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REVIEW

Selective type II TRK inhibitors overcome xDFG mutation mediated acquired resistance to the second-generation inhibitors selitrectinib and repotrectinib

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Abstract Neurotrophic receptor kinase (*NTRK*) fusions are actionable oncogenic drivers of multiple pediatric and adult solid tumors, and tropomyosin receptor kinase (TRK) has been considered as an attractive therapeutic target for "pan-cancer" harboring these fusions. Currently, two generations TRK inhibitors have been developed. The representative second-generation inhibitors selitrectinib and repotrectinib were designed to overcome clinic acquired resistance of the first-generation inhibitors larotrectinib or entrectinib resulted from solvent-front and gatekeeper on-target mutations. However, xDFG (TRKA$^{G667C/A/S}$, homologous TRKC$^{G696C/A/S}$) and some double mutations still confer resistance to selitrectinib and repotrectinib, and overcoming these resistances represents a major unmet clinical need. In this review, we summarize the acquired resistance mechanism of the first- and second-generation TRK inhibitors, and firstly put forward the emerging selective type II TRK inhibitors to overcome xDFG mutations mediated resistance. Additionally, we concluded our perspectives on new challenges and future directions in this field.

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1. Introduction

Tropomyosin receptor kinase (TRK) family including TRKA, TRKB and TRKC are well-known cell surface transmembrane receptor tyrosine kinases, and encoded by the neurotrophic receptor kinase 1, 2 and 3 genes (*NTRK1*, *NTRK2* and *NTRK3*), respectively[1,2]. Under normal physiological conditions, the binding of TRKs to neurotrophin ligands induces TRKs dimerization, phosphorylation and thereby activation of the downstream signaling pathways, including RAS/MAPKs, PI3K/AKT and PLC-γ1 pathways, which is essential for maintaining cell proliferation, differentiation and even apoptosis[3,4]. However, TRKs can be constitutively activated *via NTRK* gene fusions in the pathogenesis of human malignant cancers, in which the 3′ region of the *NTRK* gene, including the kinase domain, achieves an in-frame fusion to the 5′ sequence of fusion partner gene[5,6]. The first *NTRK* fusion gene *TPM3-NTRK1* was discovered in human colorectal carcinoma in 1982[7,8], and to date, over 100 different *NTRK* fusion partners have been identified in various cancers[9,10]. TRK proteins have been considered as attractive "pan-cancer" targets for the treatment of various cancers harboring *NTRK* fusions[11]. Because most oncogenic forms of *NTRK* fusions alter or eliminate the extracellular domain, traditional monoclonal antibody therapy is not effective. Thus, the main approach to target oncogenic *NTRK* fusions is focused on TRKs small molecule inhibitors[12–14].

Larotrectinib (**1**, Vitrakvi®) and entrectinib (**2**, Rozlytrek®) are two first-generation TRK inhibitors approved by the U.S. Food and Drug Administration (FDA) in 2018 and 2019, respectively (Fig. 1)[15–18]. Both inhibitors achieved rapid and massive clinical responses in adult and pediatric patients with advanced solid tumors harboring *NTRK* fusions[19–21]. However, drug resistances against larotrectinib and entrectinib have emerged in clinic. Point mutations in the kinase domain (KD) of TRK, including solvent-front (SF) mutations, gatekeeper (GK) mutations and xDFG motif mutations, are the main resistance mechanisms to these first-generation TRK inhibitors (Fig. 1). SF mutations located on the solvent front region of TRKs are commonly a smaller glycine substituted by arginine or glutamic acid with a larger side chain, *e.g.*, $TRKA^{G595R}$, $TRKB^{G639R}$, $TRKC^{G623R/E}$ mutations[12]. GK mutations anchor on the gatekeeper site of TRKs, which is an aromatic phenylalanine mutated as an alkyl leucine, *e.g.,* $TRKA^{F589L}$, $TRKB^{F633L}$, $TRKC^{F617L}$ [12]. The xDFG mutations map to the 'x' position of the xDFG motif with the 'x' indicating the position preceding the activation loop DFG motif. And xDFG mutations containing a small glycine are commonly replaced by cysteine, serine or alanine with large volume, *e.g.,* $TRKA^{G667C/A/S}$, $TRKB^{G709C/A/S}$ and $TRKC^{G696C/A/S}$ [12]. These point alterations in the kinase domain of TRKs cause steric hindrances and/or increase the affinity of ATP to the mutant kinases, thus compromising the binding of the first-generation TRK inhibitors[21–25].

Subsequently, second-generation TRK inhibitors were developed to overcome these resistances. The representatives, macrocycle-based selitrectinib (**3**, Loxo-195) and repotrectinib (**4**, TPX-0005), are currently undergoing phase II and phase III clinical trials, respectively (Fig. 1). Although the two drugs displayed significant clinical effects against SF and GK mutations mediated resistances[24,26,27], they showed limited efficacy against tumors with acquired xDFG mutations and compound mutations[28–30]. Moreover, several second-generation TRK inhibitors, such as taletrectinib (DS-6051b, phase II, NCT04395677)[31], PBI-200 (phase I, NCT049-01806)[32], SIM1803-1A (phase I, NCT04671849)[33] etc., are undergoing early clinical trials and the related drug resistances are not presented yet. Recently, several reviews described the resistance mechanisms of first-generation TRK inhibitors and the clinical activities of developed second-generation TRK inhibitors[12,27,34–36]. However, no review includes the detailed analysis of the resistance mechanisms of both first- and second-generation inhibitors and the potential strategies that may overcome the resistances of second-generation inhibitors. In this review, we focus on summarizing reported resistance mechanisms of the first- and second-generation TRK inhibitors and giving the insight of emerging type II TRK inhibitors that effectively address the xDFG alterations.

2. Structure and downstream signaling pathways of TRK

The full-length structures of TRKA, TRKB and TRKC are highly homologous[37,38], which are mainly composed of three parts: an extracellular domain, a transmembrane domain (TM) and an

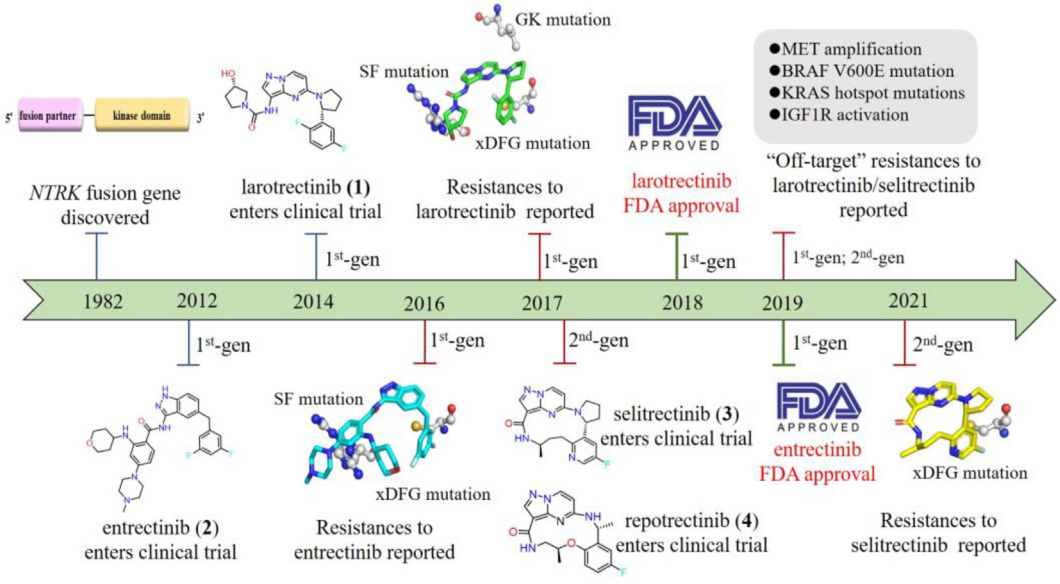

Figure 1 Development pipeline of TRK inhibitors and associated drug resistances.

