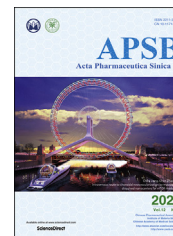




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REVIEW

Development of small molecule extracellular signal-regulated kinases (ERKs) inhibitors for cancer therapy



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Extracellular signal-regulated kinase 5 inhibitors;
Inhibition;
Selectivity

Abstract The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway is widely activated by a variety of extracellular stimuli, and its dysregulation is associated with the proliferation, invasion, and migration of cancer cells. ERK1/2 is located at the distal end of this pathway and rarely undergoes mutations, making it an attractive target for anticancer drug development. Currently, an increasing number of ERK1/2 inhibitors have been designed and synthesized for antitumor therapy, among which representative compounds have entered clinical trials. When ERK1/2 signal transduction is eliminated, ERK5 may provide a bypass route to rescue proliferation, and weaken the potency of ERK1/2 inhibitors. Therefore, drug research targeting ERK5 or based on the compensatory mechanism of ERK5 for ERK1/2 opens up a new way for oncotherapy. This review provides an overview of the physiological and biological functions of ERKs, focuses on the structure–activity relationships of small molecule inhibitors targeting ERKs, with a view to providing guidance for future drug design and optimization, and discusses the potential therapeutic strategies to overcome drug resistance.

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1. Introduction

The mitogen-activated protein kinase (MAPK) cascades are one of the major signal transduction systems that bring extracellular signals into nucleus. MAPKs are evolutionarily conserved in eukaryotes, can regulate gene expression, and are closely related to cell growth, development, differentiation, apoptosis and other functions^{1,2}. Conventional MAPKs are composed of four sub-families, namely extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases1/2/3 (JNK1/2/3), p38 isoforms (p38 $\alpha/\beta/\gamma/\delta$) and ERK5^{2,3}. They all have a Thr-Xaa-Tyr motif in their activation segment and are activated by upstream MAPK kinases (MAPKKs)⁴. There are several atypical MAPKs, including ERK3, ERK4, ERK8 and Nemo-like kinase (NLK)⁵. The structural features and biological functions of conventional MAPKs have been extensively studied (Fig. 1). Among these various signaling cascades, RAS-RAF-MEK-ERK is the most important pathway^{6,7}. This pathway is responsible for a variety of fundamental cellular processes and is therefore strictly regulated by many regulatory factors, such as scaffold proteins^{8,9}, phosphatases alternatively spliced isoforms^{10,11}, subcellular localization¹² and so on. The importance of the ERK cascade in the cells suggests that its maladjustment would be detrimental to the cells and even to the organism. It has been proved that the over-activation of various components in this pathway can induce a variety of diseases. ERK1/2 is located at the end of the pathway and is activated by MEK1/2 upstream of the pathway. Phosphorylated ERK1/2 binds to importin7, and then escorts ERK1/2 from the cytoplasm to the nucleus through the nuclear pore¹³. ERK1/2 catalyze the phosphorylation of numerous related downstream targets, including cytoplasmic and nuclear substrates such as transcription factors and regulatory molecules^{14,15}. Subsequently, early genes controlling cell proliferation are rapidly induced¹⁶. These activated proteins in turn induce the expression of other proteins that further transmit the signals to induce and regulate cell proliferation or oncogenic transformation¹⁷. The unique position of ERK1/2 in the pathway allows abnormal mutations in the upstream members cause excessive activation of ERK1/2, so inhibition of ERK1/2 activity can block the pathological effect of the pathway. A deeper understanding of the biological functions of the MAPK pathway has facilitated the development of ERK inhibitors. In recent years, a large number of selective ERK1/2 inhibitors with different mechanisms of action have been designed and synthesized, and they have been proved to have significant ERK1/2 inhibitory activity and anti-tumor cell proliferation effect. In addition, studies have shown that ERK1/2 inhibitors can overcome the acquired resistance induced by upstream kinase inhibitors, and can reverse the abnormal activation of the MAPK pathway caused by mutations in the upstream pathway^{18,19}. At present, some representative ERK1/2 inhibitors have been selected into clinical trials.

The ERK5 pathway also belongs to the conventional MAPK pathway and is activated sequentially through MEK2/3-MEK5-ERK5²⁰. The MEK5/ERK5 pathway elicits a variety of biological responses, including cell survival, proliferation and differentiation. More and more evidences show that this pathway is involved in tumor genesis and development²¹. Based on this, targeting the MEK5/ERK5 pathway is clearly one of the possible strategies to effectively block the signal transduction of this pathway and inhibit cancer growth^{22,23}. Besides, ERK5 itself can act as a transcription factor. ERK5 resembles the other MAPK family

members in its N-terminal half, but it also contains a 400-amino-acid C-terminal region, which contains a myocyte enhancer factor 2 (MEF2)-interacting domain and also a transcriptional activation domain (TAD). This region undergoes autophosphorylation, allowing ERK5 to directly regulate gene transcription^{24,25}. The TAD of ERK5 is used to activate the MEF2 family transcription factors, and then activate the endogenous nuclear receptor 4A1 (NR4A1, Nur77) gene, which further induces the transcriptional upregulation of Nur77. The whole process plays an important role in T cell apoptosis. Recent studies have found that once the ERK1/2 signal is inhibited, the ERK5 pathway will be rapidly activated, which will compensate for the maintenance of the malignant proliferation of tumor cells. The compensatory mechanism of ERK5 to ERK1/2 may be the key reason why the existing ERK1/2 inhibitors are difficult to exert efficacy in preclinical and clinical trials. Therefore, drug research based on the compensatory mechanism of ERK1/2-ERK5 is another potentially effective tumor treatment strategy²⁶.

From this perspective, we reviewed the structural characteristics and activation mechanisms of the ERK pathway in the MAPK family, introduced the mutation subtypes of this pathway as well as their pathological roles in the development, invasion and metastasis of tumors. Furthermore, we summarized the functional redundancy between ERK1 and ERK2 as well as the compensatory mechanism of ERK5 for ERK1/2. Importantly, we focused on the research progress of selective small molecule inhibitors of ERKs with different mechanisms of action, including ATP-competitive, covalent, allosteric, dual-target ERK1/2 inhibitors, ERK5 inhibitors and ERK1/5 dual-target inhibitors. We highlighted the drug design and the SAR of ERKs inhibitors from the perspective of medicinal chemistry, aiming to provide more new insights for the discovery of highly effective ERKs inhibitors. Finally, we discussed the resistance mechanisms of ERKs-targeted inhibitors and potential therapeutic strategies to combat resistance, speculated on the future directions of ERKs inhibitor design.

2. Structures and signaling pathways of ERKs

2.1. Overview of the MAPKs family

The MAPK cascades regulate a variety of normal cell functions, and they are also one of the most important carcinogenic drivers of human malignant tumors. When the MAPK cascades are abnormally regulated, they could accelerate the infinite proliferation of normal human cells, avoid apoptosis, are insensitive to growth-inhibiting signals, maintain the nutrient supply of tumor cells and promote angiogenesis, and then lead to malignant proliferation, invasion and metastasis¹⁴. The MAPK pathways includes multiple signal cascades, among which RAS-RAF-MEK-ERK pathway is one of the most mature pathways, and its disorder is also one of the important reasons for tumor malignant progression, invasion and metastasis. As shown in Fig. 1, when the extracellular stimulus binds to the receptor, the inactivated RAS releases GDP and binds to a new GTP, transforming into activated RAS, and then the GTP-loaded RAS directly contacts the RAF kinases to form active homodimers or heterodimers, composed of ARAF, BRAF, and CRAF through an intricate process, and induces catalytic activity of RAF kinases²⁷. The activated RAF can catalyze the serine phosphorylation of MEK1/2 protein through its C-terminal kinase domain, thereby further activating downstream MEK1/2. Then the kinase domain of activated MEK1/2 catalyzes the phosphorylation

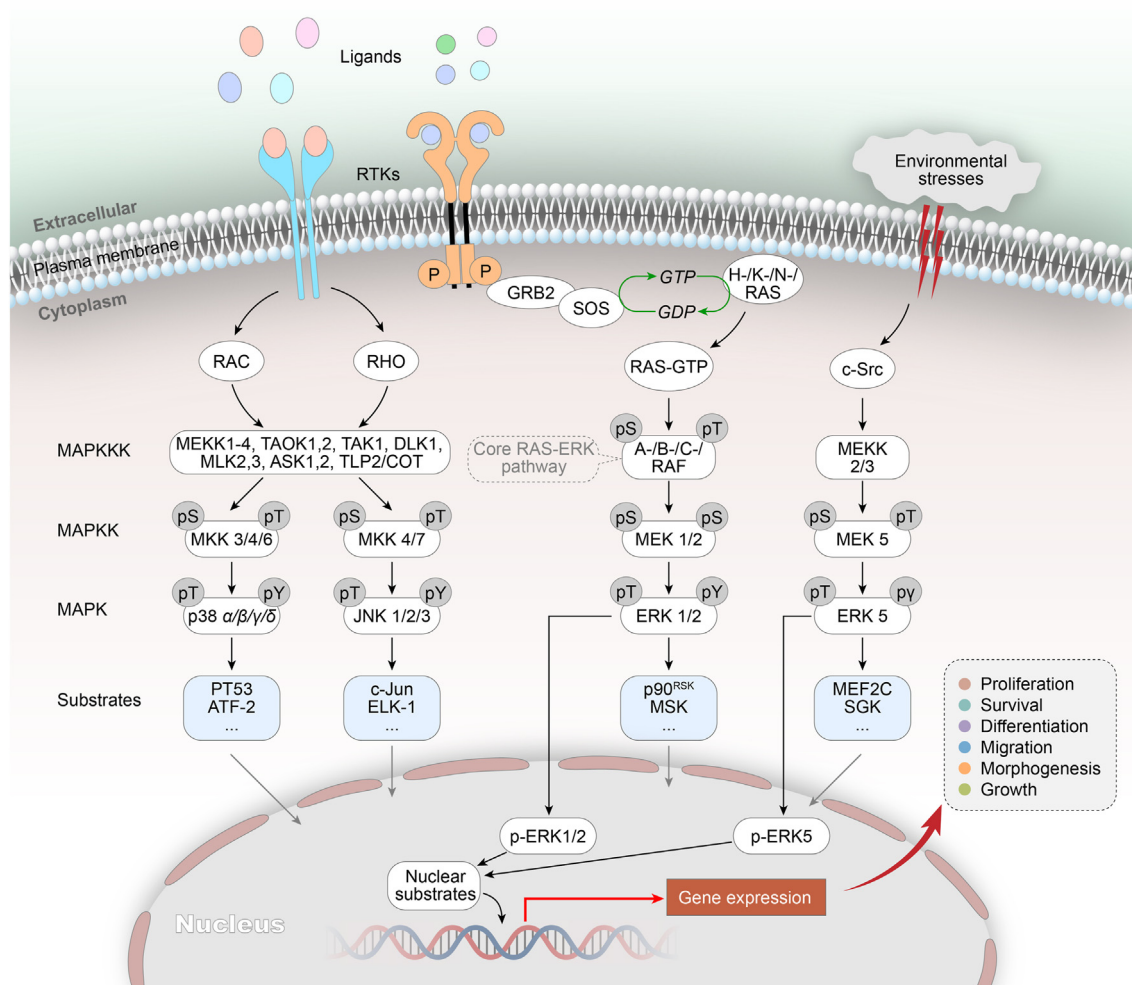


Figure 1 The MAPK cascades and the core RAS/ERK signaling pathway. Extracellular stimulus recruits the guanine nucleotide exchange factor SOS (Son of Sevenless) to induce RAS to release GDP and binds to a new GTP. Activated RAS promotes the dimerization and activation of the A-/B-/C-/RAF, which further activates downstream signals MEK1/2. The kinase domain of activated MEK1/2 catalyzes the phosphorylation of tyrosine and threonine residues in the ERK1/2 activation segment to activate ERK1/2. Eventually activated ERK1/2 enters the cell nucleus to phosphorylate a series of transcription factors and modulate many critical aspects of cell physiology.

of tyrosine and threonine residues (Thr202/Tyr204 for ERK1 and Thr185/Tyr187 for ERK2) in the activation segment of ERK1/2 and activates ERK1/2. Eventually activated ERK1/2 enters the cell nucleus, phosphorylates a series of transcription factors, regulates gene expression and triggers a series of physiological and biochemical reactions, and mediates biological processes such as cell growth, differentiation, apoptosis, and metastasis^{28–31}.

