylation has been suggested to induce subtle structural changes in the region of its DRS (Hoofnagle, Resing, Goldsmith, & Ahn, 2001), therefore Shapiro and colleagues performed a second *in silico* screen to identify compounds which potentially bind the DRS of phosphorylated ERK2 (F. Chen et al., 2006). The majority of compounds identified by this second screen were the same as those identified by the initial screen. However, the screen also revealed a group of novel compounds (**3** – Table 1), which were able to bind ERK1/2 and inhibit the phosphorylation of p90RSK and ELK-1 (F. Chen et al., 2006). Finally, Shapiro and colleagues have also applied this *in silico* screening approach to identify compounds which potentially bind to the FRS of unphosphorylated ERK2 (Samadani et al., 2015). The FRS is thought to be responsible for the interaction of ERK1/2 with a distinct set of substrates compared to the DRS, although some substrates such as ELK-1 have been shown to utilise both domains to bind to ERK1/2 (Burkhard, Chen, & Shapiro, 2011). The ERK2-FRS *in silico* screen identified a diverse range of compounds which inhibited EGF-stimulated phosphorylation of ELK-1, but not p90RSK, in HeLa cells, supporting a MOA which inhibits FRS-mediated ERK2 interactions. The binding of these compounds was modelled *in silico*, enabling the utilisation of structural information and site identification by ligand competitive saturation (SILCS) simulations to optimise the compound series. The resulting lead compound, SF-3-030 (**12** – Table 1), was shown to inhibit ELK-1 phosphorylation, the expression of ERK1/2-dependent immediate early genes and proliferation in BRAF-mutant melanoma cell lines (Samadani et al., 2015). However, SF-3-030 also contains moieties that may be widely

reactive to protein residues, such as its vinyl sulfone, which could undergo undesired covalent linkages to proteins. Again, suitable controls will need to be in place to ensure that the phenotype observed is due to prevention of ERK1/2 FRS-mediated interactions and inhibition of downstream signalling. For example, these agents should be ineffective in cells that are not addicted to ERK1/2 signalling.

In addition to the DRS and FRS, the mutagenesis of ERK2 has identified a range of other residues that could be important for its interactions with substrate or regulatory proteins (Jialin Zhang, Zhou, Zheng, & Zhang, 2003). These include a unique region within the MAP kinase insert which appears to specifically regulate interactions with MEK1/2 (Robinson, Whitehurst, Raman, & Cobb, 2002) and a leucine-rich dimerisation interface (Khokhlatchev et al., 1998; Wilsbacher et al., 2006). Therefore, if further ERK1/2 binding domains are characterised, this could facilitate the development of additional allosteric ERK1/2 inhibitors. ERK1/2 activation by phosphorylation is known to induce dimerisation, as well as substrate binding (Khokhlatchev et al., 1998). Recent molecular biology studies have shown that ERK1/2 dimers and scaffolds are required for the optimal activation of cytoplasmic, but not nuclear, substrates and that preventing ERK1/2 dimerisation is able to inhibit tumour cell proliferation and tumour development (Casar, Pinto, & Crespo, 2008). This evidence validated ERK1/2 dimerisation as a potential target for the development of novel ERK inhibitors, therefore Herrero and colleagues developed a native gel electrophoresis approach to screen for inhibitors of ERK1/2 dimerisation (Herrero et al., 2015). This screen identified DEL-22379 (**13** – Table 1) as a small molecule capable of inhibiting ERK1/2 dimerisation, without affecting ERK1/2 phosphorylation or catalytic activity. DEL-22379 inhibited the phosphorylation of ERK1/2 cytoplasmic substrates and the proliferation of tumour cell lines and xenografts harbouring RAS-ERK1/2 pathway oncogenes. Furthermore, DEL-22379 demonstrated efficacy against BRAF or MEK inhibitor resistant cells, and hypothetically could potentially retain

efficacy in cases where cell lines evolve resistance to ATP-competitive ERK1/2 kinase inhibitors (Herrero et al., 2015).

Together these academic studies have revealed that it is possible to inhibit different ERK1/2 binding interactions, advocating the continued development of allosteric approaches to inhibit ERK1/2. Such efforts have identified a range of compounds that inhibit different ERK1/2-substrate interactions, which could be of use for biochemical studies of ERK1/2 function, as well as having the potential to be developed into therapeutic agents. However, many of the molecules identified to date possess moieties that have been reported as PAINS, such as electrophilic Michael acceptors that predispose them to non-specific protein interactions. Consequently, these compounds may induce many off-target effects, complicating their use as tool compounds without proper controls in place.

6. Prospects for ERK1/2 inhibitors as monotherapy and in combination

Work described in the previous sections clearly validates the use of ERKis as a new weapon to treat tumours that are driven by mutational activation of the ERK1/2 pathway. Since mutations in ERK1/2 are quite rare in human cancer and, as far as is known, ERKis do not discriminate between mutationally activated or wild type ERK1/2 it must be anticipated that ERKi monotherapy will face the same challenges as MEKi monotherapy; the most obvious being a narrow therapeutic index arising from the inhibition of ERK1/2 signalling in both normal and tumour cells and resultant toxicity. Indeed, reports from the Phase I dose escalation and expansion study of BVD-523/ulixertinib reported similar adverse events as those previously seen with MEKi including rash, diarrhoea, fatigue and nausea (B. T. Li et al., 2017). However, this trial also revealed some encouraging results; the 9 patients (of 83) who exhibited a partial response were found to have tumours with mutations in BRAF (V600E, G469A or L485W) or NRAS, suggesting that clinical activity was related to pathway de-regulation

and ERK1/2 addiction. Indeed, the most obvious use for ERKi monotherapy is in the treatment of patients with BRAF-mutant cancer that has initially responded well to BRAFi (and is therefore addicted to ERK1/2 signalling), but has then developed resistance through 'on-pathway' mechanisms that reinstate ERK1/2 signalling.

Beyond monotherapy there is clearly a strong case for developing ERKi combinations with other ERK1/2 pathway inhibitors. As with MEKis, ERKis would be expected to antagonise the activation of ERK1/2 observed in normal cells arising from paradoxical RAF activation. Thus, BRAFi+ERKi combinations would be expected to have superior efficacy and be better tolerated than BRAFi alone in $BRAF^{V600}$ melanoma as is the case for BRAFi+MEKi. As outlined above, there is also a strong case for testing ERKi in combination with either RAFi or MEKi to mitigate ERK1/2 reactivation arising from feedback relief in tumour cells with RAS mutations. Beyond the ERK1/2 pathway, ERKi should certainly be tested with other emerging targeted agents (inhibitors of MTOR, PI3K or PKB/Akt) as well as with appropriate 'standard-of-care' cytotoxic chemotherapy in tumours with RAS mutations. A further attractive option will be to combine ERKi with inhibitors of cell survival pathways. In common with MEKi, ERKi typically exert a cytostatic effect and this is most likely due to the buffering effect of pro-survival BCL2 proteins (BCL2, BCL-X_L, MCL1) (Cook et al., 2017). A series of 'BH3 mimetics' that bind and inhibit the activity of select pro-survival proteins have already been developed and are undergoing clinical evaluation and there is already precedent that RAFi or MEKi can synergise with these agents to promote cell death in ERK-addicted tumour cells (Corcoran et al., 2013; Cragg et al., 2008; Sale & Cook, 2013). Finally, ERKi (with MEKi or BRAFi) will undoubtedly be tested in combination with immunotherapy; indeed pre-clinical models have supported the testing of BRAFi+MEKi+immunotherapy in $BRAF^{V600E}$ melanoma models (Hu-Lieskovan et al., 2015). The challenge with all these combinations will be to balance any synergy in the tumour cell with the increased toxicity arising from activity in non-tumour tissue.

Dosing, scheduling and stratification biomarkers for identifying responsive patient populations will all play a part.

Whether ERKis are used alone or in combination, experience with BRAFi or MEKi has taught us to anticipate the emergence of ERKi resistance in tumours that show initial favourable responses; indeed, *in vitro* mutagenesis studies have already anticipated potential resistance mechanisms (Goetz et al., 2014). Goetz et al subjected the ERK1 and ERK2 cDNAs to random mutagenesis in E. coli, expressed them in $BRAF^{V600E}$-positive A375 melanoma cells and selected them in the presence of the ERKi inhibitor VTX-11e, the MEKi trametinib or a MEKi+RAFi combination (trametinib + dabrafenib) (Goetz et al., 2014). ERK1/2 mutations arising from selection in the ERKi VTX-11e were also cross-resistant to SCH772984 and were found to cluster within the ATP/drug binding pocket. Indeed, subsequent experiments demonstrated that these mutant ERK1/2 proteins were refractory to ERKi, strongly suggesting that they are 'on target' resistance alleles that act by interfering with drug binding. In contrast, the ERK1/2 mutants arising from selection in RAFi/MEKi were distributed more widely throughout the ERK proteins; however, some mutations clustered in the αC helix and the common docking domain. Indeed, the $ERK2^{E322K}$ mutation that is observed as a primary mutation in squamous cell carcinomas and sustains active ERK1/2 by preventing DUSP binding was amongst these mutants. Whilst these mutants were generated 'artificially' rather than in cells they are clearly homing in on well understood vulnerabilities in protein kinases so it is highly likely that functionally similar mutations will arise in human tumours undergoing RAFi, MEKi or ERKi treatment and mitigation should be considered. In this context, it is notable that some of the ERK1/2 mutants arising during RAFi/MEKi selection were still sensitive to ERKi providing further support for intra-pathway combinations including, for example, alternate RAF/MEK and ERK dosing.

