

Review Article

Application of a macrocyclization strategy in kinase inhibitor development

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ABSTRACT

Kinases have a pivotal role in phosphorylation and kinase dysregulation has been implicated in the occurrence and progression of various diseases. By mid-2024 the Food and Drug Administration had approved 81 small-molecule kinase inhibitors targeting greater than 30 kinases, providing substantial clinical benefits for patients with cancer and other diseases. However, many reported kinase inhibitors exhibit poor target selectivity, limited solubility, and issues with drug resistance. To address these challenges medicinal chemists have used a macrocyclization strategy to optimize these molecules and three macrocyclic kinase inhibitors (lorlatinib [ALK/ROS1], pacritinib [Flt3/JAK2], and repotrectinib [TRK/ROS1]) have received FDA approval. The macrocyclization strategy is now widely used to enhance kinase inhibitory activity, overcome drug resistance caused by point mutations, improve kinase selectivity, and optimize drug-like properties. In this review we provide a comprehensive overview of the utilization of macrocyclization in the optimization of kinase inhibitors by detailing the structural modification process from lead compounds to macrocyclic molecules that has been applied in recent years. In addition, we discuss the limitations of existing macrocyclic kinase inhibitors and propose key considerations for the development of improved macrocyclic molecules. We aim for this review to offer valuable insights for future advances in kinase inhibitor development.

Keywords: Kinase inhibitor, Macrocyclization strategy, Drug design, Binding affinity, Kinase selectivity, Drug-like properties

1. INTRODUCTION

Kinases, a class of enzymes, facilitate the transfer of the γ -phosphate group from ATP to substrate proteins through a process known as phosphorylation [1]. It has been confirmed that dysregulation of the kinase phosphorylation level has a critical role in the development of various diseases, making kinases attractive targets for therapeutic intervention [2-4]. The human genome encodes 518 known kinases and the Food and Drug Administration (FDA) has approved 81 small-molecule kinase inhibitors [5]. However, with the increasing approval of kinase inhibitors, limitations have gradually emerged. For example, type I and II kinase inhibitors often exhibit poor kinase selectivity, leading to off-target toxicity [6-8]. In addition, most kinase inhibitors contain more than two aromatic rings, which can affect aqueous solubility [9]. Moreover, prolonged clinical use of these kinase inhibitors has resulted in

acquired resistance in patients due to point mutations [10, 11].

Macrocyclic kinase inhibitors, characterized by a minimum 12-membered ring structure, have attracted significant attention in recent years [12]. Due to the structural complexity and diversity, macrocyclic kinase inhibitors often display stronger binding affinity compared to acyclic kinase inhibitors [13, 14]. Moreover, the macrocyclization strategy is a powerful tool for improving kinase selectivity of acyclic inhibitors, possibly due to the semi-rigid structural features of macrocycles [15, 16]. This strategy is also used to modulate the properties of acyclic compounds, enhancing membrane permeability and oral bioavailability [17, 18].

Currently, the FDA has approved three small-molecule macrocyclic kinase inhibitors (lorlatinib, pacritinib, and repotrectinib), which specifically target the ATP binding sites of ALK/ROS1, JAK/Flt3, and NTRK/ROS1, respectively (Figure 1). Beginning with the pioneering

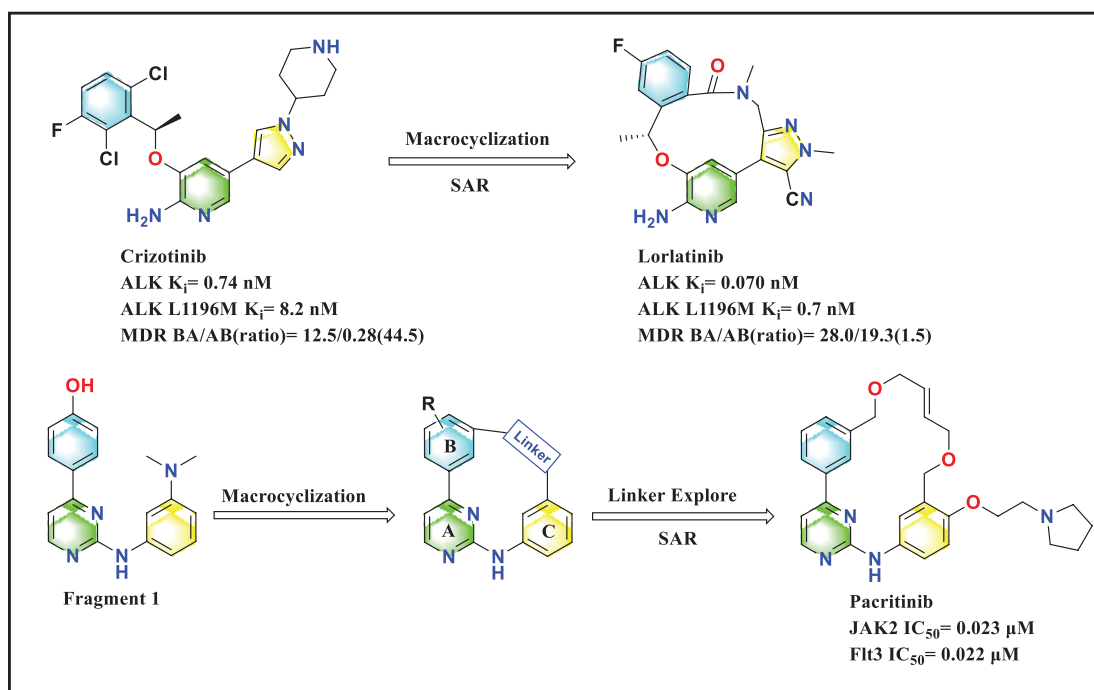


Figure 1 | Optimization process of representative macrocyclic kinase inhibitors (lorlatinib and pacritinib).

first-generation ALK/ROS1 inhibitor, crizotinib, Pfizer scientists embarked on a quest to develop third-generation ALK inhibitors. The goal was to create drugs capable of targeting ALK mutations and effectively penetrating the central nervous system [19, 20]. Guided by scaffold hopping and macrocyclization, the scientists discovered lorlatinib, a macrocyclic molecule with a potent K_i value of 0.07 nM against ALK. Lorlatinib showed superior inhibitory activities towards ALK mutants compared to crizotinib with reduced brain penetration (MDR BA/AB: 44.5 vs. 1.5). As a result, lorlatinib was approved for the treatment of ALK-positive non-small cell lung cancer (NSCLC). Pacritinib is derived from 2-aminopyrimidine fragment 1 through in-house library screening [21]. The macrocyclization strategy was used between the 2- and 4-position of the pyrimidine, making a compromised binding mode to the kinase hinge region. Various substituents by different linkers were introduced to improve the inhibitory activities against JAK2 and Flt3. Finally, researchers discovered pacritinib, which exhibits remarkable inhibitory activity against JAK2 and Flt3 with IC_{50} values of 23 nM and 22 nM, respectively. Pacritinib received FDA approval for the treatment of high-risk myelofibrosis accompanied by severe thrombocytopenia in 2022. Moreover, repotrectinib, a second generation TRK/ROS1 inhibitor, was approved by the FDA in 2023 for the treatment of adult patients with locally advanced or metastatic ROS1-positive NSCLC [22, 23]. In addition to lorlatinib, pacritinib, and repotrectinib, many other macrocyclic kinase inhibitors have entered clinical trials (Table 1). Zotiraciclib and SB1578 are pacritinib analogues, which have JAK2/Flt3 dual

inhibitory activities [24, 25]. Zotiraciclib entered phase I/II clinical trials for the treatment of NSCLC and other solid tumors and SB1578 entered a phase I clinical trial for cancer therapy. Inspired by natural products, E6201 was discovered with potent FLT3 and MAP2K1 inhibitory activity and entered phase I clinical trials for the treatment of hematologic cancers and melanoma [26].

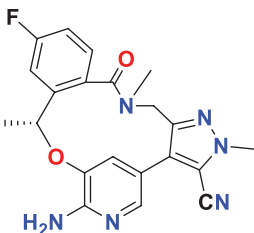
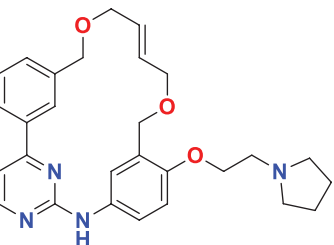
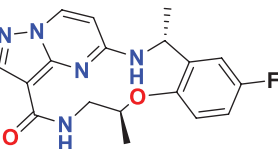
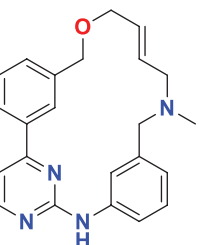
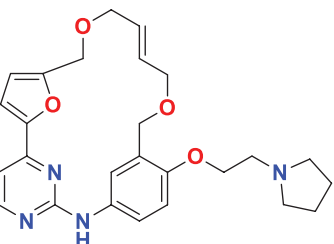
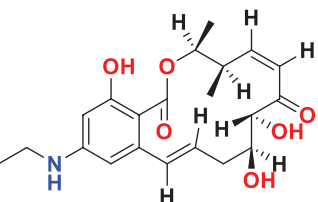
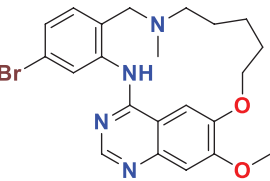
In this review we focused on the utilization of macrocyclization in kinase drug design to enhance inhibitory activity against wild-type and mutant kinases, improve the selectivity of promiscuous inhibitors, and adjust the physicochemical and pharmacokinetic (PK) properties of acyclic kinase inhibitors. Additionally, we summarized the optimization process for transforming acyclic inhibitors into macrocyclic inhibitors, discussed potential challenges during the macrocyclization process, and provided insights for the future design of macrocyclic kinase inhibitors (Figure 2).

2. IMPROVING INHIBITORY ACTIVITIES AGAINST WILD- AND MUTANT-TYPE KINASES

A kinase often adopts a fixed conformation that is favorable for inhibitor binding. The conformation of linear molecules must be adjusted when binding to the kinase domain due to molecule flexibility, which leads to entropy loss. As a result, acyclic inhibitors may exhibit decreased kinase inhibitory activity. In contrast, macrocyclic inhibitors have a constrained conformation that reduces entropy loss compared to acyclic inhibitors, thereby displaying improved kinase inhibitory activity. Importantly, the macrocyclization strategy is particularly

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Table 1 | Small molecular macrocyclic kinase inhibitors entered clinical trials.

Name	Structure	Targets	Highest Phase	Indications
Lorlatinib		ALK, ROS1	Launched	Lymphoma Neurologic Cancer Non-small Cell Lung Cancer
Pacritinib		JAK2, Flt3	Launched	Acute Respiratory Distress Syndrome Colorectal Cancer Hematopoiesis Disorders Myelofibrosis
Repotrectinib		NTRK1, ROS1	Launched	Non-Hodgkin's Lymphoma Non-small Cell Lung Cancer Solid Tumor Therapy
Zotiraciclib		CDKs, Flt3, JAK2, MAPK7	Phase I/II	Astrocytoma Glioblastoma Multiforme Hematologic
SB-1578		Flt3, JAK2, TYK2	Phase I	Cancer
E6201		Flt3, MAP2K1	Phase I	Hematologic Cancer Melanoma Psoriasis
JNJ-26483327		EGFR, ERBB2, ERBB4, Flt4, Src	Phase I	Solid tumors

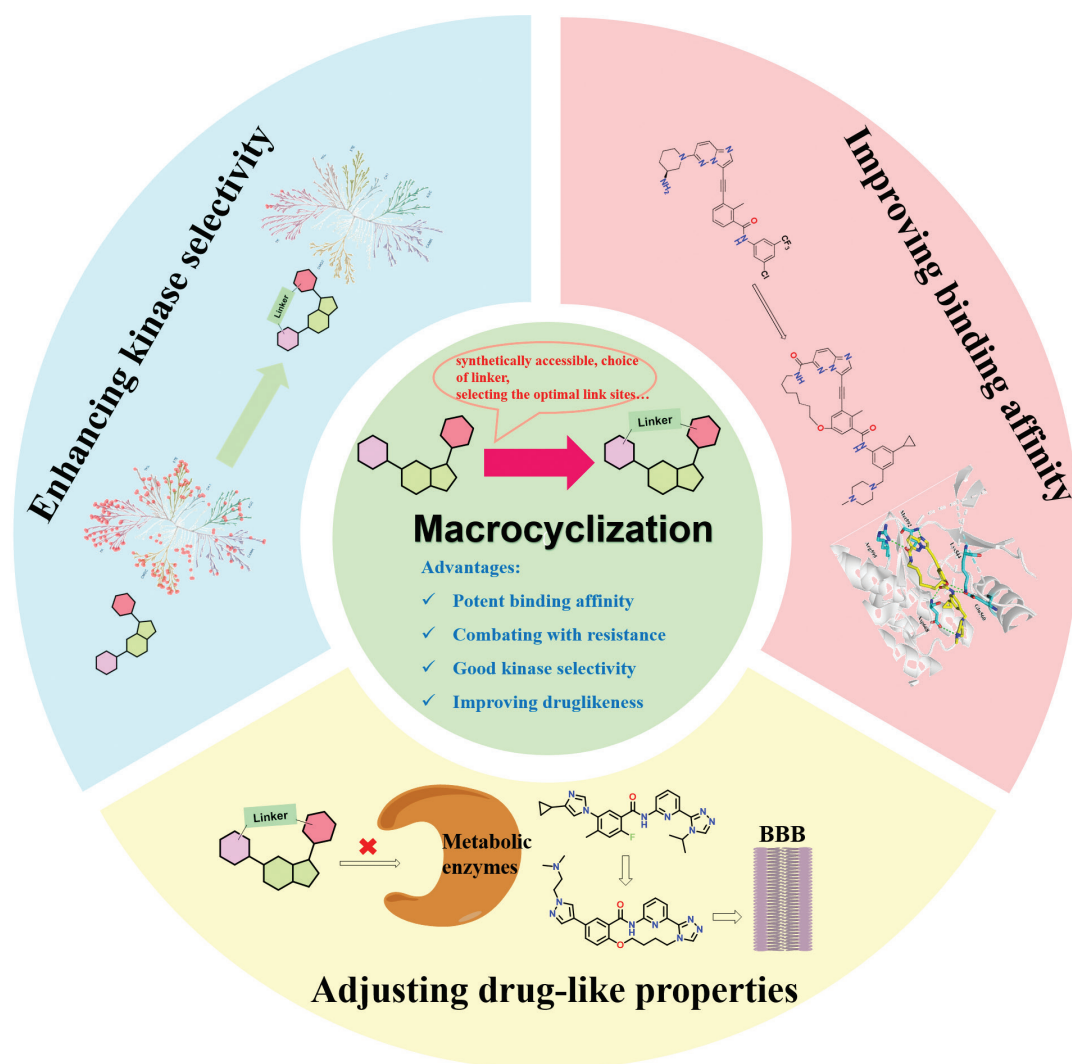


Figure 2 | Drug design strategy, advantages and potential challenges of macrocyclic kinase inhibitors.

effective in improving inhibitory activity against kinases with point mutations, which is primarily due to reduced steric hindrance with bulky amino acid residues.

2.1 Cyclin-dependent kinase (CDK) inhibitors

CDKs, which belong to the serine-threonine kinase family, function by interacting with cyclin counterparts [27, 28]. Dysregulation of the CDK-cyclin complex leads to impaired control of the cell cycle and transcription in tumor cells [29]. CDK inhibition has emerged as a promising therapeutic strategy for cancer. The FDA has approved four CDK inhibitors (abemaciclib, palbociclib, ribociclib, and trilaciclib) [30–32]. Yu et al. used generative models and structure-based drug design techniques to develop a series of highly specific macrocyclic CDK2 inhibitors (Figure 3) [33]. Using virtual screening and a fragment replacement strategy, compound 1 was identified, which was shown to have promising inhibitory activity against

CDK2/E1 with an IC_{50} value of 8.1 nM. The co-crystal structure of 1 with CDK2/cyclin E1 (PDB:8H6T) showed that the aminopyrazole moiety formed three canonical hydrogen bond interactions with Glu81 and Leu83 in the hinge region. The *cis*-cyclopentyl ring occupied the hydrophobic pocket and interacted with Phe80 through van der Waals interactions. Additionally, the carbonyl oxygen has the potential to form a hydrogen bond with Lys33, while the NH group can form a hydrogen bond with Asp145. The pyridine ring of 1 extends to the solvent-exposed region. To enhance the inhibitory potency against CDK2, a three-step workflow was used to design macrocyclic compounds derived from 1 [34]. It was found that the 6-position of the pyridine ring and the NH of the carbamate moiety are suitable for macrocyclization. The linker was studied to identify highly effective CDK2 inhibitors. During the optimization process, it was observed that macrocyclic compound 2, which features a linker

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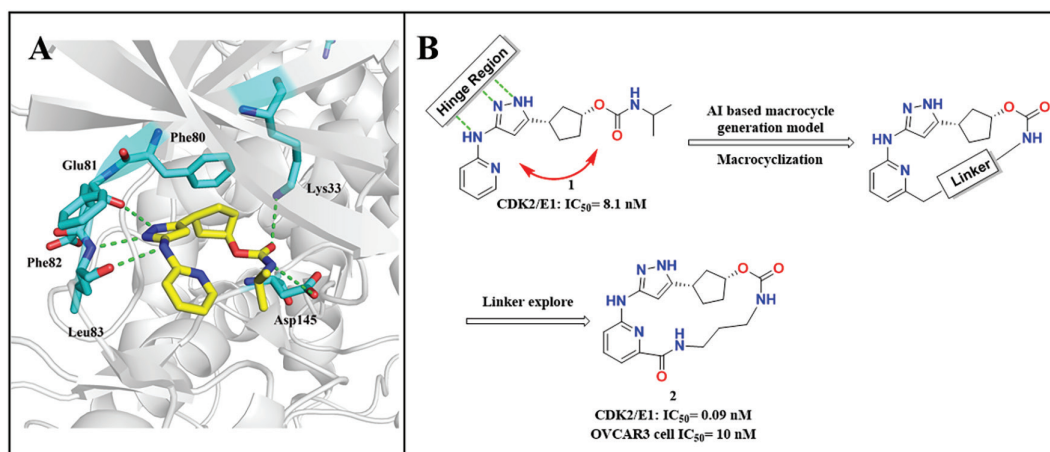


Figure 3 | (A) Co-crystal structure of compound 1 with CDK2//cyclin E1 (PDB:8H6T) [hydrogen bond interactions are green]; (B) Optimization process of pyridine-based macrocyclic CDK2 inhibitors.

with a length of six atoms, exhibits superior potency compared to compound 1 with an IC_{50} value of 0.09 nM. Furthermore, compound 2 also exhibits potent antiproliferative activity against OVCAR3 cells (IC_{50} = 10 nM).

Meng et al. designed a series of macrocyclic compounds as CDK2/4/6 inhibitors based on the macrocyclization strategy and AI-guided drug design methods (Figure 4) [35]. Compound NUV422 was selected as the lead compound and the dihedral angle scan method was used to determine the lowest energy conformation when binding to CDK2. The dihedral angle between the pyrimidine core and the 4-position phenyl ring of NUV422 was approximately 30.6° when binding to CDK2, which differed from the lowest energy conformation. The macrocyclization platform was established to identify the starting and closing points for macrocyclization. Moreover, the length and properties of various linkers were carefully selected. The macrocyclization strategy was used between the 2- and 4-position phenyl rings to fix the active binding conformation based on the predicted binding mode and macrocyclization

platform. Compound 3 was discovered with good CDK2, CDK4, and CDK6 inhibitory activities with IC_{50} values of 37.1 nM, 1.4 nM, and 1.9 nM, respectively. Then, the linker and basic group were modified to investigate the impact on bioactivity. Among the synthesized compounds, 4 displayed potent CDK2/4/6 inhibitory activities (IC_{50} values: 12.0, 1.5, and 2.9 nM, respectively) and good anti-proliferative activities against HCC1806 and OVCAR3 cells (IC_{50} values: 214.5 and 417.0 nM, respectively). Importantly, compound 4 displayed an optimized safety profile compared to NUV422.