2.2. Domain structures and biological functions of ERKs

The conventional ERK1/2/5 are encoded by the MAPK3, MAPK1 and MAPK7 genes, respectively. At present, the conventional MAPKs, especially ERK1/2 have been thoroughly researched, but little is known about the structural characteristics, regulatory mechanisms and downstream functions of atypical MAPKs. A large number of studies have shown that there are no obvious differences in functions, substrates or mechanisms between ERK1 and ERK2. On the contrary, they are functionally redundant. The sequence homology between ERK1 and ERK2 is up to 84%, while the sequence homology between ERK5 and ERK2 is 66%^{32–34}. In

terms of structures, ERK1/2/5 are composed of a relatively small N-terminal and a large C-terminal coil to form a bilobal structure, which contains multiple conservative α -helices and β -sheets (Fig. 2)^{34,35}. The N-terminal consists of five antiparallel β -sheet structures (β 1– β 5), an α -helix structure and a glycine-enriched loop structure³⁶. The C-terminal is mainly in the shape of a side helix, consisting of 6 conservative α -helix structures and 4 shorter β -sheet structures (β 6– β 9)³⁷. The structure connecting the N-terminus and the C-terminus provides an active catalytic space for the entire kinase. Its hinge region can interact with the adenine in ATP molecule through hydrogen bonds, and the P-loop region can form an important interaction with the phosphate of ATP molecule. Cyclic peptide chains activated by kinases can regulate the inactivation and activation of kinase through conformational changes^{38–40}. The activation loop peptide chain is a segment of the structure starting from DFG (Asp-Phe-Gly) and ending from APE (Ala-Pro-Glu). When the proteins are in the DFG-in conformation, the aspartic acid residue points inward to the direction of the ATP-binding site and the phenylalanine residue points to other directions, exposing the ATP-binding pocket,

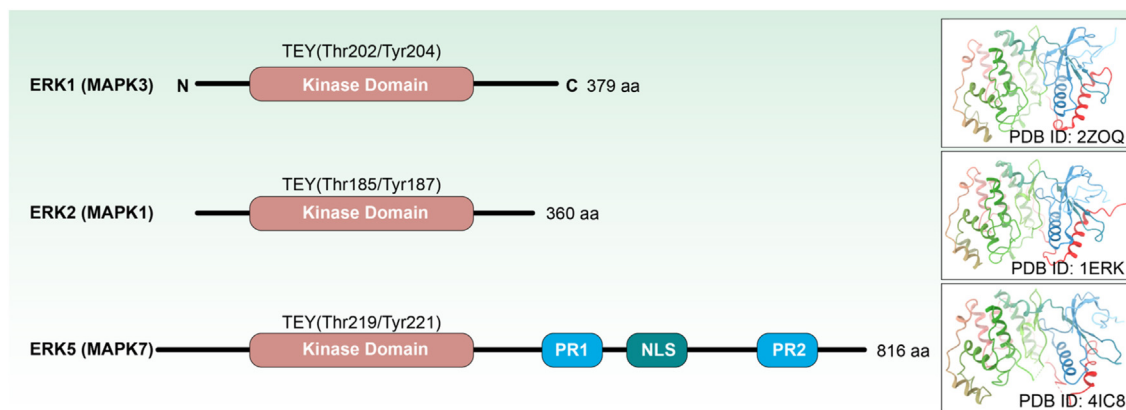


Figure 2 Schematic representation and crystal structures of ERK1 (PDB ID: 2ZOQ), ERK2 (PDB ID: 1ERK) and ERK5 (PDB ID: 4IC8). All MAPKs contain the Ser/Thr kinase domain between the N-terminal and C-terminal regions. Different additional domains are also present in some MAPKs, including a proline-rich domain 1 (PR1), proline-rich domain 2 (PR2) and nuclear localization sequence (NLS).

allowing ATP to bind and activate the kinase. When the proteins are in the DFG-out conformation, the aspartic acid residue points outward away from the ATP binding site, and the phenylalanine residue moves toward the ATP binding site to form an inactive kinase conformation, exposes the allotropic hydrophobic region near the ATP binding site⁴¹.

ERKs are located at the end of the MAPKs signaling pathway, which is a central pathway that transmits a variety of extracellular signals. A number of studies demonstrated that ERK1/2 has more than 600 substrates, including transcription factors, protein kinases and phosphatases, apoptosis regulators, etc.^{17,42}. The diversity of substrates allows ERK1/2 to mediate a variety of cellular processes in the MAPK pathway, such as regulating cell proliferation, differentiation, angiogenesis, chromatin remodeling, etc.^{43,44}. Studies have shown that the ERK1/2 pathway can regulate cell proliferation by controlling the G1/S transformation of the cell cycle. In the absence of RAS or ERK1/2, the growth of mouse embryonic fibroblasts will stagnate in the G1 phase^{45,46}. ERK1/2 can directly phosphorylate FOS, JUN, and ATF2 family members, thereby increasing their DNA-binding activity and preventing their degradation by proteasomes, thus promoting the proliferation of tumor cells⁴⁷. Besides, ERK1/2 can phosphorylate myosin light chain kinase (MLCK), calpain, focal adhesion kinase (FAK) and other proteins that regulate cell movement, thereby affecting cell migration⁴⁸. In addition to the above-mentioned protein kinase activity of ERK1/2, some reports have also confirmed its non-phosphorylation function. For example, Hu et al.⁴⁹ identified the DNA binding activity of ERK2 through *in vitro* and *in vivo* experiments, while the kinase activity of ERK2 is not necessary for DNA binding. ERK2, acts as a transcriptional repressor for interferon gamma (IFN γ)-induced genes, can prevent the binding of the transcription factor C/EBP- β in mammalian cells by directly binding to DNA in the promoter region of the IFN γ response gene. In addition, ERK1/2 has kinase-independent activity. Once activated, ERK1/2 translocates into the nucleus, interacts with lamin A and replaces the retinoblastoma (RB) protein, enabling RB to be phosphorylated by cyclin-dependent kinases and the release of E2F transcription factors to promote cell cycle entry, while these effects were independent of ERK1/2 kinase activity⁵⁰.

ERK5 is an effector kinase of the conventional three-tiered MAPK cascade. In response to several stimuli, the activated

MEKK2/3 binds to the N-terminal domain and phosphorylates Ser311 and Thr315 of MEK5, which is a dual specificity protein kinase with activity on ERK5⁵¹. Once activated, MEK5 phosphorylates two residues in the TEY sequence of ERK5. The activated ERK5 is transferred from extracellular to intracellular to perform its biological function. Current studies suggest that ERK5 is also essential for physiological processes such as cell proliferation and differentiation. In certain situations, ERK5 may provide a bypass pathway to maintain cell growth and proliferation by providing a compensatory pathway in the absence or inhibition of ERK1/2 signaling⁵². de Jong et al.⁵³ found that the deficiency of ERK1/2 in small intestinal epithelial cells would lead to insufficient nutrient absorption, disturbance of epithelial cell migration, and affect the normal function of secretory cells, but the proliferation of intestinal epithelial cells was not affected. Further studies showed that this was due to the significant increase of ERK5 phosphorylation after ERK1/2 knockout, suggesting that the ERK5 pathway may have a certain compensatory effect on the ERK1/2 pathway. The team then used human colorectal cancer cell lines HCT116 and DLD-1 with KRAS G13D heterozygous mutations to further explore the interaction between the ERK1/2 and ERK5 pathways. The results showed that after the deletion of the ERK1/2 gene or the interference of drugs, the ERK5 pathway would rapidly undergo compensatory upregulation, enabling the cell to continue to grow and proliferate. Gene excision of MYC protein impairs the growth and development of pancreatic ductal adenocarcinoma driven by KRAS mutants, while MYC over-expression leads to the formation of metastatic pancreatic ductal adenocarcinoma^{54,55}. Early studies have shown that the MAPK cascade reaction activated by RAS can phosphorylate MYC at S62 (pS62) and increase the stability of MYC protein⁵⁶. Vaseva et al.⁵⁷ applied ERK1/2 inhibitors to patients with pancreatic ductal adenocarcinoma (PDAC), but it was found that the mechanism of maintaining MYC protein stability did not depend on the ERK1/2 pathway. The results of immunoprecipitation showed that ERK5 played the same biological function as ERK1/2, and ERK5 could directly bind to MYC and regulate its S62 phosphorylation, thereby maintaining the stability of MYC protein. Subsequent Western blotting results showed that the expression level of p-ERK1/2 was effectively suppressed after silencing MEK1/2 and ERK1/2, but the expression level of p-ERK5 would increase rapidly to compensate for the biological function of ERK1/2.

3. ERKs inhibitors and their therapeutic implications

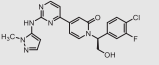
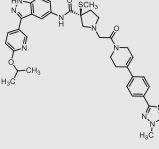
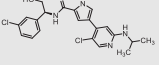
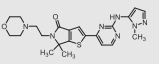
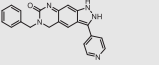
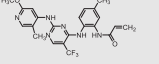
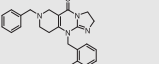
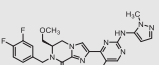
The deeper understanding of the structures and biological functions of ERKs has greatly facilitated the design and discovery of ERKs-targeting inhibitors. In the past 20 years, a large number of selective ERKs inhibitors with different mechanisms of action have been discovered. Although no selective ERKs inhibitors have been approved for the market to date, several ERK1/2 and ERK5 inhibitors have shown good anti-tumor efficacies in preclinical and clinical trials. Therefore, the development of ERKs-targeted inhibitors is an effective approach for the treatment of tumors. In this section, we summarized the status, design strategies and SARs of ERK1/2/5 inhibitors available.

3.1. ERK1/2 inhibitors in clinical trials

The ERKs inhibitors currently selected for clinical trials are shown in Table 1. Blake et al.⁵⁸ obtained a lead compound **1** of ERK2 through high-throughput screening (HTS), with an enzyme IC₅₀ of 106 nmol/L (Fig. 3A). After structural optimization, compound **2** was obtained (ERK2 IC₅₀ = 1 nmol/L), which showed a good anti-proliferation effect on HepG2 cells (IC₅₀ = 45 nmol/L). However, **2** showed high clearance rates and low oral bioavailability in multiple species⁵⁹. *In vitro* metabolic analysis found that the main metabolites of these compounds were

produced by the oxidation of C-5 and C-8 positions on the benzyl group. In addition, although the oxygen atom in urea is necessary for compound activity, the amino group does not have any effect on the protein, and its role as a hydrogen bond donor will affect the permeability and absorption of the compound. Based on these considerations, the team introduced pyridone into the original compound to remove readily metabolized C-5, C-8 and urea linker. The resulting **3** ((*S*)-14K) is a highly effective and selective ERK1/2 inhibitor (ERK2 K_i = 0.6 nmol/L). The X-ray structure of **3** binding to ERK2 (PDB ID: 4XJ0) shows that 2-aminopyrimidine interacts with the Met108 hinge region of ERK2 through a pair of hydrogen bonds. Oxygen atom on 4-THP interact with Lys114 to form a hydrogen bond, and the hydrophobic interaction in this region is also important for the compound's activity. In addition, a key water molecule forms a hydrogen bond with the pyridone carbonyl group, which also interacts with the side chains of Lys54. The methyl hydroxyl substituent interacts with the carboxylic acid of Asp167 side chain. The 3-F-4-Cl phenyl group occupies a small hydrophobic pocket under the P-loop formed by Tyr36 and C helix (Fig. 3B). These binding modes determined the strong biological activity of **3**. In the nude mouse model of HCT116 xenograft tumor, **3** showed a good inhibitory effect on tumor growth (tumor static rate was 70%) and had no significant inhibitory effect on cytochrome P450 enzymes. The oral bioavailability of this compound administered

Table 1 ERK1/2 inhibitors in clinical trials.

Inhibitor	Chemical structure	Classification/ Binding mode	Activity	Clinical phase	NCT identifier	Ref.
6 (GDC-0994)		Reversible, ATP competitive	ERK1 IC ₅₀ = 6.1 nmol/L ERK2 IC ₅₀ = 3.1 nmol/L	Phase I	NCT01875705	60
10 (MK-8353)		Reversible, ATP competitive	ERK1 IC ₅₀ = 20 nmol/L ERK2 IC ₅₀ = 7 nmol/L	Phase I	NCT01358331 NCT03745989 NCT02972034	62
11 (BVD-523, Ulixertinib)		Reversible, ATP competitive	ERK1 K _i = 0.3 nmol/L ERK2 K _i = 0.04 nmol/L	Phase I/II Phase I/II Phase I	NCT01781429 NCT02296242 NCT02608229	64
12 (LY3214996)		Reversible, ATP competitive	ERK1/2 IC ₅₀ = 5 nmol/L	Phase I/II	NCT02857270 NCT04534283	65
13 (KO-947)		NA ^a	ERK1/2 IC ₅₀ = 10 nmol/L	Phase I	NCT03051035	66
14 (CC-90003)		Covalent	ERK1/2 IC ₅₀ = 10–20 nmol/L	Phase I	NCT02313012	67
15 (ONC201)		NA	NA	Phase I/II	NCT02420795	69
16 (AZD0364)		Reversible, ATP competitive	pRSK IC ₅₀ = 5.7 nmol/L pERK IC ₅₀ = 1.7 nmol/L	Phase I	NCT04305249	70
17 (HH2710)	NA	NA	NA	Phase I/II	NCT04198818	NA
18 (LTT462)	NA	NA	NA	Phase I	NCT02711345	71

^aNA: Not available.