7. Conclusions

The clinical precedent of BRAFis and MEKis, both alone and in combination, together with pathway topology and the pivotal distributive role of ERK1/2 within the pathway provide strong rationale for developing inhibitors of ERK1/2. This has fuelled the development of a variety of novel, drug-like molecules and chemical probes that target ERK1/2. To date, attempts to develop allosteric inhibitors of ERK1/2 (dimerization inhibitors or substrate binding blockers) have not led to any drug-like molecules. Indeed, the utility of some of these probes as research tools is far from clear as many possess moieties such as electrophilic Michael acceptors that predispose to non-specific protein interactions. A critical control for these compounds will be to test their selectivity in biological systems by expressing non-drug binding mutants of ERK1/2; these should rescue biological effects if those effects are due to 'on-target' ERK1/2 inhibition. To date only two covalent ERKis have been described but these provide strong proof-of-concept that this approach may work, whilst the successful development of covalent inhibitors of BTK, EGFR/HER2 and $EGFR^{T790M}$ are compelling examples of what can be achieved. The furthest advanced ERKis are a variety of 'traditional' reversible ATP-competitive ERK1/2 inhibitors that are now undergoing various stages of clinical evaluation. These include molecules which are purely inhibitors of ERK1/2 catalytic activity and those which additionally are able to block MEK1/2-catalysed ERK1/2 T-E-Y phosphorylation. Intuitively, this latter 'dual-mechanism' seems more attractive as it may limit the extent of ERK1/2 reactivation following feedback relief. However, the biological and clinical importance of this dual mechanism remains to be seen and it is notable that the lead compound selected from the SCH772984 programme did not retain the ability to inhibit the phosphorylation of ERK1/2 (Lim et al., 2016). Finally, since the ATP-competitive ERKis do not have the exquisite selectivity, specificity even, of the allosteric MEKis, they are each likely to exhibit their own unique profile of 'off target' activity against different subsets of kinases; this unique polypharmacology may enhance efficacy or may enhance toxicity and we await the results of ongoing trials with interest.

Acknowledgements

We thank Joanne Munck, John Lyons and Tom Heightman at Astex Pharmaceuticals and members of the Cook Lab for discussions and comments on the manuscript. AK's salary is supported by Astex Pharmaceuticals through the Milner Therapeutics Consortium. SC is a consultant of Astex Pharmaceuticals. Work in the Cook lab related to this review was supported by an Institute Strategic Programme Grant awarded to the Signalling Programme at the Babraham Institute (BB/P013384/1) and grants from Worldwide Cancer Research (formerly AICR, 12-1259), Cancer Research UK (A14867), AstraZeneca and Astex Pharmaceuticals.

Conflict of interest

AK's salary is supported by Astex Pharmaceuticals through the Milner Therapeutics Consortium. SC is a consultant of Astex Pharmaceuticals. Otherwise the authors declare no conflicts of interest.

Figure Legends

Figure 1. The RAF-MEK1/2-ERK1/2 cascade.

A simplified representation of the RAS-regulated RAF–MEK1/2–ERK1/2 signalling cascade. Activated growth factor receptors recruit the guanine nucleotide exchange factor SOS (son of sevenless), to induce the release of GDP from RAS. This enables the binding of GTP, activating RAS. RAS-GTP promotes the dimerisation and activation of the RAF kinases (ARAF, BRAF and CRAF), which phosphorylate and activate MEK1 and MEK2 (MEK1/2), which in turn phosphorylate and activate the terminal kinases ERK1 and ERK2 (ERK1/2). Phosphorylation of ERK1/2 promotes their nuclear translocation allowing the phosphorylation and activation of transcription factors, including the ETS family, to regulate gene expression and modulate many

critical aspects of cell physiology. For example, ERK1/2 signalling drives the expression of many transcription factors (e.g., FOS, FRA1, EGR1) which in turn control the expression of genes involved in feedback regulation of the pathway (see below), control of cell cycle progression (CCND1, p21CIP1, p16INK4A) and control of cell survival (MCL1, BCL2, BCL-X_L). ERK1/2 also target a large number of cytoplasmic substrates enabling the pathway to regulate further processes including cell migration and apoptosis. The ERK1/2 pathway is regulated by an extensive range of negative feedback systems, allowing the fine-tuning of magnitude, duration and localisation of pathway output. These negative feedback controls can be divided into two major mechanisms. The first is the rapid ERK1/2-catalysed inhibitory phosphorylation of upstream pathway components such as BRAF, CRAF, MEK1, SOS and some receptor tyrosine kinases (RTKs) (shown in red). The second mechanism requires the *de novo* expression of negative regulators including MAP Kinase Phosphatases (MKPs or DUSPs) and Sprouty proteins (shown in green). This delayed, long term mechanism acts to dampen pathway activity following induction of the required pathway output. The ERK1/2 pathway is frequently de-regulated in cancer due to mutations in components such as BRAF, RAS, NF1, MEK or certain RTKs (indicated by yellow stars).

Figure 2. Crystal structures of a number of ERK1/2 inhibitors bound to ERK2.

(A) SCH772984 binds to the hinge region of the kinase, with the kinase adopting a "DFG-in" conformation. SCH772984 also binds within a new pocket between the *α*C helix and the P-loop of the kinase, revealed when Y36 flips into the ATP pocket, within which the terminal pyrimidine of SCH772984 makes a *π*-stacking interaction with Y64. **(B-C)** VTX-11e and GDC-0994 both bind to ERK2 in a manner consistent with Type I inhibitors, with the kinase in a "DFG-in" conformation and with Y36 outside of the ATP pocket. **(D)** Compound 10 binds to the hinge region of ERK2, presenting the electrophilic acrylamide towards C166, which subsequently reacts to form a covalent

bond, as shown in crystal structure. Main structure of the protein is shown as a ribbon in orange with certain areas highlighted for clarity, hinge – dark blue, P-loop – dark green, αC helix – violet, DFG motif – red, Y64 – purple, Y36 – light blue. Inhibitors are shown with carbon – light green, nitrogen – blue, oxygen – red, chlorine – dark green, fluorine – cyan, sulphur – yellow. Structures were taken from the PDB under the following accession codes; SCH772984 – 4QTA, VTX-11e – 4QTE, GDC-0994 – 5K4I, compound 10 – 4ZZO. The PDB structures were modified by removing all solvents and ions for clarity, before colour coding the protein and inhibitors as described above. Images were produced using Maestro 11.1 Schrödinger release 2017-4.

Figure 3. Catalytic and dual mechanism ERK1/2 inhibitors could differentially regulate the localisation of ERK1/2.

A purely catalytic ERK1/2 inhibitor should allow the nuclear accumulation of ERK1/2 since it will not prevent activating phosphorylation by MEK1/2. Indeed, in cells with wild type BRAF a catalytic ERK1/2 inhibitor should induce feedback relief, resulting in increased RAF-dependent activation of MEK, leading to further accumulation of p-ERK1/2. The phosphorylation of ERK1/2 promotes its nuclear translocation, therefore a pool of inhibitor-bound ERK1/2 might accumulate in the nucleus. In contrast, dual mechanism ERK1/2 inhibitors could disrupt the phosphorylation of ERK1/2, and reduce the nuclear translocation ERK1/2, resulting in a pool of inhibitor-bound ERK1/2 in the cytoplasm.

References

Ahronian, L. G., Sennott, E. M., Allen, E. M. V., Wagle, N., Kwak, E. L., Faris, J. E., … Corcoran, R. B. (2015). Clinical Acquired Resistance to RAF Inhibitor Combinations in BRAF-Mutant Colorectal Cancer through MAPK Pathway

Alterations. *Cancer Discovery*, *5*(4), 358–367. https://doi.org/10.1158/2159-8290.CD-14-1518

Aoki, K., Yamada, M., Kunida, K., Yasuda, S., & Matsuda, M. (2011). Processive phosphorylation of ERK MAP kinase in mammalian cells. *Proceedings of the National Academy of Sciences*, *108*(31), 12675–12680. https://doi.org/10.1073/pnas.1104030108

Arcila, M. E., Drilon, A., Sylvester, B. E., Lovly, C. M., Borsu, L., Reva, B., … Ladanyi, M. (2015). MAP2K1 (MEK1) Mutations Define a Distinct Subset of Lung Adenocarcinoma Associated with Smoking. *Clinical Cancer Research*, *21*(8), 1935–1943. https://doi.org/10.1158/1078-0432.CCR-14-2124

Aronov, A. M., Baker, C., Bemis, G. W., Cao, J., Chen, G., Ford, P. J., … Xie, X. (2007). Flipped Out: Structure-Guided Design of Selective Pyrazolylpyrrole ERK Inhibitors. *Journal of Medicinal Chemistry*, *50*(6), 1280–1287. https://doi.org/10.1021/jm061381f

Aronov, A. M., Tang, Q., Martinez-Botella, G., Bemis, G. W., Cao, J., Chen, G., … Xie, X. (2009). Structure-Guided Design of Potent and Selective Pyrimidylpyrrole Inhibitors of Extracellular Signal-Regulated Kinase (ERK) Using Conformational Control. *Journal of Medicinal Chemistry*, *52*(20), 6362–6368. https://doi.org/10.1021/jm900630q

Arvind, R., Shimamoto, H., Momose, F., Amagasa, T., Omura, K., & Tsuchida, N. (2005). A mutation in the common docking domain of ERK2 in a human cancer cell line, which was associated with its constitutive phosphorylation. *International Journal of Oncology*, *27*(6), 1499–1504.