2.2 Death-associated protein kinase-related apoptosis-inducing protein kinase 1 (DRAK1) inhibitors

DRAK1 belongs to the family of death-associated protein kinases (DAPKs) and regulates various cellular processes, including autophagy and apoptosis [36, 37]. The current understanding of DRAK1 biological functions is limited. Developing selective DRAK1 inhibitors would be meaningful for determining DRAK1 functions [37].

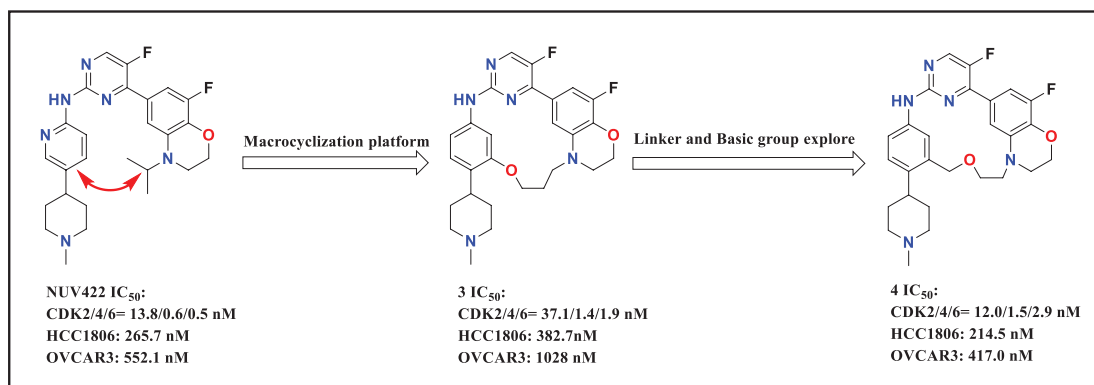


Figure 4 | Macro cyclization process of NUV422.

Kurz et al. developed a series of pyrazolo[1,5-a]pyrimidine derivatives as DRAK1 inhibitors, starting from the CK2 inhibitor, IC19 (Figure 5) [38]. Previous work suggested that the carboxylic acid motif of IC19 is crucial for CK2 inhibition and the N-benzylamide derivative, **5**, displays inhibitory activity against DRAK1 [39]. The **5** co-crystal structure with DRAK1 (PDB: 7QUE) showed that the nitrogen atom of the core forms a hydrogen bond with Ala141 in the hinge region and the oxygen atom of the carbonyl group forms a hydrogen bond with Lys90. Moreover, the benzylamide motif occupies the back pocket. Although compound **5** displays inhibitory activity against DRAK1, compound **5** inhibits other kinases. Therefore, structure-activity relationship (SAR) analysis around the benzylamide motif was performed to determine kinase inhibitory activity and selectivity. Compound **6** with the tert-butyl moiety was shown to exhibit good DRAK1 inhibitory activity ($K_D=21$ nM) and selectivity among all the synthesized compounds. The kinase selectivity assay suggested that among a panel of 468 kinases, compound **6** displayed excellent kinase selectivity at concentrations of 100 and 1000 nM. However, in a cell-based assay, compound **6** did not result in a cellular phenotype change and toxicity in GBM cell lines, which suggested that the catalytic function of DRAK1 is not required for cell survival. Therefore, the highly selective compound **6** could serve as a chemical probe for DRAK1 function research.

2.3 Dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 (DYRK1) inhibitors

Dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1A (DYRK1A) is a serine/threonine kinase that undergoes autophosphorylation at the tyrosine residue [40, 41]. DYRK1A is considered a promising therapeutic target due to a significant role in the development of neurologic disorders and cancer [42]. AC15 is a type I DYRK1A inhibitor with a 7-azaindole core and an IC_{50} value against DYRK1A of 329 nM. The co-crystal structure of AC15 with DYRK1A (PDB: 6EJ4) revealed that the nitrogen atom and the NH of the core forms two hydrogen bond interactions with Leu241 and Glu239.

Additionally, the pyridine moiety occupies the gate-keeper hydrophobic pocket and forms π - π stacking interaction [43]. The co-crystal structure of AC15 in complex with DYRK1A showed the molecule adoption of a “U-shaped” binding mode [44]. Powell et al. conducted a study in which a series of selective macrocyclic DYRK1A/B inhibitors was designed and synthesized using AC15 as the lead compound (Figure 6) [45]. The substituents located at the 3- and 5-positions in AC15 were closely positioned, enabling connection with a suitable linker. Compound **7**, among these substituents, demonstrated strong inhibitory activities against DYRK1A/B with IC_{50} values of 158 nM and 70 nM for DYRK1A and DYRK1B, respectively. To enhance kinase inhibition activity and selectivity, different substituents were introduced into the pyrazole rings at positions 3 and 5. It was observed that compound **8**, which included cyano and trifluoromethyl substitutions, demonstrated significant inhibitory activities against DYRK1A ($IC_{50}=13$ nM) and DYRK1B ($IC_{50}=19$ nM). Compound **8** demonstrated greater selectivity compared to compound **7** in the kinase selectivity assay against a panel of 468 kinases [S-Score(5): 0.16 vs. 0.03].

2.4 Fms-like tyrosine kinase 3 (Flt3) inhibitors

Flt3 is member of type III receptor tyrosine kinases and is frequently mutated in patients with acute myeloid leukemia (AML) [46-48]. Approximately 30% of AML patients harbor Flt3 mutations. There is an urgent need to develop kinase inhibitors to combat these mutations [49]. Several Flt3 small molecular inhibitors have been approved for the treatment of AML, such as midostaurin, quizartinib, and gilteritinib. However, acquired resistance caused by the emergence of secondary mutations in Flt3-ITD, such as Flt3-ITD-D835Y or Flt3-ITD-F691L, decreases the clinical response to Flt3 small molecular inhibitors [50]. Zheng et al. designed and synthesized a series of macrocyclic Flt3 inhibitors based on the Flt3 inhibitor, G-749 (Figure 7) [51]. G-749 is a type I Flt3 inhibitor that displays potent inhibitory activities against Flt3-ITD and Flt3^{F691L} [52]. The predicted binding mode of G-749 with Flt3 (PDB: 6JQR) indicated that

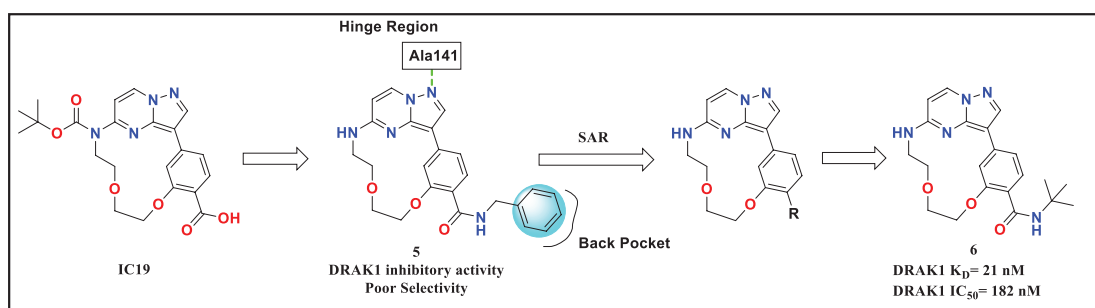


Figure 5 | Optimization process for substituents occupying the back pocket.

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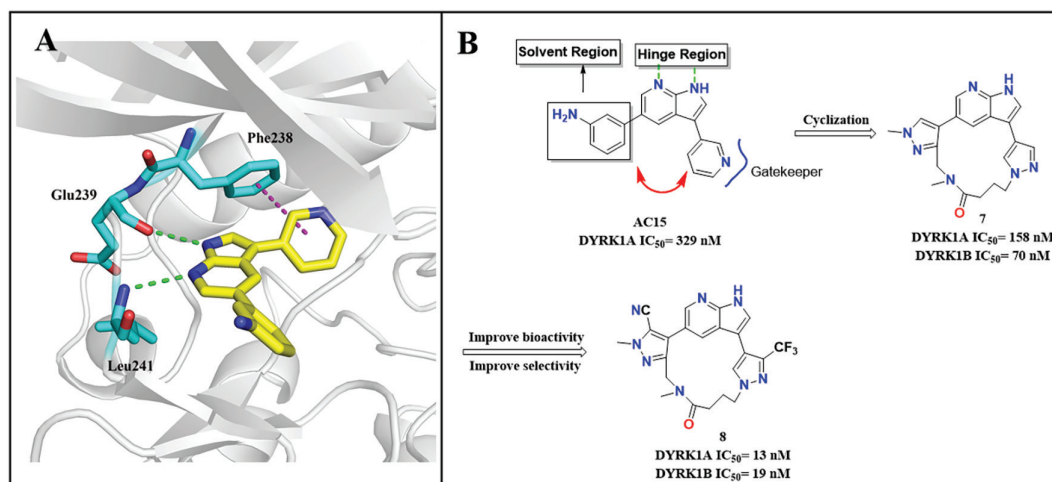


Figure 6 | (A) Co-crystal structure of AC-15 with DYRK1A (PDB: 6EJ4) [hydrogen bond interactions are green and the π - π stacking interaction is purple]; (B) Design of 7-azaindole derivatives as macrocyclic DYRK1A/B inhibitors.

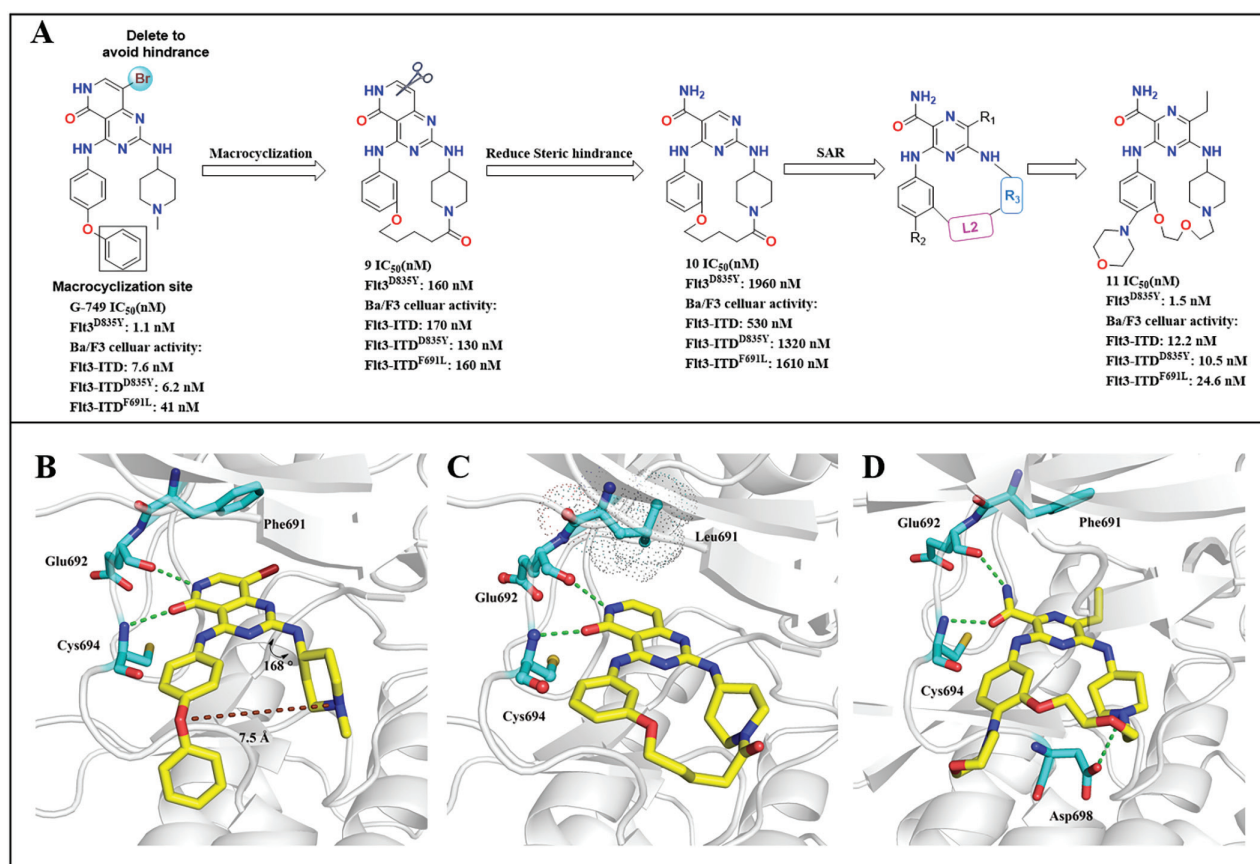


Figure 7 | (A) Optimization process of compound G-749 using macrocyclization strategy. (B) Docking result of G-749 with Flt3 (PDB: 6JQR); (C) Docking result of compound 9 with Flt3 (PDB: 6JQR); (D) Co-crystal structure of compound 11 with Flt3 (PDB: 8XB1) [hydrogen bonds are green].

the lactam of the pyrimidinone engages two hydrogen bonds with Glu692 and Cys694 in the hinge region. The di-phenyl ether moiety extends to the solvent-exposed region and the bromine atom points to the gatekeeper region. The docking pose of G-749 with Flt3 demonstrated that the pyrimidine core and the piperidine moiety adopt a dihedral angle of 168°, which displays an unfold conformation. Torsional angle scan analysis of G-749 suggested that G-749 has two local minimum energy conformations, including fold and unfold, with dihedral angles of approximately 0° and 180°. The macrocyclization strategy was used between the phenoxy and piperidine moieties to fix the unfold conformation of G-749. First, the length of the linker was explored to investigate the effect on Flt3 inhibitory activity. Compound **9**, with a 6-atom linker, displays good inhibitory activities against Flt3^{D835Y}, Flt3-ITD, and Flt3-ITD mutations (IC₅₀ values: 130–170 nM). Molecular docking of compound **9** with Flt3 suggested that the pyrimidinone moiety of **9** might cause potential steric hindrance with the mutated Leu691. Therefore, the core of **9** was changed to a smaller pyrimidine-5-carboxamide scaffold, resulting in compound **10**. However, compound **10** displays a 10-fold decrease in activity against Flt3 mutants, possibly because compound **10** does not occupy the hydrophobic pocket formed by Cys828, Asp829, Lys644, and Val675. Thus, the pyrimidine core was replaced with pyrazine and different small hydrophobic substituents were introduced at the R₁ position to investigate the inhibitory activities against Flt3 mutants. In addition, different hydrophilic groups and linkers were introduced to the R₂, L₂, and R₃ positions to determine the effects on the inhibitory activities against Flt3 mutants. Among all synthesized compounds, compound **11** displays the most potent inhibition of Flt3 mutants (IC₅₀ values: 1.5–24.6 nM). The co-crystal structure of **11** with wild-type Flt3 (PDB: 8XB1) indicated that the carboxamide moiety forms two hydrogen bonds with Glu692 and Cys694 in the hinge region and the nitrogen atom of piperidine moiety forms an extra hydrogen bond with Asp698. The kinase selectivity assay of **11** demonstrated that **11** displays good selectivity among 378 kinases. Moreover, **11** displays moderate PK properties (SD Rats, P.O. 10 mg/kg: C_{max}: 204.64 ng/mL, AUC_(0-last): 3201.72 ng·h/mL) and good *in vivo* anti-tumor activity without potential toxicity.

2.5 Met inhibitors

c-Met is a receptor tyrosine kinase that is activated when hepatocyte growth factor is bound, resulting in the formation of receptor dimers and auto-phosphorylation [53, 54]. Once activated, c-Met can initiate various downstream signaling pathways, including the MAPK signaling pathway, PI3K/AKT axis, and STAT3 pathway [55, 56]. Alterations in the Met gene have been identified as the main cause of NSCLC [57] and gastric cancer [58]. Thus, the development of c-Met kinase inhibitors holds great promise as a potential strategy for cancer

therapy [59]. Wang et al. discovered a series of potent c-Met kinase inhibitors known as 2-(2-(quinolin-6-yl)ethyl)pyridazin-3(2*H*)-one derivatives using molecular hybridization and macrocyclization strategies (Figure 8) [60]. The co-crystal structure of tepotinib with c-Met (PDB: 4R1V) revealed a U-shaped binding mode [61]. The nitrogen atom of pyrimidine forms a hydrogen bond interaction with Met1160 in the hinge region, the pyridazinone moiety engages in a π -stacking interaction with Tyr1230 in the activation loop, and the carbonyl group forms a hydrogen bond interaction with Asp1222. Similarly, the co-crystal structure of NVP-BVU972 with c-Met (PDB: 3QTI) [62] indicated that the quinoline part interacts with the hinge region, while the imidazopyridazine moiety extends to the activation loop and engaged in a π -stacking interaction with Tyr1230 that is similar to tepotinib. A molecular hybridization strategy was used to obtain 2-(2-(quinolin-6-yl)ethyl)pyridazin-3(2*H*)-one derivatives using tepotinib and NVP-BVU972 as references. Among the synthesized compounds, **12** exhibited significant inhibitory activity against c-Met and was shown to have anti-proliferation activity in cells. The co-crystal structure of compound **12** with c-Met (PDB: 7Y4U) revealed that the quinoline moiety acts as a hinge binder, while the pyridazinone part interacts with Tyr1230 and Asp1222 in the activation loop. Additionally, the pyrazole moiety extends to the solvent-exposed region. The X-ray analysis of **12** in complex with c-Met (PDB: 7Y4U) demonstrated that the CH₂CH₂ linker assumes a folded conformation, which is characterized by a dihedral angle of 76.3°. A macrocyclization strategy was used involving the pyrazol ring and the benzamide portion to stabilize the active conformation of **12** with c-Met. It can be inferred that a linker length of 4–5 carbon atoms would be suitable based on an approximate distance of 6.9 Å between the methylpyrazole and formamide groups. Compound D6808, with a 4-carbon atom length, demonstrated enhanced inhibition activity against c-Met and Hs746T cancer cells with IC₅₀ values of 2.9 and 0.7 nM, respectively. The kinase selectivity assay showed that D6808 exhibits exceptional selectivity among a panel of 373 wild-type kinases, only displaying inhibitory activities against Axl and TRKs (IC₅₀: Axl: 401.3 nM; TRKA: 437.2 nM; TRKB: 1386.0 nM; TRKC: 203.9 nM). D6806 demonstrated superior anti-proliferative activity against Ba/F3 cells transformed with various Tpr-Met fusion proteins (IC₅₀ values: 1–39 nM) compared to tepotinib (IC₅₀ values: 1.5–46.7 nM) in a cell-based assay. However, D6808 exhibited poor PK properties (p.o. 10.0 mg/kg, F: 2.02%; i.p. 10 mg/kg F: 33.91%) and showed weak inhibition on the hERG potassium channel (hERG IC₅₀ = 27.72 μ M), possibly due to its high lipophilicity. Therefore, further optimization focusing on improving solubility is necessary.