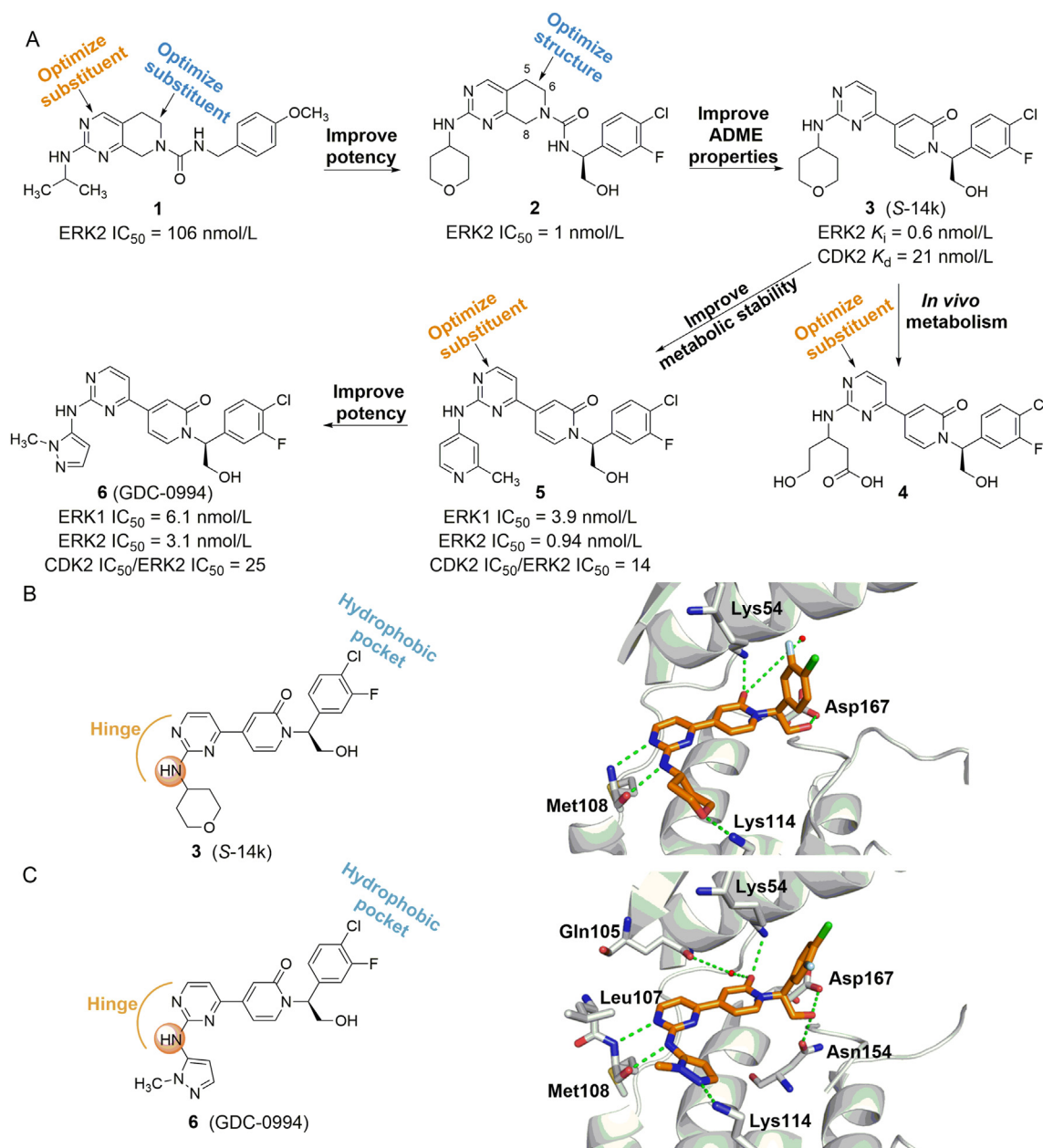


Figure 3 (A) Discovery and design procedure of the ATP competitive ERK1/2 inhibitor **6** (GDC-0994) by combing HTS strategy and medicinal chemistry. (B) X-ray structure of compound **3** (orange) bound to ERK2 (PDB ID: 4XJ0). Hydrogen-bonding interactions are illustrated with green dashed lines. (C) X-ray structure of **6** (orange) bound to ERK2 (PDB ID: 5K4I).

at 5 mg/kg in rats ($F = 44\%$) and dogs ($F = 46\%$) was superior to **2** (rat: $F = 11\%$, dog: $F = 26\%$). Further studies found that compound **3** is mainly metabolized on the 4-THP loop to form hydroxyl acid (**4**) in human, mouse, dog, and cynomolgus monkey species, suggesting that THP partial substitution is the key to improve metabolic stability and enhance efficiency⁶⁰. **5** obtained by replacing THP with 2-methylpyridine had an IC_{50} of 0.94 nmol/L for ERK2, but it failed to show excellent pharmacokinetic characteristics in rats. Replacement of THP with *N*-methylpyrazole (**6**, GDC-0994) retained the kinase inhibitory activity (ERK2 $IC_{50} = 3.1$ nmol/L) and significantly improved the antitumor effect. Similar to the binding mode of compound **3**-ERK2, the 2-aminopyrimidine on **6** interacts with the hinge region

Met108 and Leu107 of ERK2 to form a pair of hydrogen bonds (Fig. 3C). The 3-F-4-Cl phenyl group binds to a hydrophobic pocket under the glycine-rich loop, which is formed by the side chain of Tyr36. Similar to the oxygen in THP, 5-aminopyrazole interacts with Lys114 to form a hydrogen bond (PDB ID: 5K4I). **6** has entered clinical trials due to its excellent bioactivity and has now completed a phase I dose-escalation clinical trial in patients with locally advanced or metastatic solid tumors (NCT01875705). Results showed that 15 of 45 patients (33%) had the best overall response rate, and two patients with BRAF mutated colorectal cancer had a partial response. Overall, **6** is expected to be a marketed ERK1/2-targeted inhibitor due to its excellent clinical trial results and acceptable safety profile.

Boga et al.⁶¹ screened hit compound **7** using the Automated Ligand Identification System (ALIS) and then modified it to obtain an ATP-competitive ERK1/2 inhibitor **8** (SCH772984) with IC₅₀ values of 4 and 1 nmol/L for ERK1 and ERK2, respectively (Fig. 4A). In BRAF V600E mutated human melanoma cell line LOXIMv1 (LOX), **8** not only binds inactive ERK1/2 to block phosphorylation activation of kinases but also promotes dephosphorylation of active kinases. In addition, this compound exhibited good anti-tumor cell proliferation effects in several xenograft mouse models with KRAS or BRAF mutant cell lines⁶¹. However, pyrrolidinamide in **8** is easily hydrolyzed by proteolytic enzymes, which leads to poor pharmacokinetic properties⁶². The introduction of a steric hindrance group on the 3-position of **8** pyrrolidine will greatly increase the bioavailability of the new compound **9** (rat/dog *F* = 70/75). The authors further optimized **9** along this line and found that **10** (MK-8353) was the most effective ERK1/2 inhibitor (ERK1/2 IC₅₀ = 20/7 nmol/L)⁶². In human malignant melanoma A2058 cells with BRAF V600E mutation, **10** can decrease the expression levels of p-ERKs and p-RSK.

The excellent selectivity of **10** against ERK1/2 is mainly attributed to its ability to induce conformational changes of glycine-rich rings in ERK1/2. As shown in Fig. 4B, the indazole ring of **10** was inserted into the ATP binding pocket to simulate the hydrogen bond formed by adenine to the hinged region of ERK, as well as the hydrogen bond to the carbonyl group of Asp104 and the amino group on Met106. The nitrogen on pyrrolidine also forms a strong hydrogen bond with catalytic Lys52, which requires the deprotonic form of amino nitrogen. Tyr34 is folded under the Gly-rich ring and is stacked with the pyrrolidine ring. This rearrangement forms a cavity, which is occupied by the methyltriazole on the compound and is stacked with Tyr62. The 3(*S*)-thiomethoxy group points to the binding region composed of Asn152 and Cys164 residues, which can maintain a strong binding with ERK (PDB ID: 6DCG). The extensive

interactions of **10** with ERK contribute to its potency and selectivity against other kinases. **10** has also achieved good results in clinical trials. In phase I clinical trial of advanced/metastatic solid tumors (NCT01358331), this compound was well tolerated by patients with BRAF V600E mutant melanoma and showed anti-tumor activity⁶³. **10** is currently in phase I clinical study of advanced malignant tumors and colorectal cancer (NCT03745989, NCT02972034).

Germann et al.⁶⁴ reported an orally effective, ATP-competitive ERK1/2 inhibitor **11** (BVD-523, ulixertinib) with *K_i* values of 0.3 and 0.04 nmol/L for ERK1 and ERK2, respectively (Table 1). **11** has shown good antitumor activity and low toxicity in human BRAF V600E mutated A375 malignant melanoma cells and Colo205 colorectal cancer cells, and KRAS G12C mutated Miapaca-2 pancreatic cancer heteroimplant tumor animal models. Currently, **11** has completed a phase I/II clinical trial in patients with advanced malignancy (NCT01781429), a phase I/II clinical trial in patients with acute myeloid leukemia or myelodysplastic syndrome (NCT02296242), and a phase I clinical trial in patients with metastatic pancreatic cancer (NCT02608229). Bhagwat et al.⁶⁵ developed a novel selective ERK1/2 inhibitor, **12** (LY3214996), with an IC₅₀ value of 5 nmol/L for both ERK1 and ERK2 in biochemical assays. Oral administration of **12** significantly inhibited tumor growth and was well tolerated in BRAF or NRAS mutated melanoma, colorectal, lung, and pancreatic cancers. In addition, **12** also exhibited antitumor activity in A375 melanoma transplantation models resistant to the BRAF inhibitor vemurafenib. Currently, **12** alone or in combination with other drugs is undergoing phase I/II clinical trials in patients with advanced and metastatic cancer (NCT02857270, NCT04534283). Burrows et al.⁶⁶ found that the selective ERK1/2 inhibitor **13** (KO-947) has anti-tumor activity in both cells and *in vivo*, with an IC₅₀ value of 10 nmol/L against ERK1/2. At low nanomolar concentration, **13** can block the proliferation of tumor cells caused by the

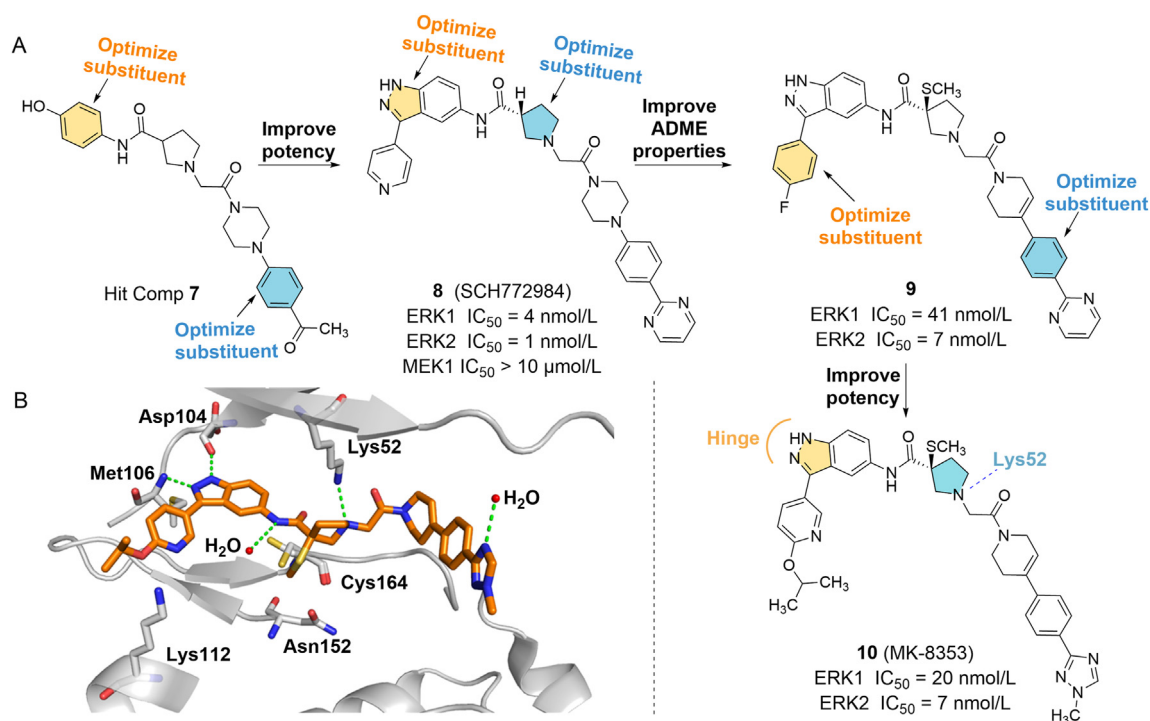


Figure 4 (A) Chemical structure and design strategy for compound **10**. (B) X-ray crystal structure of **10** in complex with ERK2, green dotted lines indicate hydrogen bonds (PDB ID: 6DCG).

abnormal ERK signal transduction and MAPK pathway. The good pharmacokinetic properties of **13** enable it to achieve optimal antitumor activity through intermittent administration. It can be used for the treatment of colorectal, gastric, and cervical cancers with RAS and BRAF mutations. **13** is now undergoing its first human phase I study of advanced unresectable or metastatic tumors (NCT03051035). **14** (CC-90003) is a covalent and irreversible inhibitor of ERK1/2, which has a strong inhibitory effect on ERK1/2 with IC_{50} values ranging from 10 to 20 nmol/L⁶⁷. The drug showed anti-proliferative activity in KRAS and BRAF mutant tumor models. Mita et al.⁶⁸ reported the first study on the treatment of patients with RAS or BRAF mutated tumors with **14**. Compound **14** have completed a phase I clinical trial in patients with advanced refractory BRAF or RAS mutation malignancies (NCT02313012). The dual-targeting serine/threonine protein kinase (AKT) and ERK1/2 inhibitor **15** (ONC201) have a significant effect in the treatment of lymphoma and at present is in the clinical phase I/II trial (NCT02420795)⁶⁹. **16** (AZD0364), a selective ERK1/2 inhibitor developed by AstraZeneca, demonstrated high cellular potency (pRSK IC_{50} = 5.7 nmol/L, pERK IC_{50} = 1.7 nmol/L) as well as excellent physicochemical and pharmacokinetic properties. It has a good therapeutic effect on non-small cell lung cancer (NSCLC) and other solid tumors⁷⁰. This compound was introduced to China by Antengene Corporation on November 6, 2019. **17** (HH2710) was developed by Haihe Pharma in collaboration with a team led by Ding Jian from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, has demonstrated good antitumor activity in preclinical trials. The capsule obtained the phase I/II clinical trial license (NCT04198818) of US Food and Drug Administration (FDA) in September 2019 and gained domestic clinical trial permission on November 4, 2019. **17** is currently in clinical trials for malignancies with abnormal MAPK signaling pathways. In addition, Novartis developed undisclosed compound **18** (LTT462, CLXH254X2102, MedGen UID: 925271) as a novel ATP competitive ERK inhibitor⁷¹. A phase I dose study (NCT02711345) of oral **18** in adult patients with advanced solid tumors with changes in the MAPK pathway has been completed. Since the development of ERK1/2 inhibitors is later than other upstream inhibitors of the MAPK signal pathway, compared with a large number of clinical trials involving RAF and MEK inhibitors, there are only about 35 clinical trials related to ERK1/2 inhibitors (Table 1). However, with the emergence of resistance to RAF and MEK inhibitors, as well as a deeper understanding of the ERK pathway, the discovery and development of ERKs inhibitors has gradually attracted the attention of researchers, and small molecule compounds targeting ERKs will also usher in a blowout.