Baell, J., & Walters, M. A. (2014). Chemistry: Chemical con artists foil drug discovery. *Nature News*, *513*(7519), 481. https://doi.org/10.1038/513481a

Bagdanoff, J. T., Jain, R., Han, W., Poon, D., Lee, P. S., Bellamacina, C., & Lindvall, M. (2015). Ligand efficient tetrahydro-pyrazolopyridines as inhibitors of ERK2 kinase. *Bioorganic & Medicinal Chemistry Letters*, *25*(17), 3626–3629. https://doi.org/10.1016/j.bmcl.2015.06.063

Bagdanoff, J. T., Jain, R., Han, W., Zhu, S., Madiera, A.-M., Lee, P. S., … Poon, D. (2015). Tetrahydropyrrolo-diazepenones as inhibitors of ERK2 kinase. *Bioorganic & Medicinal Chemistry Letters*, *25*(18), 3788–3792. https://doi.org/10.1016/j.bmcl.2015.07.091

Baillie, T. A. (2016). Targeted Covalent Inhibitors for Drug Design. *Angewandte Chemie International Edition*, *55*(43), 13408–13421. https://doi.org/10.1002/anie.201601091

Balmanno, K., & Cook, S. J. (2008). Tumour cell survival signalling by the ERK1/2 pathway. *Cell Death and Differentiation*, *16*(3), 368. https://doi.org/10.1038/cdd.2008.148

Bardwell, L. (2006). Mechanisms of MAPK signalling specificity. *Biochemical Society Transactions*, *34*(5), 837–841. https://doi.org/10.1042/BST0340837

Bauer, R. A. (2015). Covalent inhibitors in drug discovery: from accidental discoveries to avoided liabilities and designed therapies. *Drug Discovery Today*, *20*(9), 1061–1073. https://doi.org/10.1016/j.drudis.2015.05.005

Blake, J. F., Burkard, M., Chan, J., Chen, H., Chou, K.-J., Diaz, D., … Schwarz, J. B. (2016). Discovery of (S)-1-(1-(4-Chloro-3-fluorophenyl)-2-hydroxyethyl)-4-(2-((1-methyl-1H-pyrazol-5-yl)amino)pyrimidin-4-yl)pyridin-2(1H)-one (GDC-0994), an Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) Inhibitor in Early Clinical Development. *Journal of Medicinal Chemistry*, *59*(12), 5650–5660. https://doi.org/10.1021/acs.jmedchem.6b00389

Blüthgen, N., & Legewie, S. (2008). Systems analysis of MAPK signal transduction. *Essays In Biochemistry*, *45*, 95–108. https://doi.org/10.1042/bse045095

Bollag, G., Hirth, P., Tsai, J., Zhang, J., Ibrahim, P. N., Cho, H., … Nolop, K. (2010). Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*, *467*(7315), 596–599. https://doi.org/10.1038/nature09454

Bos, J. L. (1989). ras Oncogenes in Human Cancer: A Review. *Cancer Research*, *49*(17), 4682–4689.

Boston, S. R., Deshmukh, R., Strome, S., Priyakumar, U. D., MacKerell, A. D., & Shapiro, P. (2011). Characterization of ERK Docking Domain Inhibitors that Induce Apoptosis by Targeting Rsk-1 and Caspase-9. *BMC Cancer*, *11*, 7. https://doi.org/10.1186/1471-2407-11-7

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radzlejewska, E., Morgenbesser, S. D., … Yancopoulos, G. D. (1991). ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, *65*(4), 663–675. https://doi.org/10.1016/0092-8674(91)90098-J

Burkhard, K. A., Chen, F., & Shapiro, P. (2011). Quantitative Analysis of ERK2 Interactions with Substrate Proteins ROLES FOR KINASE DOCKING DOMAINS AND ACTIVITY IN DETERMINING BINDING AFFINITY. *Journal of Biological Chemistry*, *286*(4), 2477–2485. https://doi.org/10.1074/jbc.M110.177899

Buscà, R., Christen, R., Lovern, M., Clifford, A. M., Yue, J.-X., Goss, G. G., … Lenormand, P. (2015). ERK1 and ERK2 present functional redundancy in

tetrapods despite higher evolution rate of ERK1. *BMC Evolutionary Biology*, *15*, 179. https://doi.org/10.1186/s12862-015-0450-x

Buscà, R., Pouysségur, J., & Lenormand, P. (2016). ERK1 and ERK2 Map Kinases: Specific Roles or Functional Redundancy? *Frontiers in Cell and Developmental Biology*, *4*, 53. https://doi.org/10.3389/fcell.2016.00053

Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., … Arkinstall, S. (1998). Catalytic Activation of the Phosphatase MKP-3 by ERK2 Mitogen-Activated Protein Kinase. *Science*, *280*(5367), 1262–1265. https://doi.org/10.1126/science.280.5367.1262

Cargnello, M., & Roux, P. P. (2011). Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiology and Molecular Biology Reviews*, *75*(1), 50–83. https://doi.org/10.1128/MMBR.00031-10

Carlson, S. M., Chouinard, C. R., Labadorf, A., Lam, C. J., Schmelzle, K., Fraenkel, E., & White, F. M. (2011). Large-Scale Discovery of ERK2 Substrates Identifies ERK-Mediated Transcriptional Regulation by ETV3. *Sci. Signal.*, *4*(196), rs11-rs11. https://doi.org/10.1126/scisignal.2002010

Casar, B., Pinto, A., & Crespo, P. (2008). Essential Role of ERK Dimers in the Activation of Cytoplasmic but Not Nuclear Substrates by ERK-Scaffold Complexes. *Molecular Cell*, *31*(5), 708–721. https://doi.org/10.1016/j.molcel.2008.07.024

Caunt, C. J., & McArdle, C. A. (2010). Stimulus-induced uncoupling of extracellular signal-regulated kinase phosphorylation from nuclear localization is dependent on docking domain interactions. *J Cell Sci*, *123*(24), 4310–4320. https://doi.org/10.1242/jcs.076349

Caunt, C. J., Sale, M. J., Smith, P. D., & Cook, S. J. (2015). MEK1 and MEK2 inhibitors and cancer therapy: the long and winding road. *Nature Reviews Cancer*, *15*(10), 577–592. https://doi.org/10.1038/nrc4000

Chaikuad, A., Koch, P., Laufer, S., & Knapp, S. (2017). Targeting the Protein Kinases Cysteinome. *Angewandte Chemie (International Ed. in English)*. https://doi.org/10.1002/anie.201707875

Chaikuad, A., Tacconi, E. M. C., Zimmer, J., Liang, Y., Gray, N. S., Tarsounas, M., & Knapp, S. (2014). A unique inhibitor binding site in ERK1/2 is associated with slow binding kinetics. *Nature Chemical Biology*, *10*(10), 853–860. https://doi.org/10.1038/nchembio.1629

Chen, F., Hancock, C. N., Macias, A. T., Joh, J., Still, K., Zhong, S., … Shapiro, P. (2006). Characterization of ATP-independent ERK inhibitors identified through in silico analysis of the active ERK2 structure. *Bioorganic & Medicinal Chemistry Letters*, *16*(24), 6281–6287. https://doi.org/10.1016/j.bmcl.2006.09.038

Chen, H., Guo, R., Zhang, Q., Guo, H., Yang, M., Wu, Z., … Chen, L. (2015). Erk signaling is indispensable for genomic stability and self-renewal of mouse embryonic stem cells. *Proceedings of the National Academy of Sciences*, *112*(44), E5936–E5943. https://doi.org/10.1073/pnas.1516319112

Chen, J., Fujii, K., Zhang, L., Roberts, T., & Fu, H. (2001). Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK–ERK independent mechanism. *Proceedings of the National Academy of Sciences*, *98*(14), 7783–7788. https://doi.org/10.1073/pnas.141224398

Claro, R. A. de, McGinn, K. M., Verdun, N., Lee, S.-L., Chiu, H.-J., Saber, H., … Pazdur, R. (2015). FDA Approval: Ibrutinib for Patients with Previously

Treated Mantle Cell Lymphoma and Previously Treated Chronic Lymphocytic Leukemia. *Clinical Cancer Research*, *21*(16), 3586–3590. https://doi.org/10.1158/1078-0432.CCR-14-2225

Cohen-Armon, M., Visochek, L., Rozensal, D., Kalal, A., Geistrikh, I., Klein, R., … Seger, R. (2007). DNA-Independent PARP-1 Activation by Phosphorylated ERK2 Increases Elk1 Activity: A Link to Histone Acetylation. *Molecular Cell*, *25*(2), 297–308. https://doi.org/10.1016/j.molcel.2006.12.012

Cook, S. J., Stuart, K., Gilley, R., & Sale, M. J. (2017). Control of cell death and mitochondrial fission by ERK1/2 MAP kinase signalling. *The FEBS Journal*, doi: 10.1111/febs.14122. [Epub ahead of print]. https://doi.org/10.1111/febs.14122

Corcoran, R. B., Cheng, K. A., Hata, A. N., Faber, A. C., Ebi, H., Coffee, E. M., … Engelman, J. A. (2013). Synthetic Lethal Interaction of Combined BCL-XL and MEK Inhibition Promotes Tumor Regressions in KRAS Mutant Cancer Models. *Cancer Cell*, *23*(1), 121–128. https://doi.org/10.1016/j.ccr.2012.11.007

Corcoran, R. B., Dias-Santagata, D., Bergethon, K., Iafrate, A. J., Settleman, J., & Engelman, J. A. (2010). BRAF Gene Amplification Can Promote Acquired Resistance to MEK Inhibitors in Cancer Cells Harboring the BRAF V600E Mutation. *Sci. Signal.*, *3*(149), ra84-ra84. https://doi.org/10.1126/scisignal.2001148

Courcelles, M., Frémin, C., Voisin, L., Lemieux, S., Meloche, S., & Thibault, P. (2013). Phosphoproteome dynamics reveal novel ERK1/2 MAP kinase substrates with broad spectrum of functions. *Molecular Systems Biology*, *9*(1), 669. https://doi.org/10.1038/msb.2013.25

Cragg, M. S., Jansen, E. S., Cook, M., Harris, C., Strasser, A., & Scott, C. L. (2008). Treatment of B-RAF mutant human tumor cells with a MEK inhibitor requires Bim and is enhanced by a BH3 mimetic. *The Journal of Clinical Investigation*, *118*(11), 3651–3659. https://doi.org/10.1172/JCI35437

Crews, C. M., Alessandrini, A., & Erikson, R. L. (1992). The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science*, *258*(5081), 478–480. https://doi.org/10.1126/science.1411546

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., … Futreal, P. A. (2002). Mutations of the BRAF gene in human cancer. *Nature*, *417*(6892), 949–954. https://doi.org/10.1038/nature00766

Deng, Y., Shipps, G. W., Cooper, A., English, J. M., Annis, D. A., Carr, D., … Samatar, A. A. (2014). Discovery of Novel, Dual Mechanism ERK Inhibitors by Affinity Selection Screening of an Inactive Kinase. *Journal of Medicinal Chemistry*, *57*(21), 8817–8826. https://doi.org/10.1021/jm500847m

Dombi, E., Baldwin, A., Marcus, L. J., Fisher, M. J., Weiss, B., Kim, A., … Widemann, B. C. (2016). Activity of Selumetinib in Neurofibromatosis Type 1–Related Plexiform Neurofibromas. *New England Journal of Medicine*, *375*(26), 2550–2560. https://doi.org/10.1056/NEJMoa1605943

Dougherty, M. K., Müller, J., Ritt, D. A., Zhou, M., Zhou, X. Z., Copeland, T. D., … Morrison, D. K. (2005). Regulation of Raf-1 by Direct Feedback Phosphorylation. *Molecular Cell*, *17*(2), 215–224. https://doi.org/10.1016/j.molcel.2004.11.055

Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., & Saltiel, A. R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade.