2.6 Leucine-rich repeat kinase 2 (LRRK2) inhibitors

LRRK2 is a member of the ROCO protein family and has vital role in the pathogenesis of Parkinson's disease (PD)

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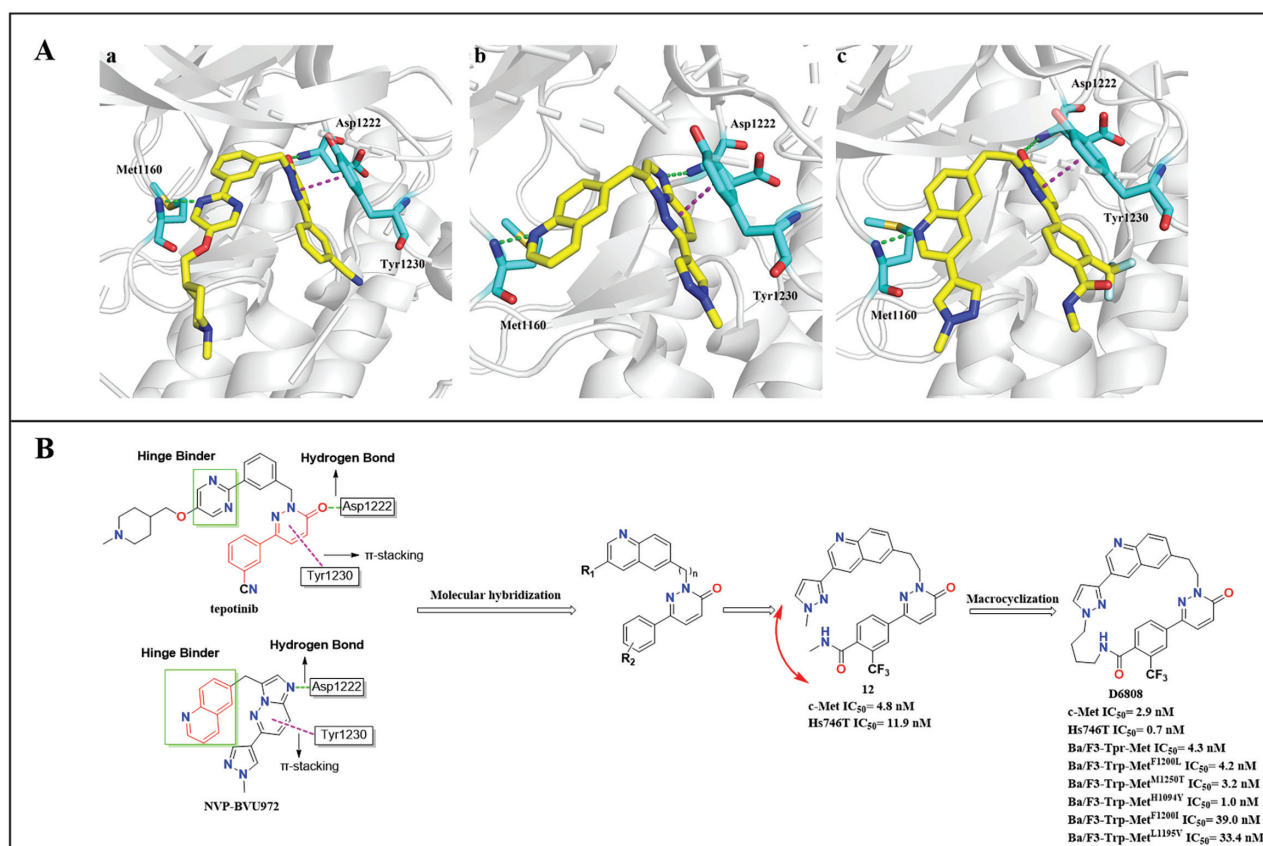


Figure 8 | (A) a: Co-crystal structure of tepotinib with c-Met (PDB: 4R1V); b: Co-crystal structure of NVP-BVU972 with c-Met (PDB: 3QTI); c: Co-crystal structure of compound 12 with c-Met (PDB: 7Y4U) [hydrogen bond interactions are green and the π -stacking interaction is purple]; (B) Discovery of D6808 using molecular hybridization and macrocyclization strategies.

[63, 64], which is attributed to involvement in encouraging α -synuclein aggregation by impairing the autophagy system [65]. The LRRK2^{G2019S} mutation has been identified in both familial and sporadic PD cases [66], so developing ATP-competitive inhibitors that target wild- and mutate-type LRRK2 has become a promising strategy for PD therapy [67]. Despite the identification of numerous LRRK2 inhibitors, poor blood-brain barrier (BBB) permeability restricted the application of LRRK2 inhibitors [67, 68]. Kim et al. designed and synthesized a series of pyrimidine-based macrocycles with the aim of improving efficacy and BBB permeability of HG-10-102-01 (Figure 9) [69]. The predicted binding mode of HG-10-102-01 with LRRK2 (PDB: 7LI4) suggested that the aminopyrimidine moiety forms two hydrogen bonds with Ala1950 in the hinge region and the oxygen atom of the amide moiety forms an extra hydrogen bond with Arg1957. The docking result indicated that the three hydrogen bonds are important for maintaining LRRK2 inhibitory activity and the aminopyrimidine and amide moieties can be retained. Because the morpholine moiety extends into the solvent-exposed region, this part can be removed during the design of macrocycles. The

macrocyclization strategy was used to connect the methylamino and amide moieties, and alkyl linkers of different lengths were introduced. First, the length of the linker was explored and the compounds with a three or four alkyl chain were shown to display moderate inhibitory activities against the wild-type and G2019S mutant of LRRK2. Then, different substituents were introduced to the structure to determine the impact on bioactivity. Compound 13 displayed the most potent inhibitory activities against LRRK2 (IC_{50} =0.6 nM) and LRRK2^{G2019S} (IC_{50} =0.2 nM). Compound 13 showed excellent activity against G2019S mutant (IC_{50} =6.3 nM) in the cell-based assay. Compound 13 was shown to downregulate the p-LRRK2 level in mouse brain in vivo, suggesting that compound 13 is a candidate for further research.

2.7 Phosphoinositide 3-kinase (PI3K) inhibitors

PI3K is a lipid kinase activated by membrane and tyrosine kinase receptors [70, 71]. The PI3K/AKT/mTOR signaling pathway has a crucial role in various cellular processes, including cell growth, proliferation, differentiation, and survival [72, 73]. Dysregulation of the PI3K/AKT/mTOR pathway has been implicated in tumor development,

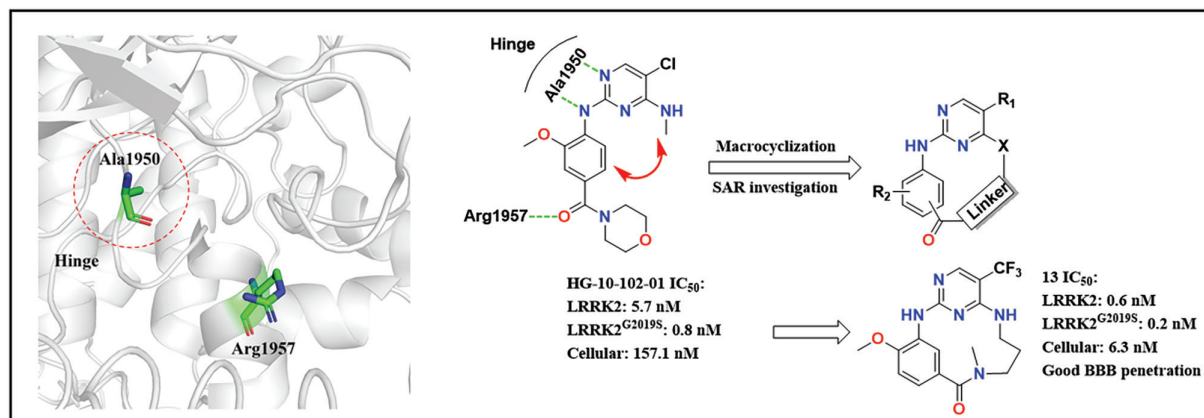


Figure 9 | Macrocyclization process of HG-10-102-01 was undertaken to improve bioactivity and blood-brain barrier (BBB) penetration.

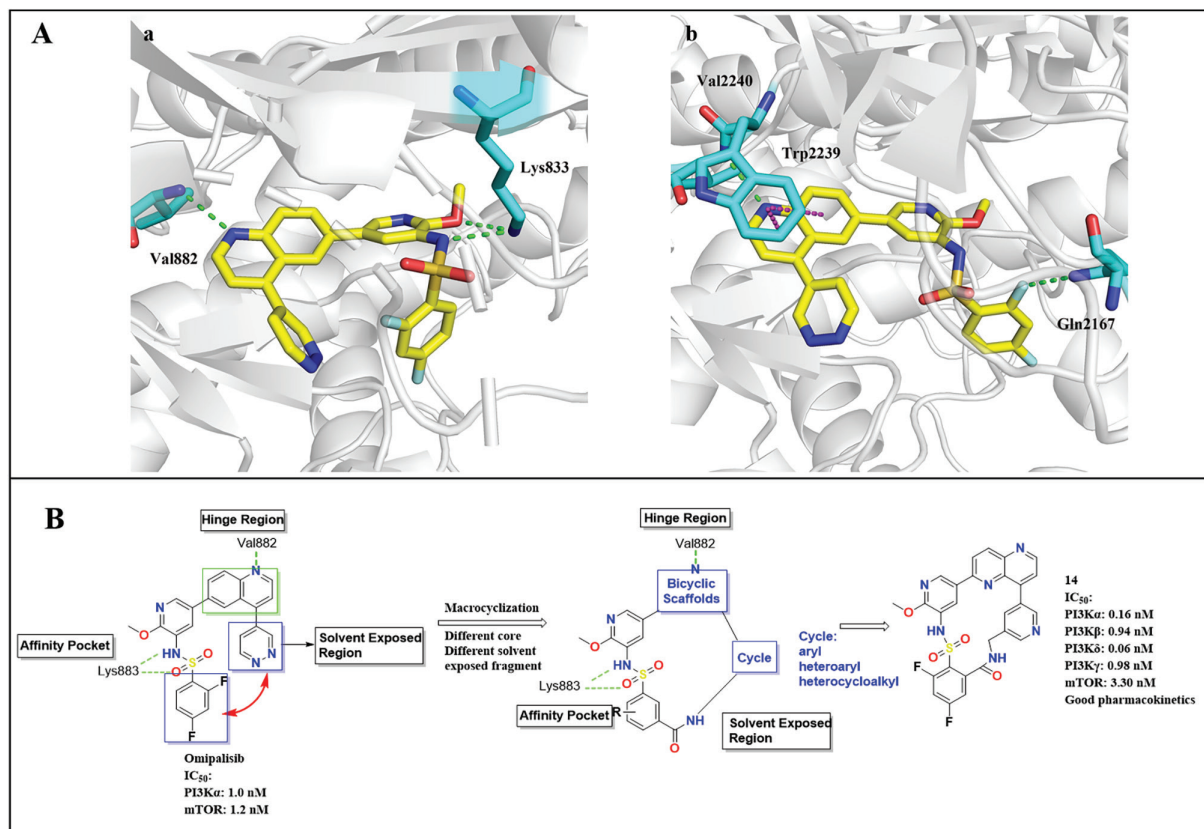


Figure 10 | (A) a: Co-crystal structure of omipalisib with PI3K γ (PDB: 3L08); b: Proposed binding mode of omipalisib with mTOR (PDB: 4JSX) [hydrogen bond interactions are green and the π -stacking interactions are purple]; (B) Design of macrocyclic PI3K/mTOR dual inhibitors based on omipalisib.

making inhibition of PI3K a potential strategy for cancer therapy [74]. Building upon the inspiration from omipalisib [75], a dual PI3K/mTOR inhibitor, Alvarez et al. developed a series of macrocyclic compounds that simultaneously target PI3K and mTOR (Figure 10) [76].

The co-crystal structure of omipalisib with PI3K γ (PDB: 3L08) [77] revealed that the nitrogen atom of the quinoline moiety forms a hydrogen bond with Val882 in the hinge region. Additionally, the pyridosulfonamide part extends to the affinity pocket and forms hydrogen bonds

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with Lys833 and Asp841. The pyridazine moiety at the 4-position of quinoline extends into the solvent region, allowing for good kinase selectivity. Furthermore, the difluorophenyl component has been shown to be positioned at the solvent front in PI3K. Molecular docking analysis of omipalisib with mTOR (PDB: 4JSX) revealed a comparable binding mode to PI3K γ . Based on these observations, a macrocyclization strategy was used to connect the pyridazine and difluorophenyl moieties using an amide linker. Additionally, bicyclic scaffolds featuring nitrogen atoms capable of hydrogen bonding at the same position were studied. Furthermore, various substituents, such as aryl, heteroaryl, and heterocycloalkyl moieties, were utilized for these scaffolds. Among the synthesized compounds, compound **14**, a macrocyclic compound with a 1,5-naphthyridine core, exhibits remarkable kinase inhibitory activity. Compound **14** displays an IC_{50} value of 0.8 nM for PI3K α and 3.3 nM for mTOR. The kinase selectivity assay revealed that compound **14** exhibits better selectivity compared to omipalisib (selectivity score: 0.025). Additionally, compound **14** demonstrated significant inhibitory activity against PI3K β , PI3K δ , and PI3K γ , with IC_{50} values of 0.94, 0.06, and 0.98 nM, respectively. Mechanistic studies indicated that compound **14** downregulates p-Akt, P70S6K, and S6 in the MCF-7 cell line. Notably, compound **14** exhibits excellent PKs (p.o. 10 mg/kg C_{max} = 1565.8 ng/mL; F = 84.6%) and a low drug-drug interaction risk (CYP3A4: IC_{50} > 30 μ M), making compound **14** a promising candidate for further research.

PI3K δ , which is primarily expressed in leukocytes, has shown promise as a target for cancer therapy [78, 79]. Selective inhibition of PI3K δ is an effective and low-toxicity therapeutic strategy [80]. Spencer et al. discovered

a range of benzoxazine derivatives that exhibit strong inhibitory activity against PI3K δ (Figure 11) [81]. The binding mode of compound **15** with PI3K δ was determined by X-ray crystallography (PDB: 6ZAA). The analysis revealed that the oxygen atom in the core formed a hydrogen bond with Val828 in the hinge region. Additionally, the phenyl ring exhibits a π - π stacking interaction with Trp760, while the *N*-methylpiperazine moiety extends towards the solvent front. The co-crystal structure revealed a distance of approximately 5.0 Å between the carbonyl oxygen and the sulfonamide, indicating sufficient space to accommodate a linker for attaching both ends of the molecule. Initially, compound **15** was simplified to compound **16**, which exhibits an IC_{50} value of 2511 nM against PI3K δ . Subsequently, a series of acyclic and macrocyclic compounds with different linkers were synthesized. Notably, acyclic compounds **17** and **18** demonstrated promising inhibitory activity against PI3K δ with IC_{50} values of 631 and 20 nM, respectively. The corresponding macrocyclic compounds (**19** and **20**) exhibit even higher activity compared to the original compounds with IC_{50} values of 50 and 16 nM, respectively. Because the linkers consist of hydrophobic alkyl chains, the macrocyclic compounds exhibit higher lipophilicity and lower LipE but aqueous solubility is still preserved. Compound **19** demonstrated improved *in vivo* PK properties compared to the acyclic compound (*in vivo* Cl: 12.3 mL/min/kg, $T_{1/2}$ = 1.5 h).

2.8 Provirus integration in Maloney murine leukemia virus-1 (Pim-1) inhibitors

Pim-1 is a serine/threonine kinase that can be activated by the PI3K/AKT and JAK/STAT signal pathways [82, 83].

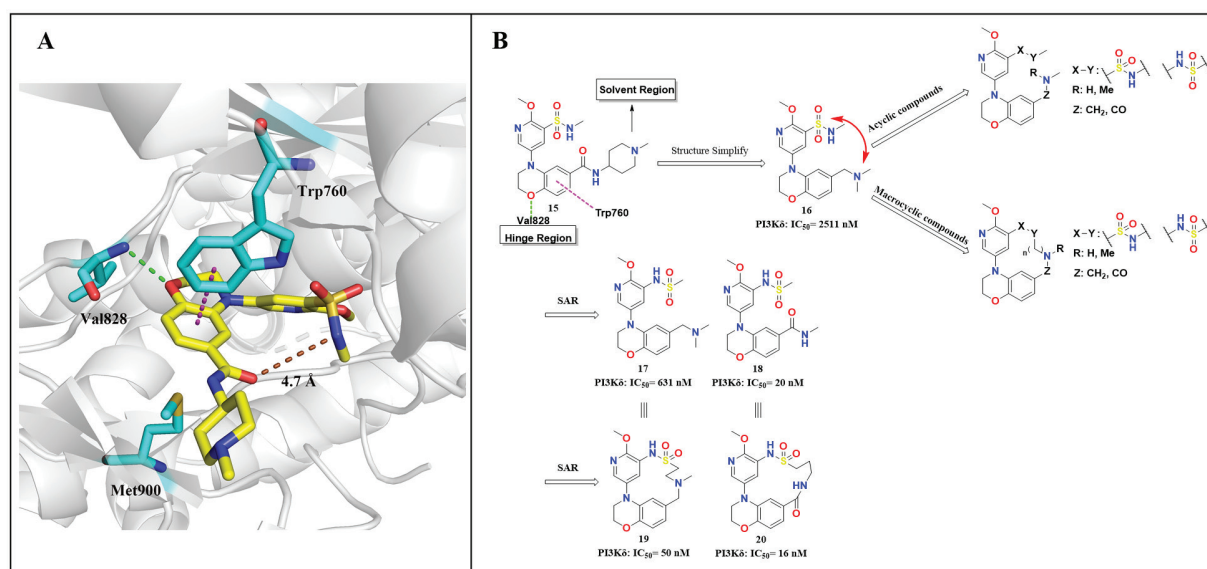


Figure 11 | (A) Co-crystal structure of compound **15** with PI3K δ (PDB: 6ZAA) [hydrogen bond is green]; **(B)** Optimization of macrocyclic benzoxazine derivatives as potent PI3K δ inhibitors.

Once activated, the Pim-1 kinase phosphorylates proteins, such as BAD, 4E-BP1, MDR1, and Myc, thereby contributing to tumor cell proliferation and mediating resistance [84]. Consequently, Pim-1 represents a potential target for low-toxicity cancer treatment [85]. Xu et al. conducted a study in which a range of macrocyclic compounds were designed and synthesized using 10-DEBC and compound **21** as a starting point (Figure 12) [86]. The aim of the Xu et al. study [86] was to enhance the kinase inhibitory activity and physicochemical properties. Unlike other kinases, Pim-1 kinase has a proline residue in the hinge region, resulting in ATP forming only one hydrogen bond interaction with Pim-1 in the hinge region [87]. The co-crystal structure of compound **21** with Pim1 (PDB: 6L16) [88] showed that the nitrogen atom of the benzo[*b*]pyridine[4,3-*e*][1, 4]oxazine core potentially forms a

hydrogen bond with the conserved Lys67. Additionally, the basic NH_2 group of compound **21** can form a hydrogen bond interaction with Asp131. The macrocyclization strategy was used between the basic group and the phenyl ring of the core. It is worth mentioning that preserving the basic group is crucial for maintaining the inhibitory activity against Pim-1 kinase. Among all synthesized compounds, compound **22** exhibits the most potent Pim-1 inhibitory activity ($\text{IC}_{50} = 35.13 \text{ nM}$) and cell anti-proliferative activity (MDA-MD-231 [$\text{IC}_{50} = 8.15 \text{ }\mu\text{M}$]). However, the macrocyclic compound, **22**, has weaker Pim-1 inhibitory activity compared to the acyclic compound, **21** (Pim-1 [$\text{IC}_{50} = 1.7 \text{ nM}$]). This difference may be attributed to replacement of the NH_2 moiety with a secondary amine, leading to a decline in the ability to form hydrogen bonds with acidic amino residues.