intracellular kinase domain (KD) (Fig. 2A). The extracellular domain of TRK consists of two cysteine-rich clusters (C1−2), three leucine-rich regions (LRR1−3) and two immunoglobulin-like domains (Ig1, Ig2), which are responsible for ligand recognition and binding[39−41]. The KD of TRK possesses the similar architectural features to other protein kinases, which contains one N-terminal lobe mainly composed of β-strands, one C-terminal lobe constituted of α-helices and a hinge linker bridging these two lobes[42]. Although TRKA, TRKB, TRKC share similar tertiary protein structure, their extracellular neurotrophin binding ligands are different. TRKA binds preferentially to nerve growth factor (NGF)[2,42], TRKB specifically binds to brain-derived neurotrophic factor (BDNF)[43,44], while TRKC selectively binds to neurotrophin-3 (NT-3)[45,46]. Extracellular neurotrophin ligands binding to TRK receptors results in receptor dimerization, autophosphorylation and eventually activates the kinase activity of TRK (Fig. 2B)[3,4]. The NGF binding to TRKA triggers the activating of downstream rat sarcoma oncogene (RAS)-mitogen-activated protein kinase (MAPK) signaling pathways, controlling cell growth and proliferation[2]. The NGF/TRKA pathway also plays an important role in mediating chronic pain, inflammation, itch and the survival and differentiation of sympathetic and sensory neurons[47]. The BDNF-TRKB signaling causes the activation of RAS-extracellular-signal-regulated kinase (ERK), phospholipase C-γ1 (PLC-γ1), phosphatidylinositol-3-kinase (PI3K) pathways, regulating neural cell plasticity, survival and metabolism[43]. The NT-3 binding to TRKC stimulates PI3K-v-AKT murine thymoma viral oncogene homolog (AKT) pathway, which is critical for sustaining cell survival and preventing apoptosis[45]. However, TRK aberrations, including *NTRK* fusion, mutation and TRK over-expression, can abnormally activate TRK in a neurotrophin ligands-independent manner, leading continuously activation of downstream PI3K/AKT, RAS/MAPK, and PLC-γ1 pathways and high risks of carcinogenesis[14,48].

3. *NTRK* fusions

NTRK fusions are the most common mechanisms of oncogenic TRK activation. Fusions involving all three *NTRK* genes have been identified as oncogenic drivers in a broad range of pediatric and adult tumors[5,6,12]. These fusions arise from inter- or intrachromosomal rearrangements, in which the 3′ region of *NTRK* gene containing kinase domain is fused with the 5′ fusion partner gene[5,49]. The expressed *NTRK* fusion protein commonly lacks an extracellular ligand binding domain, but contains a 5′ gene partner encoding an oligomerization domain, such as coiled-coil domains, zinc finder domains or WD repeats, which are required for full activation of downstream kinases (Fig. 3). Driving by the 5′ upstream partner, the *NTRK* chimeric oncoprotein can be constitutively activated through a ligand-independent manner, leading persistent activation of TRK downstream signaling pathways and high risks of oncogenesis[50−52].

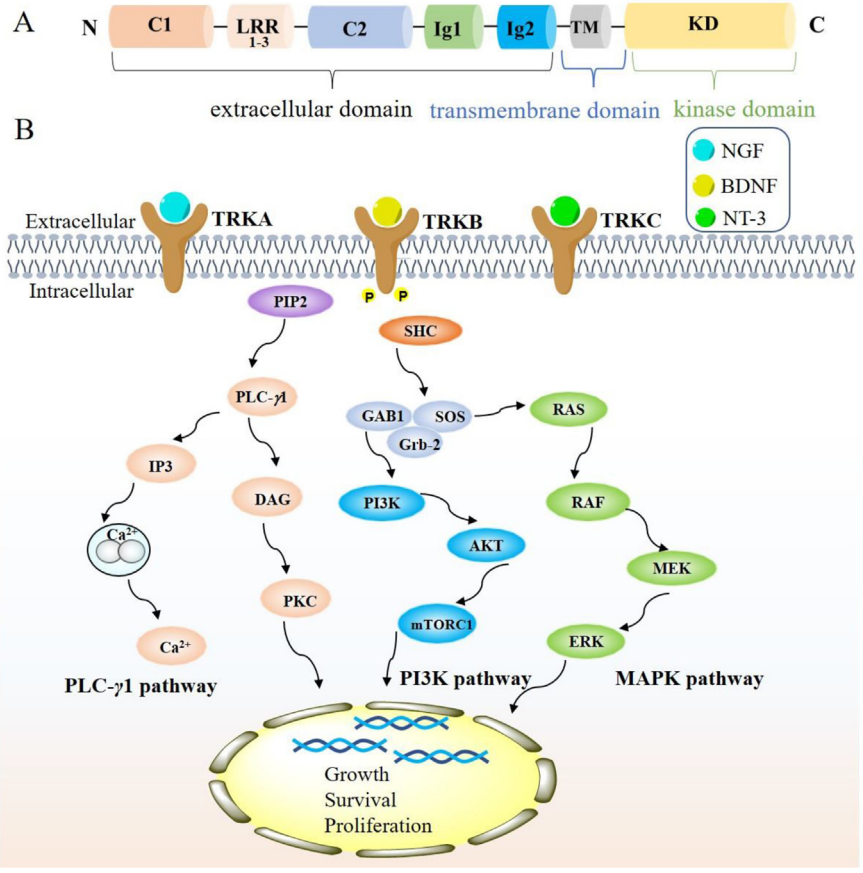

Figure 2 Overview of the structure and major signal transduction pathways of TRK. (A) A cartoon shows three domains of TRK: an extracellular domain containing two cysteine-rich clusters (C1, C2), three leucine-rich regions (LRR1−3), two immunoglobulin-like domains (Ig1, Ig2); a transmembrane domain (TM) and an intracellular kinase domain (KD). (B) TRK mediates the activation of RAS−RAF−MEK−ERK, PI3K−AKT, and PLC-γ1−DAG−PKC signaling pathways.