Proceedings of the National Academy of Sciences of the United States of America, *92*(17), 7686–7689.

Eblen, S. T., Slack-Davis, J. K., Tarcsafalvi, A., Parsons, J. T., Weber, M. J., & Catling, A. D. (2004). Mitogen-Activated Protein Kinase Feedback Phosphorylation Regulates MEK1 Complex Formation and Activation during Cellular Adhesion. *Molecular and Cellular Biology*, *24*(6), 2308–2317. https://doi.org/10.1128/MCB.24.6.2308-2317.2004

Freeman, A. K., Ritt, D. A., & Morrison, D. K. (2013). Effects of Raf Dimerization and Its Inhibition on Normal and Disease-Associated Raf Signaling. *Molecular Cell*, *49*(4), 751–758. https://doi.org/10.1016/j.molcel.2012.12.018

Frémin, C., Saba-El-Leil, M. K., Lévesque, K., Ang, S.-L., & Meloche, S. (2015). Functional Redundancy of ERK1 and ERK2 MAP Kinases during Development. *Cell Reports*, *12*(6), 913–921. https://doi.org/10.1016/j.celrep.2015.07.011

Friday, B. B., Yu, C., Dy, G. K., Smith, P. D., Wang, L., Thibodeau, S. N., & Adjei, A. A. (2008). BRAF V600E Disrupts AZD6244-Induced Abrogation of Negative Feedback Pathways between Extracellular Signal-Regulated Kinase and Raf Proteins. *Cancer Research*, *68*(15), 6145–6153. https://doi.org/10.1158/0008-5472.CAN-08-1430

Germann, U., Furey, B., Roix, J., Markland, W., Hoover, R., Aronov, A., … Welsch, D. J. (2015). Abstract 4693: The selective ERK inhibitor BVD-523 is active in models of MAPK pathway-dependent cancers, including those with intrinsic and acquired drug resistance. *Cancer Research*, *75*(15 Supplement), 4693–4693. https://doi.org/10.1158/1538-7445.AM2015-4693

Gilley, R., March, H. N., & Cook, S. J. (2009). ERK1/2, but not ERK5, is necessary and sufficient for phosphorylation and activation of c-Fos. *Cellular Signalling*, *21*(6), 969–977. https://doi.org/10.1016/j.cellsig.2009.02.006

Giroux, S., Tremblay, M., Bernard, D., Cardin-Girard, J.-F., Aubry, S., Larouche, L., … Charron, J. (1999). Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. *Current Biology*, *9*(7), 369–376. https://doi.org/10.1016/S0960-9822(99)80164-X

Goetz, E. M., Ghandi, M., Treacy, D. J., Wagle, N., & Garraway, L. A. (2014). ERK Mutations Confer Resistance to Mitogen-Activated Protein Kinase Pathway Inhibitors. *Cancer Research*, *74*(23), 7079–7089. https://doi.org/10.1158/0008-5472.CAN-14-2073

Good, M. C., Zalatan, J. G., & Lim, W. A. (2011). Scaffold Proteins: Hubs for Controlling the Flow of Cellular Information. *Science*, *332*(6030), 680–686. https://doi.org/10.1126/science.1198701

Gross, S., Rahal, R., Stransky, N., Lengauer, C., & Hoeflich, K. P. (2015). Targeting cancer with kinase inhibitors. *The Journal of Clinical Investigation*, *125*(5), 1780–1789. https://doi.org/10.1172/JCI76094

Hancock, C. N., Macias, A., Lee, E. K., Yu, S. Y., MacKerell, & Shapiro, P. (2005). Identification of Novel Extracellular Signal-Regulated Kinase Docking Domain Inhibitors. *Journal of Medicinal Chemistry*, *48*(14), 4586–4595. https://doi.org/10.1021/jm0501174

Hari, S. B., Merritt, E. A., & Maly, D. J. (2013). Sequence Determinants of a Specific Inactive Protein Kinase Conformation. *Chemistry & Biology*, *20*(6), 806–815. https://doi.org/10.1016/j.chembiol.2013.05.005

Hatzivassiliou, G., Haling, J. R., Chen, H., Song, K., Price, S., Heald, R., … Belvin, M. (2013). Mechanism of MEK inhibition determines efficacy in mutant KRAS- versus BRAF-driven cancers. *Nature*, *501*(7466), 232–236. https://doi.org/10.1038/nature12441

Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B. J., Anderson, D. J., Alvarado, R., … Malek, S. (2010). RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature*, *464*(7287), 431–435. https://doi.org/10.1038/nature08833

Heidorn, S. J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvas, I., Dhomen, N., … Marais, R. (2010). Kinase-Dead BRAF and Oncogenic RAS Cooperate to Drive Tumor Progression through CRAF. *Cell*, *140*(2), 209–221. https://doi.org/10.1016/j.cell.2009.12.040

Herrero, A., Pinto, A., Colón-Bolea, P., Casar, B., Jones, M., Agudo-Ibáñez, L., … Crespo, P. (2015). Small Molecule Inhibition of ERK Dimerization Prevents Tumorigenesis by RAS-ERK Pathway Oncogenes. *Cancer Cell*, *28*(2), 170–182. https://doi.org/10.1016/j.ccell.2015.07.001

Holderfield, M., Deuker, M. M., McCormick, F., & McMahon, M. (2014). Targeting RAF kinases for cancer therapy: BRAF-mutated melanoma and beyond. *Nature Reviews Cancer*, *14*(7), 455. https://doi.org/10.1038/nrc3760

Holderfield, M., Merritt, H., Chan, J., Wallroth, M., Tandeske, L., Zhai, H., … Nagel, T. E. (2013). RAF Inhibitors Activate the MAPK Pathway by Relieving Inhibitory Autophosphorylation. *Cancer Cell*, *23*(5), 594–602. https://doi.org/10.1016/j.ccr.2013.03.033

Hoofnagle, A. N., Resing, K. A., Goldsmith, E. J., & Ahn, N. G. (2001). Changes in protein conformational mobility upon activation of extracellular regulated

protein kinase-2 as detected by hydrogen exchange. *Proceedings of the National Academy of Sciences*, *98*(3), 956–961. https://doi.org/10.1073/pnas.98.3.956

Howe, L. R., Leevers, S. J., Gómez, N., Nakielny, S., Cohen, P., & Marshall, C. J. (1992). Activation of the MAP kinase pathway by the protein kinase raf. *Cell*, *71*(2), 335–342. https://doi.org/10.1016/0092-8674(92)90361-F

Hu, S., Xie, Z., Onishi, A., Yu, X., Jiang, L., Lin, J., … Zhu, H. (2009). Profiling the Human Protein-DNA Interactome Reveals ERK2 as a Transcriptional Repressor of Interferon Signaling. *Cell*, *139*(3), 610–622. https://doi.org/10.1016/j.cell.2009.08.037

Hu-Lieskovan, S., Mok, S., Moreno, B. H., Tsoi, J., Robert, L., Goedert, L., … Ribas, A. (2015). Improved antitumor activity of immunotherapy with BRAF and MEK inhibitors in BRAFV600E melanoma. *Science Translational Medicine*, *7*(279), 279ra41-279ra41. https://doi.org/10.1126/scitranslmed.aaa4691

Ishii, N., Harada, N., Joseph, E. W., Ohara, K., Miura, T., Sakamoto, H., … Sakai, T. (2013). Enhanced Inhibition of ERK Signaling by a Novel Allosteric MEK Inhibitor, CH5126766, That Suppresses Feedback Reactivation of RAF Activity. *Cancer Research*, *73*(13), 4050–4060. https://doi.org/10.1158/0008-5472.CAN-12-3937

Johannessen, C. M., Boehm, J. S., Kim, S. Y., Thomas, S. R., Wardwell, L., Johnson, L. A., … Garraway, L. A. (2010). COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature*, *468*(7326), 968–972. https://doi.org/10.1038/nature09627

Joseph, E. W., Pratilas, C. A., Poulikakos, P. I., Tadi, M., Wang, W., Taylor, B. S., … Rosen, N. (2010). The RAF inhibitor PLX4032 inhibits ERK signaling and

tumor cell proliferation in a V600E BRAF-selective manner. *Proceedings of the National Academy of Sciences*, *107*(33), 14903–14908. https://doi.org/10.1073/pnas.1008990107

Jung, K.-Y., Samadani, R., Chauhan, J., Nevels, K., Yap, J. L., Zhang, J., … Fletcher, S. (2013). Structural Modifications of (Z)-3-(2-aminoethyl)-5-(4-ethoxybenzylidene)thiazolidine-2,4-dione that Improve Selectivity for the Inhibition of Melanoma Cells Containing Active ERK Signaling. *Organic & Biomolecular Chemistry*, *11*(22), 3706–3732. https://doi.org/10.1039/c3ob40199e

Karnoub, A. E., & Weinberg, R. A. (2008). Ras oncogenes: split personalities. *Nature Reviews Molecular Cell Biology*, *9*(7), 517. https://doi.org/10.1038/nrm2438

Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., & Cobb, M. H. (1998). Phosphorylation of the MAP Kinase ERK2 Promotes Its Homodimerization and Nuclear Translocation. *Cell*, *93*(4), 605–615. https://doi.org/10.1016/S0092-8674(00)81189-7

Khozin, S., Weinstock, C., Blumenthal, G. M., Cheng, J., He, K., Zhuang, L., … Pazdur, R. (2017). Osimertinib for the Treatment of Metastatic EGFR T790M Mutation–Positive Non–Small Cell Lung Cancer. *Clinical Cancer Research*, *23*(9), 2131–2135. https://doi.org/10.1158/1078-0432.CCR-16-1773

Kidger, A. M., & Keyse, S. M. (2016). The regulation of oncogenic Ras/ERK signalling by dual-specificity mitogen activated protein kinase phosphatases (MKPs). *Seminars in Cell & Developmental Biology*, *50*, 125–132. https://doi.org/10.1016/j.semcdb.2016.01.009

Kriegsheim, A. von, Baiocchi, D., Birtwistle, M., Sumpton, D., Bienvenut, W., Morrice, N., … Kolch, W. (2009). Cell fate decisions are specified by the

dynamic ERK interactome. *Nature Cell Biology*, *11*(12), 1458. https://doi.org/10.1038/ncb1994

Kubiniok, P., Lavoie, H., Therrien, M., & Thibault, P. (2017). Time-resolved Phosphoproteome Analysis of Paradoxical RAF Activation Reveals Novel Targets of ERK. *Molecular & Cellular Proteomics*, *16*(4), 663–679. https://doi.org/10.1074/mcp.M116.065128

Lamba, V., & Ghosh, I. (2012). New directions in targeting protein kinases: focusing upon true allosteric and bivalent inhibitors. *Current Pharmaceutical Design*, *18*(20), 2936–2945.