3.2. ERK1/2/5 inhibitors in pre-clinical trials

Among the whole ERKs family, ERK1/2/5 is the most fully studied, and its protein structures and biological functions have been extensively discussed and confirmed. Therefore, the current research on ERKs inhibitors is mainly focused on ERK1/2/5. In this section, the research status of current ERK1/2/5 will be summarized. According to the binding site of ERK1/2, ERK1/2 inhibitors can be divided into ATP competitive inhibitors and allosteric inhibitors. Inhibitors that target the ATP binding site of the active kinase are defined as ATP competitive inhibitors. At present, drug discovery based on the ATP binding site of ERKs

have been widely researched. In addition, covalent and allosteric inhibitors of ERK1/2 have been designed and synthesized in recent years with the development of covalent and allosteric inhibitor design strategies. Different from the weak bond between the ATP competitive inhibitors and kinases, covalent inhibitors can usually bind more firmly to the residues of the target protein through covalent bonds to achieve long-term inhibition of the target protein. Therefore, covalent inhibitors have the advantages of high biochemical efficiency, long action time, low dosage and administration frequency, etc. Besides, researchers also aimed at the allosteric site located on the back of the ATP binding pocket of ERK protein, developing the ERK1/2 allosteric inhibitors. However, ERK1/2 inhibitors are prone to develop resistance after a period of monotherapy, one of the mechanisms of which is the potential bypass pathway provided by ERK5. It can rescue the proliferation and weaken the effect of ERK1/2 inhibitors after the ERK1/2 signal conduction is cancelled. As a consequence, drug research targeting ERK5 is another potential strategy for tumor treatment. Moreover, pharmacological inhibition of the ERK cascade abrogates the negative feedback of ERK, leading to the feedforward activation of some receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR). The increased expression of RTKs further causes the activation of alternative pathways such as the PI3K/AKT pathway, which can sustain the survival of tumor cells. Based on this phenomenon, developing inhibitors that can simultaneously target ERK1/2 and related proteins is an effective method and important strategy to improve the anti-tumor effects and overcome the drug resistance. Therefore, at the end of this section, we summarized some inhibitors that can target ERK1/2 and other kinases at the same time, showing strong inhibition of ERK pathway and good biological activity. The dual-targeting strategy is expected to achieve better cancer treatment effects.

3.2.1. ATP competitive ERK1/2 inhibitors

Among many kinase inhibitors, ATP competitive inhibitors have attracted much attention because of their high affinity and clear sites of action. By competitively binding with ATP, small molecules can block the catalytic process of kinases and further interfere with the transmission of cell signals to achieve the therapeutic purpose. Similar to other kinase inhibitors, drug design and discovery based on the ATP binding site of ERKs have been widely studied, and many mature strategies for the design of ERKs inhibitors have been developed. In 2007, Aronov et al.⁷² obtained a small-molecule ERK inhibitor (**19**, ERK K_i = 2.3 μ mol/L) through HTS (Fig. 5A). The X-ray structure of the drug-ERK complex shows that (PDB ID: 2OJG) the pyrazolium ring of **19** forms two hydrogen bonds with the hinge region of ERK, which is the active group of the compound. The carbonyl group on the amide forms a hydrogen bond with Lys52, the phenyl group on the pyrazole forms a hydrophobic interaction with the glycine-rich ring Val37, and the amino group on the pyrrole forms a hydrogen bond with the side chain carbonyl group of Gln103 on the gatekeeper residue (Fig. 5B). Based on this structural information, the author optimized the structure by retaining the core group pyrazolopyrimidine, introducing small lipophilic substituents on the phenyl group, and extending the amide to the salt bridge region at the ATP site between Lys52 and Asp165 to occupy additional space. They found that the introduction of chlorine on the phenyl group was the most active (three times that of **19**), and optimization of the amide to benzamide (**20**) yielded the greatest improvement in the K_i of

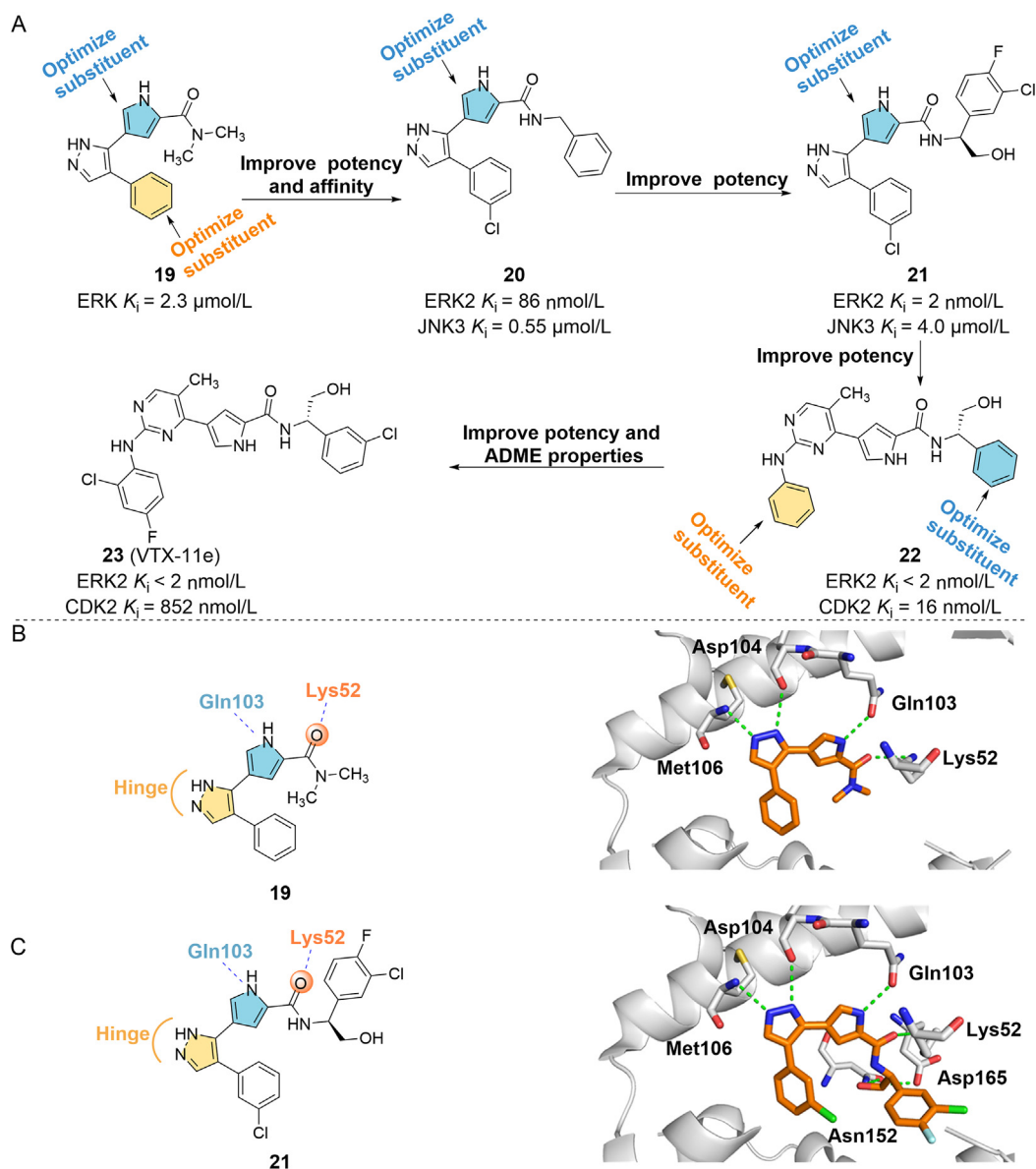


Figure 5 (A) Discovery of compound **23** from the lead compound **19** to improve its potency and affinity to ERK1/2. (B) Crystal structure of **19** bound to ERK2 (PDB ID: 2OJG). (C) Binding mode of compound **21** in complex with ERK2 (PDB ID: 2OJJ).

ERK2 ($K_i = 86$ nmol/L). The 3-chloro-substituted group of **20** contacts with Val37, and the benzyl group is partially directed to the glycine ring and forms hydrophobic interaction with the C δ of the Lys52 side chain, which may explain its high ERK inhibitory activity. The open space near the *m/p* position of the benzyl group suggests that the addition of groups on the benzyl group may be more conducive to the interaction with this region (PDB ID: 2OJI). Among the optimized structures, the (*S*)-enantiomer (**21**) is the most effective compound, and the K_i of ERK2 is 2 nmol/L, which is 43 times stronger than that of **20**. In the crystal structure of the **21**/ERK2 complex (PDB ID: 2OJJ), the four hydrogen bonds previously observed also exist, and the hydroxyl group on the phenylglycinol structure forms two other hydrogen bonds with the carboxylate oxygen of Asp165 and the formamide oxygen of the side chain of Asn152 at the salt bridge region of the ATP site. These six hydrogen bonds are anchored at the active site of ERK2, which greatly improves the activity of **21** (Fig. 5C)⁷².

However, the main disadvantage of the pyrazolyl pyrrole series compounds is still insufficient cell viability ($IC_{50} = 0.54$ μ mol/L in the Colo205 cell proliferation test). The author continued to improve the pyrazole moiety of **21** to increase the cell activity of the compound⁷³. Finally, the lead compound **22**, a potent pyrimidinylpyrrole ERK inhibitor ($K_i < 2$ nmol/L), was found in the aminopyrimidine library. The cell activity of the compound has been greatly improved ($IC_{50} = 29$ nmol/L in the HT29 cell proliferation test), but the selectivity was significantly reduced. Among the many compounds modified based on **22**, **23** (VTX-11e) is a selective ERK2 inhibitor with high antiproliferative activity, and its K_i value is less than 2 nmol/L. **23** showed significant anti-tumor activity ($IC_{50} = 48$ nmol/L) in HT29 cell proliferation experiments, and has good pharmacokinetic characteristics (rat: $F = 65\%$, $t_{1/2} = 3$ h; mice: $F = 67\%$, $t_{1/2} = 4.4$ h)⁷³.

Ohuri et al.⁷⁴ used computer-aided drug design (CADD) to screen a pyrazopyridine derivative **24** (FR180204), with IC_{50}

values of 510 nmol/L ($K_i = 310$ nmol/L) and 330 nmol/L ($K_i = 140$ nmol/L) for ERK1 and ERK2, respectively. The co-crystal structure analysis with ERK2 showed that **24** could bind to the ATP pocket, and two hydrogen bonds are formed between the 3-amino group of the pyrazolopyridazine ring and the carbonyl groups of Gln105 and Asp106. In addition, hydrogen bonds were also observed between the nitrogen atom at position 2 of the ring and the main chain amide group of Met108, as well as the nitrogen atom at position 3 of the pyrazolopyrimidine ring and the amino group of Lys54 (Fig. 6A). Ward et al.⁷⁵ described a method of using in-house kinase selectivity panel data as a start point to find potent and selective ERK1/2 inhibitors, followed by the quick optimization according to the binding mode of the compound. Eventually, it was found that compound **25** has excellent biological activity, the K_M for ERK1/2 is less than 0.3 nmol/L, and it shows good anti-tumor effects in the A375 cell xenograft model (Fig. 6B). Hairtman et al.⁷⁶ used X-ray crystallography and biophysical fragment screening methods to find a highly selective ERK1/2 inhibitor **26**, with an IC_{50} of 3.0 nmol/L for ERK1/2. This compound has an inhibitory effect on BRAF V600E mutant A375 cells ($IC_{50} = 4.9$ nmol/L) and Colo205 cells ($IC_{50} = 7.5$ nmol/L). **27** is a tetrahydropyrrole-diazepam derivative obtained through a series of structure-based drug design and screening methods, with an IC_{50} of 0.19 nmol/L for ERK2⁷⁷. **28**, which has a K_i of 0.6 nmol/L for ERK2, can inhibit tumor growth in xenograft mouse model by intragastric administration without significant toxicity (Fig. 6B)⁵⁹.

As an effective ATP-competitive ERK1/2 inhibitor, **11** was chosen as a lead compound in a structure design, and a novel series of isoindolin-1-one derivatives were synthesized as ERK inhibitors. Among them, **29** (Fig. 7) had good inhibitory effects on ERK1 ($IC_{50} = 1.5$ nmol/L) and ERK2 ($IC_{50} = 0.7$ nmol/L), and showed considerable *in vivo* antitumor efficacy in an HCT-116 xenograft model ($IC_{50} = 154.97$ nmol/L)⁷⁸. Aly et al.⁷⁹ synthesized and identified novel series of potential ERK inhibitors which blocked ERK in an ATP-competitive manner. Radioactive kinase assay results showed that **30** and **31** exhibited pronounced ERK inhibitory activity, they inhibited ERK1 with IC_{50} values of 0.5 and 0.19 μ mol/L, and inhibited ERK2 with IC_{50} of 0.6 and 0.16 μ mol/L, respectively. In addition, the two compounds inhibited the proliferation of the BRAF mutant A375 melanoma

cell line with IC_{50} of 3.7 and 0.13 μ mol/L, respectively. Burdick et al.⁸⁰ screened a small fragment library *via* an NMR-based saturation transfer difference (STD) assay and a surface plasmon resonance (SPR) assay to find novel ERK1/2 inhibitors. The improvement of the fragment hit compound obtained in the screen led to the discovery of a novel and potent ERK1/2 inhibitor **32** (ERK2 LC3K $IC_{50} = 37$ nmol/L). In the crystal structure of the complex of **32** and ERK2 (PDB ID: 4QPA), the N at position 4 of pyrrolopyrazine forms a hydrogen bond with Leu107. In addition, piperidine N and pyrazole N also form hydrogen bonds with Lys114 and Lys54, respectively (Fig. 7A). Li et al.⁸¹ reported the discovery and optimization of a series of indazole amide based ERK1/2 kinase inhibitors *via* structure-based drug design and kinase screen. The optimized **33** demonstrated remarkable inhibition of ERK1/2 enzyme activity (ERK1 $IC_{50} = 19.4$ nmol/L, ERK2 $IC_{50} = 9.5$ nmol/L), growth of BRAF mutant HT29 cells (Cell $IC_{50} = 0.48$ μ mol/L) and ERK signaling in HT29 cells (Fig. 7B).