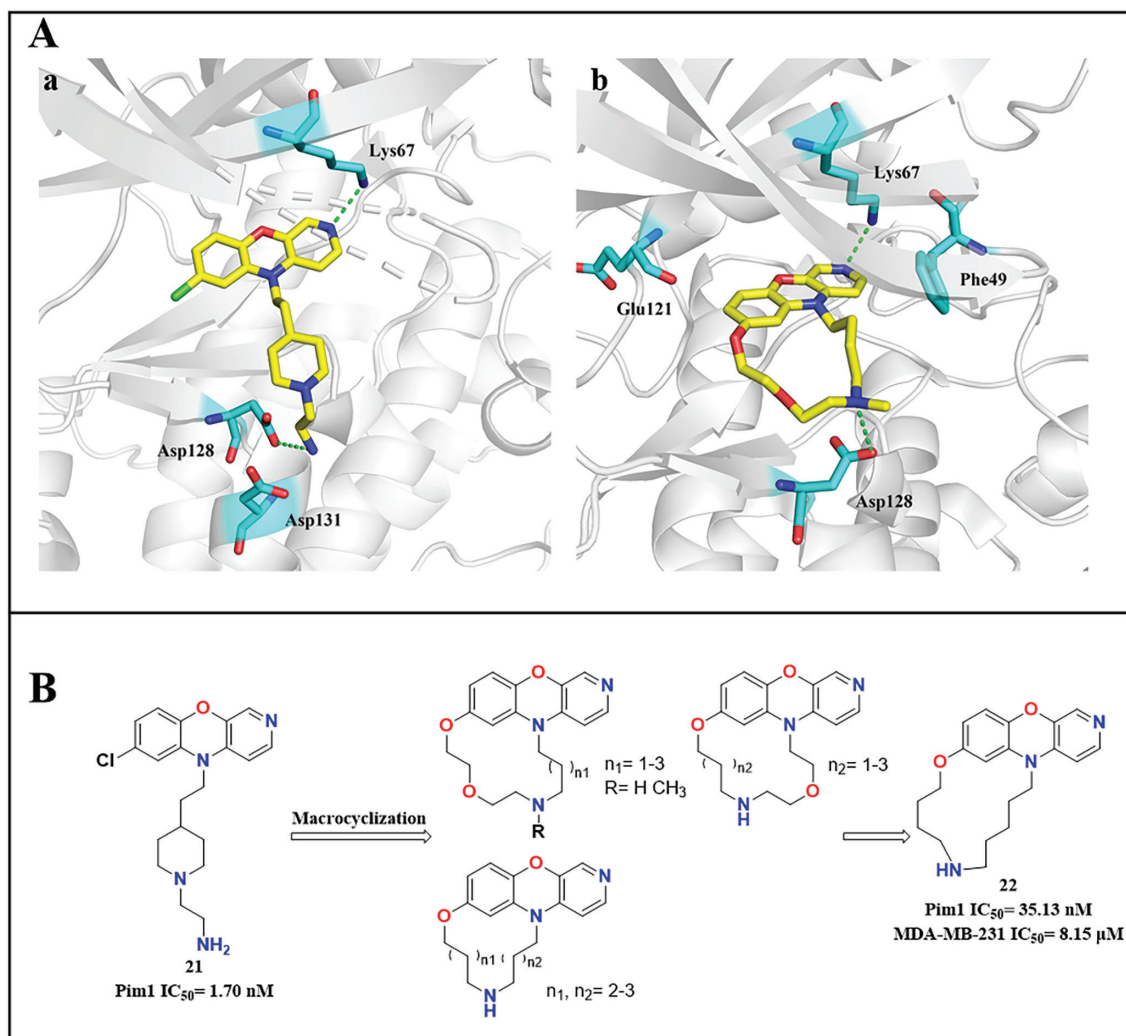


Figure 12 | (A) a: Co-crystal structure of compound **21** with Pim-1 (PDB: PDB: 6L16); b: Co-crystal structure of compound **22** with Pim-1 (PDB: PDB: 7XSV) [hydrogen bond interactions are green]; (B) Design of macrocyclic benzo[*b*]pyridine[4,3-*e*][1, 4]oxazine derivatives as Pim-1 inhibitors.

Review Article

2.9 Tropomyosin receptor kinases (TRK) inhibitors

TRKs are members of the transmembrane receptor tyrosine kinase family and are activated by neurotrophins, a family of growth factors [89-91]. Although larotrectinib and entrectinib have demonstrated favorable clinical responses, instances of acquired resistance have been reported [92]. These cases often involve specific point mutations in amino acids, such as TRKA^{G595R} and TRKC^{G623R} solvent front mutations, TRKA^{G667C} and TRKC^{G696A} xDFG-motif mutations, and the TRKA^{F589L} gatekeeper mutation [93, 94]. The emergence of novel TRK inhibitors is crucial in addressing the issue of point mutations in TRKs. Wang et al. reported a series of macrocycle-based type II TRK inhibitors that effectively overcome point mutations in TRKs (Figure 13) [95]. Starting from 3-(imidazo[1,2-a]pyrazin-3-ylethynyl)-2-methylbenzamide **23**, which has poor

pharmacokinetic properties, the scaffold hopping strategy was used to develop compound **24**. Compound **24** is able to maintain inhibitory activity against TRKs. However, compound **24** exhibits weak activity against the steroidogenic factor (SF) mutation, TRKA^{G595R} (IC₅₀ = 2608 nM) and Ba/F3-CD74-TRKA^{G595R} cells (IC₅₀ = 0.77 μM). The predicted binding mode of compound **24** with TRKC (PDB: 6KZD) suggested that the N-1 of the imidazo[1,2-b]pyridazine core formed a hydrogen bond interaction with Met620 in the hinge region and the NH of amide formed a hydrogen bond interaction with Lys572. The central toluene ring formed π - π stacking interactions with gatekeeper Phe617. The 3-aminopiperidine moiety extends to the solvent front and the bulky piperidine may form a steric clash with Arg623 (the same as Arg595 in TRKA). A macrocyclization strategy was used to design novel type II TRK inhibitors for

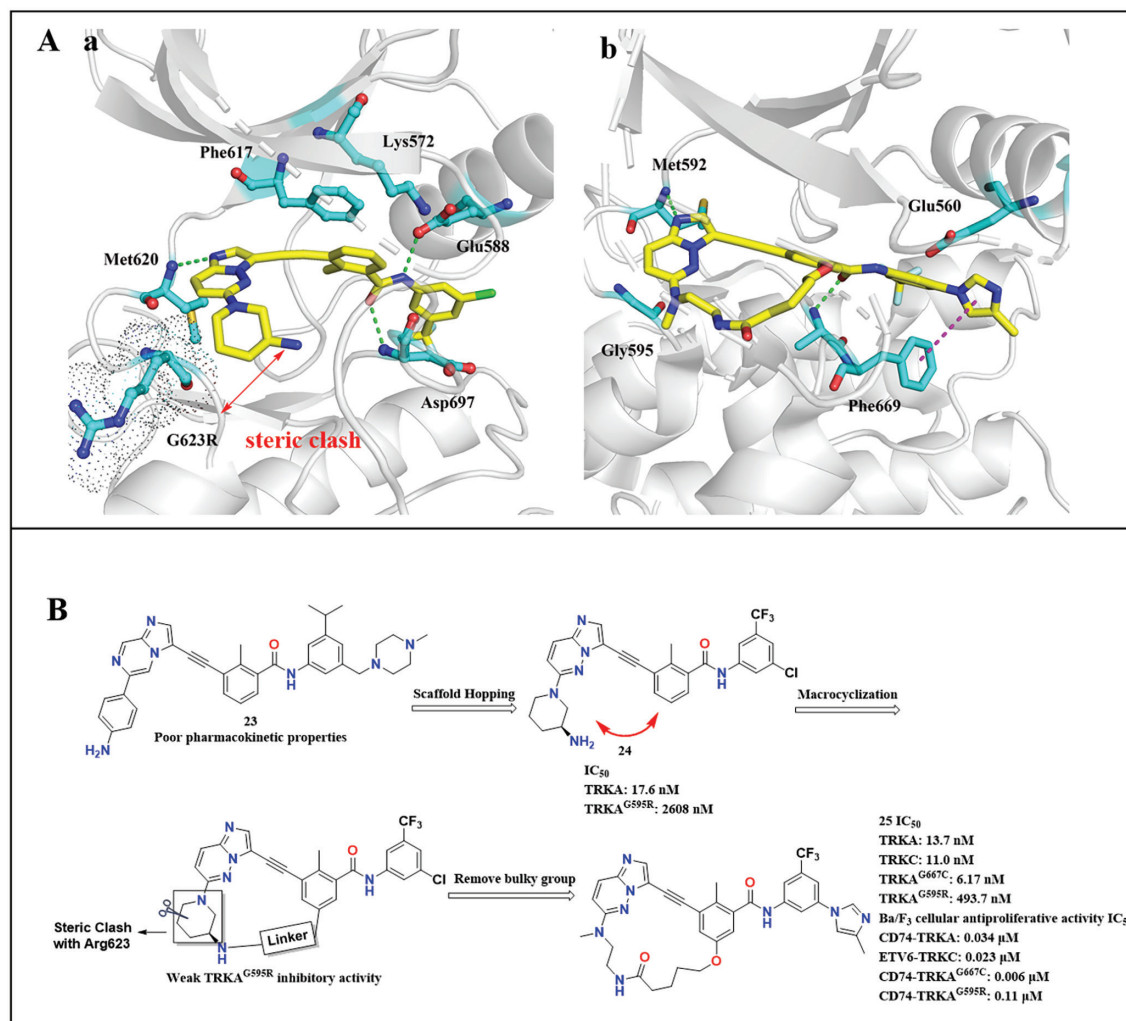


Figure 13 | (A) a: The predicted binding mode of compound **24** with TRKC (PDB: 6KZD); **Right:** co-crystal structure of compound **25** with TRKA (PDB: 7XAF) [hydrogen bond interactions are green and the π - π stacking interaction is purple]; **(B)** Design of macrocyclic Type II TRK inhibitors using scaffold hopping and macrocyclization strategy.

combating SF mutations by reducing steric hindrance with Arg595 according to the docking results. Initially, the central toluene ring and piperidine were connected using alkyl chains of varying lengths. Although the compounds retained inhibitory activity against WT TRKs and TRKA^{G667C}, the inhibitory activity against TRKA^{G595R} was weak. This inhibitory activity was attributed to the bulky piperidine moiety, which was replaced with the smaller methylethanediamine to prevent steric clash with Arg595. Compound **25** exhibited potent inhibitory activity against TRKA^{G595R} (TRKA^{G595R}: IC₅₀=493.7 nM; CD74-TRKA^{G595R} IC₅₀=110 nM) while also retaining effects against wild-type TRKs and xDFG mutant TRKs (TRKA: IC₅₀=13.7 nM; TRKA^{G667C}: IC₅₀=11.0 nM). A kinase selectivity assay indicated that compound **25** exhibits exceptional selectivity against TRKs within the kinome. However, pharmacokinetic studies conducted in SD rats showed that compound **25** displays poor pharmacokinetic profiles upon oral administration. Compound **25** exhibits a low exposure curve (AUC_(0-t)=51.56 ng·h/mL) and a low maximum concentration (C_{max}: 19.13 ng/mL), which is possibly due to high lipophilicity.

Although compound **25** displays high inhibitory activities against various DFG-mutations, compound **25** displays moderate activities against SF and gatekeeper

mutations (TRKA^{G595R} IC₅₀=493.7 nM, TRKA^{F589L} IC₅₀=2100 nM). The predicted binding mode of compound **25** with TRKA^{G595R} indicated that the methylethanediamine linker exists as a steric hindrance with the mutant, Arg595. Thus, the linker should be optimized to reduce spatial collisions. In addition to avoiding steric hindrance with bulky amino residues, forming new interactions with mutated amino residues is an effective strategy to overcome acquired mutations. Thus, the ethylamine moiety was replaced by an *N*-methylamide group to make hydrogen bond interactions with Arg595 (Figure 14). Moreover, the length of the linker was changed to determine the impact on bioactivity. The resulting compound **26**, with a 7-membered carbon linker, achieved potent TRK^{G595R} inhibitory activity (IC₅₀=63.35 nM), which serves as a starting point for further optimization. Then, SAR analysis of the tail part was performed, which showed that compound **27** with a cyclopropyl moiety displays the best inhibitory activities against both wild-type and mutant variants of TRKs (IC₅₀=1.42–6.77 nM). The co-crystal structure of compound **27** with TRKA^{G595R} (PDB: 8J5X) and TRKA^{F589L} (PDB: 8J5W) were obtained to understand the binding mode of compound **27** with TRKs. Compound **27** binds to the ATP binding pocket of TRKA mutants in a type II

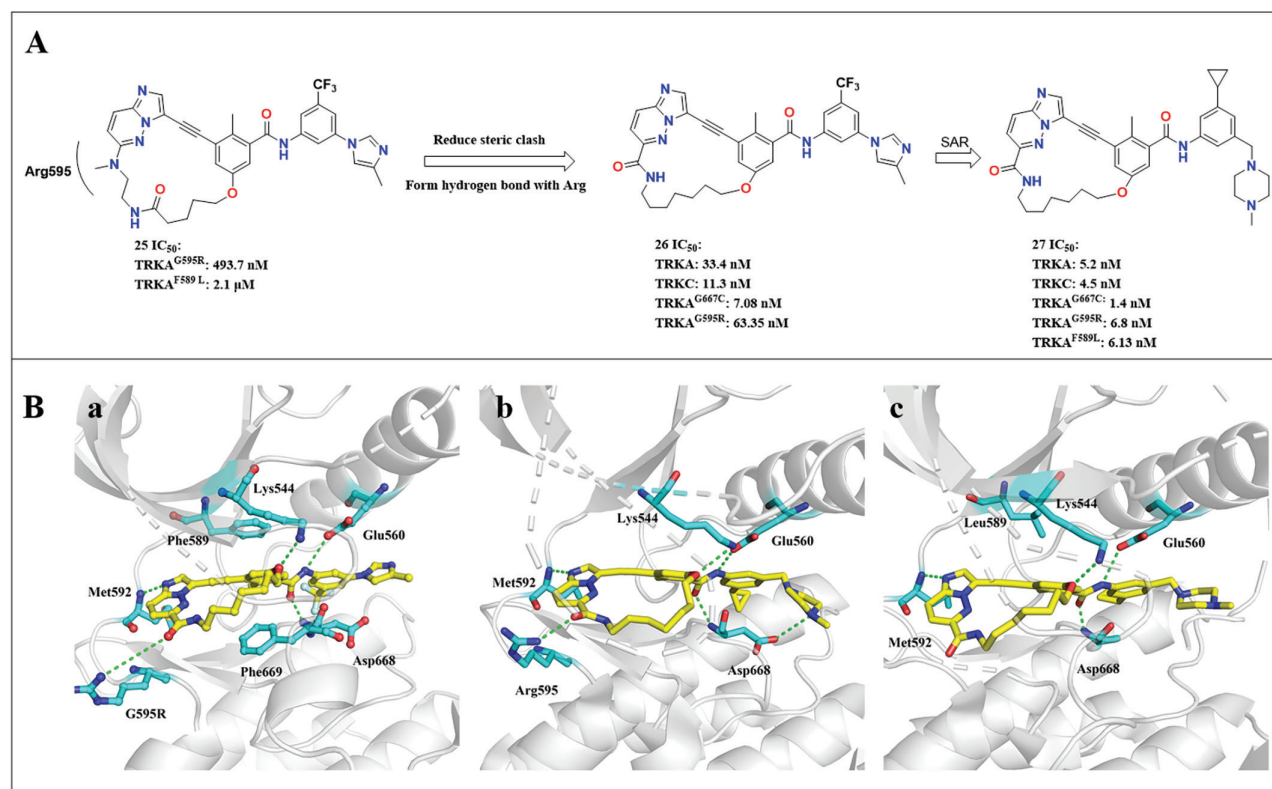


Figure 14 | (A) Optimization process of the macrocyclic TRK inhibitor, compound **25**; (B) a: The predicted binding mode of **26** with TRKA^{G595R}; b: The co-crystal structure of **27** with TRK^{G595R} (PDB: 8J5X); c: The co-crystal structure of **27** with TRK^{F589L} (PDB: 8J5W) [hydrogen bonds are green].

Review Article

mode. The nitrogen atom of imidazo[1,2-*b*]pyridazine core forms a hydrogen bond with Met592 in the hinge region. The oxygen atom of the benzamide moiety and methylene methylpiperazine part forms hydrogen bonds with Asp668 and the oxygen atom at the linker of compound **27** forms a hydrogen bond with Lys544. It was noted that the amide group of the linker forms key hydrogen bond with a mutated amino residue, Arg595, as predicted, which provided a major contribution to overcoming the SF mutation. The middle methylphenyl group has a favored interaction with the mutated Leu589 to avoid steric hindrance for the gatekeeper mutation, TRKA^{F589L}. Compound **27** displayed excellent kinase selectivity in a panel of 378 WT kinases. Compound **27** displayed good anti-proliferation activity against wild-type and mutated TRKs without potential cytotoxicity based on a cell-based assay. Importantly, compound **27** showed improved pharmacokinetic profiles in ICR mice compared to compound **25** (P.O. 10 mg/kg; C_{max} =95.2 ng/mL, $AUC_{(0-t)}$ =501 ng·h/mL). The anti-cancer efficacy of compound **27** at 50 mg/kg *in vivo* was tested in Ba/F3-CD74-TRKA^{G595R} and Ba/F3-CD74-TRKA^{G667C} xenografts and the results indicated that compound **27** displays robust *in vivo* anti-cancer activity (TGI=78% and 72%). This research demonstrated that the linker of the macrocyclic compounds could be designed to make new interactions with the target to improve the bioactivity of macrocyclics [96].

Larotrectinib (LOXO-101) is a first-generation inhibitor of TRKs that exhibits potent inhibitory activities against TRKA/B/C (IC_{50} = 5-11 nM) [97]. Although it shows a high clinical response, acquired resistances to LOXO-101 have been observed with prolonged treatment [98]. In accordance with LOXO-101, Li et al. conducted a study in which they designed and synthesized

a range of macrocyclic compounds to counteract TRK SF mutations [99] (Figure 15). The predicted binding mode of LOXO-101 with TRKA^{G595R} indicated that the nitrogen atom on the pyrazole ring potentially establishes a hydrogen bond interaction with Met592 in the hinge region. The difluorophenyl part extends to the hydrophobic pocket formed by Phe521 and Asp668. The hydroxy pyrrolidine moiety extends to the solvent region and the bulky pyrrolidine part forms a steric hindrance with Arg595. To avoid steric hindrance with Arg595, the bulky hydroxypyrrolidinyl moiety was removed. The pyrrolidinyl group was replaced with an aliphatic group using a ring-opening strategy to simplify the structure. A macrocyclic strategy was used to counteract xDFG mutations of TRK. Among the synthesized compounds, compound **28** exhibits the highest inhibitory effects against both wild-type TRKA (inhibitory rate at 500 nM: 102.7%) and TRKA^{G595R} (IC_{50} : 13.1 nM). Compound **28** demonstrated a superior inhibitory effect against the Ba/F3-LMNA-NTRK1-G595R cell line compared to LOXO-101 (0.646 μ M vs. 2.495 μ M) based on cell-based assays.

To address acquired resistance, second-generation macrocyclic inhibitors (TPX-0005 [100] and LOXO-195 [101]) were developed. These inhibitors have shown efficacy in inducing remission in patients who have experienced treatment failure with larotrectinib. Building upon TPX-0005 and LOXO-195, Liu et al. designed and synthesized a series of pyrazolo[1,5-*a*]pyrimidine-based macrocyclic compounds as potent pan-TRK inhibitors (Figure 16) [102]. These compounds exhibited excellent physicochemical properties and good oral PKs, making the compounds promising candidates for further study. The predicted binding mode of TPX-0005 with TRKA (PDB: 4YNE) indicated that the nitrogen atom on the pyrazole ring formed a hydrogen bond with Met592

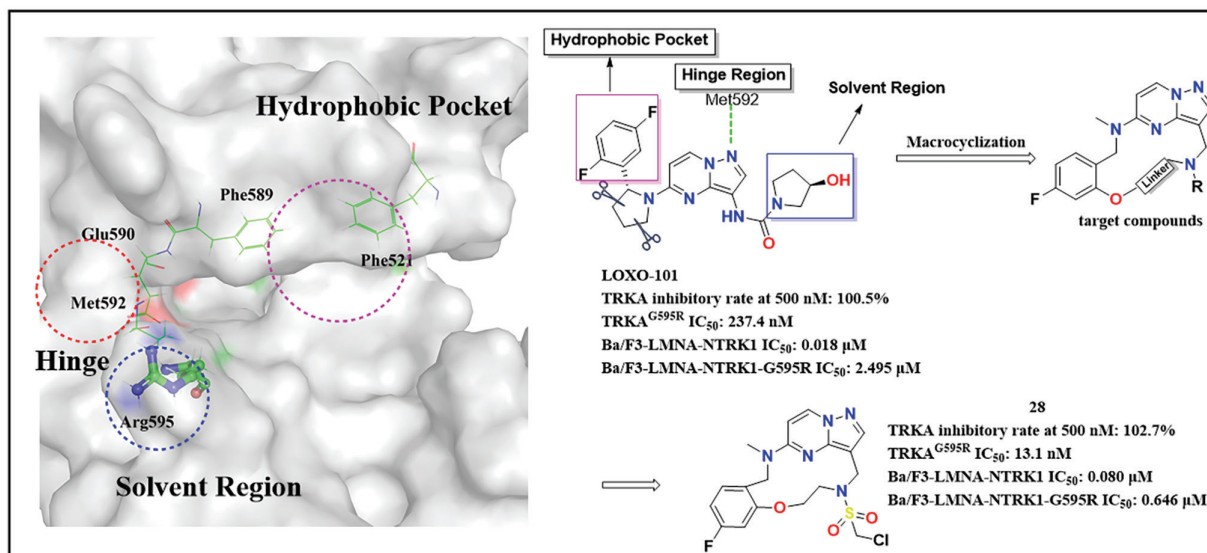


Figure 15 | Design of macrocyclic TRK inhibitors based on LOXO-101 aimed to improve activity against SF mutations.