Lawrence, M. S., Stojanov, P., Mermel, C. H., Robinson, J. T., Garraway, L. A., Golub, T. R., … Getz, G. (2014). Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature*, *505*(7484), 495. https://doi.org/10.1038/nature12912

Lebraud, H., Wright, D. J., East, C. E., Holding, F. P., O'Reilly, M., & Heightman, T. D. (2016). In-gel activity-based protein profiling of a clickable covalent ERK1/2 inhibitor. *Molecular BioSystems*, *12*(9), 2867–2874. https://doi.org/10.1039/C6MB00367B

Lebraud, H., Wright, D. J., Johnson, C. N., & Heightman, T. D. (2016). Protein Degradation by In-Cell Self-Assembly of Proteolysis Targeting Chimeras. *ACS Central Science*, *2*(12), 927–934. https://doi.org/10.1021/acscentsci.6b00280

Leevers, S. J., & Marshall, C. J. (1992). Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. *The EMBO Journal*, *11*(2), 569–574.

Li, B. T., Janku, F., Patel, M. R., Sullivan, R. J., Flaherty, K., Buchbinder, E. I., … Infante, J. R. (2017). First-in-class oral ERK1/2 inhibitor Ulixertinib (BVD-

523) in patients with advanced solid tumors: Final results of a phase I dose escalation and expansion study. *Journal of Clinical Oncology*, *35*(15_suppl), 2508–2508. https://doi.org/10.1200/JCO.2017.35.15_suppl.2508

Li, D., Ambrogio, L., Shimamura, T., Kubo, S., Takahashi, M., Chirieac, L. R., … Wong, K.-K. (2008). BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene*, *27*(34), 4702–4711. https://doi.org/10.1038/onc.2008.109

Li, Q., Al-Ayoubi, A., Guo, T., Zheng, H., Sarkar, A., Nguyen, T., … Zhang, S. (2009). Structure–activity relationship (SAR) studies of 3-(2-amino-ethyl)-5-(4-ethoxy-benzylidene)-thiazolidine-2,4-dione: Development of potential substrate-specific ERK1/2 inhibitors. *Bioorganic & Medicinal Chemistry Letters*, *19*(21), 6042–6046. https://doi.org/10.1016/j.bmcl.2009.09.057

Lidke, D. S., Huang, F., Post, J. N., Rieger, B., Wilsbacher, J., Thomas, J. L., … Lenormand, P. (2010). ERK Nuclear Translocation Is Dimerization-independent but Controlled by the Rate of Phosphorylation. *Journal of Biological Chemistry*, *285*(5), 3092–3102. https://doi.org/10.1074/jbc.M109.064972

Lim, J., Kelley, E. H., Methot, J. L., Zhou, H., Petrocchi, A., Chen, H., … Siliphaivanh, P. (2016). Discovery of 1-(1H-Pyrazolo[4,3-c]pyridin-6-yl)urea Inhibitors of Extracellular Signal-Regulated Kinase (ERK) for the Treatment of Cancers. *Journal of Medicinal Chemistry*, *59*(13), 6501–6511. https://doi.org/10.1021/acs.jmedchem.6b00708

Lito, P., Saborowski, A., Yue, J., Solomon, M., Joseph, E., Gadal, S., … Rosen, N. (2014). Disruption of CRAF-Mediated MEK Activation Is Required for

Effective MEK Inhibition in KRAS Mutant Tumors. *Cancer Cell*, *25*(5), 697–710. https://doi.org/10.1016/j.ccr.2014.03.011

Little, A. S., Balmanno, K., Sale, M. J., Newman, S., Dry, J. R., Hampson, M., … Cook, S. J. (2011). Amplification of the Driving Oncogene, KRAS or BRAF, Underpins Acquired Resistance to MEK1/2 Inhibitors in Colorectal Cancer Cells. *Sci. Signal.*, *4*(166), ra17-ra17. https://doi.org/10.1126/scisignal.2001752

Little, A. S., Smith, P. D., & Cook, S. J. (2013). Mechanisms of acquired resistance to ERK1/2 pathway inhibitors. *Oncogene*, *32*(10), 1207–1215. https://doi.org/10.1038/onc.2012.160

Liu, Q., Sabnis, Y., Zhao, Z., Zhang, T., Buhrlage, S. J., Jones, L. H., & Gray, N. S. (2013). Developing Irreversible Inhibitors of the Protein Kinase Cysteinome. *Chemistry & Biology*, *20*(2), 146–159. https://doi.org/10.1016/j.chembiol.2012.12.006

Long, G. V., Stroyakovskiy, D., Gogas, H., Levchenko, E., de Braud, F., Larkin, J., … Flaherty, K. (2014). Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *The New England Journal of Medicine*, *371*(20), 1877–1888. https://doi.org/10.1056/NEJMoa1406037

Martin, G. A., Viskoohil, D., Bollag, G., McCabe, P. C., Crosier, W. J., Haubruck, H., … McCormick, F. (1990). The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell*, *63*(4), 843–849. https://doi.org/10.1016/0092-8674(90)90150-D

Masoumi-Moghaddam, S., Amini, A., & Morris, D. L. (2014). The developing story of Sprouty and cancer. *Cancer and Metastasis Reviews*, *33*(2–3), 695–720. https://doi.org/10.1007/s10555-014-9497-1

Meloche, S., & Pouysségur, J. (2007). The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene*, *26*(22), 3227–3239. https://doi.org/10.1038/sj.onc.1210414

Mendoza, M. C., Er, E. E., & Blenis, J. (2011). The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends in Biochemical Sciences*, *36*(6), 320–328. https://doi.org/10.1016/j.tibs.2011.03.006

Montagut, C., & Settleman, J. (2009). Targeting the RAF–MEK–ERK pathway in cancer therapy. *Cancer Letters*, *283*(2), 125–134. https://doi.org/10.1016/j.canlet.2009.01.022

Morris, E. J., Jha, S., Restaino, C. R., Dayananth, P., Zhu, H., Cooper, A., … Samatar, A. A. (2013). Discovery of a Novel ERK Inhibitor with Activity in Models of Acquired Resistance to BRAF and MEK Inhibitors. *Cancer Discovery*, *3*(7), 742–750. https://doi.org/10.1158/2159-8290.CD-13-0070

Müller, S., Chaikuad, A., Gray, N. S., & Knapp, S. (2015). The ins and outs of selective kinase inhibitor development. *Nature Chemical Biology*, *11*(11), 818–821. https://doi.org/10.1038/nchembio.1938

Nabet, B., Ó Broin, P., Reyes, J. M., Shieh, K., Lin, C. Y., Will, C. M., … Licht, J. D. (2015). Deregulation of the Ras-Erk Signaling Axis Modulates the Enhancer Landscape. *Cell Reports*, *12*(8), 1300–1313. https://doi.org/10.1016/j.celrep.2015.06.078

Nguyen, L. K., Matallanas, D. G., Romano, D., Kholodenko, B. N., & Kolch, W. (2015). Competing to coordinate cell fate decisions: the MST2-Raf-1 signaling device. *Cell Cycle*, *14*(2), 189–199. https://doi.org/10.4161/15384101.2014.973743

O'Donnell, A., Odrowaz, Z., & Sharrocks, A. D. (2012). Immediate-early gene activation by the MAPK pathways: what do and don't we know? *Biochemical Society Transactions*, *40*(1), 58–66. https://doi.org/10.1042/BST20110636

Ohori, M., Kinoshita, T., Okubo, M., Sato, K., Yamazaki, A., Arakawa, H., … Fujii, T. (2005). Identification of a selective ERK inhibitor and structural determination of the inhibitor–ERK2 complex. *Biochemical and Biophysical Research Communications*, *336*(1), 357–363. https://doi.org/10.1016/j.bbrc.2005.08.082

Ohori, M., Kinoshita, T., Yoshimura, S., Warizaya, M., Nakajima, H., & Miyake, H. (2007). Role of a cysteine residue in the active site of ERK and the MAPKK family. *Biochemical and Biophysical Research Communications*, *353*(3), 633–637. https://doi.org/10.1016/j.bbrc.2006.12.083

Ojesina, A. I., Lichtenstein, L., Freeman, S. S., Pedamallu, C. S., Imaz-Rosshandler, I., Pugh, T. J., … Meyerson, M. (2014). Landscape of genomic alterations in cervical carcinomas. *Nature*, *506*(7488), 371–375. https://doi.org/10.1038/nature12881

Park, J. W., Liu, M. C., Yee, D., Yau, C., van 't Veer, L. J., Symmans, W. F., … Berry, D. A. (2016). Adaptive Randomization of Neratinib in Early Breast Cancer. *New England Journal of Medicine*, *375*(1), 11–22. https://doi.org/10.1056/NEJMoa1513750

Plotnikov, A., Zehorai, E., Procaccia, S., & Seger, R. (2011). The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1813*(9), 1619–1633. https://doi.org/10.1016/j.bbamcr.2010.12.012