Bagdanoff et al.⁸² reported a potent, selective and orally bioavailable ATP-competitive ERK1/2 inhibitor (compound **34**, ERK2 $IC_{50} = 0.0139$ nmol/L) that effectively inhibits pERK signaling and has demonstrated tumor growth inhibition in multiple MAPK-activated cancer cells and xenograft models (Fig. 8). Importantly, compound **34** demonstrated broad efficacy targeting multiple known mechanisms of resistance to BRAF and MEK inhibitors⁸³. Li et al.⁸⁴ found a series of thiazololactam compounds as effective ERK1/2 inhibitors. Compounds **35** and **36** showed significant ERK2 kinase inhibitory activity (ERK2 IC_{50} values of 0.49 and 0.31 nmol/L, respectively) and anti-tumor cell proliferation activity (HT29 cell IC_{50} values of 0.022 μ mol/L and 21 nmol/L, respectively)⁸⁵. Further optimization of the structure showed that the new compound **37** had stronger inhibitory activity when the spiro skeleton was removed (ERK2 $IC_{50} = 0.05$ nmol/L, HT29 cell $IC_{50} = 0.05$ nmol/L)⁸⁶. Compound **38** is a substituted fusedbicyclic derivative, which has good inhibitory activity to ERK1 and ERK2, with IC_{50} values of 3.8 and 1.0 nmol/L, respectively. Compound **38** significantly inhibited the proliferation of Colo205 tumor cells *in vitro* in tumor cells (Colo205 cell $IC_{50} = 10$ nmol/L)⁸⁷. Xu et al.⁸⁸ reported the preparation and use of a class of isoindolinone ERK inhibitors, which have certain selectivity for ERK kinase. Compounds **39** and **40** were the most

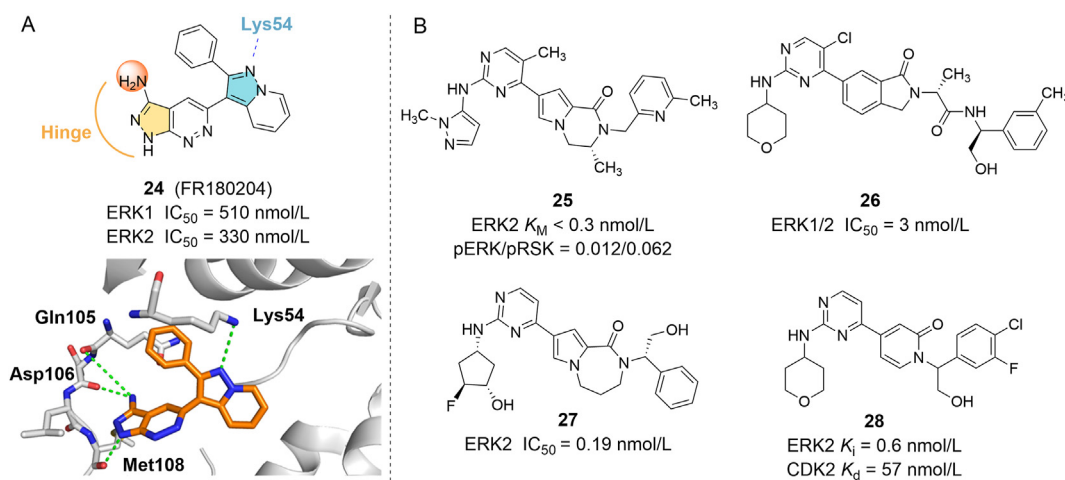


Figure 6 (A) X-ray crystal structure of **24** binding to the active site of ERK2. Hydrogen bonds were highlighted in green dashed lines (PDB ID: 1TVO). (B) Chemical structures of ATP competitive ERK inhibitors **24–28** that discovered by adopting a CADD strategy.

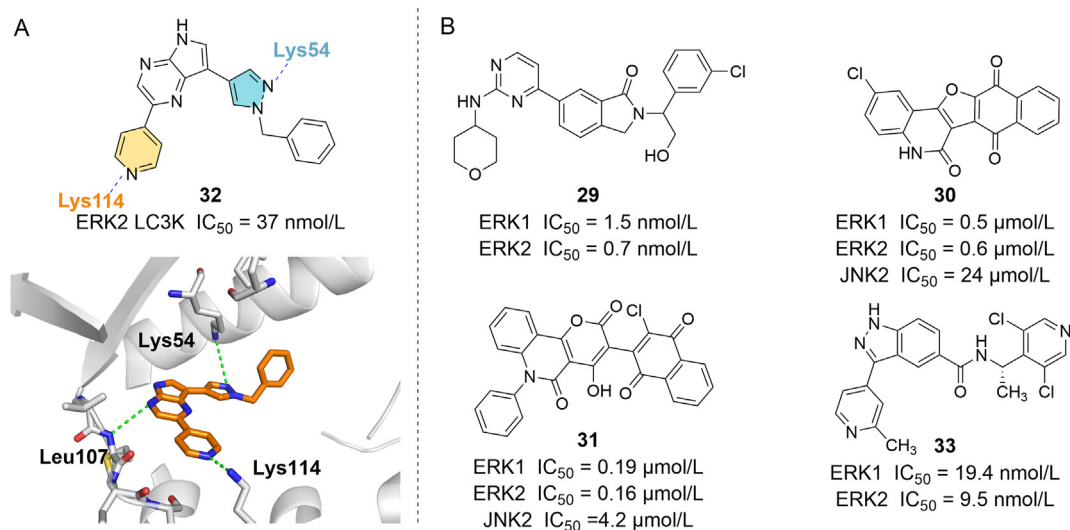


Figure 7 (A) X-ray crystal structure of **32** in the active site of ERK2 with hydrogen bond interactions to the key residues highlighted in green dashed lines (PDB ID: 4QPA). (B) Chemical structures of ATP competitive ERK inhibitors **29–33**.

effective ERK2 inhibitors, with IC_{50} of 0.7 and 1.2 nmol/L, respectively. In the *in vivo* experiments of the HCT-116 cell xenograft model, compound **39** showed strong anti-tumor activity, with a tumor inhibition rate of 71%.

The above inhibitors inhibited ERK activity by competitively binding to ATP sites, thereby exerting anti-tumor effects. However, there are still deficiencies in applying such inhibitors to the clinic. The high degree of homology in the ATP-binding pockets of protein kinases results in low target selectivity of these inhibitors. Moreover, the concentration of ATP in the cells is much higher than the concentration of the drugs, which makes it

necessary for the drug to have sufficient affinity and selectivity to compete with ATP. In addition, the experience of resistance to upstream MAPK inhibitors has led to the design of more effective compounds with different mechanisms of action.

3.2.2. Covalent selective ERK1/2 inhibitors

In recent years, there has been increasing interest in the development of covalent inhibitors of kinases. Covalent interaction can reduce off-target effects or permanently inhibit the activity of target proteins until re-synthesis of the target proteins. Therefore, given the advantages of covalent inhibitors, some

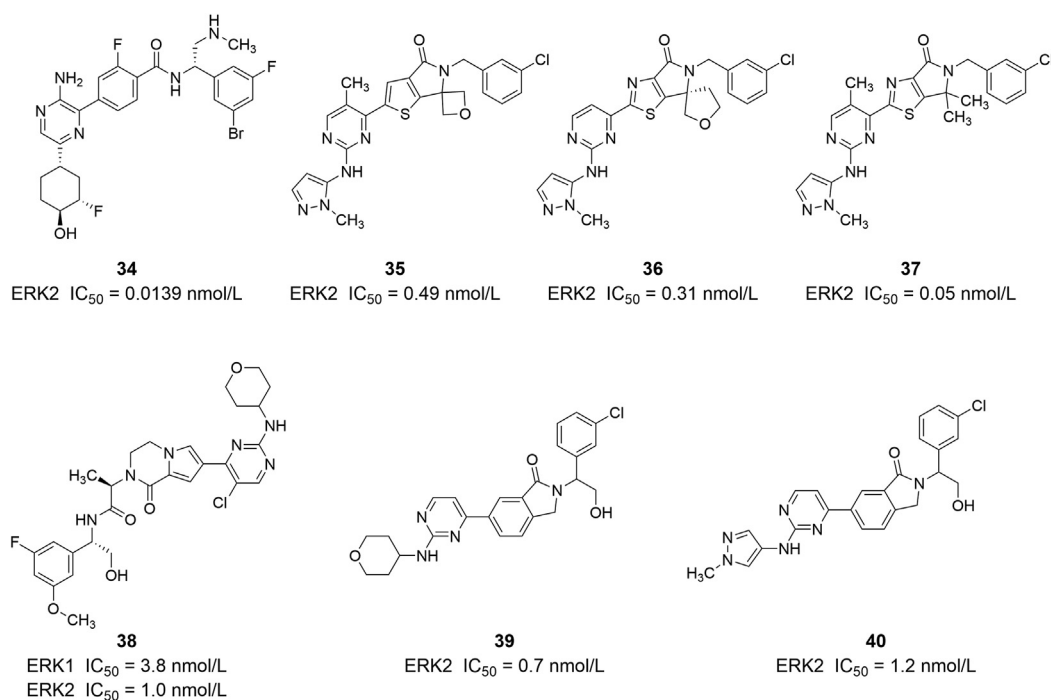


Figure 8 Chemical structures of ERK1/2 inhibitors **34–40**.

progresses have been made in the design and development of ERK1/2 covalent inhibitors. Liu et al.⁸² conducted a systematic analysis of kinase histones and found that more than 200 kinases, including ERK1/2, had cysteine residues that could produce covalent effects near the ATP binding sites. The cysteine residue of ERK1/2, located behind the ATP binding pocket and before the DFG region, can be conveniently used for the design of covalent inhibitors.

Ward et al.⁸³ identified a group of non-covalent ERK1/2 inhibitors *via* HTS. Three target compounds were synthesized by molecular modeling and covalent docking of these published inhibitors, which showed weak ERK2 inhibitory activity and anti-A375 melanoma cell proliferation. Among them, **41** (ERK2 K_M ATP = 0.37 μ mol/L) formed key hydrogen bonds with Leu107 and Met108 residues in the hinge region of ERK2 (PDB ID: 4ZZM). The sulfonamide group interacted with the bound H₂O and formed a complex hydrogen bond network with Asp167, Lys54 and Gln105 (Fig. 9B). The covalent bond between **41** and Cys166 (PDB ID: 2OJI) was observed in the electron density. The authors then used ERK2 biochemical analysis for internal screening and synthesized a series of reversible inhibitors with higher activity (Fig. 9A). The crystal structure (PDB ID: 4ZZN) showed that the pyridine group in **42** (ERK2 K_M ATP = 0.26 μ mol/L) formed two important

hydrogen bonds with Met108 located in the hinge region. It has been identified that crystal water in the complex can directly interact with the amide group of **42** and form a hydrogen bond network with Glu71, Gln105 and Asp167. The intrinsic reactivity of these compounds to glutathione is reduced and the half-life is prolonged compared with the alkylsulfonamide series. When a double bond was introduced into the structure, the new covalent inhibitor **43** (ERK2 K_M ATP = 8.5 nmol/L, PDB ID: 4ZZO) was observed to have a very similar binding pattern to **42**. The introduction of N into the benzene ring of **43** enhanced the activity of the new compound **44** (ERK2 K_M ATP = 3.1 nmol/L) on Cys166 and also increased the reactivity of acrylamide to glutathione. When the 5-position of pyrimidine of **43** was replaced by trifluoromethyl, the obtained **45** maintained high inhibitory activity against ERK2 (ERK2 K_M ATP = 6.9 nmol/L). The IC₅₀ of **45** in the A375 cell anti-proliferation test is 53 nmol/L, which is a potential *in vivo* tool compound for mouse research⁸³.

Ohuri et al.⁸⁴ designed and synthesized an ERK2 covalent inhibitor **46** (FR148083) with an enzyme IC₅₀ of 80 nmol/L (Fig. 10). The co-crystal structure shows that this compound can form covalent bonds with Cys166, and can form key intermolecular hydrogen bonds with Met108 in the hinge region, and intermolecular hydrogen bonds with Ser153, Lys114 and Asn154. It

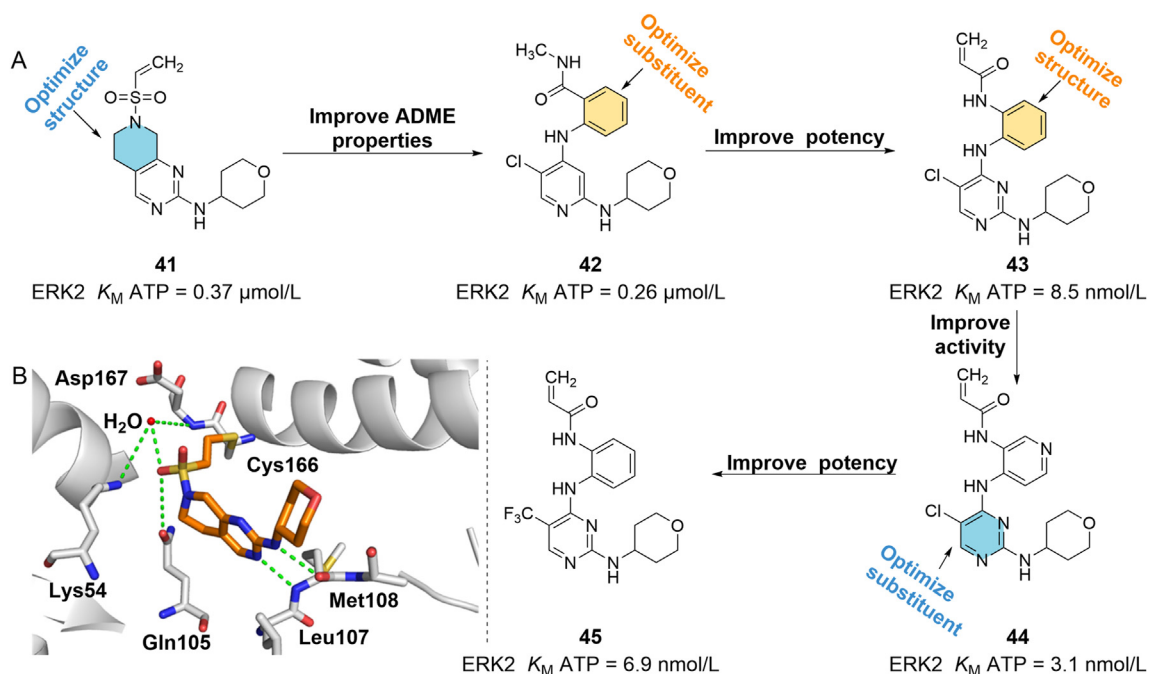


Figure 9 (A) Discovery and design procedure of the ERK1/2 covalent inhibitor **45** by combing HTS strategy and medicinal chemistry. (B) Cocystal structure of compound **41** in complex with ERK2, green dotted lines indicate hydrogen bonds, red sphere indicate water molecule (PDB ID: 4ZZM).