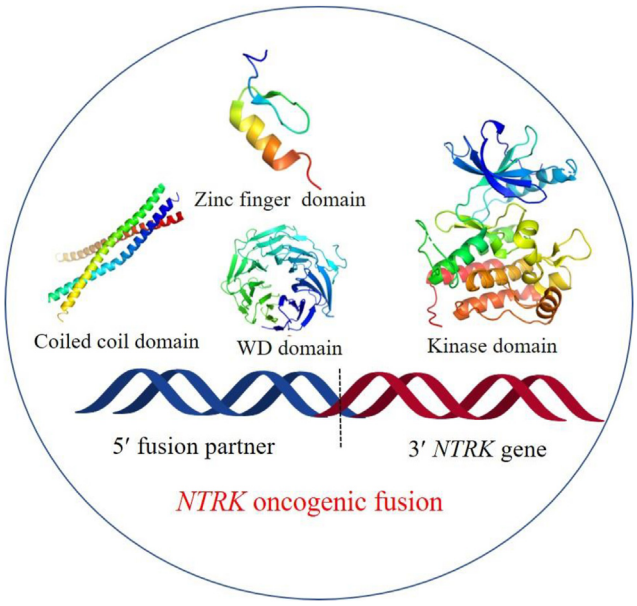

Figure 3 The structure of *NTRK* fusion gene.

Since the first *NTRK* fusion protein *TPM3-NTRK1* was detected in colon cancer in 1982, over 100 different 5′ fusion partners have been identified in more than 20 types of human tumors[8,10,12,53,54]. Among three *NTRK* isoforms, *NTRK1* and *NTRK3* fusions are widely distributed in a series of different cancer types, while *NTRK2* fusions are mainly detected in central nervous system (CNS) tumors[54–57]. *NTRK* fusions are the major oncogenic driving events in some rare tumors, such as infantile fibrosarcoma (IFS)[58], secretory breast cancer (SBC)[59], mammary analog secretory carcinoma (MASC)[60], and congenital mesoderm nephroma (CMN)[61]. The prevalence of *NTRK* fusions can reach 90% or higher in these rare tumors, with the *ETV6-NTRK3* fusion diagnosed most[6,62–65], while *NTRK* fusions are found at low incidence (<5%) in a series of common cancer types, such as lung cancer, breast cancer, colorectal cancer, pancreatic cancer etc.[21,12,66,67]. In addition to *NTRK* fusions, other TRK aberrations have also been found in some tumors, including TRK point mutations[68–72], splice variants[48,68,73–75], and TRK overexpression[76–78].

4. Resistances to the first-generation TRK inhibitors

DFG motif, a highly conserved three amino acids (Asp-Phe-Gly) in the activation loop, directly affects the conformation of kinases. In an active or DFG-in conformation, the Asp of DFG motif directs the ATP binding site. In an inactive or DFG-out conformation, the Asp points outward the ATP binding site and the Phe of DFG motif flips into the ATP active site to form an accessible hydrophobic back pocket[79]. TRK small molecule inhibitors can be classified into three different types, namely type I, type II and type III, according to their different binding modes with TRK kinases. Inhibitors that bind to the ATP-binding site of kinase with an active conformation (DFG-in) are defined as type I inhibitors[80], such as the first- and second-generation TRK inhibitors larotrectinib, entrectinib, selitrectinib, repotrectinib, taletrectinib etc.[14]. Inhibitors that bind to the ATP-binding site and an adjacent hydrophobic back pocket of kinase with an inactive conformation (DFG-out) are classified as type II inhibitors, *e.g.*, multi-targeted kinase inhibitors cabozantinib, foretinib, ponatinib[80]. Inhibitors that bind to a hydrophobic pocket distant from the ATP-binding site of kinase are defined as type III inhibitors, which can induce conformational changes to regulate the kinase activity[80].

Despite the initial marked tumor shrinkage achieved by the treatment with larotrectinib and entrectinib[19–21], unfortunately, duration of response was eventually limited by acquired resistances. Tumor sequencing or sequential circulating tumor DNA (ctDNA) obtained from patient's tumor specimen or plasma at progression revealed that point mutations in the KD of TRKs are the main mechanisms of acquired resistances[21–25]. There are three major point mutations in the KD of TRKs: solvent-front (SF) mutations, gatekeeper (GK) mutations as well as xDFG motif mutations (Fig. 4A–C)[19,22]. SF mutations located on the solvent front region of TRKs are commonly a smaller glycine substituted by arginine or glutamic acid with a larger side chain, *e.g.,* TRKA^{G595R}, TRKB^{G639R}, TRKC$^{G623R/E}$ mutations (Fig. 4D), which are paralogous to ALK^{G1202R} and ROS1^{G2032R} mutations[81]. Structural modeling indicates that the large side chain of mutant arginine or glutamic acid introduce steric hindrance with the hydroxypyrrolidine group of larotrectinib or methylpiperazine motif of entrectinib (Fig. 5B–C)[22,24,26]. Besides, biochemical analyses demonstrate that the TRKA^{G595R} mutation leads the increased binding affinity of ATP to the mutant kinase (K_m 6 μmol/L for TRKA^{G595R} *vs.* 51 μmol/L for TRKA), thus improving the intrinsic kinase activity[24]. Both steric hindrances and increased intrinsic kinase activity impair the binding of larotrectinib and entrectinib.

The GK mutations anchor on the gatekeeper site of TRKs, which is an aromatic phenylalanine mutated as a alkyl leucine, *e.g.,* TRKA^{F589L}, TRKB^{F633L}, TRKC^{F617L} (Fig. 4E)[12]. Structural modeling indicates that the mutant leucine may generate steric clash with pyrrole fragment in larotrectinib and hinder its binding (Fig. 5B)[12,82]. Whereas entrectinib is a methylene group at this position, which effectively avoids steric hindrance with the mutated Leu589 (Fig. 5C)[83]. The xDFG mutations locate on the activate loop of kinase, which contains a small glycine replaced by cysteine, serine or alanine with large volume, *e.g.,* TRKA$^{G667C/A/S}$, TRKB$^{G709C/A/S}$ and TRKC$^{G696C/A/S}$ (Fig. 4F)[12]. These mutations cause potential steric clashes between the difluorophenyl groups of larotrectinib and entrectinib and mutated residues (Fig. 5B–C)[22,24,36].

In order to overcome the acquired point mutations of larotrectinib and entrectinib, great efforts have been made in exploiting the second-generation TRK inhibitors[12,14]. The rigid macrocyclic selitrectinib (**3**) and repotrectinib (**4**) are two representative second-generation TRK inhibitors, which are undergoing phase II and phase III clinical studies, respectively. Selitrectinib is a selective pan-TRKs inhibitor[24], while repotrectinib is a multi-targeted TRK/anaplastic lymphoma kinase (ALK)/c-ros oncogene 1 (ROS1) inhibitor[26]. Selitrectinib and repotrectinib are designed by a cyclized conformationally restricted strategy based on the scaffold of larotrectinib, thus avoiding steric hindrances with the mutated residues and reducing the binding entropy penalty (Fig. 5A)[24,26]. Selitrectinib and repotrectinib effectively induce visible tumor regression in patients with the SF acquired resistance mutations (*e.g.,* TRKA^{G595R}, TRKC^{G623R} and TRKC^{G623E}) relapsed from the treatment of larotrectinib (**1**) or entrectinib (**2**)[23,24,26,84,85]. An X-ray cocrystal structure of repotrectinib with TRKA^{G595R} demonstrates that it precisely anchors in the ATP-binding pocket without any extra motifs extending to the solvent area to clash with the bulky Arg595 (Fig. 5E)[24,83]. The docking model of selitrectinib with TRKA^{G595R} indicates that it displays similar binding mode to that of repotrectinib (Fig. 5D).