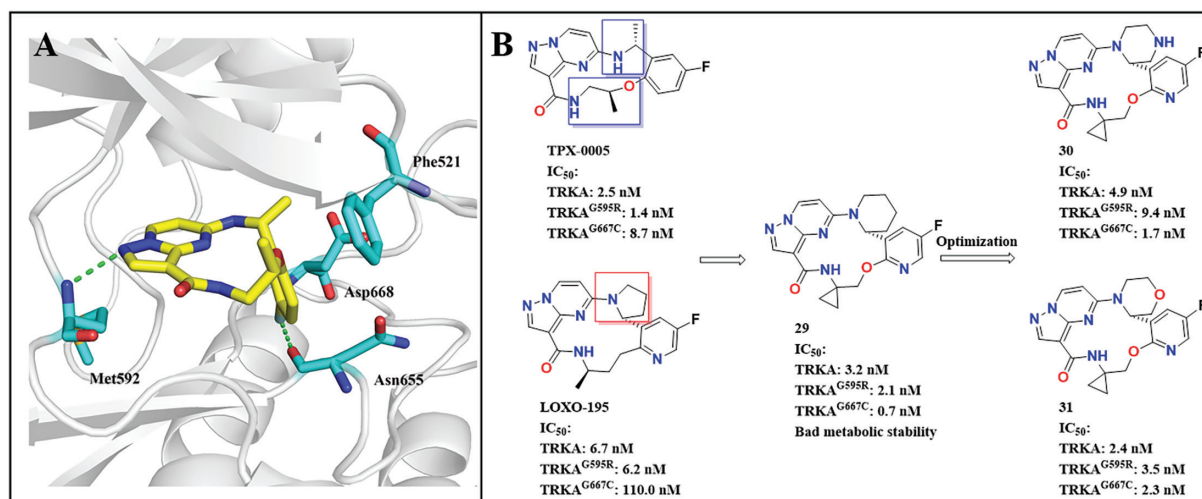


Figure 16 | (A) Predicted binding mode of TPX-0005 with TRKA (PDB: 4YNE) [hydrogen bond interaction is green]. (B) Optimization of macrocyclic TRK inhibitors based on LOXO-195 and TPX-0005 aimed at improving anti-SF mutation activity.

in the hinge area and the fluorophenyl occupied the hydrophobic cavity consisting of Phe521, Leu657, Gly667, and Asp668. Importantly, the fluorine atom forms a key hydrogen bond interaction with Asn655. The researchers hypothesized that replacing the TPX-0005 linker could help determine TRK inhibitory activity. During the optimization process, compound **29**, which has a piperidine substitution, was shown to exhibit potent TRKA activity with an IC₅₀ value of 3.2 nM. Furthermore, compound **29** exhibits strong inhibitory activities against TRKA^{G595R}, TRKA^{G667C}, TRKC, ALK, and OS1, with IC₅₀ values of 3.2, 2.1, 0.7, 0.2, 18.5, and 0.2 nM, respectively. *In vitro* PK evaluation of compound **29** indicated rapid metabolism in humans, rats, and dogs with metabolic rates of 242, 980, and 268 ml/min/kg, respectively. The fast metabolism of compound **29** may be attributed to oxidation on the piperidine ring. Therefore, to address this issue the piperidine ring was substituted with piperazine or morpholine, resulting in the synthesis of compounds **30** and **31**. Compounds **30** and **31** exhibit potent TRKA inhibitory activity with a decline in inhibitory activity against ALK (ALK IC₅₀ values: compound **32**, 88.9 nM; compound **33**, 182.0 nM), suggesting improved selectivity. Notably, compounds **30** and **31** demonstrated enhanced metabolic stability compared to compound **29** (MMS: compound **30**, 29-74 ml/min/kg; compound **31**, 10.7-28.8 ml/min/kg). Because compound **31** displays weaker hERG inhibitory activity compared to compound **30** (13.0 μM vs. 6.7 μM), compound **31** was further evaluated as a pre-clinical drug candidate for its efficacy and PKs *in vivo*.

3. IMPROVING SELECTIVITY IN KINASE INHIBITORS THROUGH MACROCYCLIZATION

Because type I and II kinase inhibitors bind to the highly conserved ATP-binding pocket, small-molecule kinase

inhibitors often exhibit limited selectivity within the kinase. This lack of selectivity results in undesirable side effects and restrict clinical application. With kinase inhibitors widely used in clinical settings, acquired mutations caused by point mutations and the activation of negative feedback pathways have emerged frequently, driving the development of next-generation kinase inhibitors. For example, afatinib is a second-generation EGFR inhibitor with excellent inhibitory activity against the EGFR^{T790M} mutation. However, due to the poor selectivity between EGFR^{WT} and EGFR^{T790M}, side effects, such as rash and diarrhea caused by afatinib, limit its clinical application [103]. Therefore, it is imperative to attain selectivity between the kinase wild and mutation types. The macrocyclization strategy can effectively stabilize the active conformation of acyclic compounds, thereby enhancing selectivity. This approach is frequently used to improve the specificity of acyclic compounds. Importantly, macrocyclic compounds often demonstrate similar selectivity towards homologous kinases, as well as wild-type and mutant kinases.

3.1 Bone morphogenic protein receptor type-II (BMPR2) kinase inhibitor

BMPR2 is a serine/threonine receptor kinase that belongs to the tyrosine kinase-like (TKL) group [104, 105]. BMPR2 has a crucial role in the transcription of BMP target genes and is involved in activating several signal pathways, such as ERK, MAPK, LIMK, NOTCH, and Wnt [106]. Overactivation of BMP signaling has been associated with various pathologic conditions, such as pulmonary arterial hypertension, cancer, and Alzheimer's disease [107]. Thus, targeting BMPR2 could be a potential therapeutic strategy. Amrhein et al. synthesized a series of macrocyclic compounds derived from the promiscuous kinase inhibitor, compound

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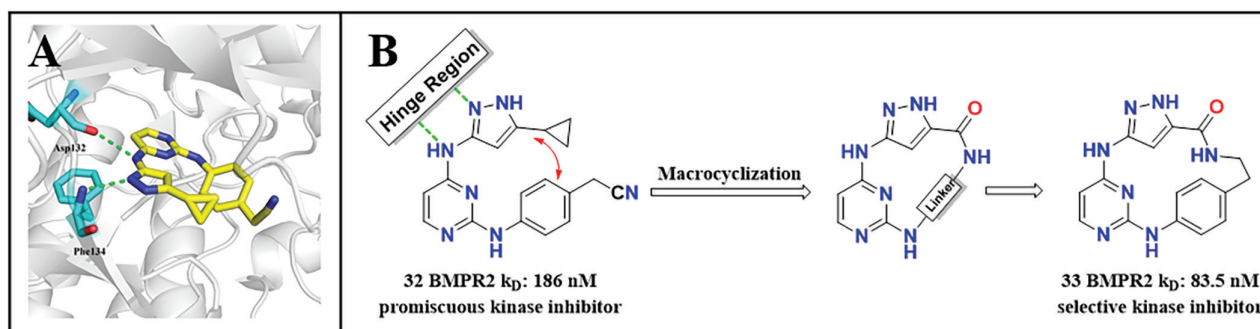


Figure 17 | (A) Co-crystal structure of compound 32 with VRK1 (PDB: 3OP5) [hydrogen bond interactions are green]; **(B)** Macrocyclization of the promiscuous kinase inhibitor, compound 32 to gain the selective BMPR2 inhibitor, compound 33.

32 [108] (**Figure 17**). Compound **32** contains a 3-amino-1H-pyrazole moiety that has a crucial role in forming hydrogen bond interactions with the hinge region of the kinase domain. The co-crystal structure of compound **32** with VRK1 (PDB: 3OP5) [109] showed that the pyrimidine moiety extends into the hydrophobic pocket, while the phenyl and cyclopropane parts are located in the solvent region. To investigate BMPR2 binding affinity, various linkers were used to macrocyclize the pyrazole moiety and the 2-position of the pyrimidine ring. Compound **33** was shown to have the highest binding affinity to BMPR2 (k_D =83.5 nM) and notable selectivity against GSK3A (IC_{50} =11 μ M) and GSK3B (IC_{50} =34 μ M). The kinase selectivity assay showed that compound **33** displays favorable selectivity against BMPR2 ($S35$ = 0.01) in a panel of 90 kinases. This study suggested that macrocyclization can be an effective strategy to enhance the selectivity profile of a promiscuous kinase inhibitor.

3.2 CDK inhibitors

As a key regulator of RNA transcription, cyclin-dependent kinase 9 (CDK9) has been an emerging target for cancer therapy [110, 111]. Importantly, inhibition of CDK9 downregulates the protein level of tumorigenic proteins, such as Mcl-1 [112]. Currently, dozens of CDK9 inhibitors have entered clinical trials for the treatment of hematologic cancers and solid tumors [113]. Wu et al. designed and synthesized a series of macrocyclic CDK9 inhibitors for the treatment of osimertinib-resistant NSCLC based on the 2-aminopyrimidine scaffold [114] (**Figure 18**). Because Mcl-1 has a vital role in the proliferation and differentiation of lung cancer and contributes to resistance to EGFR inhibitors, the researchers reasoned that inhibiting CDK9 could downregulate the Mcl-1 protein level, indicating that CDK9 inhibitors have potential in treating lung cancer with EGFR mutations. The phenotypic screening approach was used to identify compounds with CDK9 inhibitory and anti-proliferation activities against lung cancer cell lines. Among the compounds with CDK9 inhibitory and anti-proliferation activities, compound **34** displayed significant CDK9

inhibitory activity and anti-proliferation activity (IC_{50} : CDK9: 3.1 nM, HCC827: 340.0 nM, H1975: 254.4 nM.). However, compound **34** displayed high *in vivo* toxicity, possibly due to poor kinase selectivity, and needs to be optimized to improve kinase selectivity. The predicted binding mode of compound **34** with CDK9 (PDB: 6Z45) suggested that the amino triazine made two hydrogen bond interactions with Cys106 in the hinge region, while the *para*-fluorine group formed a hydrogen bond interaction with Lys48. Additionally, a π -stacking interaction were formed with Phe103 and Ile25 for the two substituted phenyl rings and the benzamide moiety formed a hydrogen bond interaction with Ile25. The macrocyclization strategy was used between the two phenyl rings. First, a linker with a 6-atom length was introduced to compound **34** and afforded compound **35**. Compound **35** has strong CDK9/CycT1 inhibitory (IC_{50} =1.1 nM) and anti-proliferation activity against HCC827 cell lines (IC_{50} =31 nM). However, due to the poor selectivity among CDK2/CycA2 (IC_{50} =3.2 nM), compound **37** has *in vivo* toxicity. The linker and hydrophilic group of compound **35** were replaced to investigate the inhibitory activity and selectivity of compounds. Finally, compound **36** with an 8-atom length linker and bulky morpholinyl has potent CDK9/CycT1 inhibitory activity (IC_{50} =3.2 nM) and good selectivity against CDK2/CycA2 (IC_{50} =209 nM). Moreover, compound **36** has strong cytotoxicity in HCC875 (IC_{50} = 315 nM) and H1975 cell lines (IC_{50} = 174 nM) and induces approximately 75% downregulation of Mcl-1 at 500 nM. In a selectivity assay with a panel of 80 kinases, compound **36** displayed good kinase selectivity at a concentration of 500 nM. The PK studies revealed that compound **36** has acceptable exposure in SD rats when administered intravenously at a dose of 2 mg/kg (AUC=1078 μ g·h/L). However, the oral absorption of compound **36** was poor, resulting in low oral bioavailability (F = 8%). Compound **36** has strong anti-proliferation activity against lung cancer cell lines and inhibits the growth of patient-derived organoids.

CDK7 forms the CDK-activating kinase (CAK) complex with cyclin H and MAT1, which subsequently exert downstream effects on other CDKs involved in cell cycle

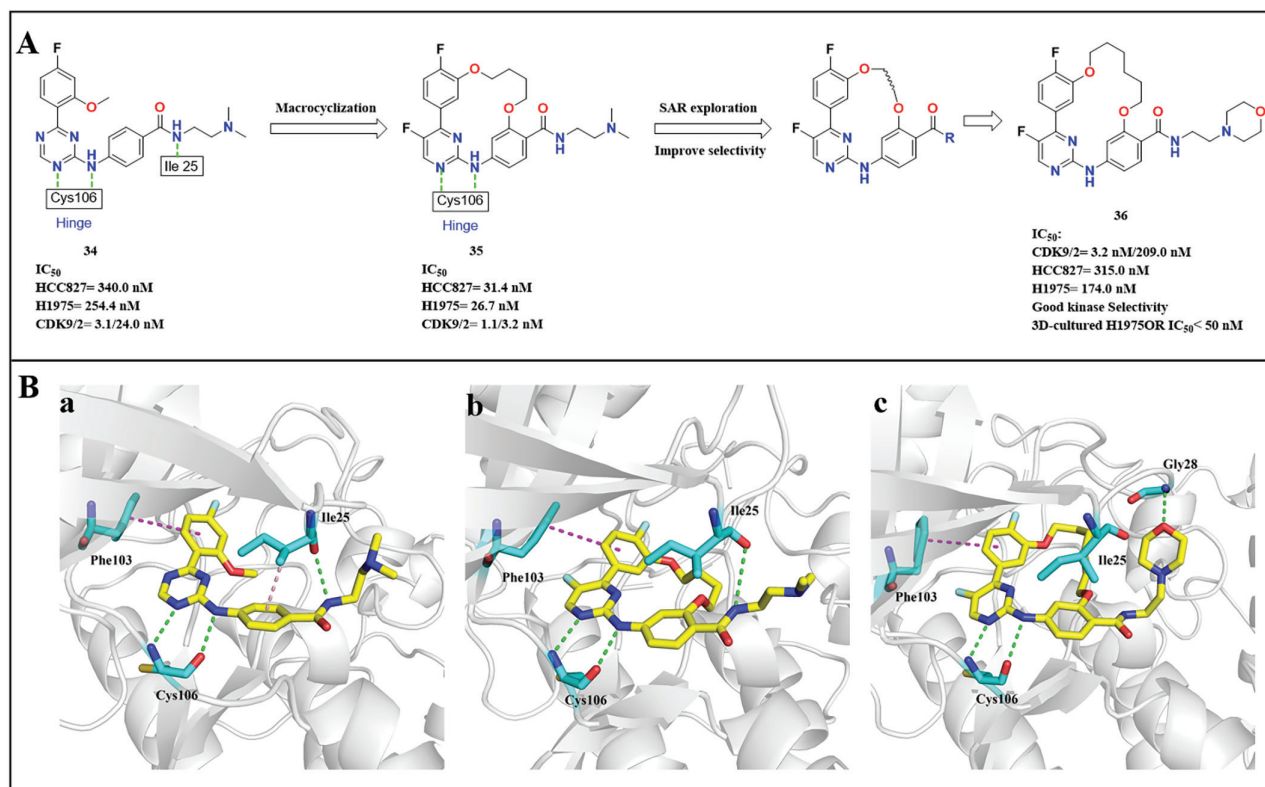


Figure 18 | (A) Design of macrocyclic CDK9 inhibitors based on compound 34; (B) a: Predicted binding mode of compound 36 with CDK9 (PDB: 6Z45); b: Predicted binding mode of compound 35 with CDK9 (PDB: 6Z45); c: Predicted binding mode of compound 36 with CDK9 (PDB: 6Z45) [hydrogen bonds are green].

control [115, 116]. As an activator of the cell cycle and transcription, CDK7 has become a promising target for cancer therapy [117]. However, the ATP-binding site of CDKs is highly conserved, making the discovery of selective CDK7 inhibitors extremely challenging. Niu et al. designed and synthesized a series of pyrazolo[1,5-*a*] [1,3,5]triazine-based macrocycles as selective CDK7 inhibitors [118] (Figure 19). CT7001 is a pyrazolopyrimidine derivative with potent CDK7 inhibitory activity and displays 15-fold greater selectivity against CDK2, the closest isoform to CDK7 [119]. The crystal structure of CT7001 with CDK7 (PDB: 7B5Q) and CDK2 (PDB: 5JQ5) suggested that the benzylamine of CT7001 adopts a ring up conformation in CDK7 [120] and ring down conformation in CDK2 [121]. The researchers hypothesized that the macrocyclization strategy could be used between the benzyl and piperidine moieties in CT7001 to restrict the conformation and specifically bind to CDK7. First, alkyl linkers with different lengths were introduced to CT7001 to determine the impact on CDK7 inhibitory activity and selectivity. Interestingly, the 16-membered ring displayed potent CDK7 inhibitory activity and selectivity, whereas the 14-membered ring reversed the selectivity from CDK7-to-CDK2/5. The potent CDK7 inhibitory activity and selectivity of the 16-membered ring compound 37 were explained by molecular dynamics (MD)

simulations. The results showed that the 16-membered compound 37 displayed a lower root mean square deviation (RMSD) value, a more stabilized hydrogen bond network, and tighter binding when docking to CDK7. The SAR of 14- and 16-membered ring compounds indicated that the 16-membered macrocycles possessed a broader SAR. However, the macrocycles displayed poor stability in mouse/human liver microsomes. The researchers installed a hydroxyl group on the alkyl linker to modulate the physiochemical properties and improve stability. The two enantiomers (compounds 38 and 39) exhibited similar CDK7 inhibitory activity and cellular potency. Moreover, compounds 38 and 39 displayed improved metabolic stability in mouse/human liver microsomes. However, neither compound 38 nor 39 was able to provide detectable plasma exposure after oral gavage at a dose of 10 mg/kg. So, further optimization should be performed to improve the properties of compounds 38 and 39.