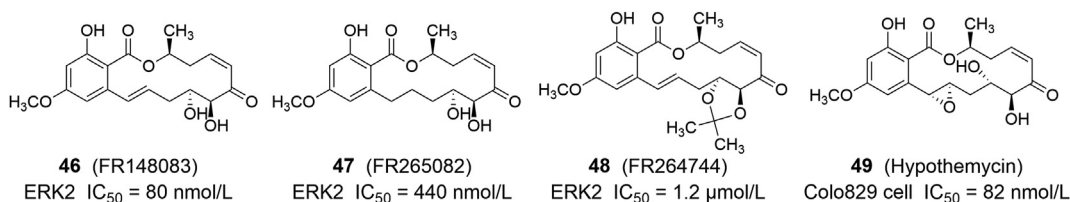


Figure 10 Chemical structures of ERK1/2 covalent inhibitors **46–49**.

also formed hydrophobic forces with Ile31, Val39, Ala52 and Leu156, which jointly maintained the stability of the compound binding to the protein. In addition to **46**, other derivatives such as **47** (FR265082, ERK2 IC₅₀ = 440 nmol/L) and **48** (FR264744, ERK2 IC₅₀ = 1.2 μmol/L) also showed inhibitory effects on ERK⁸⁴. **49** (hypothemycin) can covalently bind with Cys166 of ERK2, and has a strong anti-proliferative effect against the BRAF V600E mutant Colo829 cell lines (IC₅₀ = 82 nmol/L)⁸⁵.

Although covalent inhibitors have great potential, they have not been successful in clinical trials. Currently only **14** (CC-90003) is in phase I clinical trials, but it was terminated in 2014 in a locally advanced malignancy trial since the maximum tolerated dose results were not enough to support further administration trials. Therefore, in the development of covalent ERK1/2 inhibitors with clinical efficacy, attention should be paid not only to the biological activity of the candidate drugs but also to the toxicity and safety risks caused by the irreversible binding characteristics of the covalent inhibitors.

3.2.3. ERK1/2 allosteric inhibitors

In the search for ERK-specific inhibitors, in addition to targeting the catalytic site of ERK1/2, allosteric ERK1/2 inhibitors were developed by targeting the allosteric region located on the dorsum of the ATP-binding pocket of ERK proteins (Table 2). ERK1/2 has more than 600 substrates and contains two main substrate binding sites, called D-Recruitment Site (DRS) and F-Recruitment Site (FRS). DRS is a peptide-binding groove extending on the back of MAPK, while FRS is a shallow hydrophobic groove adjacent to the SER/THR-pro binding site^{17,86,87}. The structural basis of the interaction between the DRS structure of ERK and its docking sequence has been resolved by X-ray crystallography⁸⁸. By means of computer simulation, it has been confirmed that compounds bound to DRS and ATP sites have significant differences in binding patterns and inhibition mechanisms, etc. Targeting DRS or FRS has specific ERK1/2 recognition ability. In 2005, Hancock et al.⁸⁹ screened more than 800,000 small molecules and obtained 7 non-ATP competitive ERK inhibitors by the CADD method, which inhibited the phosphorylation site of ERK on RSK-1 (Thr573) by more than 25%. Further targeting experiments showed that **50** and **51** could directly interact with ERK2. In 2006, Chen et al.⁹⁰ designed a group of ERK1/2 allosteric inhibitors that can bind to ERK1/2 and inhibit the phosphorylation of p90RSK and ELK-1. Compounds **52–56** were effective inhibitors of cell proliferation with IC₅₀ values within 50 μmol/L. **53** and **56** inhibited ELK-1 by 70% and p90RSK-1 by 50% in HeLa cells, and their K_d values against ERK2 were 1.3 and 1.6 μmol/L, respectively. In 2009, Li et al.⁹¹ synthesized a series of analogs of lead compound **51**. Through SAR analysis, they found that **57** obtained by moving the ethoxy group from position 4 to position 2 on the benzene ring had the best ERK1/2 inhibition and anti-tumor cell proliferation activity. In 2011, Boston et al.⁹² designed and synthesized several derivatives of **51**. Through the combination of MAC-BITS fingerprint and Tanimoto similarity index calculation method, 10 compounds similar to the lead compound **51** were identified from a database containing more than 1 million compounds, of which **58**, **59** and **60** showed good anti-proliferative activity in HeLa cells. The fluorescence quenching rates of ERK2 in the presence of these three compounds were 18%, 15% and 13%, respectively, indicating that these compounds inhibited

Table 2 ERK1/2 allosteric inhibitors.

Inhibitor	Chemical structure	Activity	Ref.
50		NA	89
51		A549 lung carcinoma cell IC ₅₀ = 25 μmol/L	89
52		HeLa cell IC ₅₀ = 5.0 μmol/L	90
53		HeLa cell IC ₅₀ < 2 μmol/L	90
54		HeLa cell IC ₅₀ = 5–10 μmol/L	90
55		HeLa cell IC ₅₀ = 25–50 μmol/L	90
56		HeLa cell IC ₅₀ = 10–25 μmol/L	90
57		NA	91
58		NA	92
59		NA	92
60		NA	92
61		ERK2 IC ₅₀ = 9.9 μmol/L	93
62 (SF-3-029)		AP-1 IC ₅₀ = 5–10 μmol/L	94
63 (SF-3-030)		AP-1 IC ₅₀ = 5–10 μmol/L	94
64 (DEL-22379)		ERK1/2 dimerization IC ₅₀ = 500 nmol/L	95

cell proliferation by interacting with ERK2. In addition, **59** and **60** showed a better kinase inhibitory effect than **51** in kinase activity assay. Sammons et al.⁹³ identified a novel inhibitor **61** by screening a synthetic combinatorial library containing more than 30 million compounds. This compound can target ERK DRS, thereby inhibited ERK2 from phosphorylating a DRS-targeting substrate. In addition, the compound can block ERK2 activation in a dose-dependent manner by a constitutively active MAPK/ERK kinase 1 (MEK1) mutant, with an IC_{50} of 9.9 $\mu\text{mol/L}$.

DRS exists in both the active and inactive conformation of ERK1/2, but FRS exists only in the biphosphorylated active conformation of ERK1/2. Since ERK1/2 in the activated state is involved in tumor cell proliferation, targeting FRS may be a more effective ERKs allosteric inhibitors design strategy. Samadani et al.⁹⁴ obtained a class of molecules through virtual screening, which can interact with FRS, and inhibit the proliferation of melanoma cells containing BRAF mutation or activated ERK1/2 signal. Based on site identification by ligand competitive saturations (SILCs), **62** (SF-3-029) and **63** (SF-3-030) were the most effective inhibitors with the activator protein-1 (AP-1) promoter where IC_{50} values between 5 and 10 $\mu\text{mol/L}$.

In addition to designing allosteric inhibitors for FRS, Herrero et al.⁹⁵ established a method to detect ERK dimerization by native PAGE electrophoresis. Using this technique, the authors discovered a small molecule inhibitor DEL-22379 (compound **64**) that can inhibit ERK1/2 dimerization (with an IC_{50} of 500 nmol/L). **64** did not alter ERK phosphorylation and therefore had no effect on ERK kinase activity. Authors identified the DEL-22379 binding site in the groove of the ERK2 dimerization interface and speculated that **64** inhibited ERK dimerization by docking onto the dimerization interface, thus preventing the interaction between the two ERK molecules. Among tumor cell lines harboring mutated

BRAF, **64** had the greatest cell inhibitory potential. In addition, **64** induced significant apoptotic responses and prevented tumor growth in xenografts of melanoma and colorectal cancer cell lines. In general, the above allosteric inhibitors do not interact with the ATP binding pocket, so they can be used in combination with ATP competitive inhibitors to improve the efficacy of drug therapy and overcome the disadvantages of small molecule inhibitors such as low selectivity, off-target and drug resistance.

3.2.4. Discovery and design of small molecule inhibitors of ERK5

The development of ERK5 inhibitors is relatively rare and mainly focuses on the design and discovery of ATP-competitive inhibitors (Figs. 11 and 12). Gray's group⁹⁶ at Harvard Medical School synthesized a library of analogs for ATP-competitive polo-like kinase 1 (PLK1) inhibitor **65** (BI-2536) during the development of PLK1 inhibitors. More than 400 kinases were used to screen the library to find the potential kinase targets of these compounds. The authors found that extending the six-membered aliphatic ring of 2-amino pyrido [2,3-*d*] pyrimidine from **65** to a seven-member aliphatic ring containing an *o*-aminobenzoic acid resulted in a loss of PLK1 inhibitory activity, but the new compound showed some biochemical activity against ERK5. On this basis, a series of competitive ATP inhibitors targeting ERK5 were synthesized, such as **66** (ERK5 IC_{50} = 0.19 $\mu\text{mol/L}$), **67** (XMD8-92, ERK5 IC_{50} = 0.24 $\mu\text{mol/L}$) and **68** (ERK5 IC_{50} = 0.13 $\mu\text{mol/L}$) (Fig. 11A). Among them, **67** and **68** had excellent pharmacokinetic characteristics in rats and mice, with oral bioavailability of 68.6% and 86.6%, respectively⁹⁷. **67** has been used as a tool compound to inhibit protein activity in promyelocytic leukemia. In the *in vivo* experiments of the HeLa cell xenograft model, **67**

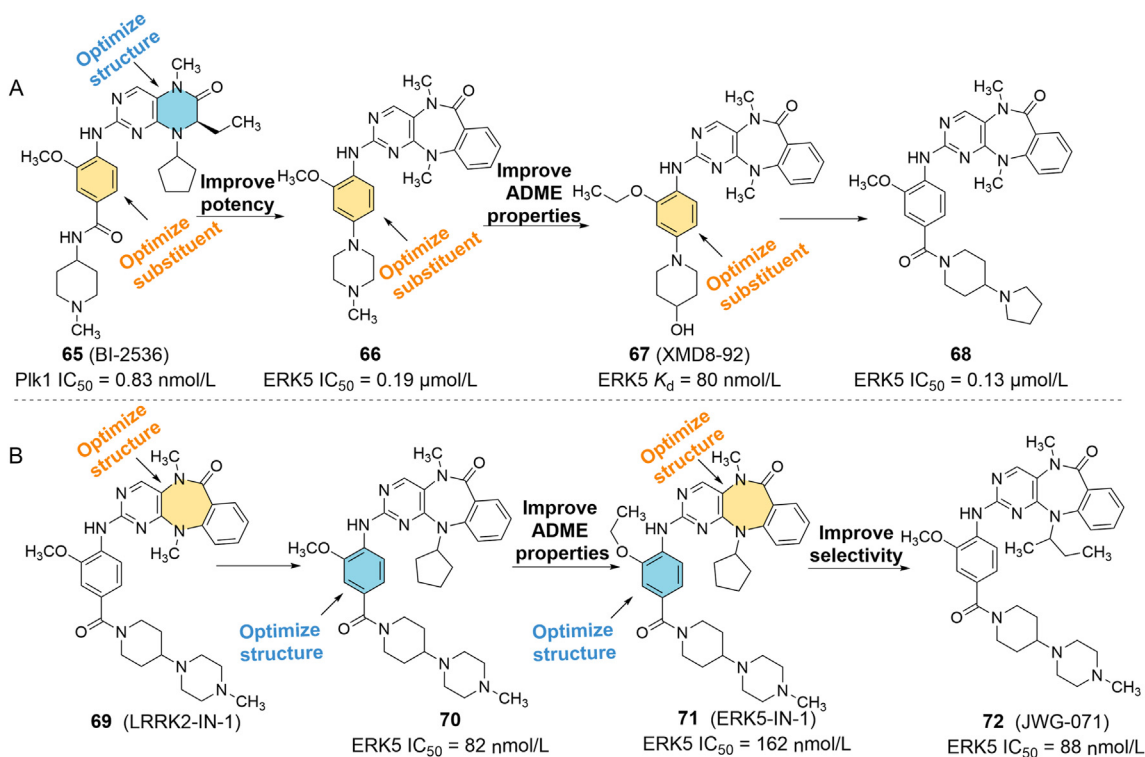


Figure 11 (A) Optimization of ERK5 inhibitor **67** with high activity and selectivity based on compound **65**. (B) Optimization of ERK5 inhibitor **72** with high activity and selectivity based on compound **69**.