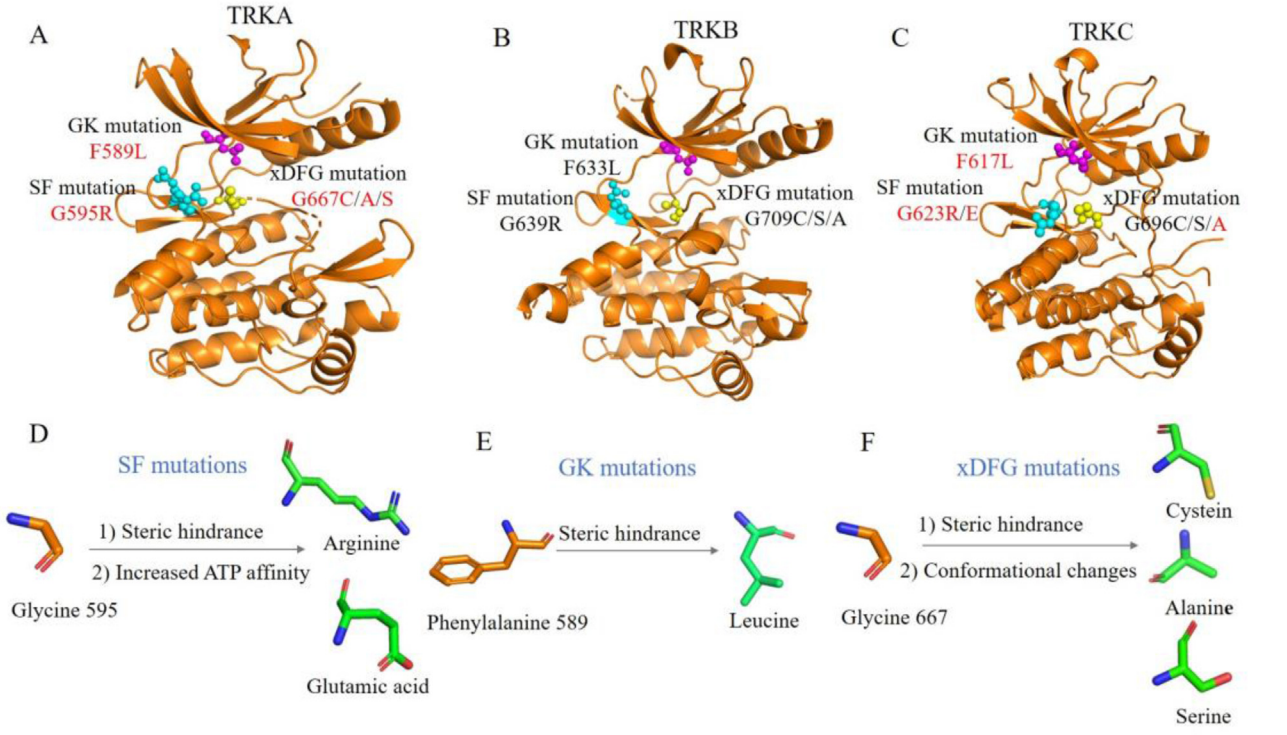

Figure 4 Representative point mutations in the KD of TRKA (PDB code 7VKN) (A); TRKB (PDB code 4ASZ) (B); TRKC (PDB code 6KZD) (C). The solvent-front (SF), gatekeeper (GK), xDFG substitutions are shown in cyan, magenta, yellow spheres, respectively. Mutations detected in patients are shown in red font. Resistance mechanisms caused by SF mutations (D); GK mutations (E); xDFG mutations (F).

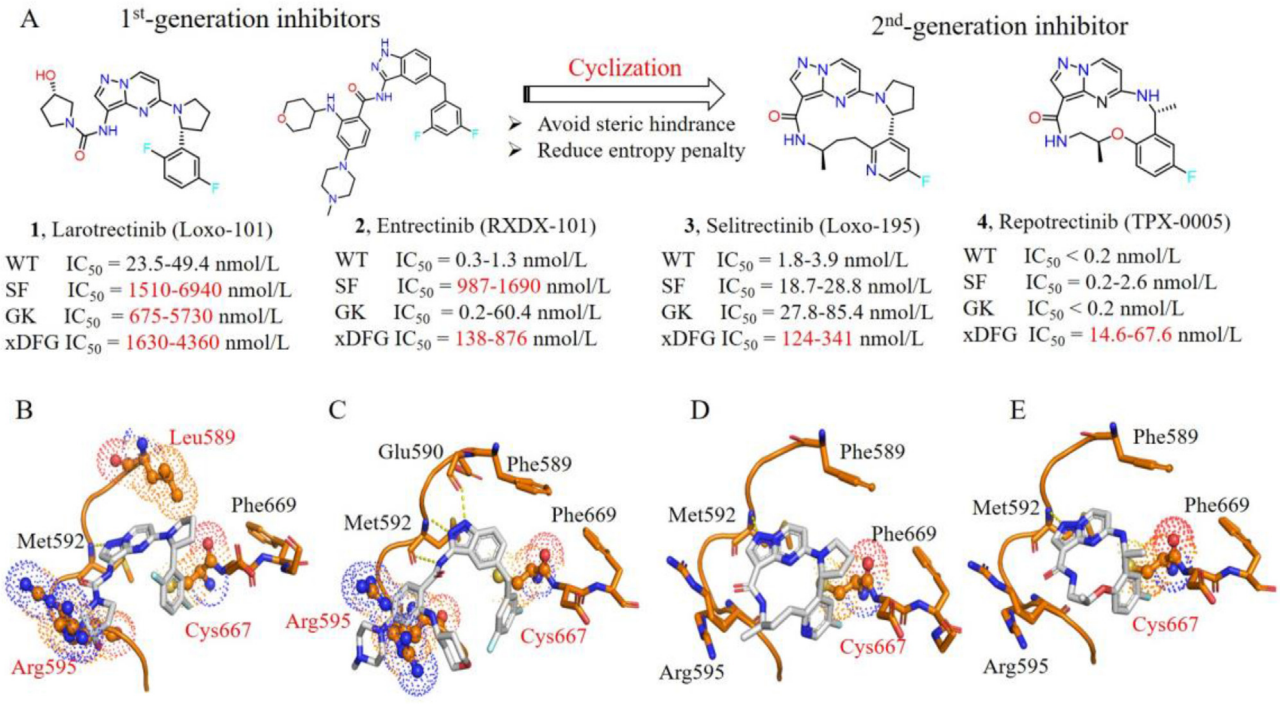

Figure 5 Chemical structures, cell-proliferation inhibitory activities and binding modes of the first- and second-generations TRKs inhibitors. (A) Chemical structures of larotrectinib, entrectinib, selitrectinib, repotrectinib and their cell-proliferation inhibitory activities against Ba/F3 cells engineered with wild-type (WT), solvent-front (SF) mutant, gatekeeper (GK) mutant and xDFG mutant TRKs. Structural models showing steric clashes between solvent-front (G595R), gatekeeper (F589L) or xDFG (G667C) mutations and larotrectinib (B); entrectinib (C); selitrectinib (D); repotrectinib (E) (generated from PDB code 7VKN). Steric clashes are highlighted in spheres.