Compound 40 was obtained during the optimization process of selective CDK7 inhibitors that displayed activity against CDK2 (K_i : CDK2/cyclin E: 3.2 nM) and CDK5 (K_i : CDK5/p25: 4.4 nM). With the aim of improving inhibitory activity and selectivity, Niu et al. developed a series of macrocycles that selectively target CDK2 based on macrocyclic compound 40 [122] (Figure 20). First, different

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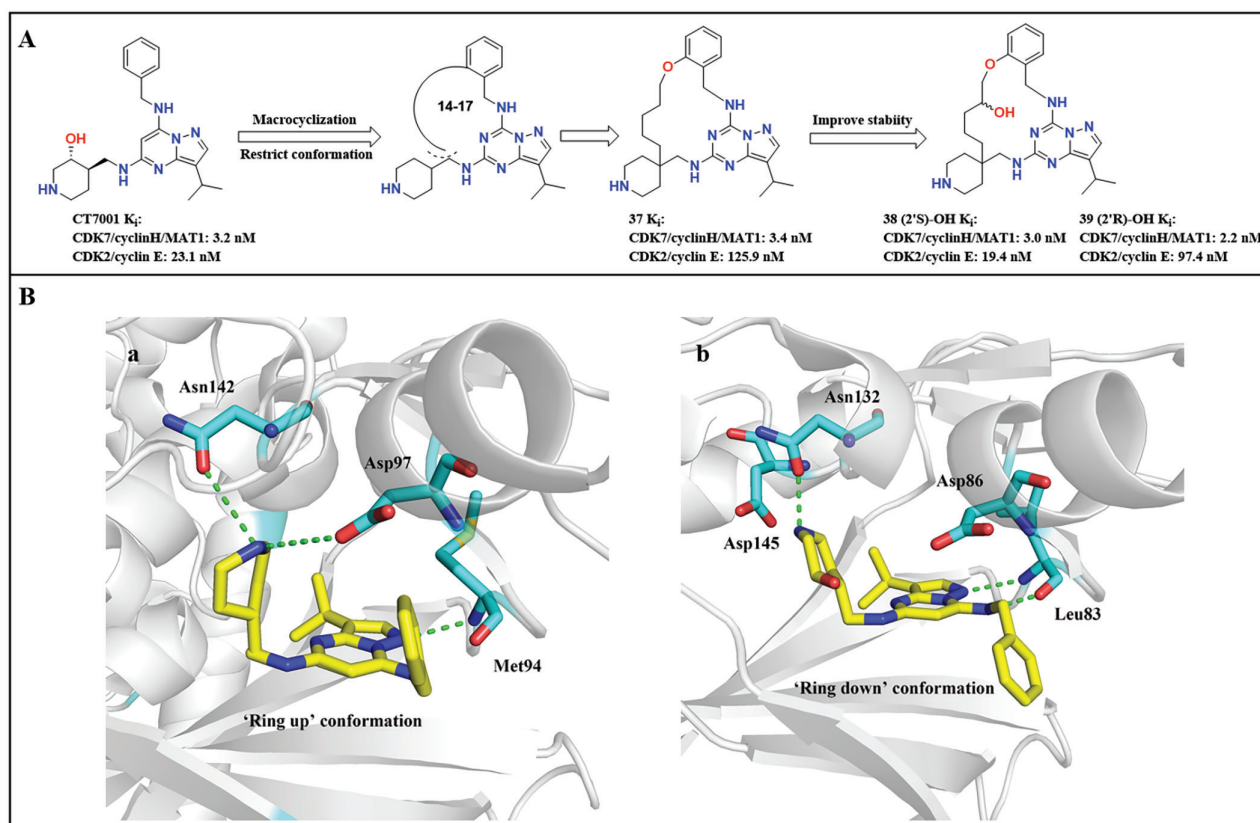


Figure 19 | (A) Optimization process of selective CDK7 inhibitors using a macrocyclization strategy; (B) a: Co-crystal structure of CT7001 with CDK7 (PDB: 7B5Q); b: Co-crystal structure of CT7001 with CDK2 (5JQ5) [hydrogen bonds are green].

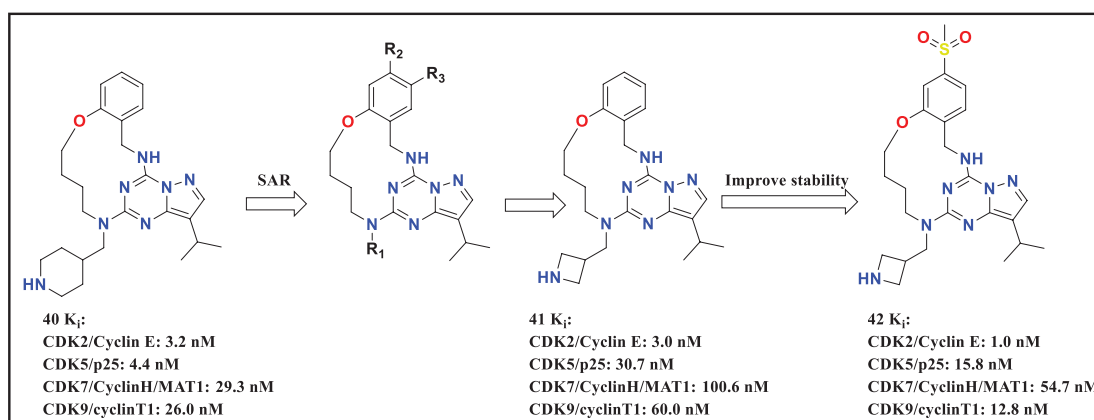


Figure 20 | Optimization of pyrazolo[1,5-a][1,3,5]triazine-based macrocyclic CDK2 inhibitors.

types of substituents were introduced to the benzene ring to investigate the impact on CDK2 enzyme activity and selectivity. The results indicated that when the bulky substituents were introduced to the R_2 or R_3 position, CDK2 inhibitory effects decreased. Then, different basic groups were introduced to the R_1 position to explore the impact on CDK2 inhibitory activity. It was found that

the CDK2 inhibitory activity and selectivity improved when cyclobutylamine was introduced (compound **41**: K_i : CDK2/CycE1: 3.0 nM). The MD simulation assay was performed to explain why the CDK5 inhibitory activity declined when the piperidine moiety was replaced with cyclobutylamine. The results demonstrated that compound **40** formed a more stable complex with lower

binding free energy than compound **41**, which was consistent with better inhibitory activity against CDK5 than compound **41**. However, compound **41** displayed poor stability in mouse/human liver microsomes, so different electron-withdrawing groups were introduced to the benzene ring to improve stability. Among all synthesized compounds, compound **42** displayed improved CDK2 inhibitory activity and microsomal stability, which deserved further investigation. These examples demonstrated that MD simulation investigations help analyze changes in bioactivity when the length of the linker is altered, thereby guiding the design of macrocycles to be more efficient and accurate. This finding makes MD simulation a valuable tool for studying kinase inhibitory activity and selectivity. Additionally, the introduction of different substituents in the linker can modulate the properties of macrocycles, which is a feasible strategy for optimizing these compounds.

3.3 Epidermal growth factor receptor (EGFR) inhibitors

The EGFR is a receptor tyrosine kinase that acts as an oncogenic driver in some lung tumors [123–125]. The discovery of mutations in EGFR has led to the development of the next generation of EGFR inhibitors [126]. Specifically, the EGFR^{del19/L858R/T790M/C797S} mutant kinase variant, which arises in second-line patients progressing on osimertinib, is no longer responsive to any of the currently approved EGFR inhibitors [127]. Therefore, it is important to develop selective inhibitors that target EGFR^{del19/L858R/T790M/C797S} [128]. Engelhardt et al. identified compound **43** as the starting point for discovering potent selective EGFR^{L858R/T790M/C979S} inhibitors by screening compounds with EGFR^{L858R/T790M/C979S} inhibitory activity and no inhibitory activity against EGFR^{WT} (Figure 21) [129]. Compound **43** displays moderate inhibitory activity against EGFR^{del19/T790M/C797S} with an IC₅₀ of 250 nM and excellent kinase selectivity against

238 kinases. The co-crystal structure of compound **43** with EGFR^{L858R/T790M} (PDB: 6S9B) suggested that the NH of benzimidazole and the carbonyl formed hydrogen bond interaction with Met793 in the hinge region and the pyridine substituent in the R₂ position binds to the Lys745 in the phosphate region of the pocket. Importantly, the torsion angle between the two aromatic rings in compound **43** is approximately 40°. Introduction of a methoxy group to the pyridine ring obtained compound **44** with excellent kinase inhibitory activity (EGFR^{d19/T790M/C797S} IC₅₀ < 3.0 nM). However, compound **44** displays poor kinase selectivity (BaF3-EGFR^{WT} IC₅₀ = 700 nM). Using a phenyl group to replace the second pyridine ring and abolish the hydrogen bond interaction between the compound and Lys745, the researchers obtained compound **45** to improve selectivity. Compound **45** displays good EGFR^{d19/T790M/C797S} inhibitory activity (IC₅₀ = 35 nM). The co-crystal structure of compound **45** with EGFR^{L858R/T90M} suggested that the compound binds to the protein with an expected dihedral angle of the bis-aryl system of approximately 20°. In addition, the distance to the Lys745 is sufficiently large. To stabilize the active conformation of the compounds, a macrocyclization strategy was used. Among the synthesized compounds, BI-4020 with a smaller pyrazole substitution was shown to display excellent kinase inhibitory activity (p-EGFR^{d19/T790M/C797S} IC₅₀ = 0.6 nM). In addition, BI-4020 displayed good kinase selectivity in a panel of 393 kinases. Moreover, BI-4020 showed better DMPK properties compared to osimertinib (Table 2). In a PC-9^{del19/T790M/C797S} xenograft model, BI-4020 suppressed tumor growth and displayed better anti-tumor activity than osimertinib (osimertinib, p.o., 25 mg/kg, TGI=6%; BI-4020, p.o., 10 mg/kg, TGI=121%). The discovery of BI-4020 indicated that the macrocyclization strategy could be used to fix the active conformation, which reduced entropy loss and improved the bioactivity of acyclic compounds.

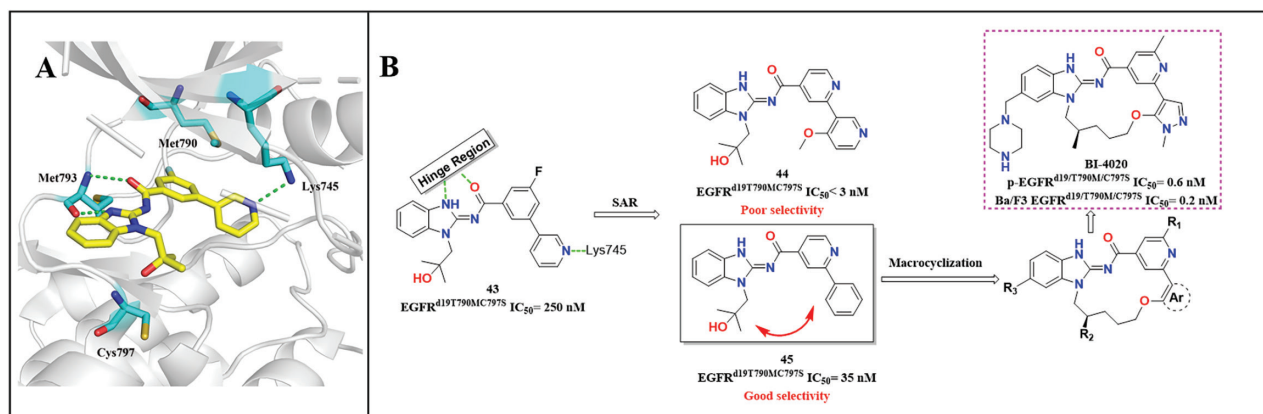


Figure 21 | (A) Co-crystal structure of compound **43** with EGFR^{L858R/T790M} (PDB: 6S9B) [hydrogen bond interactions are green]; (B) Discovery process of the macrocyclic EGFR^{d19/T790M/C797S} inhibitor, BI-4020.

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Table 2 | DMPK properties of compounds BI-4020 and osimertinib.

Compound	Solubility at pH 6.8 (µg/mL)	Metabolic-stable hepatocytes (%QH)		PPB-free fraction		Permeability pampa assay Papp A-B [cm/s] at pH 7.4
		Mouse	Human	Mouse	Human	
BI-4020	95	56	47	1.2	7.5	5.3×10^{-6}
Osimertinib	86	83	27	<0.1	2.5	2.7×10^{-6}

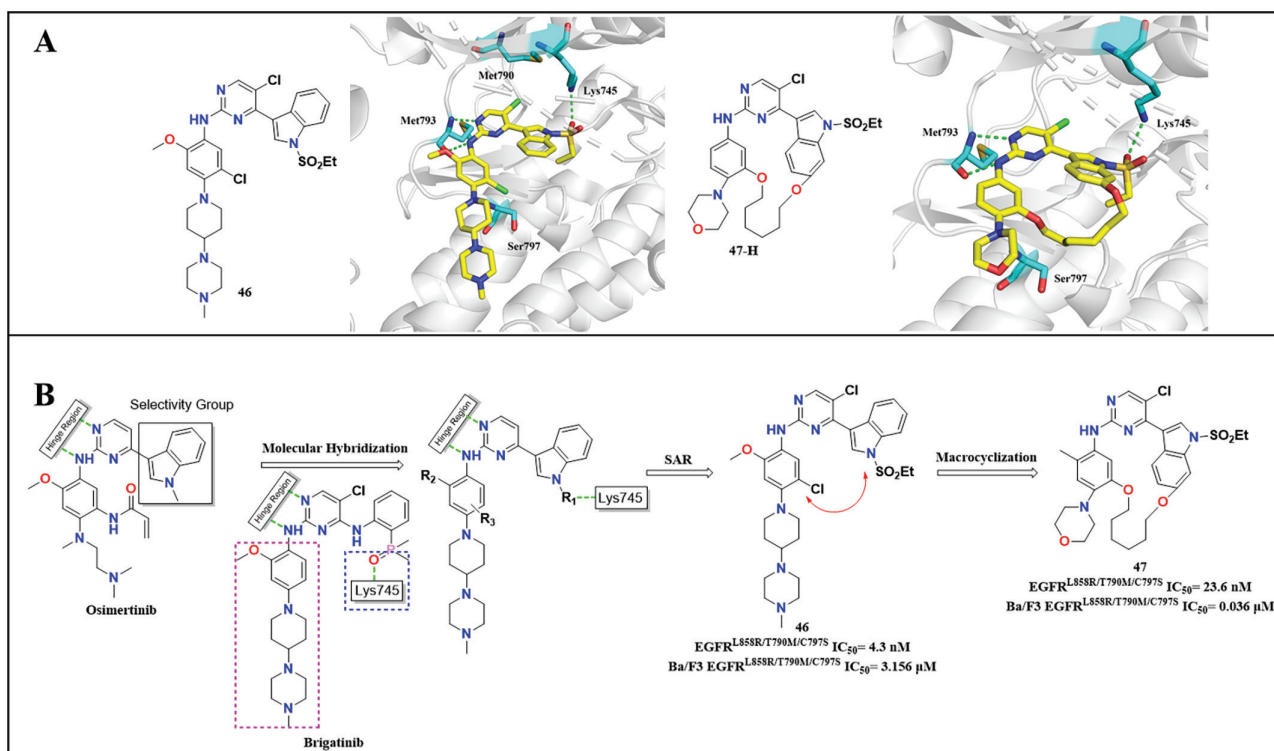


Figure 22 | (A) Co-crystal structure of compound 46 with EGFR^{T790M/C797S} (PDB: 7VRE) and compound 47-H with EGFR^{T790M/C797S} (PDB: 7VRA) [hydrogen bond interactions are green]. (B) Optimization of EGFR^{d19/T790M/C797S} and EGFR^{L858R/T790M/C797S} inhibitors using molecular hybridization and macrocyclization strategies.

Chen et al. designed and synthesized a series of macrocyclic 2,4-disubstituted pyrimidine derivatives as potent EGFR mutant inhibitors [130]. By hybridizing the general scaffold of brigatinib with the selectivity contributing 3-indolyl moiety of osimertinib, a series of 4-[1-(sulfonyl)-1*H*-indol-3-yl] pyrimidine derivatives were designed as new potential EGFR^{C797S} inhibitors (Figure 22). Importantly, the introduction of the sulfonyl group aimed to make hydrogen bond interaction with Lys745, which increased inhibitory activity against EGFR^{C797S} mutants. Among the synthesized compounds, compound 46 displays the most potent inhibitory activity against EGFR mutants (EGFR^{d19T/T790M/C797S} IC₅₀=25.6 nM, EGFR^{L858R/T790M/C797S} IC₅₀=4.3 nM) and good selectivity against EGFR^{WT} (IC₅₀=816 nM). Unfortunately, compound 46 displays weak cell inhibitory activity (Ba/F3 EGFR^{L858R/T790M/C797S} IC₅₀=3.156 µM). The co-crystal

structure of compound 46 with EGFR^{T790M/C797S} (PDB: 7VRE) suggested that the NH and nitrogen atom of pyrimidine forms a hydrogen bond interaction with Leu792 and Met793 in the hinge region and one oxygen atom of the ethyl sulfonate moiety formed a hydrogen bond with the NH₂ group of Lys745. Importantly, the dihedral of the pyrimidine core and the 4-(3-indolyl) substituent was -30.9°, which adopted an unflipped conformation. A macrocyclization strategy was used between the aniline ring and the indole phenyl ring to lock the minimum energy unflipped conformation of compounds. Among the synthesized compounds, compound 47 displays excellent inhibitory activity against EGFR^{L858R/T790M/C797S} and displays good selectivity against EGFR^{WT}. Compound 47 displays strong inhibitory activity and selectivity against Ba/F3 EGFR^{d19/T790M/C797S} and Ba/F3 EGFR^{L858R/T790M/C797S} with IC₅₀s of 36 and 52 nM,

respectively, in a cell-based assay. Unfortunately, the oral pharmacokinetics of compound **47** is poor (p.o. 5 mg/kg, C_{max} =134.6 ng/mL, F=11.9%), possibly due to the introduction of the lipophilicity linker. Therefore, the lipophilic linker could be replaced by an ethylene glycol-containing linker to modulate the lipophilicity of the compounds.

Osimertinib is a second-generate EGFR inhibitor with 200-fold greater selectivity against EGFR^{WT} that was approved for the treatment of patients with an EGFR^{T790M} mutation [131]. Shen et al. [132] designed and synthesized a series of macrocyclic compounds based on osimertinib using different alkyl chains as linkers (Figure 23). The connecting sites of the macrocyclic compounds were selected at the nitrogen atom in the 1-position of the indole ring and the NH group of the acrylamide moiety. Bioactivity assay results indicated that the 17-membered macrocyclic compound **48** displayed good inhibitory activities against EGFR^{L858R/T790M} and EGFR^{d746-750/T790M}, the IC_{50} s of which were 6.49 and 0.22 nM, respectively. Compound **48** displayed excellent anti-proliferative activities against Ba/F3 EGFR^{del19/T790M} and Ba/F3 EGFR^{del19/T790M/C797S} cell lines in the cell-based assay with IC_{50} s of 35 nM and 695 nM, which were comparable to osimertinib (Ba/F3 EGFR^{del19/T790M} IC_{50} =33 nM, Ba/F3 EGFR^{del19/T790M/C797S} IC_{50} =859 nM).

Gefitinib [133] and erlotinib [134] are quinazoline-based EGFR wild-type inhibitors that are used for first-line treatment of NSCLC patients with EGFR exon 19 deletions (EGFR^{d19}) and EGFR^{L858R} mutations. Amrhein et al. designed and synthesized a series of quinazoline-based macrocyclic EGFR^{d19/L858R} selective inhibitors (Figure 24) to avoid the undesired side effects caused by inhibition of wild-type EGFR [135]. The co-crystal structure of gefitinib with EGFR^{L858R} (PDB: 2ITZ) [136] indicated that the nitrogen atom at the 1-position of quinazoline forms a hydrogen bond interaction with Met793 in the hinge region and the 4-position phenyl ring occupies the gatekeeper hydrophobic area. The morpholine extends to the solvent region. The macrocyclization strategy was used between the 6-position of the quinazoline

core and the 4-position phenyl ring. Among the synthesized compounds, compound **50**, bearing a trimethylene glycol linker, displays excellent kinase inhibitory effects, the IC_{50} of which against EGFR^{del19}, EGFR^{L858R}, and EGFR^{L858R/T790M} were 0.3, 0.7, and 102.8 nM, respectively. Unfortunately, compound **50** displayed poor selectivity against EGFR^{WT} (Ba/F3 EGFR^{WT} IC_{50} =14.6 nM) in a cell-based assay. Compared to compound **50**, compound **49** displayed weaker inhibitory activities against EGFR mutants (EGFR^{del19} IC_{50} : 119.1 nM, EGFR^{L858R} IC_{50} : 820.8 nM) but in cell-based assay compound **49** displayed excellent selectivity against EGFR^{WT} (Ba/F3 EGFR^{WT} IC_{50} : > 1×10^4 nM; Ba/F3 EGFR^{del19} IC_{50} : 197.5 nM; Ba/F3 EGFR^{L858R} IC_{50} : 385.6 nM). The kinase selectivity assay suggested that compared to acyclic compounds, compound **49** displayed extraordinary selectivity among 468 kinds of kinases at a concentration of 1 μ M. Compound **49** only displayed weak inhibitory activity against EGFR^{WT} and moderate inhibitory activity against EGFR mutants (EGFR^{d19}, EGFR^{d747-752}, EGFR^{L858R}). Although compound **49** displayed no activity against the gatekeeper mutation, compound **49** could still act as a tool compound to explore other EGFR mutants.