Poulikakos, P. I., Persaud, Y., Janakiraman, M., Kong, X., Ng, C., Moriceau, G., … Solit, D. B. (2011). RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature*, *480*(7377), 387–390. https://doi.org/10.1038/nature10662

Poulikakos, P. I., Zhang, C., Bollag, G., Shokat, K. M., & Rosen, N. (2010). RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature*, *464*(7287), 427–430. https://doi.org/10.1038/nature08902

Pratilas, C. A., Taylor, B. S., Ye, Q., Viale, A., Sander, C., Solit, D. B., & Rosen, N. (2009). V600EBRAF is associated with disabled feedback inhibition of RAF–MEK signaling and elevated transcriptional output of the pathway. *Proceedings of the National Academy of Sciences*, *106*(11), 4519–4524. https://doi.org/10.1073/pnas.0900780106

Queirolo, P., Picasso, V., & Spagnolo, F. (2015). Combined BRAF and MEK inhibition for the treatment of BRAF-mutated metastatic melanoma. *Cancer Treatment Reviews*, *41*(6), 519–526. https://doi.org/10.1016/j.ctrv.2015.04.010

Ritt, D. A., Monson, D. M., Specht, S. I., & Morrison, D. K. (2010). Impact of Feedback Phosphorylation and Raf Heterodimerization on Normal and Mutant B-Raf Signaling. *Molecular and Cellular Biology*, *30*(3), 806–819. https://doi.org/10.1128/MCB.00569-09

Robinson, F. L., Whitehurst, A. W., Raman, M., & Cobb, M. H. (2002). Identification of Novel Point Mutations in ERK2 That Selectively Disrupt Binding to MEK1. *Journal of Biological Chemistry*, *277*(17), 14844–14852. https://doi.org/10.1074/jbc.M107776200

Rodríguez, J., Calvo, F., González, J. M., Casar, B., Andrés, V., & Crespo, P. (2010). ERK1/2 MAP kinases promote cell cycle entry by rapid, kinase-independent

disruption of retinoblastoma–lamin A complexes. *The Journal of Cell Biology*, *191*(5), 967–979. https://doi.org/10.1083/jcb.201004067

Saba-El-Leil, M. K., Vella, F. D. J., Vernay, B., Voisin, L., Chen, L., Labrecque, N., … Meloche, S. (2003). An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. *EMBO Reports*, *4*(10), 964–968. https://doi.org/10.1038/sj.embor.embor939

Sale, M. J., & Cook, S. J. (2013). The BH3 mimetic ABT-263 synergizes with the MEK1/2 inhibitor selumetinib/AZD6244 to promote BIM-dependent tumour cell death and inhibit acquired resistance. *Biochemical Journal*, *450*(2), 285–294. https://doi.org/10.1042/BJ20121212

Samadani, R., Zhang, J., Brophy, A., Oashi, T., Priyakumar, U. D., Raman, E. P., … Shapiro, P. (2015). Small-molecule inhibitors of ERK-mediated immediate early gene expression and proliferation of melanoma cells expressing mutated BRaf. *Biochemical Journal*, *467*(3), 425–438. https://doi.org/10.1042/BJ20131571

Schirmer, A., Kennedy, J., Murli, S., Reid, R., & Santi, D. V. (2006). Targeted covalent inactivation of protein kinases by resorcylic acid lactone polyketides. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(11), 4234–4239. https://doi.org/10.1073/pnas.0600445103

Schulze, A., Lehmann, K., Jefferies, H. B. J., McMahon, M., & Downward, J. (2001). Analysis of the transcriptional program induced by Raf in epithelial cells. *Genes & Development*, *15*(8), 981–994. https://doi.org/10.1101/gad.191101

Shapiro, P. S., Whalen, A. M., Tolwinski, N. S., Wilsbacher, J., Froelich-Ammon, S. J., Garcia, M., … Ahn, N. G. (1999). Extracellular Signal-Regulated Kinase Activates Topoisomerase IIα through a Mechanism Independent of

Phosphorylation. *Molecular and Cellular Biology*, *19*(5), 3551–3560. https://doi.org/10.1128/MCB.19.5.3551

Sipthorp, J., Lebraud, H., Gilley, R., Kidger, A. M., Okkenhaug, H., Saba-El-Leil, M., … Heightman, T. D. (2017). Visualization of Endogenous ERK1/2 in Cells with a Bioorthogonal Covalent Probe. *Bioconjugate Chemistry*, *28*(6), 1677–1683. https://doi.org/10.1021/acs.bioconjchem.7b00152

Sturm, O. E., Orton, R., Grindlay, J., Birtwistle, M., Vyshemirsky, V., Gilbert, D., … Kolch, W. (2010). The Mammalian MAPK/ERK Pathway Exhibits Properties of a Negative Feedback Amplifier. *Sci. Signal.*, *3*(153), ra90-ra90. https://doi.org/10.1126/scisignal.2001212

Tiacci, E., Park, J. H., De Carolis, L., Chung, S. S., Broccoli, A., Scott, S., … Tallman, M. S. (2015). Targeting Mutant BRAF in Relapsed or Refractory Hairy-Cell Leukemia. *New England Journal of Medicine*, *373*(18), 1733–1747. https://doi.org/10.1056/NEJMoa1506583

Tiacci, E., Trifonov, V., Schiavoni, G., Holmes, A., Kern, W., Martelli, M. P., … Falini, B. (2011). BRAF Mutations in Hairy-Cell Leukemia. *New England Journal of Medicine*, *364*(24), 2305–2315. https://doi.org/10.1056/NEJMoa1014209

Trahey, M., & McCormick, F. (1987). A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science*, *238*(4826), 542–545. https://doi.org/10.1126/science.2821624

Ünal, E. B., Uhlitz, F., & Blüthgen, N. (2017). A compendium of ERK targets. *FEBS Letters*, *591*(17), 2607–2615. https://doi.org/10.1002/1873-3468.12740

Villanueva, J., Vultur, A., Lee, J. T., Somasundaram, R., Fukunaga-Kalabis, M., Cipolla, A. K., … Herlyn, M. (2010). Acquired Resistance to BRAF Inhibitors

Mediated by a RAF Kinase Switch in Melanoma Can Be Overcome by Cotargeting MEK and IGF-1R/PI3K. *Cancer Cell*, *18*(6), 683–695. https://doi.org/10.1016/j.ccr.2010.11.023

Ward, R. A., Bethel, P., Cook, C., Davies, E., Debreczeni, J. E., Fairley, G., … Zhai, B. (2017). Structure-Guided Discovery of Potent and Selective Inhibitors of ERK1/2 from a Modestly Active and Promiscuous Chemical Start Point. *Journal of Medicinal Chemistry*. https://doi.org/10.1021/acs.jmedchem.7b00267

Ward, R. A., Colclough, N., Challinor, M., Debreczeni, J. E., Eckersley, K., Fairley, G., … Yeung, K. (2015). Structure-Guided Design of Highly Selective and Potent Covalent Inhibitors of ERK1/2. *Journal of Medicinal Chemistry*, *58*(11), 4790–4801. https://doi.org/10.1021/acs.jmedchem.5b00466

Warne, P. H., Vician, P. R., & Downward, J. (1993). Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature*, *364*(6435), 352–355. https://doi.org/10.1038/364352a0

Whitty, A., & Kumaravel, G. (2006). Between a rock and a hard place? *Nature Chemical Biology*, *2*(3), 112–118. https://doi.org/10.1038/nchembio0306-112

Wilhelm, S. M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., … Trail, P. A. (2004). BAY 43-9006 Exhibits Broad Spectrum Oral Antitumor Activity and Targets the RAF/MEK/ERK Pathway and Receptor Tyrosine Kinases Involved in Tumor Progression and Angiogenesis. *Cancer Research*, *64*(19), 7099–7109. https://doi.org/10.1158/0008-5472.CAN-04-1443

Wilsbacher, J. L., Juang, Y.-C., Khokhlatchev, A. V., Gallagher, E., Binns, D., Goldsmith, E. J., & Cobb, M. H. (2006). Characterization of Mitogen-

Activated Protein Kinase (MAPK) Dimers. *Biochemistry*, *45*(44), 13175–13182. https://doi.org/10.1021/bi061041w

Wu, P., Clausen, M. H., & Nielsen, T. E. (2015). Allosteric small-molecule kinase inhibitors. *Pharmacology & Therapeutics*, *156*, 59–68. https://doi.org/10.1016/j.pharmthera.2015.10.002

Yang, S.-H., Sharrocks, A. D., & Whitmarsh, A. J. (2013). MAP kinase signalling cascades and transcriptional regulation. *Gene*, *513*(1), 1–13. https://doi.org/10.1016/j.gene.2012.10.033

Yap, J. L., Worlikar, S., MacKerell, A. D., Shapiro, P., & Fletcher, S. (2011). Small-Molecule Inhibitors of the ERK Signaling Pathway: Towards Novel Anticancer Therapeutics. *ChemMedChem*, *6*(1), 38–48. https://doi.org/10.1002/cmdc.201000354

Zhang, J., Yang, P. L., & Gray, N. S. (2009). Targeting cancer with small molecule kinase inhibitors. *Nature Reviews Cancer*, *9*(1), 28–39. https://doi.org/10.1038/nrc2559

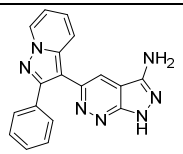
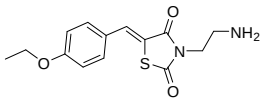
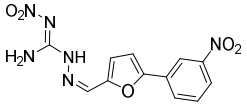
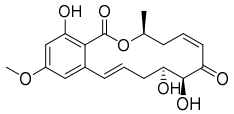
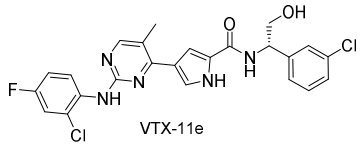
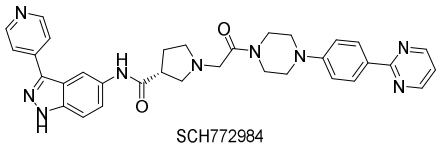
Zhang, J., Zhou, B., Zheng, C.-F., & Zhang, Z.-Y. (2003). A Bipartite Mechanism for ERK2 Recognition by Its Cognate Regulators and Substrates. *Journal of Biological Chemistry*, *278*(32), 29901–29912. https://doi.org/10.1074/jbc.M303909200