Besides, selitrectinib and repotrectinib with a compact macrocyclic structure can better accommodate GK mutations and show improved *in vitro* kinase inhibitory activity against TRKA^{F589L} mutation, compared with larotrectinib (Fig. 5A)[24,83]. However, structural modeling indicates that the mutated Cys667 still generates steric hindrances with the fluoroaromatic group in selitrectinib and repotrectinib and hinders their binding (Fig. 5D–E)[28,29].

5. Emerging resistances to selitrectinib and repotrectinib

Despite the superior activities of selitrectinib and repotrectinib against a variety of SF and GK alterations *in vitro* and *in vivo*, acquired drug resistances still emerged. Clinical trials and *in vitro* experimental models indicate multiple resistance mechanisms of selitrectinib and repotrectinib, which can be classified into two categories: TRK-dependent and TRK-independent resistance mechanisms. The former involves acquired xDFG mutations and compound mutations (such as SF/xDFG, SF/GK compound mutations)[28,29,36,86], while the latter is associated to abnormal activation of TRK bypass signaling, such as MAPK pathway[30,85].

5.1. TRK-dependent resistance mechanisms

5.1.1. xDFG mutations

It was reported that the xDFG mutations in the KD of TRKs, *e.g.,* TRKA$^{G667C/A/S}$ alterations, result in acquired resistances to selitrectinib in breast and colorectal cancer patients[28]. One patient harboring a *TPM3-NTRK1*-fused TRKA^{G595R}-mutant sarcoma progressed on selitrectinib therapy. Target next-generation sequencing (NGS) assays based on the tumor samples revealed the acquisition of TRKA^{G667C} mutation and loss of TRKA^{G595R} mutation[28]. Another patient with *LMNA-NTRK1*-fused, TRKA^{G595R}-mutant breast cancer developed resistance to selitrectinib after 2 months of treatment. Cell-free DNA (cfDNA) collected from plasma samples and molecular profiling using NGS platform demonstrated a novel TRKA^{G667C} alteration[28]. Besides, a TRKA^{G595R} mutated primary colorectal cancer cell line developed resistance to repotrectinib following chronic drug exposure due to an emerged TRKA^{G667C} mutation[28]. CellTiter-Glo-based proliferation and Western blot assays confirmed that repotrectinib fails to suppress cell growth and TRK-activated downstream signaling, suggesting that the xDFG mutation TRKA^{G667C} may also confer acquired resistance to it[28]. The decreased potencies of selitrectinib and repotrectinib against xDFG alterations have also confirmed by *in vivo* cell proliferation inhibitory activities with IC_{50} values of 124−341 nmol/L and 14.6−67.6 nmol/L against Ba/F3 cells engineered with xDFG mutant TRKs, respectively, which are more than 68-fold less potent than their wide-type counterpart (Fig. 5A)[28,83]. Besides, taletrectinib, another second-generation TRK inhibitor, also shows poor inhibitory efficacy towards TRKA^{G667C} mutation with an IC_{50} value of 304.1 nmol/L against Ba/F3 *TPM3-NTRK1*^{G667C} cells[31].

Although the cyclization strategy effectively avoids steric clashes in the solvent front region, the macrocycle inhibitors selitrectinib and repotrectinib do not essentially change the binding conformation of the fluoroaromatic group in the original first generations. Similar to the resistance mechanisms of larotrectinib and entrectinib[14,16], the mutated residues (*e.g.,* alanine, cysteine or serine) at Gly667 in TRKA still cause steric impedances with the fluoroaromatic group and disturb the binding of selitrectinib and repotrectinib (Fig. 5D–E)[28,29]. Cocco E et al. reported that the xDFG mutations (TRKA^{G667C} or TRKC^{G696C}) induced conformational changes of TRK to preferentially adopt a DFG-out state[28,29], thus favorable for binding with type II inhibitors (Fig. 6). Interestingly, several multi-targeted type II inhibitors, *e.g.,* cabozantinib, foretinib, ponatinib, indeed display high potencies against TRK xDFG alterations *in vitro* and *in vivo*[28,29]. In a word, the steric clashes and conformational change resulted from the xDFG mutations will abrogate the binding of most type I-based 1st- and 2nd-generation TRK inhibitors and sensitize to type II inhibitors, providing insights into rational type II drug design to address recalcitrant resistant alterations.

5.1.2. Compound mutations

In addition to the xDFG mutations, some compound mutations were also detected in patients after the treatment of selitrectinib[28,86]. A patient with a *LMNA-NTRK1*^{G595R}-mutant colorectal cancer developed resistance to selitrectinib after 11 months of treatment. Targeted next-generation sequencing of the resistant tumor revealed a novel TRKA^{G667A} mutation and retained TRKA^{G595R} mutation. RNA sequencing indicated that TRKA^{G595R} and TRKA^{G667A} mutations are located on the same allele in *cis* with similar allele frequencies (33% and 27%, respectively)[28]. Selitrectinib and repotrectinib display decreased inhibitory activities against TRKA$^{G595R/G667C}$ (SF/xDFG) compound mutation with IC_{50} values of 596 and 205 nmol/L, respectively[28]. Besides, a patient with *ETV6-NTRK3* fused infantile fibrosarcoma underwent resistance to selitrectinib due to a new TRKC^{F617L} alteration in *cis*-form with TRKC^{G623R}, which was definitized as SF/GK compound mutation[86]. Selitrectinib displays weak antiproliferative activity against Ba/F3 cells expressed TRKA$^{G595R/F589L}$ alteration with an IC_{50} value of 468 nmol/L, which is 100-fold less potent than that of wild-type[83]. Although the resistance mechanisms of compound mutations have not been illustrated in detail, there is an urgent need to exploit novel TRK inhibitors to address recalcitrant xDFG or compound mutations.

5.2. TRK-independent resistance mechanisms

Similar to other receptor tyrosine kinase inhibitors, off-target alterations also mediated acquired resistances to TRK inhibitors[30,87,88]. Previous studies have demonstrated acquired BRAF^{V600E}, KRAS^{G12D} mutations as well as MET amplification render resistance to the 1st-generation inhibitors larotrectinib and entrectinib in clinic[30]. These off-target alterations are predicated to TRK-independently activate downstream MAPK signaling pathways and cannot be adequately addressed by the 2nd-generation TRK inhibitor selitrectinib alone. Furthermore, the emerged KRAS$^{G12A/V/D}$ substitutions can also cause resistance to patients who receive selitrectinib[30,85]. Combination therapy of selitrectinib and a MET inhibitor crizotinib can achieve marked tumor shrinkage in a cholangiocarcinoma patient harboring *NTRK*-fusion and MET amplification[30].