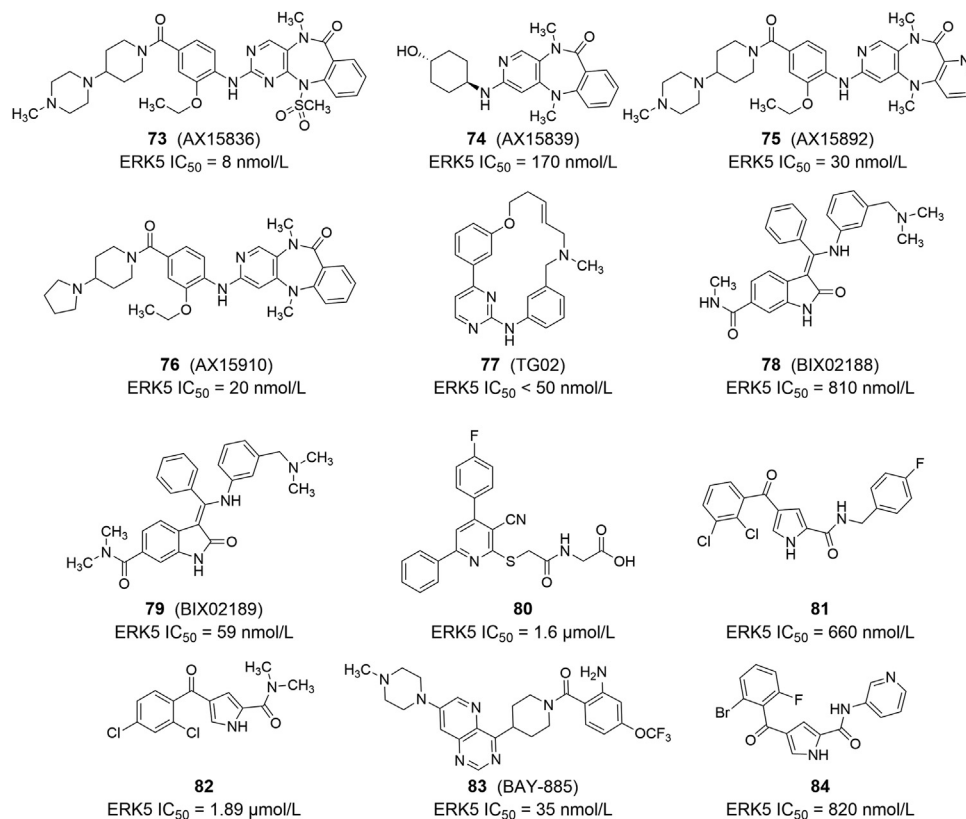


Figure 12 Chemical structures of ERK5 inhibitors **73–84**.

showed strong anti-tumor activity, with a tumor inhibition rate of 95%⁹⁸.

Later, the team synthesized leucine-rich repeat kinase 2 (LRRK2) inhibitor **69** (LRRK2-IN-1) using the 2-amino pyrido [2,3-*d*] pyrimidine scaffold and accidentally found that this compound can inhibit epidermal growth factor (EGF)-mediated autophosphorylation of ERK5 (EC₅₀ = 0.16 μmol/L). They went on to develop selective ERK5 inhibitors **70** and **71** (ERK5-IN-1) with IC₅₀ values of 82 and 162 nmol/L for ERK5, respectively. **71** has excellent pharmacokinetic characteristics, with an F value of 90% after oral administration in mice⁹⁹. In a recent SAR discussion of these compounds, the team found that replacing the nitrogen methyl groups on the seven-membered nitrogen heterocycle with progressively increasing alkyl groups can affect the conformation of the rings and can modulate the spatial and hydrophobic interactions between the rings and bromodomain-containing protein 4 (BRD4), resulting in a reduced affinity for BRD4 and LRRK2, but almost have no effect on ERK5. For instance, the selective ERK5 inhibitor **72** (JWG-071, ERK5 IC₅₀ = 88 nmol/L) is obtained by substitution of methyl to sec-butyl (Fig. 11B)¹⁰⁰. Subsequently, Lin et al.¹⁰¹ synthesized a series of derivatives of **67** (compounds **73–76**), which are also ATP competitive ERK5 inhibitors with IC₅₀ values ranging from 8 to 170 nmol/L, among which **73** (AX15836) has the best anti-tumor cell proliferation effect (Fig. 12). **77** (TG02) is an oral multi-kinase inhibitor that effectively inhibits the proliferation of multiple myeloma cell lines. The compound also inhibits the activity of ERK5 with an IC₅₀ less than 50 nmol/L¹⁰². Tataka et al.¹⁰³ conducted HTS of Boehringer Ingelheim compounds and obtained two potent MEK5/ERK5 inhibitors **78** (BIX02188) and **79**

(BIX02189). Both compounds exhibited certain inhibitory effects on ERK5, and **79** (IC₅₀ = 59 nmol/L) had better activity than **78** (IC₅₀ = 810 nmol/L). Myers et al.¹⁰⁴ used the same method to find four different series of compounds, and obtained compounds **80–82** after structural optimization, which showed good ERK5 inhibitory activity. Similarly, Nguyen et al.¹⁰⁵ found a highly selective ERK5 inhibitor, **83** (BAY-885), through HTS and synthesis optimization. Enzyme activity assay showed that the IC₅₀ of this compound against ERK5 was 35 nmol/L, and it also exhibited strong ERK5 inhibitory activity (IC₅₀ = 115 nmol/L) and transcriptional inhibitory effect (IC₉₀ = 691 nmol/L) in SN12C-MEF2 cell line. **84** was screened and synthesized by Myers et al.¹⁰⁶ and showed submicromolar activity against ERK5 (IC₅₀ = 820 nmol/L), which could effectively reduce the tumor volume of xenograft mouse model of human ovarian cancer cells with an inhibition rate of 57%.

The ERK5 signaling pathway plays an important role in a variety of cell life activities. Although some progresses have been made in the study of the biological function of the ERK5 pathway and the development of ERK5 selective inhibitors, it is still insufficient compared with ERK1/2. Further study and understanding of the ERK5 signaling pathway will facilitate the design of more effective ERK5 inhibitors for clinical treatment.

3.2.5. Dual inhibitors of ERK1/2 and other kinases

In drug design and development, the design of inhibitors targeting two or more targets simultaneously is an effective strategy for disease treatment and anti-drug resistance. Studies have shown that phosphatidylinositol 3-kinase (PI3K)/AKT and ERK1/2 pathways are both key signal transduction pathways that regulate

cell survival, proliferation and activity. Their abnormal activation can promote cell survival and unlimited proliferation, resulting in the occurrence of tumors and malignant progress. Thus, the design and development of targeted PI3K/AKT-ERK1/2 inhibitors will be an effective strategy for anti-tumor drug discovery¹⁰⁷. In 2013, Allen et al.⁶⁹ found a dual AKT-ERK1/2 inhibitor **15** (ONC201, also known as NSC350625 or TIC10) in the screening of compounds that induce tumor necrosis factor. It has good properties and can penetrate the blood–brain barrier, and has been used in 15 clinical I/II trials of various advanced tumor treatments¹⁰⁸. **85** (AEZS-136) is a dual-targeted PL3K-ERK1/2 inhibitor based on pyridine pyrazine scaffold, which inhibits ERK1/2 ($IC_{50} = 50$ nmol/L) and PI3K ($IC_{50} = 100$ nmol/L) through ATP competition mechanism, and induces dephosphorylation of MAPK and PI3K/AKT pathways in cells. Locatelli et al.¹⁰⁹ studied the anti-proliferative effect of **85** on more than 40 human tumor cell lines, and evaluated the physicochemical properties and ADME properties of the compound, indicating that it has good tolerance and significant anti-tumor activity (Fig. 13). Gao et al.¹¹⁰ synthesized a series of novel indole substituted compounds *via* multicomponent reaction, and the most activated compound **86** was chosen to study the antitumor activities to hepatocellular carcinoma. The result showed that **86** significantly induced the cell cycle arrest and apoptosis of HepG2 ($IC_{50} = 9.1$ μ mol/L) by targeting AKT and ERK1/2 pathways. Moreover, they found that oral administration of (2-hydroxypropyl)- β -cyclodextrin (HBC)-hosted **86** effectively inhibited tumor growth and prolonged the survival time of tumor-bearing mice without any obvious side effects. Awadallah et al.¹¹¹ described the synthesis of 1,2,4-trisubstituted imidazolinone derivatives, and the new compounds were designed as dual p38 α MAPK and ERK1/2 inhibitors. The kinase inhibition assay revealed that compounds **87** and **88** show excellent activity in the nanomolar range, with IC_{50} values of 4.5 and 4.7 nmol/L against p38 α MAPK, 25.0 and 24.0 nmol/L against ERK1, and 3.2 and 3.5 nmol/L against ERK2, respectively. Moreover, the two compounds elicited high antiproliferative activity at a sub-micromolar level against breast, prostate and melanoma cells. Except for ERK, vascular endothelial growth factor receptor (VEGFR) and Abelson murine leukemia-1 (Abl-1) had been respectively identified as potential drug targets. Jin et al.¹¹² discovered series of novel

compounds as active scaffolds against VEGFR2, ERK2 and Abl-1 kinases through the combination of support vector machine, similarity searching and molecular docking. The representative compound **89** seemed promising candidates for enzymic activity, compound **89** at 50 μ mol/L inhibited 11.05% of VEGFR2 activity, 10.86% of ERK2 activity and 6.71% of Abl-1 activity, respectively. In addition, **89** induced significant HepG2 cells apoptosis, with an IC_{50} value of 11.3 μ mol/L, and displayed better anti-proliferative abilities against K562 cells ($IC_{50} = 4.5$ μ mol/L).

The Aurora protein kinase family (Aurora A/B/C) can modulate many aspects of mammalian cell division. When the function of the protein is impaired, it may lead to the occurrence of tumors. Studies have found that they are also overexpressed in a variety of tumor types, and are closely related to the malignant progression and prognosis of tumors¹¹³. Defaux et al.¹¹⁴ developed a family of Aurora A/B inhibitors using the novel (7-aryl-1,5-naphthyridin-4-yl) ureas skeleton, which exhibited excellent Aurora A/B inhibitory activity in the nanomolar range (e.g., compound **90**: Aurora A $IC_{50} = 13$ nmol/L, Aurora B $IC_{50} = 107$ nmol/L), but all of which were deficient in antitumor cell proliferation. In the optimization of the structure, the authors found that shifting the urea group from position 4 to position 2 of the ring could maintain the compound's inhibitory activity against Aurora A/B, and also showed an inhibitory effect on the ERK pathway¹¹⁵. Based on this structure, a series of ERK and Aurora dual-target inhibitors were synthesized. Among them, representative **91** had an IC_{50} of 30 nmol/L for ERK2 and 579 nmol/L for Aurora B, and its potency was more than 10 times higher than that of other compounds in the same series (Fig. 14A). **91** binds well to the ATP pocket of ERK2 (PDB ID: 3I5Z). When the urea part extends to the Gln103 residue in the hinge region, it is observed to form a key hydrogen bond with the Met106 of ATP hinge residue. The benzyl group enhances affinity by forming a close hydrophobic interaction with Tyr34 and Val37 residues in the P-loop sequence. In addition, the newly synthesized compound showed strong anti-proliferative activity against five cancer cell lines (colorectal cancer cell lines HCT116, MDA-MB468 breast, PC3 prostate, A549 NSCLC, U87MG CNS), which was significantly improved compared with the previous structural system¹¹⁵.

ERK1 and ERK5 have pivotal roles in melanoma, colorectal cancer and other cancers. As previously mentioned, under some circumstance, ERK5 may provide a bypass route that can rescue cell proliferation when ERK1 signaling is suppressed. Due to the compensatory mechanism of ERK5 for ERK1/2, the currently developed ERK1 and ERK5 inhibitors have failed to meet clinical expectations. Therefore, Wang et al.²⁶ proposed a new therapeutic strategy to overcome the compensatory mechanism of ERK5 in specific tumors by jointly targeting ERK1 and ERK5, and successfully developed the first selective dual-target inhibitor of ERK1 and ERK5 (Fig. 14B). Multiple docking strategies were used to screen for lead inhibitors of ERK1 and ERK5. First, 212,255 compounds in the SPECS library were screened by Lipinski's rule of five, and 97,360 small molecules were obtained. Accurate docking analysis yielded 231 and 208 compounds targeting ERK1 and ERK5, respectively, all with scores greater than 30. Then, the overlapping and docking patterns of the two groups of compounds were analyzed, and 15 candidate molecules were screened. Further filtering by bioassay, **92** was identified as a lead compound targeting ERK1/5. Although **92** has a docking pattern similar to the previous inhibitors, some hydrogen bond interactions with the hinge region are required to stabilize the compound's binding to ERK. With this in mind, the author

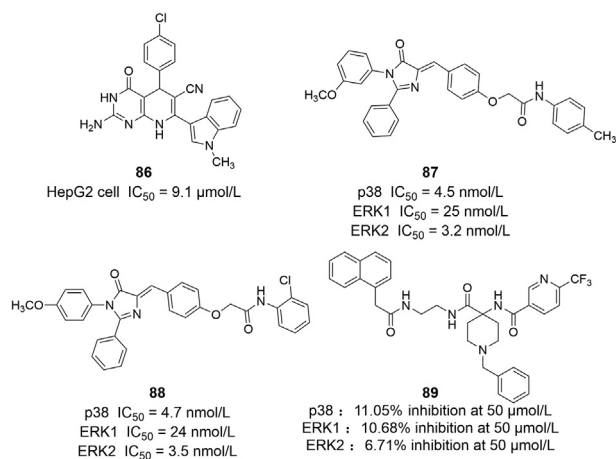


Figure 13 Chemical structures of ERK1/2 dual-targeting inhibitors **86–89**.