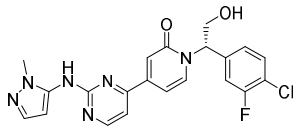
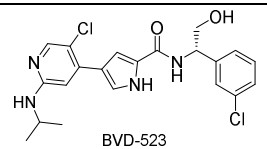
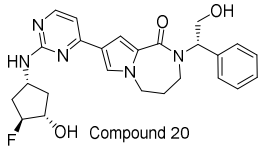
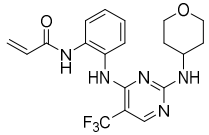
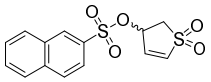
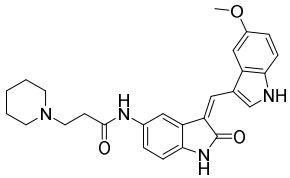
Zhao, Z., Wu, H., Wang, L., Liu, Y., Knapp, S., Liu, Q., & Gray, N. S. (2014). Exploration of Type II Binding Mode: A Privileged Approach for Kinase Inhibitor Focused Drug Discovery? *ACS Chemical Biology*, *9*(6), 1230–1241. https://doi.org/10.1021/cb500129t

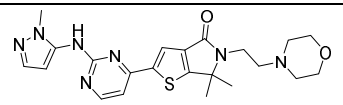
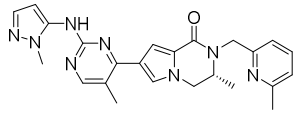
Zhou, G., Bao, Z. Q., & Dixon, J. E. (1995). Components of a New Human Protein Kinase Signal Transduction Pathway. *Journal of Biological Chemistry*, *270*(21), 12665–12669. https://doi.org/10.1074/jbc.270.21.12665

Zhu, H. Y., Desai, J., Deng, Y., Cooper, A., Wang, J., Shipps, J., … Windsor, W. (2015). Discovery of hydroxyaniline amides as selective Extracellular Regulated Kinase (Erk) inhibitors. *Bioorganic & Medicinal Chemistry Letters*, *25*(7), 1627–1629. https://doi.org/10.1016/j.bmcl.2015.01.049

Table 1. Small molecule inhibitors of ERK1/2

ERK inhibitor		Structure	Year Reported	Developer	Classification/ Binding Mode	*In vitro* IC_{50} for ERK2 (nM)	Notes	Clinical progression	References
FR180204	1	 FR180204	2005	Astellas Pharmaceuticals	Reversible, ATP competitive – Catalytic	330		Pre-clinical	(Ohori et al., 2005)
Compound 76	2	 Compound 76	2005	Shapiro group, University of Maryland	Allosteric	Not relevant	ERK1/2 D-recruitment site (DRS, also known as common docking (CD)-domain) inhibitor	Research tool	(Hancock et al., 2005) Development of compound 76: (Boston et al., 2011; Jung et al., 2013; Li et al., 2009)
Compound 89	3	 Compound 89	2006	Shapiro group, University of Maryland	Allosteric	Not relevant	ERK1/2 DRS inhibitor, targeted at active structure of ERK2	Research tool	(Chen et al., 2006)
FR148083	4	 FR148083	2007	Astellas Pharmaceuticals	Covalent	80‡		Pre-clinical	(Ohori et al., 2007)
VTX-11e	5	 VTX-11e	2009	Vertex Pharmaceuticals	Reversible, ATP competitive - Catalytic	<2		Pre-clinical	(Aronov et al., 2009)
SCH772984	6	 SCH772984	2011	Merck	Reversible, ATP competitive - Dual-mechanism	1	Clinical analogue MK-8353 (SCH900353) in Phase I trials.	Pre-clinical / Phase I (MK-8353)	(Deng et al., 2014; Lim et al., 2016; Morris et al., 2013; Zhu et al., 2015) ClinicalTrials.gov Identifier:

									NCT01358331
GDC-0994	7	 GDC-0994	2013	Genentech / Array BioPharma	Reversible, ATP competitive – Catalytic *	3.1		Phase I	(Blake et al., 2014, 2016; Ren et al., 2015; Robarge et al., 2014) ClinicalTrials.gov Identifier: NCT01875705
BVD-523 (Ulixertinib)	8	 BVD-523	2013	Biomed Valley Discoveries	Reversible, ATP competitive – Catalytic *	<0.3	Developed from Vtx-11e compound series.	Phase I	(Germann et al., 2015; Li et al., 2017) ClinicalTrials.gov Identifier: NCT01781429
CC-90003	9	N/A	2014	Celgene	Covalent	Not available		Phase I	(Mita et al., 2017) ClinicalTrials.gov Identifier: NCT02313012
Compound 20	10	 Compound 20	2015	Novartis	Reversible, ATP competitive – Catalytic *	0.19	Cyclized variants of the Vtx-11e compound series.	Pre-clinical	(Bagdanoff et al., 2015a, 2015b)
AZ13767370 (Compound 13)	11	 Compound 13	2015	AstraZeneca	Covalent	6.9‡		Pre-clinical	(Ward et al., 2015)
SF-3-030	12	 SF-3-30	2015	Shapiro group, University of Maryland	Allosteric	Not relevant	ERK1/2 F-recruitment site (FRS) inhibitor	Research tool	(Samadani et al., 2015)
DEL-22379	13	 DEL-22379	2015	Crespo group, University of Cantabria	Allosteric	Not relevant	ERK1/2 dimerisation inhibitor	Research tool	(Herrero et al., 2015)

LY3214996[†]	14	 LY3214996	2016	Eli Lily	Not available	5		Phase I	(Bhagwat et al., 2017) ClinicalTrials.gov Identifier: NCT02857270
LTT462	15	N/A	2016	Novartis	Not available	Not available		Phase I	ClinicalTrials.gov Identifier: NCT02711345
Compound 35	16	 Compound 35	2017	AstraZeneca	Reversible, ATP competitive - Dual-mechanism	<0.3	Small changes in the compound series altered the binding profile between catalytic and dual mechanism ERK inhibitors.	Pre-clinical	(Ward et al., 2017)
KO-947	17	N/A	2017	Kura Oncology	Not available	10		Phase I	(Burrows et al., 2017) ClinicalTrials.gov Identifier: NCT03051035