6. Type II TRK inhibitors overcome xDFG mutations

6.1. Multi-targeted type II inhibitors

The majority of the 1st- and 2nd-generation TRK inhibitors are type I inhibitors and bind in the DFG-in active conformation of TRK[13,14]. However, the xDFG mutations stabilize the kinases in DFG-out inactive conformation, thus sensitizing the binding of type II TRK inhibitors[28,29]. Some multi-targeted type II kinase inhibitors, *e.g.,* cabozantinib (**5**), altiratinib (**6**), foretinib (**7**) and

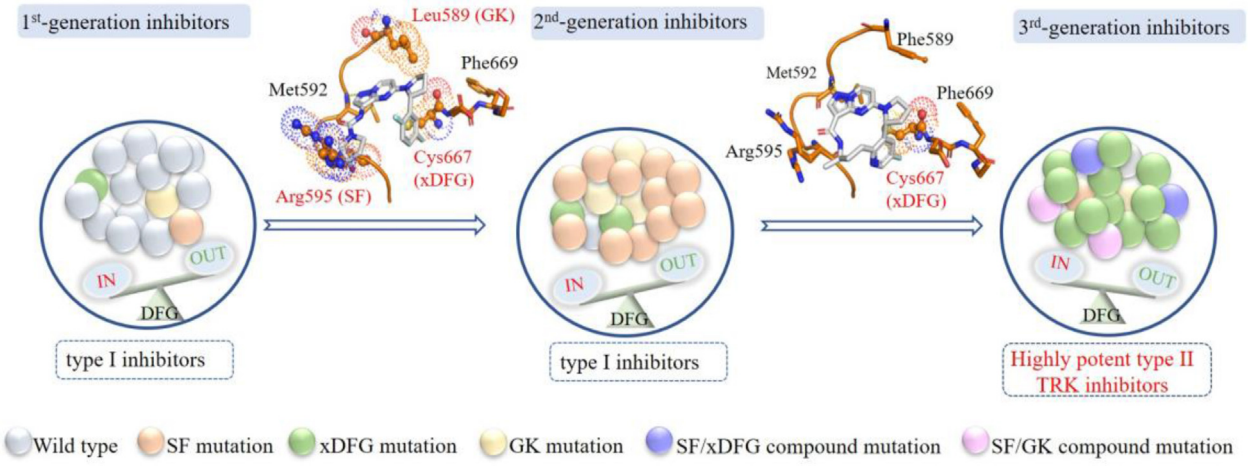

Figure 6 Proposed therapeutical pipeline for *NTRK* fusion cancers. Upon diagnosed, tumors mainly harbor wild-type *NTRK* fusion cells. These wide-type kinases adopt a DFG-in active conformation and type I based 1st-generation TRK inhibitors larotrectinib or entrectinib are approved for use. Subsequently developed point mutations resistances and SF alterations dominate. The kinases remain in DFG-in active conformation and type I based 2nd-generation inhibitors are applied. At progression, the emerged xDFG mutations and some compound mutations cause steric clashes, impeding the binding of type I based 2nd-generation inhibitors, and induce the kinase preferring a DFG-out inactive conformation, favoring the binding of type II inhibitors. Each colored ball represents a distinct clone. Steric clashes are highlighted in spheres.

ponatinib (**8**), preferentially bind to TRK xDFG mutations and exhibit strong *in vitro* and *in vivo* inhibitory activities (Fig. 7). Cabozantinib, a multi-kinase inhibitor for hepatocyte growth factor receptor (c-Met), vascular endothelial growth factor receptor 2 (VEGFR2), and rearranged during transfection (RET)[89], displays strong biochemical and cellular inhibitory activities against $TRKA^{G667C}$ and Ba/F3-*TPM3-NTRK1* cells with IC_{50} values of 1 and 99 nmol/L, respectively. Furthermore, cabozantinib exhibits potent and durable tumor progression inhibition in selitrectinib resistant ($TRKA^{G667C}$) patient derived xenografts (PDX) model[28]. Currently, cabozantinib is undergoing multiple phase II trials in patients with RET fusion-positive advanced non-small cell lung cancer (NSCLC) and those with other genotypes: *ROS1* or *NTRK* fusions or increased MET or AXL activity (NCT01639508). Altiratinib, regarded as a c-Met, tunica intima endothelial kinase 2 (TIE2) and VEGFR2 kinase inhibitor[90], suppresses the growth of Ba/F3-*TPM3-NTRK1*^{F589L} and Ba/F3-*TPM3-NTRK1*^{G667C} cells with IC_{50} values of 18.3 and 1.8 nmol/L, respectively. Besides, foretinib and ponatinib also exhibit potent kinase inhibitory efficacies against $TRKA^{G667C}$ mutation ($IC_{50} < 1$ nmol/L)[28,91]. More importantly, cabozantinib, foretinib and ponatinib can strongly inhibit $TRKA^{G595R/G667C}$ compound mutation with IC_{50} values below 5 nmol/L, while corresponding IC_{50} values of selitrectinib and repotrectinib are 596 and 205 nmol/L, respectively[28]. Although the aforementioned type II inhibitors can potently inhibit xDFG alterations *in vitro* and *in vivo*, substantial off-target inhibition of these agents may cause poor plasma exposures and high frequency of side effects. It is desired that the focus is put on exploitation of highly selective type II TRK inhibitors.