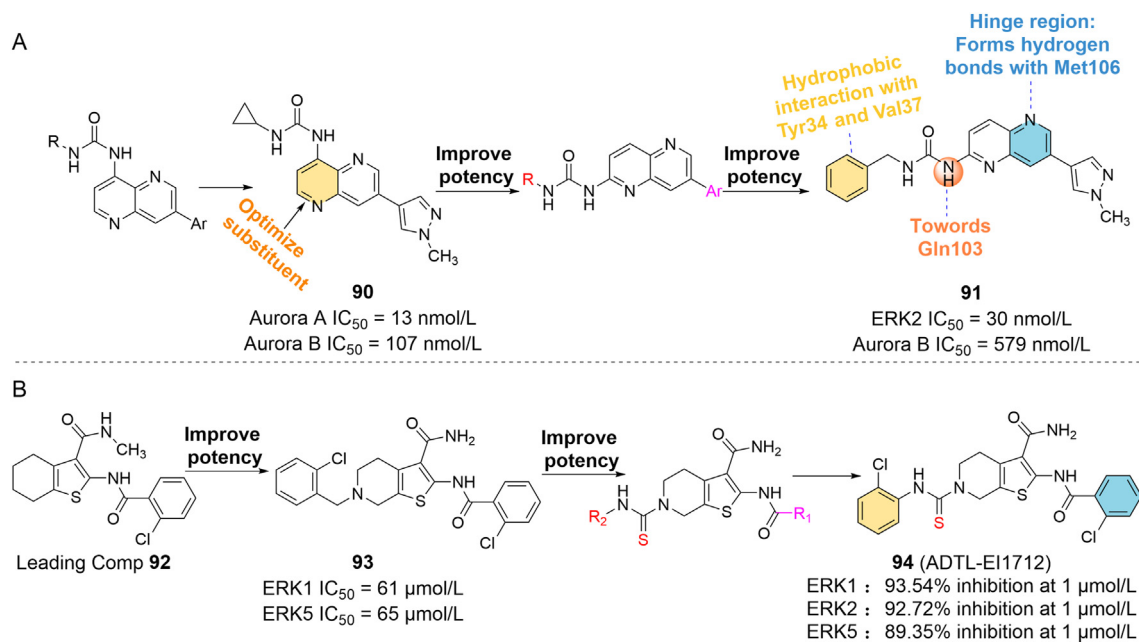


Figure 14 (A) Design strategy used from the novel (7-aryl-1,5-naphthyridin-4-yl) ureas skeleton to the dual ERK2 and Aurora B kinases inhibitor **91**. (B) Design strategy used from the leading compound **92** to the dual-target inhibitor of ERK1 and ERK5, **94**, with high potency through a series of optimizations.

replaced the six-membered ring hydrocarbon in **92** with a piperidine ring and filled the hydrophobic region by adding suitable substituents on the nitrogen atom. In the first series of synthesized inhibitors, the *ortho*-position of the R_1 group (benzyl) in the representative **93** was replaced by the electron-withdrawing group chlorine, which extended the core group of **93** toward the hinged region, thereby enhancing the kinase inhibitory activity (ERK1 IC_{50} = 61 μ mol/L, ERK5 IC_{50} = 65 μ mol/L) and anti-proliferative activity. But **93** still cannot form a stabilizing effect in the hinge area and hydrophobic channel. The author hypothesized that replacing the amide by introducing thiourea and urea groups might enhance the interaction with this region, so the R_1 chlorophenyl group was retained and the R_2 group was modified, and then the second series of compounds were synthesized. The results of bioactivity showed that the compound exhibited better enzyme inhibition activity when R_2 was a halogen-substituted phenyl, especially when R_2 was a 2-chlorophenyl (compound **94**, ADTL-EI1712, inhibition rates of ERK1/2/5 at 1 μ mol/L were 93.54%, 92.72% and 89.35%, respectively). **94** also showed higher antiproliferative activity. In addition, the author found that **94** can induce regulated cell death accompanied by autophagy in MKN-74 cells. In summary, **94** is a valuable chemical probe or lead compound for the study of the functional relationship between ERK1 and ERK5 and the discovery of more novel anti-tumor drug candidates based on its structure.

At present, dual-target drug design has become a hot research area for cancer therapy. The basic principle of developing such drugs is to overcome the problems of poor efficacy and drug resistance that often occur when using single-target inhibitors. These inhibitors work together with ERKs and another kinase, showing stronger ERK pathway inhibition and biological activity, so a dual-targeted strategy is expected to achieve better cancer treatment outcomes.

4. Conclusions and future perspectives

Mutations and disorders of protein kinases play a crucial role in the pathogenesis of many human diseases. These kinases have become one of the most important drug targets in the past few decades. With the continuous research, MAPK cascade inhibitors have been demonstrated to have significant effects in the treatment of a variety of cancers. At present, several BRAF and MEK inhibitors have been approved by the FDA for clinical treatment, but drug resistance frequently appeared soon after, severely limiting the clinical therapeutic effect of MAPK inhibitors, and becoming one of the major challenges in drug design and discovery of MAPK inhibitors. On the contrary, ERKs showed higher stability compared to other upstream MAPKs. Besides, ERKs are located at the end of the MAPK pathway, receive information from MEK1/2, and then passing it to hundreds of substrates downstream. In-depth research on the structure and physiological functions of ERKs has promoted the development of various novel small-molecule inhibitors targeting ERKs.

At present, more and more ERK1/2 inhibitors have been developed, and representative compounds have entered clinical trials¹¹⁶. However, there are problems with these inhibitors, the main drawback with all these inhibitors is that their effect is limited. Besides, they tend to develop resistance after a period of administration^{17,19}. The main reason for drug resistance is that the existing ERK1/2 inhibitors can inhibit the activity of ERK1/2 in the cytoplasm, and completely block the cytoplasmic function of ERK1/2, which also leads to the blockade of its negative feedback loop. When the ERK cascade is stimulated by extracellular signals, it causes the activation of the negative feedback loop, thereby balancing the activity of the cascade by reducing the expression of upstream components. The reduction of the feedback loop will induce the overactivation of the upstream components of the

cascade, leading to the development of drug resistance. Therefore, it should be of great value to design inhibitors that specifically block nuclear ERK activity without affecting cytoplasmic ERK function. To this end, Seger et al.¹¹⁷ designed a nuclear translocation sequence (NTS)-derived myristoylated phosphopeptide (EPE peptide), which can block the interaction between importin7 and ERK1/2, thereby inhibiting the nuclear translocation of ERK1/2 without affecting the cytoplasmic processes it induces, including negative feedback loops. EPE peptide induces apoptosis of BRAF melanoma cells, inhibits the survival of various cancer cells, but has no effect on non-transformed, immortalized cells, suggesting that EPE peptide may only specifically kill malignant tumor cells.

In addition, the blockade of RAS-ERK cascade also leads to the activation of alternative pathways, such as PI3K-AKT¹¹⁸. PI3K-AKT is one of the major pathways that it is often over-activated due to the lack of ERK negative feedback loops. Straussman et al.¹¹⁹ proposed that the tumour micro-environment confers innate resistance to therapy. Immunohistochemistry assay confirmed that stromal cells from patients with BRAF mutant melanoma express hepatocyte growth factor (HGF), the secretion of HGF leads to the activation of the HGF receptor MET and further overactivation of the AKT pathway, which leads to the development of drug resistance. The dual inhibition of RAF with HGF or MET resulted in reversal of drug resistance, suggesting that RAF inhibitors combined with HGF or MET inhibitors is a potential therapeutic strategy for BRAF mutant melanoma.

ERK5 activation is a compensatory mechanism for inhibition of the RAF-MEK1/2-ERK1/2 pathway. Blockage of ERK negative feedback leads to feedforward activation of some RTKs like EGFR, and the increasing expression of RTKs further activates the MEK5-ERK5 pathway¹²⁰. In addition, inhibition of the above negative feedback induced overactivation of the PI3K/AKT pathway can also lead to an increase in ERK5 signaling¹²¹. While the activation of ERK5 itself causes the activation of AKT, which in turn enhances the ERK5 signal^{122,123}. When the ERK1/2 cascade is inhibited, the positive feedback loop between ERK5 and AKT enables ERK5 to be rapidly activated to play a compensatory role in maintaining malignant proliferation of tumor cells. Based on this, we proposed a novel therapeutic strategy to overcome the above-mentioned compensatory mechanism in specific tumor types by co-targeting both ERK1 and ERK5, and identified compound **94** (ADTL-EI1712) was the first selective dual-targeted inhibitor of ERK1 and ERK5 (inhibition rates of ERK1/2/5 at 1 $\mu\text{mol/L}$ were 93.54%, 92.72% and 89.35%, respectively), and had potent antitumor effects *in vitro* and *in vivo*²⁶. Overall, these results warrant the potential of this dual-target inhibitor as a new candidate drug that conquers compensatory mechanism in certain tumor types. Nevertheless, the mechanism of interaction between ERK1/2 and ERK5 pathways has not been fully elucidated. Therefore, it is necessary to further explore the compensation mechanism mentioned above to strengthen the theoretical basis of ERK1/2 and ERK5 pathway combined targeted therapy, so as to achieve more effective tumor treatment.

All upstream kinases of the ERK signaling cascade were frequently mutated in cancer¹⁴. Although the ERK molecule was highly active in most cancers, oncogenic mutations in ERK itself were very rare¹²⁴. Unlike many known mutations in RAS, RAF and MEK, almost all known mutations in ERK are discovered in the laboratory and only a few have been identified in cancer patients. For instance, the ERK2 E320K mutation (mutations ID: COSM461148) appeared in dozens of patients with cervical and

head and neck carcinoma, but the E320K mutation did not affect the innate catalytic properties of the kinase¹²⁵. In addition, in a single cancer case reported in Catalogue of Somatic Mutations in Cancer (COSMIC), an endometrial cancer patient was shown to have an R84H mutation (mutations ID: COSM4875436) in the ERK1 gene, which was also identified in screening for drug-resistant ERK2 molecules¹²⁶. The only oncogenic ERK mutant found in ERK to date is ERK1 R84S, which was generated on the basis of a mutation in the yeast ortholog Mpk1/Slt2. This mutation may imbue ERK with intrinsic activity and potentially drive tumor growth^{127,128}. Therefore, activation of ERK mutations, particularly R84 mutations, will prove to be clinically significant. Activation mutations of ERK may not be completely carcinogenic or pathogenic, but may contribute to disease, and a large number of mutations may cause ERK resistant to inhibitors¹²⁹. Although ERK1/2 mutations are rarer than BRAF or MEK1/2 mutations, it is foreseeable that monotherapy of ERKs inhibitors may face the same challenges as other MAPK inhibitors. In this regard, existing studies have shown that multi-targeting inhibitors that simultaneously target ERKs and other proteins such as mTOR, PI3K, PKB and AKT, or combining ERKs inhibitors with upstream inhibitors of MAPK or other anti-tumor drugs is an effective method and an important strategy to improve the anti-tumor effect and overcome the problem of drug resistance^{130–133}. For example, the combination of compound **6** with the MEK inhibitor cobimetinib significantly enhanced the antitumor activity of KRAS and BRAF mutant tumor models¹³⁰. Carlino et al.¹³⁴ found that although ERKs inhibitors were proved to have the effect of anti-proliferation of drug-resistant melanoma cells, ERK-dependent negative feedback was alleviated, and RAS, as well as PI3K/AKT signals were reactivated due to ERKs inhibition, resulting in the inability of melanoma cells to apoptosis. While the combined inhibition of ERK/PI3K and mTOR can effectively inhibit all melanoma cell models resistant to ERK inhibitors.

The similarity of the structure and sequence of the kinase ATP binding pocket makes selective inhibition of different kinases a formidable challenge. To solve the problem of low selectivity of ATP competitive inhibitors, Li et al.¹³⁵ designed and developed a series of novel fourth-generation small molecule EGFR inhibitors, which could occupy both the ATP-binding pocket of EGFR and the allosteric site near the pocket generated by the outward shift of αC helices. This dual effect enhanced the binding affinity of the inhibitor to EGFR^{L858R/T790M/C797S}. Inspired by this study, we can look for binding allosteric sites near the ATP binding sites of ERK and design ERK inhibitors that can bind ATP pocket and allosteric sites at the same time, so as to improve the low selectivity of current ATP competitive ERK inhibitors. In addition, covalent kinase inhibitors have received extensive attention due to their potential of high selectivity, sustained target inhibition, better ligand efficiency, and overcoming drug resistance. Therefore, the development of ATP competitive/covalent kinase inhibitors using the same strategy is another feasible approach. Moreover, compared with a single inhibitor, the combination of different inhibitors on the same target may produce significant therapeutic effects compared with a single inhibitor. Since ERK allosteric inhibitor acts on allosteric sites outside the ATP binding pocket, the combined application of ERK allosteric inhibitor and ATP competitive inhibitor may enhance the inhibitory effect on ERK, and may effectively overcome the shortcoming of low selectivity of ATP competitive inhibitor.

Compared with small molecule inhibitors that only inhibit protein activity, proteolysis targeting chimera (PROTAC) has

attracted extensive attention in the field of drug discovery due to its advantages in selectively inducing intracellular protein degradation¹³⁶. PROTAC is a technology that uses small molecules to induce the ubiquitination of target proteins and achieve targeted protein degradation through the ubiquitination proteasome pathway¹³⁷. On the basis of this theory, we can design a PROTAC that can target ERK protein and induce the interaction between ERK protein and E3 ubiquitin ligase to realize the non-natural ubiquitination of ERK, and the ubiquitinated ERK will be further recognized and degraded by the proteasome. Under the condition of reasonable design and full optimization, PROTAC technology may theoretically achieve better selectivity compared than traditional ERK inhibitors.

In recent years, many breakthroughs have been made in tumor immunotherapy, and the combination of inhibiting ERKs immunotherapy will also provide a new strategy for the clinical treatment of tumors¹³⁸. In conclusion, great progress has been made in the research on the molecular function, mechanism of action, biological process and drug discovery of ERKs protein. The development of inhibitors targeting ERKs, dual-targeting inhibitors, and the combination of drugs or other therapeutic methods will have a positive and profound impact on the clinical treatment of cancer.

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Author contributions

Liang Ouyang, Guan Wang and Lan Zhang conceived the project and supervised the project. Xiaoli Pan and Junping Pei summed up the literature and drafted the manuscript. Aoxue Wang was involved in drawing the figures. Wen Shuai, Lu Feng, Faqian Bu and Yumeng Zhu collected and organized the inhibitors. Guan Wang and Lan Zhang proofread the structures and figures. Liang Ouyang revised the manuscript. All authors approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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