3.4 Hematopoietic progenitor kinase (HPK1) inhibitors

HPK1 belongs to the serine/threonine kinase family and acts as a negative regulator of T-cell activation [137, 138]. Activation of HPK1 leads to phosphorylation of the adaptor protein, SLP76, which subsequently destabilizes the TCR signaling complex and inhibits T-cell function [139]. A preclinical study suggested that inhibiting HPK1 in syngeneic tumor models could promote tumor immune surveillance and enhance the synergistic effect with checkpoint inhibitors [140]. Thus, inhibition of HPK1 is a promising strategy for cancer immunotherapies [141]. GLK (MAP4K3) is structurally similar to HPK1 but acts as a positive regulator of T-cell activation. It is important to achieve selectivity between HPK1 and GLK [142]. Wang et al. designed and synthesized a series of macrocyclic pyrimidine derivatives as potent and selective HPK1

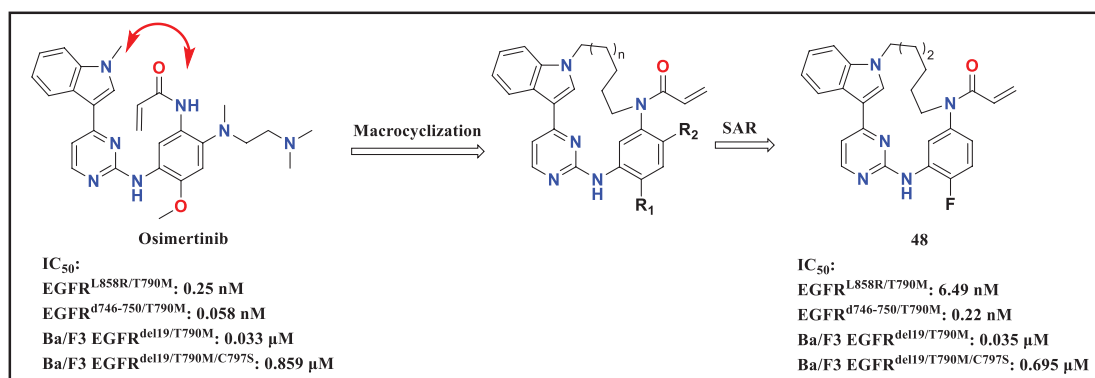


Figure 23 | Discovery of macrocyclic derivatives bearing aniline pyrimidine scaffolds as EGFR^{L858R/del19} inhibitors.

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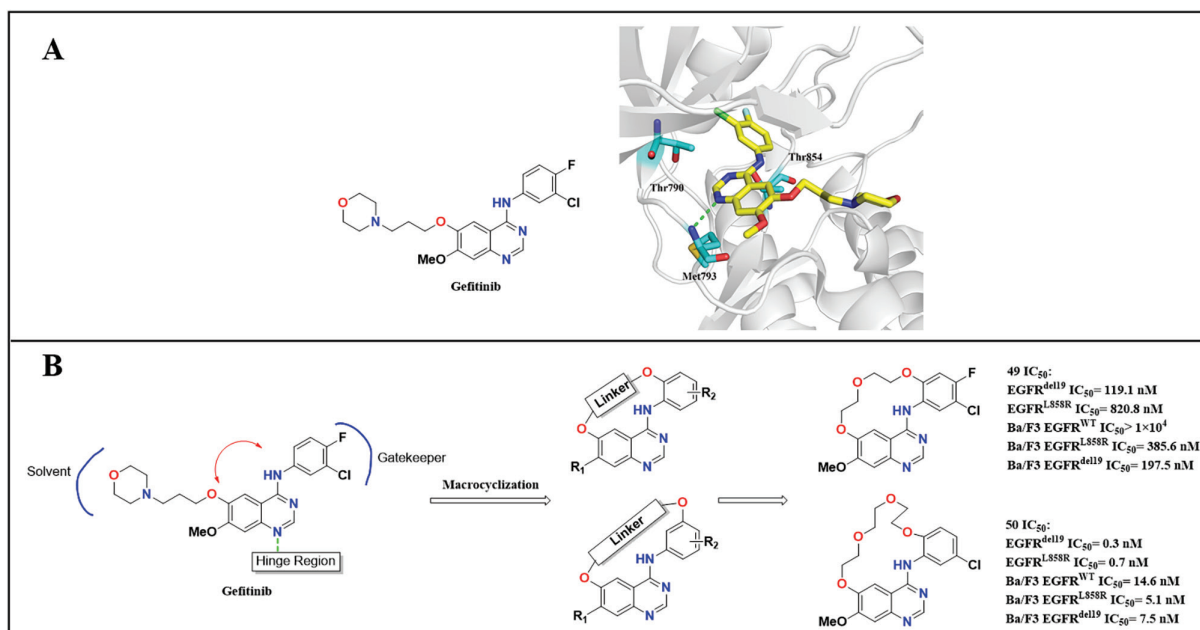


Figure 24 | (A) Co-crystal structure of gefitinib with EGFR^{L858R} (PDB: 2ITZ); (B) Design of quinazoline-based macrocyclic derivatives as selective EGFR^{L858R/del19} inhibitors based on gefitinib.

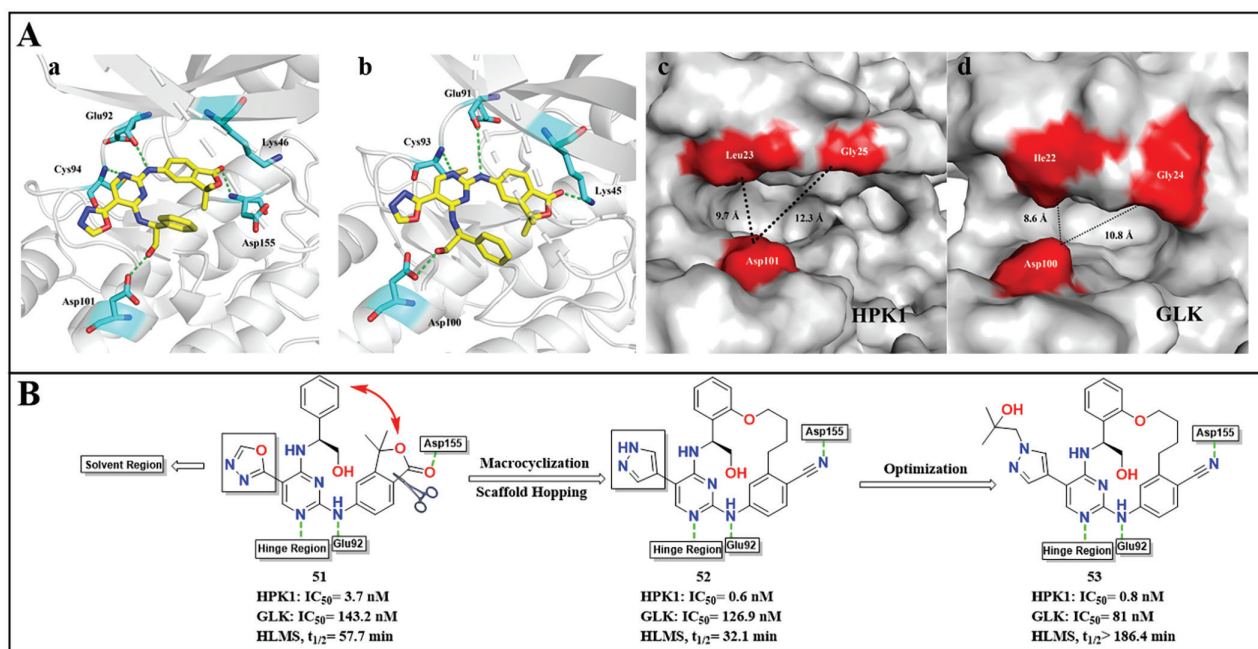


Figure 25 | (A) a,b: Binding modes of compound 51 to HPK1 (PDB: 7KAC) and GLK (PDB: 5J5T). c,d: Comparison of the solvent-exposed region size of HPK1 and GLK; (B) Optimization of the selective HPK1 inhibitor by a macrocyclization strategy using compound 51 as the lead compound.

inhibitors [143] (Figure 25). Compound 51 is a selective HPK1 inhibitor, the IC_{50} s of which against HPK1 and GLK are 3.7 and 143.2 nM, respectively. However, compound 51 also displays inhibitory activities against IRAK4, Fms,

Flt3, and c-kit, and compound 51 shows poor stability in human liver microsomes ($T_{1/2}$ = 57.7 min). Researchers first explored the selectivity mechanism underlying compound 51 based on molecular docking (GLK PDB:

5J5T) and co-crystal structure (HPK1 PDB: 7KAC [144]). The results indicated that compound **53** shares a similar binding pose for HPK1 and GLK. The nitrogen atom at the 1-position of the pyrimidine ring forms a hydrogen bond interaction with the hinge region of HPK1(Cys94) and GLK(Cys93). The NH group forms an extra hydrogen bond interaction with gatekeeper residue (Glu92 in HPK1 and Glu91 in GLK). The -OH moiety forms a hydrogen bond interaction with Asp101 in the solvent-exposed region (Asp101 in HPK1 and Asp100 in GLK). Through sequence alignment, researchers found that the width of the entry to the active site differed. Specifically, HPK1 has a wider entrance, while GLK has a narrower entrance in the solvent-exposed region. The narrow distance makes it easier to generate steric clashes between compound **51** and GLK, which could explain the selectivity of compound **51**. However, due to the flexible phenyl ring, compound **51** can fit GLK by undergoing a conformational change with limited loss in inhibitory activity, while compound **51** only shows 30-fold greater selectivity between GLK and HPK1. A macrocyclization strategy was used between the 2- and 4-position phenyl ring to reduce the flexibility of the phenyl ring and increase the steric hindrance with GLK. Importantly, the lactone moiety of compound **51** was replaced by a cyano group to retain a hydrogen bond acceptor and simplify the structure. Finally, the oxadiazole moiety was replaced by a volume like pyrazole for synthesis accessibility. The length of the linker was investigated, which showed that compound **52** with a 5-atom linker exhibits excellent HPK1 inhibitory activity with an IC_{50} of 0.6 nM. However, compound **52** displays poor human liver microsome stability ($T_{1/2}=32.1$ min). To improve the metabolic stability, different substituent groups were introduced to the N-1 position of the pyrazole ring. Compound **53** with a 2-hydroxy-2-methylpropyl moiety displays excellent HPK1 inhibitory activity ($IC_{50}=0.8$ nM) and stability ($T_{1/2} > 186.4$ min) (Table 3). Importantly, compound **53** displays selectivity against GLK ($IC_{50}=81.0$ nM [101.3-fold]). In a panel of 28 kinases, compound **53** showed better selectivity compared to compound **51**. Compound **53** showed good PK properties when administered via gastric gavage to mice and beagles. Compound **53** demonstrated a synergistic

effect with anti-PD-1 in MC38 and CT26 syngeneic models, which indicated that compound **53** has great potential in immunotherapy. This finding indicates that when a kinase inhibitor targets two homologous kinases, the macrocyclization strategy can be used to enhance the selectivity of the inhibitor and reduce the risk of side effects.

3.5 Mammalian sterile 20-like serine/threonine protein kinase (MST)3 inhibitor

The MSTs include five related proteins (MST1, MST2, MST3, MST4, and YSK1) [145-147]. MSTs regulate cell functions, such as proliferation and migration. MST3 is primarily located in the cytoplasm, is expressed in various tissues, and has a vital role in cell growth and apoptosis [148]. Dysregulation of MST3 has been confirmed to trigger high-grade tumors [149]. However, there are no isoform-selective inhibitors for MST3 and it is meaningful to develop highly selective MST3 inhibitors to validate the role of MST3 in tumorigenesis. Amrhein et al. developed a series of 3-aminopyrazole-based macrocycles as selective MST3 inhibitors [150] (Figure 26). Starting from the promiscuous kinase inhibitor, compound **54**, the macrocyclization strategy was applied between the cyano and cyclopropyl groups to pursue selective MST3 inhibitors. The differential scanning fluorimetry (DSF) assay was used to assay the binding affinity and selectivity of the macrocycles. Amrhein et al. [150] reported that the quinazoline derivative, compound **55**, led to high stabilization of MST3 and MST4 with ΔT_m values of 7.5 and 7.4 °C, respectively. Moreover, compound **55** displays good selectivity against MST1 and MST2. Compound **55** displays good inhibitory activity with EC_{50} values of 76 and 106 nM against permeabilized cells and intact cells, respectively, in a cell-based assay. The co-crystal structure of compound **55** with MST3 (PDB: 8QLQ) suggested that the aminopyrazole moiety forms three hydrogen bonds with Glu112 and Leu114 and the quinazoline part forms water-mediated hydrogen bonds with Asp121. The amide of the linker forms a hydrogen bond with Asp174. Importantly, the aliphatic linker is stabilized by hydrophobic interactions on both sides of the ring and contributes to the potency of compound **55**. Moreover, the binding of compound **55** with MST3 leads to large structural reorganization, suggesting

Table 3 | Pharmacokinetic properties of compound 53.

Compound 53	Mice		Beagles	
	1 mg/kg (i.v.)	20 mg/kg (i.g.)	1 mg/kg (i.v.)	20 mg/kg (i.g.)
$T_{1/2}(h)$	1.3	3.7	8.1	6.2
$T_{max}(h)$		0.8		1.0
$C_{max}(ng/mL)$	1118.0	2048.0	1811.2	539.3
$AUC_{(0-inf)}(ng\cdot h/mL)$	860.0	8528.0	3132.2	4094.9
F (%)		49		27

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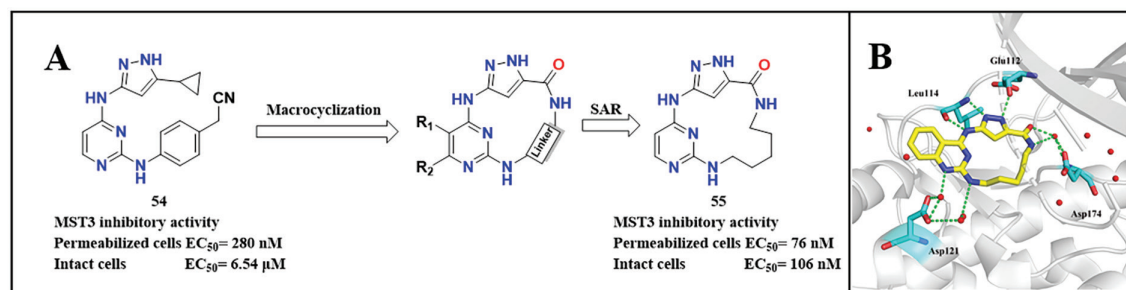


Figure 26 | (A) The macrocyclization strategy was used to yield compound 55 as an MST3 selective inhibitor; (B) Co-crystal structure of compound 55 with MST3 (PDB: 8QLQ) [hydrogen bonds are green].

that the residues beyond the binding site that facilitate the concerted induced-fit movements may also be key for the good selectivity.

4. IMPROVING DRUG-LIKE PROPERTIES THROUGH MACROCYCLIZATION

In addition to enhancing inhibitory activity and kinase selectivity, macrocyclization is a promising strategy for improving the drug-like properties of acyclic kinase inhibitors. Compared to linear molecules, macrocyclic compounds typically have a smaller molecular volume that may contribute to improved permeability. Moreover, the restricted conformation of macrocyclic compounds can reduce binding with metabolizing enzymes, thereby improving metabolic stability. Structurally, macrocyclic compounds often feature a hydrophobic or hydrophilic

linker, which enables modulation of molecular properties. Furthermore, various strategies have been used to improve the DMPK properties of macrocycles, including linker exploration, introducing hydrophilic groups, and designing prodrugs.

4.1 Apoptosis signal-regulating kinase 1 (ASK1) inhibitors

ASK1 is a mitogen-activated protein kinase kinase kinase that has a critical role in the cellular stress response by modulating inflammation and apoptosis [151, 152]. Regulation of ASK1 activity is a feasible strategy to treat neurologic disorders [153]. Himmelbauer et al. designed and synthesized a series of macrocyclic compounds with a low efflux ratio (ER) and increased CNS penetration [154] starting with GS-4997 [155], a pyridine benzamide-based ASK1 inhibitor (IC_{50} = 5.9 nM; **Figure 27**). The

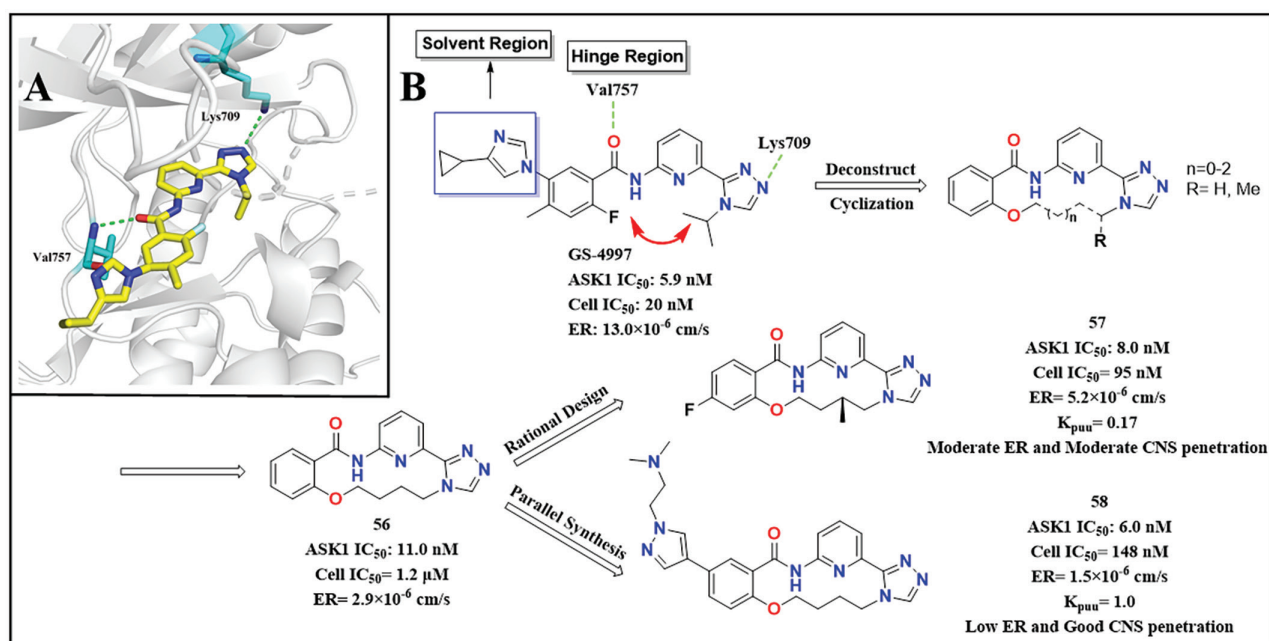


Figure 27 | (A) The co-crystal structure of GS-4997 with ASK1 (PDB: 6OYT) [hydrogen bonds are green]; (B) Optimization of macrocyclic ASK1 inhibitors utilizing GS-4997 as a lead compound to enhance central nervous system (CNS) permeability.

co-crystal structure of GS-4997 with ASK1 (PDB: 6OYT) indicated that the carbonyl group of the amide forms a hydrogen bond with Val757 in the hinge region and the nitrogen atom of the triazole forms a hydrogen bond with the catalytic Lys709, while the cyclopropyl-imidazole moiety extends to the solvent-exposed region. An intramolecular hydrogen bond between the amide NH group and the fluorine atom was observed, and this interaction contributed to locking the conformation of GS-4997 into a favorable binding pose. By deconstructing the solvent-exposed substituent and macrocyclization strategy, a series of macrocyclic compounds were designed and synthesized to increase CNS penetration. Compound **56** displays good ASK1 inhibitory activity ($IC_{50}=11$ nM) and moderate ASK1 autophosphorylation inhibitory activity (cell IC_{50} : 1.2 μ M). Importantly, compared to GS-4997, compound **56** displays lower ER (2.9×10^{-6} cm/s). Introduction of a methyl group at the butylene linker and fluorine atom at the salicylate part affords compound **57** increased activity and stability (ASK1 inhibition IC_{50} : 8.0 nM, cell IC_{50} : 95 nM; RLM/HLM: 90/16 mL/min/kg). Compared to GS-4997, the CNS penetration of compound **57** increased significantly (ER: 13.0×10^{-6} vs 5.2×10^{-6} cm/s), possibly due to the introduction of a lipophilicity linker. Additionally, a parallel synthesis strategy was used to discover a strong ASK1 inhibitor with excellent CNS-like properties by modulating the lipophilicity of the compounds. Among the synthesized compounds, compound **58** displays good ASK1 inhibitory activity ($IC_{50}=6.0$ nM) with a low efflux ratio (ER: 1.5×10^{-6} cm/s). These results demonstrate that the introduction of a lipophilic linker modulates the physicochemical properties of the acyclic compound and macrocyclization is a feasible strategy for optimizing CNS-like drugs.