6.2. *Selective type II TRK inhibitors*

More recently, diverse selective TRK type II inhibitors that can overcome acquired resistance mutations were developed. Li et al.[92] reported a series of selective type II TRK inhibitors with a quinazoline scaffold based on compound **9**, which is potent against TRKs ($IC_{50} = 0.2-2$ nmol/L) and Aurora A/B ($IC_{50} = 6/13$ nmol/L) with poor oral bioavailability[93]. Structure analysis indicated that the thiazole ring of **9** forms crucial hydrogen bond interactions with Lys162 and Asp274 on Aurora A, respectively, while corresponding residues on TRKA are Phe589 and Phe669 (Fig. 8A). In order to improve its TRK selectivity, the thiazole ring of **9** was replaced by a phenyl group to reduce the hydrogen interactions with Aurora A and increase a $\pi-\pi$ interaction with TRKs. The resulting compound **10** indeed shows decreased activity against Aurora A but greatly lost potency towards TRKA[92]. Considering the back hydrophobic pocket of TRKA is more spacious than that of Aurora A, diverse substituents were introduced to the terminal phenyl ring of **10** to occupy the hydrophobic pocket of TRKA. The final compound **11** with 3-CF_3/6-OCH_3 phenyl group exhibits improved kinase inhibitory activity for TRKs (IC_{50} ranging from 5 to 29 nmol/L) and high selectivity over Aurora A/B with IC_{50} values of 454.2 and 548.4 nmol/L, respectively. Moreover, **11** potently inhibits $TRKA^{G667C}$, $TRKA^{F589L}$, $TRKC^{G696A}$ alterations as well as $TRKA^{G595R/G667C}$ with IC_{50} values of 13.3, 10.4, 22.5 and 19.6 nmol/L, respectively, while exhibiting moderate activities against $TRKA^{G595R}$ and $TRKC^{G623R}$ with IC_{50} values of 151.4 and 75.4 nmol/L, respectively[92]. The docking mode of **11** with TRKA illustrates that it anchors the ATP-binding pocket in a type II binding mode with the quinazoline core and urea moiety forming tight hydrogen bonds with Met592, Glu560 and Asp668. The middle phenyl group forms additional $\pi-\pi$ interactions with Phe589 and Phe669. The terminal substituted phenyl group occupies the back pocket of TRKA and makes favorable hydrophobic interactions with surrounding residues (Fig. 8B). Most importantly, **11** exhibits acceptable *in vivo* pharmacokinetics (PK) profiles in rats with good oral absorption ($AUC_{0-inf} = 3012$ ng/mL × h), moderate half-life ($t_{1/2} = 5.1$ h) and reasonable oral bioavailability ($F = 27.2\%$). **11** shows good *in vivo* antitumor efficacy in KM12 xenograft mouse model with TGI value of 64% at a dosage of 75 mg/kg three times per week by orally administered[92].

Pan et al.[94] screened their in-house kinase inhibitors-like compound library and identified a lead compound **12**, which has moderate inhibitory activities for TRKs (IC_{50} ranging from 0.211 to 0.516 μmol/L) and weak potency for $TRKC^{G623R}$ ($IC_{50} = 1.352$ μmol/L) (Fig. 9A). In order to improve its potency

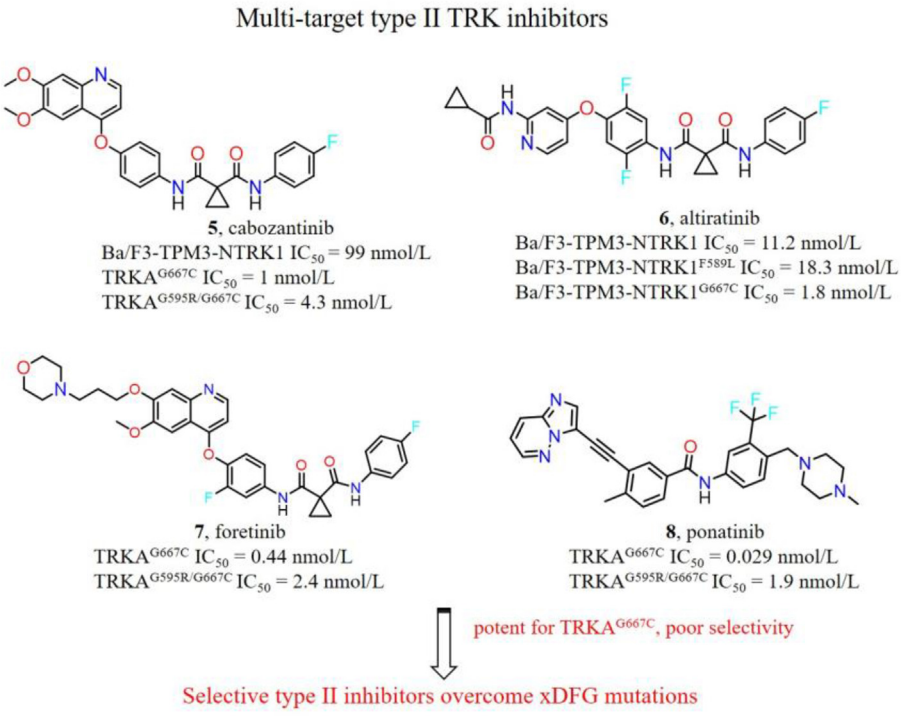

Figure 7 Chemical structures of multi-targeted type II TRK inhibitors and their kinase or cellular inhibitory activities towards wild-type (WT) or mutant TRK.

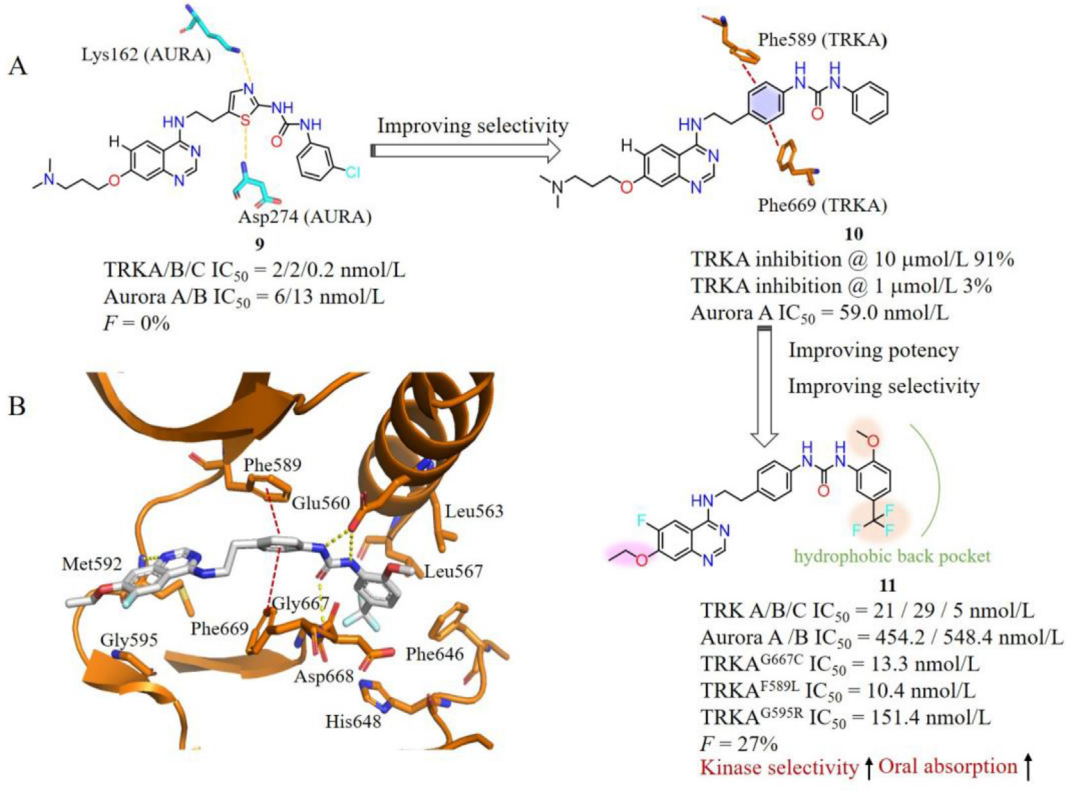

Figure 8 (A) Optimization process of compound **11**. (B) The binding mode of **11** with TRKA (docking based on PDB code 6PL1). **11** and key residues are showed in sticks, hydrogen bonds are presented as yellow dashed lines, and $\pi-\pi$ interactions are presented as red dashed lines.