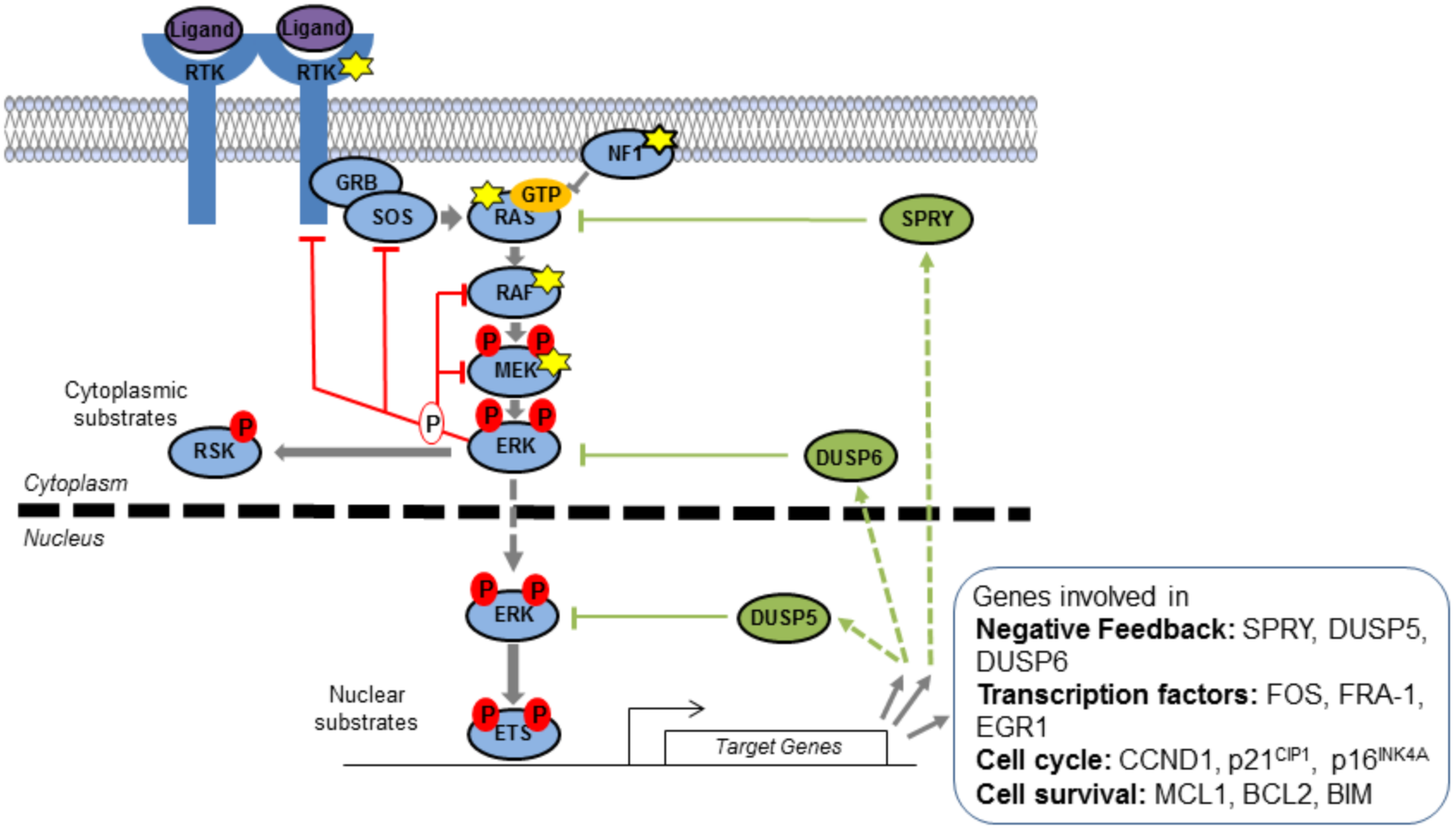
Notes

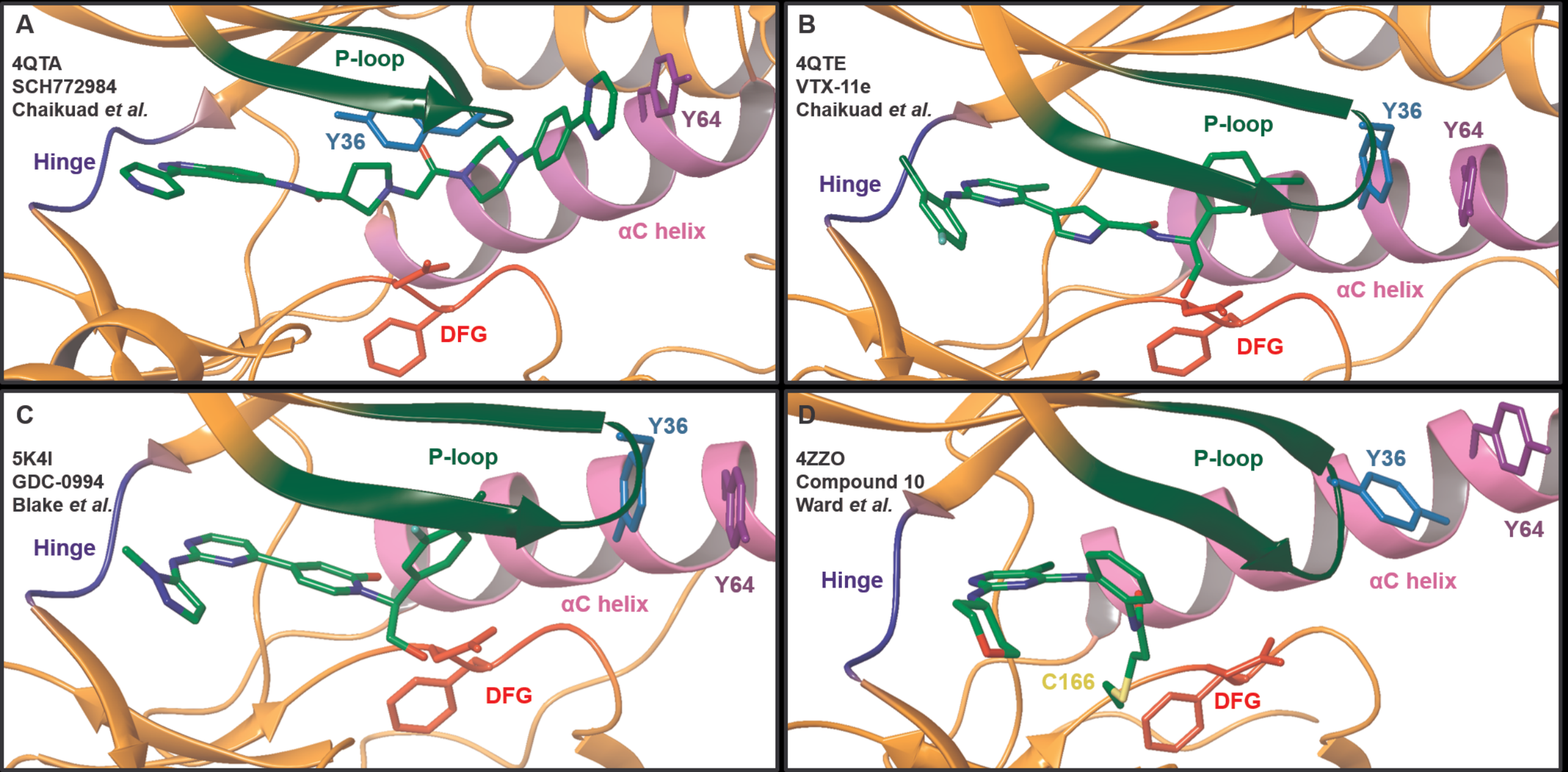
The year reported is based on the earliest publication or clinical trial start date of the compound in question.

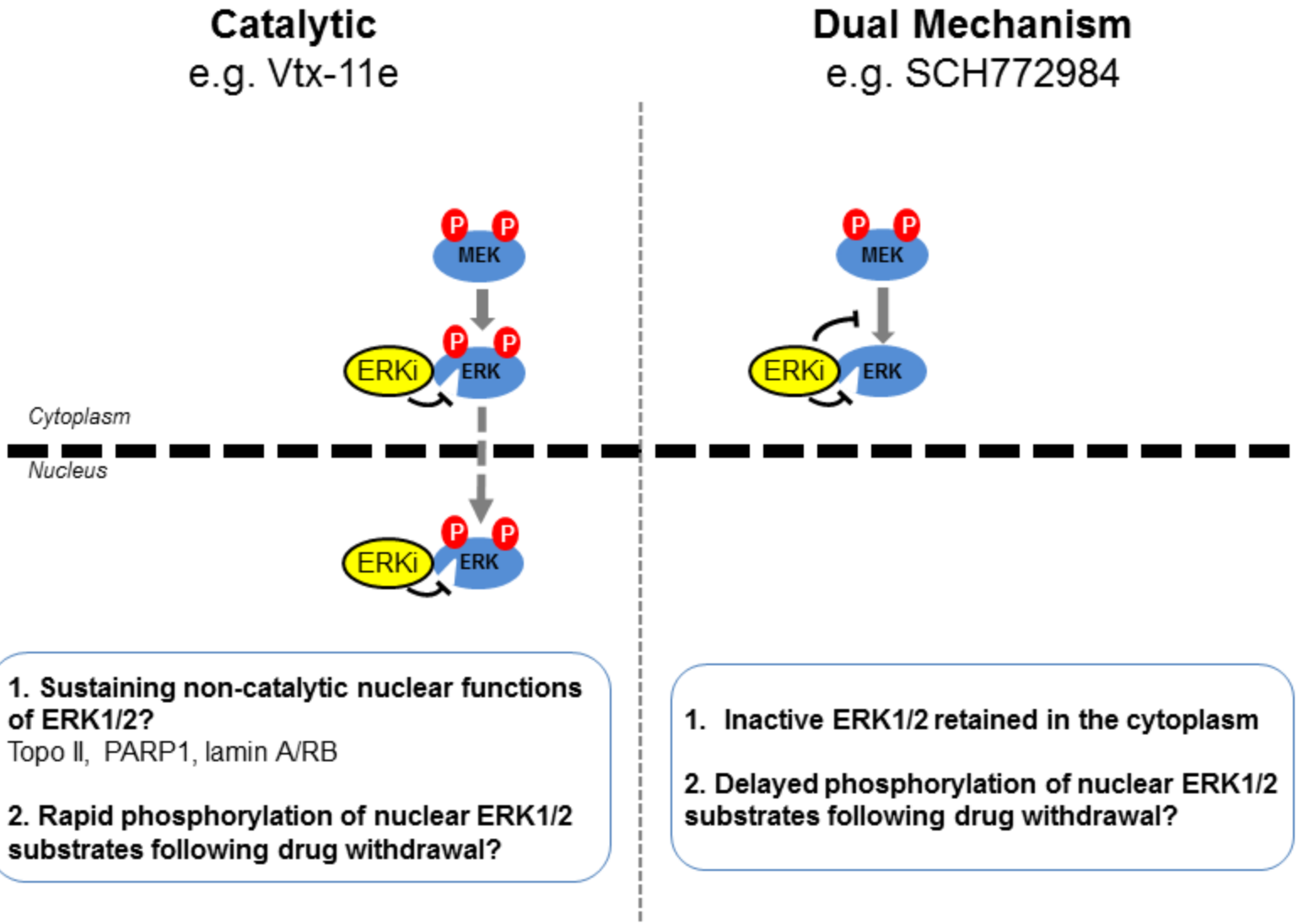
*No evidence these compounds are able to inhibit the phosphorylation of ERK and therefore possess a "dual mechanism", however limited *in vivo* biological data has been published for these compounds.

[‡] Due to their nature as covalent inhibitors, IC_{50} values cannot be compared between assays, as the time of incubation will affect the relative potency of the compounds.

[†] Structure of LY3214996 is taken from Selleck Chem, which corresponds to a structure disclosed within an Eli Lily patent, however, to our knowledge the structure has not been disclosed in any primary literature.


Ligand
Ligand
RTK
RTK
NF1
GRB
SOS
GTP
RAS
SPRY
RAF
P
P
MEK
Cytoplasmic
substrates
P
P
P
RSK
ERK
DUSP6
Cytoplasm
Nucleus
P
P
ERK
DUSP5
Nuclear
substrates
P
P
ETS
Target Genes
Genes involved in
Negative Feedback: SPRY, DUSP5, DUSP6
Transcription factors: FOS, FRA-1, EGR1
Cell cycle: CCND1, p21CIP1, p16INK4A
Cell survival: MCL1, BCL2, BIM


A
4QTA
SCH772984
Chaikuad et al.
P-loop
Y36
Y64
Hinge
αC helix
DFG
B
4QTE
VTX-11e
Chaikuad et al.
P-loop
Y36
Y64
Hinge
αC helix
DFG
C
5K4I
GDC-0994
Blake et al.
Y36
P-loop
Hinge
Y64
αC helix
DFG
D
4ZZO
Compound 10
Ward et al.
P-loop
Y36
Y64
Hinge
αC helix
C166
DFG


Catalytic
e.g. Vtx-11e
Dual Mechanism
e.g. SCH772984
P
P
MEK
ERKi
ERK
Cytoplasm
Nucleus
1. Sustaining non-catalytic nuclear functions of ERK1/2?
Topo II, PARP1, lamin A/RB
2. Rapid phosphorylation of nuclear ERK1/2 substrates following drug withdrawal?
1. Inactive ERK1/2 retained in the cytoplasm
2. Delayed phosphorylation of nuclear ERK1/2 substrates following drug withdrawal?