4.2 CDK2 inhibitors

Despite showing potent inhibitory effects against CDK2, compound **2** exhibits relatively poor permeability and a high ER. Further optimization of the linker in compound

2 was performed to identify macrocyclic CDK2 inhibitors with improved properties (Figure 28). Eventually, compound QR-6401, which has a *trans*-cyclobutyl ring, displayed good CDK2/E1 inhibitory activity with increased permeability (Table 4). Additionally, compound QR-6401 showed good selectivity against related kinases and acceptable PK properties (Table 5) (Rat, p.o. 5 mg/kg: $C_{max}=213$ ng/mL, $AUC_{last}=765$ ng·h/mL, $F=50\%$). In an *in vivo* anti-tumor efficacy study using an OVCAR3 ovarian cancer xenograft model, compound QR-6401 demonstrated excellent anti-tumor activity when administered orally at a dose of 50 mg/kg for 28 days, resulting in a TGI of 78%. This case suggests that when macrocycles exhibit unsatisfactory properties, exploring the intramolecular linker can help improve the PK profiles.

4.3 DYRK2 inhibitors

The azaindole-based compound **9** exhibited potent inhibitory activities against DYRK1A and DYRK1B but the pharmacokinetic properties were poor. It is worth

Table 4 | Stability and membrane permeability of compounds **2** and QR-6401.

Compd.	Human liver microsomal stability $T_{1/2}$ (h)	Papp A to B (10^{-6})/efflux ratio
2	64	0.9/13
QR-6401	150	3.2/13

Table 5 | PK properties of QR-6401.

QR-6401	Mouse	
	20 mg/kg (p.o.)	100 mg/kg (p.o.)
T_{max} (h)	0.19	0.083
C_{max} (ng/mL)	803	5527
$AUC_{(0-last)}$ (ng·h/mL)	758	5587

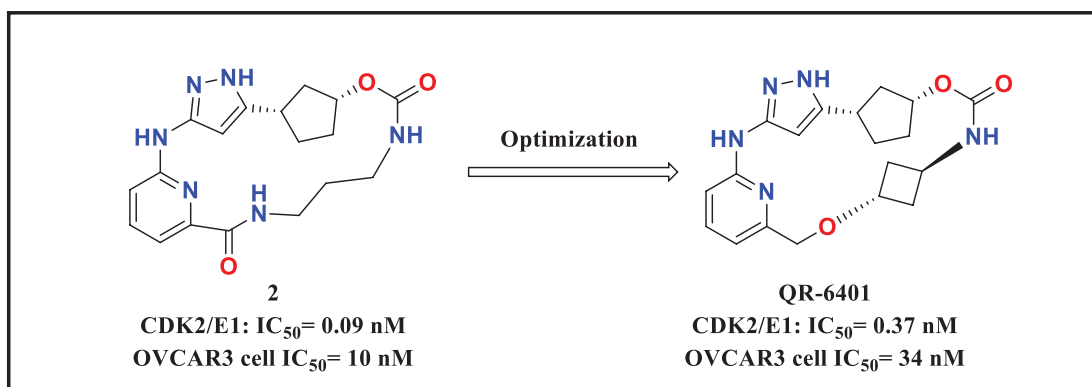


Figure 28 | Linker exploration of compound **2** to improve pharmacokinetic properties.

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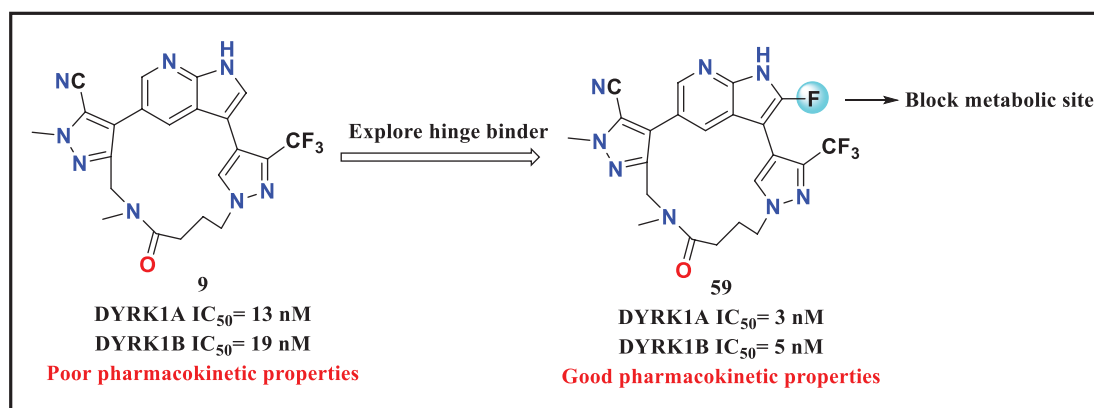


Figure 29 | Optimization of pharmacokinetic properties of the macrocyclic DYRK2 inhibitor, compound 9.

Table 6 | PK properties of compounds 9 and 59.

Compd.	Administration	Dose (mg/kg)	$T_{1/2}$ (h)	AUC_{inf} (ng·h/mL)	Cl (mL/min/kg)	C_{max} (μM)	F (%)
9	IV	5	0.3±0.1	504.9±59.6	166.6±20.0	n.d.	
	PO	10	2.0±1.0	23.6±8.1	n.d.	11.6±3.4	1.1
59	IV	2	1.2±0.9	8947.2±4389.5	5.9±5.4	34.1±20.7	
	PO	10	2.4±0.7	5770.3±654.8	29.3±4.4	6.4±1.8	12.3

noting that the 2-position of the azaindole ring was identified as the labile site. Therefore, a fluorine atom was introduced at the 2-position of the azaindole ring in compound 9 to yield compound 59 (Figure 29). As expected, compound 59 showed improved pharmacokinetic properties compared to compound 9 (Table 6). Moreover, introduction of the fluorine atom enhanced the potency of hydrogen bonding interactions between the hinge binder and the protein, leading to enhanced inhibitory activity against DYRK1A (IC_{50} =3 nM) and DYRK1B (IC_{50} =5 nM).

4.4 Spleen tyrosine kinase (SYK) inhibitors

SYK is a non-receptor cytoplasmic protein tyrosine kinase expressed mostly in hematopoietic cells [156, 157]. The B cell receptor activates SYK and subsequently activates downstream signaling pathways, such as PI3K δ and BTK [158]. SYK is an attractive target for autoimmune disorders [159] and hematologic cancers [160]. Grimster et al. designed and synthesized a series of macrocyclic 2,4-disubstituted pyrimidine derivatives as potent SYK inhibitors based on previous research [161] (Figure 30). Compound 61 is a potent SYK inhibitor that displays no hERG inhibition (SYK IC_{50} =0.19 nM, hERG IC_{50} > 40 μM). Unfortunately, compound 61 possesses suboptimal PK profiles and is not suitable for further development (Mouse: p.o. 1 mg/kg, F=7%) [162]. The previous work demonstrated that compound 60 displays a U-shaped conformation under the solution state and the length between the amide methyl group and

the dimethylamine moiety is approximately 4 Å. Thus, the amide and dimethylamine parts could be linked via different linkers to gain macrocyclic compounds with improved properties. The results suggested that the compounds with a 5-atom length linker display potent SYK inhibitory activity, especially compound 62, the SYK IC_{50} of which is 0.13 nM. Compared with acyclic compound 60, compound 62 displayed increased metabolic stability, perhaps due to the increased steric hindrance of the dimethylamine moiety. However, compound 62 displays strong hERG inhibitory activity, the IC_{50} value of which is 0.85 μM. Polar groups were introduced to the nitrogen atom at the 1-position of the indazole ring to decrease lipophilicity, thereby reducing hERG inhibition. Among the synthesized compounds, compound 63 displays weak hERG inhibition (IC_{50} > 39 μM) and excellent kinase selectivity (inhibition of 4 kinases in a panel of 379 kinases). However, due to the alcoholic hydroxyl, compound 63 displays unfavorable pharmacokinetic profiles (p.o., F=0.9%). Thus, the ester prodrug strategy was used to mask the alcohol hydroxyl group to obtain compound 64, which displays improved PK profiles (p.o., F=12%) compared to compound 63.

5. CONCLUSION AND PERSPECTIVE

Although the development of kinase inhibitors has made significant progress in disease treatment, continued use in clinical trials has revealed certain limitations. Following the FDA approval of lorlatinib, pacritinib, and

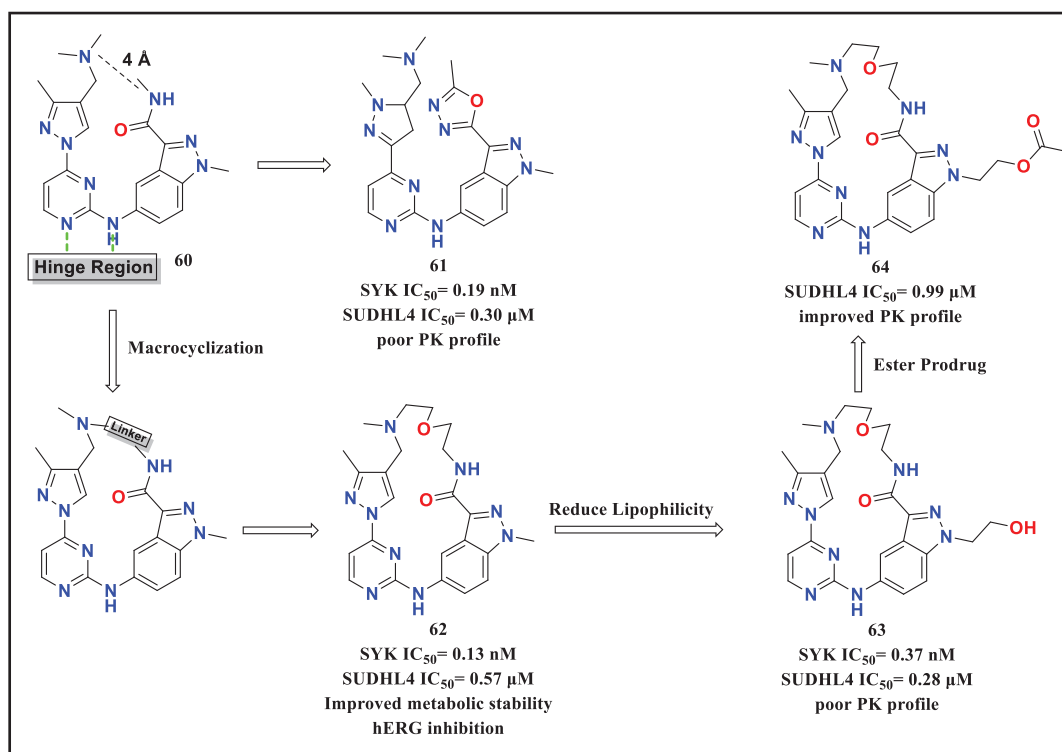


Figure 30 | Optimization of macrocycle-based ester prodrugs as SYK inhibitors with improved pharmacokinetic profiles.

reprotrectinib, utilization of the macrocyclization strategy has gained considerable momentum in the optimization of kinase inhibitors. Compared to conventional small molecule kinase inhibitors, macrocyclic kinase inhibitors offer several advantages. When binding to the kinase domain, macrocyclic compounds with limited conformational flexibility can reduce entropy loss and potentially improve binding affinity. An example of this is the discovery process of the EGFR^{del19/T790M/C797S} inhibitor, BI-4020, in which a macrocyclization strategy was used to stabilize the acyclic inhibitor active conformation in the EGFR kinase domain. Additionally, the introduced linker has the potential to form new interactions with the kinase domain, thereby enhancing the inhibitory activity of the macrocyclic inhibitors. The optimization process of the selective BMPR2 inhibitor has shown that using a macrocyclization strategy improves the kinase selectivity of promiscuous inhibitors. This improvement may be attributed to the limited range of conformations available for macrocyclic inhibitors. Moreover, the use of macrocyclic compounds has been shown to be beneficial in achieving inhibitory activity against point mutant kinases. This finding is primarily attributed to the reduction in steric hindrance with the corresponding amino acid residues. For example, LOXO-101, a second-generation TRK inhibitor, exhibits excellent inhibitory activity against TRK WT. However, LOXO-101 has weak bioactivity against TRK solvent front mutants, possibly

due to the steric hindrance caused by the bulky Arg residue (TRKA^{G595R} IC_{50} =237.4 nM). To address this finding, Li et al. [99] used a scaffold hopping and macrocyclization strategy to design a series of macrocyclic TRK inhibitors based on LOXO-101. As a result, the inhibitory activity against TRKA^{G595R} significantly improved (TRKA^{G595R} IC_{50} = 13.1 nM). In addition, the identification of lorlatinib, a macrocyclic ASK1, and SYK inhibitors has demonstrated that designing macrocyclic kinase inhibitors also improves drug-like characteristics, such as enhanced stability, membrane permeability, and PK properties.

While macrocyclization has gained recognition as a successful approach for designing kinase inhibitors, there are certain challenges that might hinder application of macrocyclization, including synthetic availability, poor PK properties, and uncertain target binding patterns. The synthesis of macrocyclic compounds typically requires complex chemical reaction conditions. For example, constructing the macrocyclic structure often involves multi-step reactions, each of which demands precise control over reaction conditions, such as temperature, solvent, and reaction time. Owing to the considerable ring strain within the macrocyclic molecules, reaction intermediates are inclined to decompose or undergo side reactions. Moreover, for macrocyclic compounds containing special functional groups or complex structures, traditional synthesis methods may not meet the requirements. Amrhein et al. reviewed the synthetic

Review Article

opportunities and challenges for macrocyclic kinase inhibitors [163]. Amrhein et al. [163] summarized the commonly used chemical reactions during the synthesis of macrocyclic molecules, including ring-closing metathesis (RCM), nucleophilic substitution, amide coupling, and Pd-catalyzed reactions. In addition to traditional synthetic methods, new strategies are being devised to increase yields beyond traditional chemical reactions. These strategies include exploring new chemical ligation methods, such as click chemistry or photocatalytic reactions, to simplify the synthesis process and utilizing computational chemistry to predict and design synthetic routes. Additionally, the synthesis of macrocyclic compounds can also be achieved through template-directed synthesis. By introducing an appropriate template molecule, this approach guides the reactive groups to react in a specific orientation. This not only helps to overcome the challenges posed by ring strain but also effectively reduces the occurrence of side reactions, making the synthesis process more efficient and controllable.

Macrocyclic kinase inhibitors might exhibit unfavorable PK characteristics, such as poor permeability and low bioavailability, which limit efficacy. Some macrocyclic molecules may have poor solubility in water, which can limit absorption and distribution within the body. Additionally, the macrocyclic structure may be susceptible to degradation and metabolism by enzymes in the body, leading to reduced drug stability. This property can affect the efficacy and duration of drug action. For example, despite demonstrating potent c-Met inhibitory activity and anti-proliferative effects, D6806 exhibits poor pharmacokinetic properties and shows inhibition of the hERG potassium channel, possibly due to its high lipophilicity. Therefore, the impact on the PK properties

of these compounds should be considered during macrocyclization design. Several strategies can be used to improve the PK properties of macrocyclic kinase inhibitors. Appropriate functional groups can be introduced to optimize solubility, permeability, and stability, such as a PEG-containing linker. In addition to structural modifications, using drug delivery systems, such as liposomes or polymer nanoparticles, or developing prodrugs are feasible strategies to enhance the PK properties of macrocyclic inhibitors.

The macrocyclization strategy can be used to improve inhibitory activities against targets, but the SAR of macrocyclic compounds are sometimes unclear. Compared to acyclic compounds, macrocyclic kinase inhibitors often display reduced entropy loss and show more potent bioactivities. Therefore, it is crucial to determine the principles of entropy reduction. Determining the binding mode of the target to the macrocyclic inhibitor is essential to guide the design of macrocyclic inhibitors. During the design process of macrocyclic compounds, molecular docking and MD simulations can be used to predict and elucidate the possible binding modes of compounds with their targets, thereby guiding further compound design. Moreover, structural biology techniques, such as X-ray crystallography or NMR, can be used to precisely understand how macrocyclic compounds interact with their targets. These methods can help us determine the binding mode of macrocyclic compounds with their targets and identify the principles for reducing entropy loss (Figure 31).

The macrocyclization strategy has been widely adopted in recent years to modify the structure of kinase inhibitors. The development of macrocyclic drugs faces several limitations, including synthetic challenges,

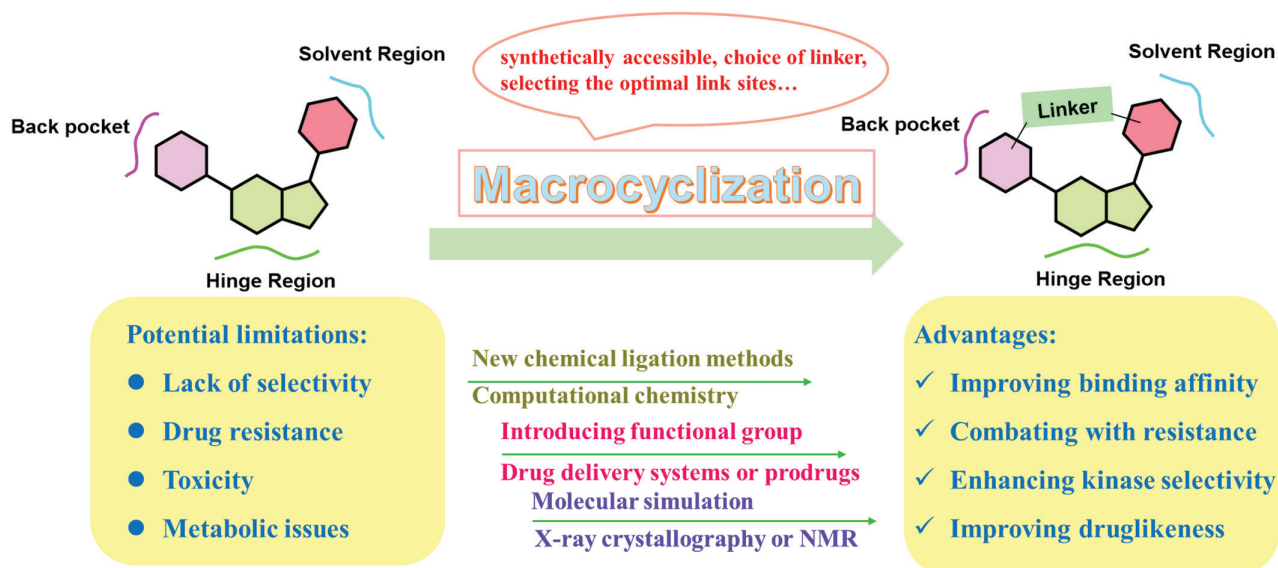


Figure 31 | Discovery process, advantages, and challenges for macrocyclic kinase inhibitors.

biological activity and selectivity, PK, and bioavailability, as well as toxicity and safety. However, with the continuous advances in chemical synthesis techniques and biotechnologies, these limitations are expected to be gradually overcome. Macrocyclic drugs are poised to have an increasingly important role in future drug development. We are convinced that this strategy can be effectively applied to various domains beyond kinase inhibitors, leading to the development of macrocyclized compounds that exhibit enhanced bioactivity and superior properties compared to their acyclic counterparts.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Review Article

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