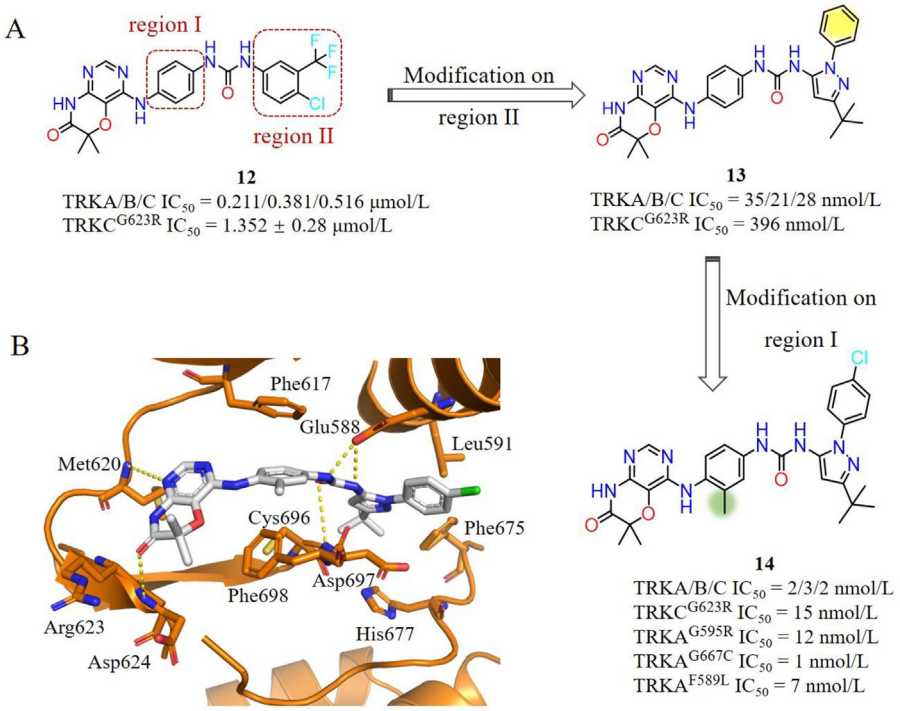

Figure 9 (A) Optimization process of compound **14**. (B) The binding mode of compound **14** with TRKC$^{G623R/G696C}$ kinase (generated based on PDB code 6KZD). **14** and key residues are showed in sticks and the hydrogen bonds are presented as yellow dashed lines.

against TRKs, the researchers have carried out abundant structural optimizations to increase additional interactions with two spacious pockets in TRKs. One pocket is surrounding residues Phe589 and Phe669 (region I), and another is a big hydrophobic back pocket partly occupied by the "tail" 4-chloro-3-(trifluoromethyl) phenyl moiety of **12** (region II) (Fig. 9A). Firstly, they replaced the 4-chloro-3-(trifluoromethyl) phenyl group of **12** with diverse hydrophobic groups to fill the region II pocket. Compound **13**, with a 3-(*tert*-butyl)-1-phenyl-1*H*-pyrazole hydrophobic "tail" interacting with Leu591, Phe675, His677 and Asp697, displays improved activities against TRKA^{WT} and TRKC^{G623R} with IC_{50} values of 35 and 396 nmol/L, respectively (Fig. 9B). Further modifications on the middle phenyl moiety (region I) yielded compound **14** with a *meta*-methyl, which displays low nanomolar inhibitory activities against both WT and various TRK mutants (TRKA^{G595R}, TRKC^{G623R}, TRKA^{G667C} and TRKA^{F589L}) (IC_{50} ranging from 0.5 to 26 nmol/L) in biochemical and cellular levels (Fig. 9A). The binding mode of compound **14** with mutant TRKC$^{G623R/G696C}$ shows that **14** occupies the ATP-binding pocket in a type II mode without any steric hindrances with mutant Arg623 and Cys696 (Fig. 9B). Compound **14** displays evident tumor growth inhibition in Ba/F3-*TEL-TRKA* and Ba/F3-*TEL-TRKC*^{G623R} allograft mouse models without apparent toxicity[94].

Our group has also developed several selective TRK inhibitors in recent years[95–99]. Compound **15** is a potent ABL (IC_{50} = 2.1 nmol/L) and TRKs (IC_{50} ranging from 4.4 to 9.4 nmol/L) inhibitor developed in our laboratory[100]. Switching the methyl group in the middle phenyl moiety from 4′-position to 2′-position effectively improves the TRK selectivity over ABL. The representative compound **16** shows digital nanomolar inhibitory activities against TRKs while sparing ABL (Fig. 10). However, the poor pharmacokinetic properties of **16** hinders its further development[95,101]. In order to exploit novel selective TRK inhibitors, scaffold hopping strategy was employed to obtain a series of imidazo[1,2-*b*]pyridazine derivatives based on **16**. The resulting compound **17** displays nanomolar inhibitory activities against both WT and G667C mutant, but less potent against the G595R mutant (Ba/F3-*CD74-TRKA*^{G595R} IC_{50} = 0.77 μmol/L) (Fig. 10). Structural modeling suggested that the aminopiperidine group of **17** causes steric hindrance with mutated Arg623 (Fig. 11A)[98]. Considering that cyclized conformationally restricted type I inhibitors (*e.g.,* selitrectinib or repotrectinib) effectively overcome SF mutants, a series of macrocycle-based type II molecules with different sizes were designed. Compound **18** bearing an 8-atom linker displays a minor activity improvement against TRKA^{G595R} with an IC_{50} value of 0.68 μmol/L for Ba/F3-*CD74-TRKA*^{G595R} cells. In order to further reduce the steric hindrance with SF mutation, the aminopiperidine of **18** was replaced by a smaller size methylethanediamine yielding compound **19**, which shows increased activity against TRKA^{G595R} with an IC_{50} value of 0.55 μmol/L. **19** with a freely rotating oxygen atom at the linker position effectively prevents intramolecular hydrogen bond between the two amide groups in **18** and maintains key hydrogen bond interactions with Glu560 and Asp668 (Fig. 11B). Introducing extra hydrophilic groups pointing towards the solvent-accessible area generated the representative compound **20**, which demonstrates high potencies towards WT and various TRKs variants with IC_{50} values of 110 and 6 nmol/L against Ba/F3-*CD74-TRKA*^{G595R} and Ba/F3-*CD74-TRKA*^{G667C} cells. The binding model of **20** with TRKA^{G595R} indicated that it occupies the ATP binding pocket with a type II binding mode, the imidazo[1,2-*b*]pyridazine core and carbonyl of amide form key hydrogen bond interactions with Met592 and Asp668, respectively. Methylimidazole ring points to the solvent region and forms an additional $\pi-\pi$ interaction with the flipped Phe669. Methylethanediamine is far away from the mutant Arg595, which can explain the improved potency for the SF mutations (Fig. 11B)[98]. More importantly, such

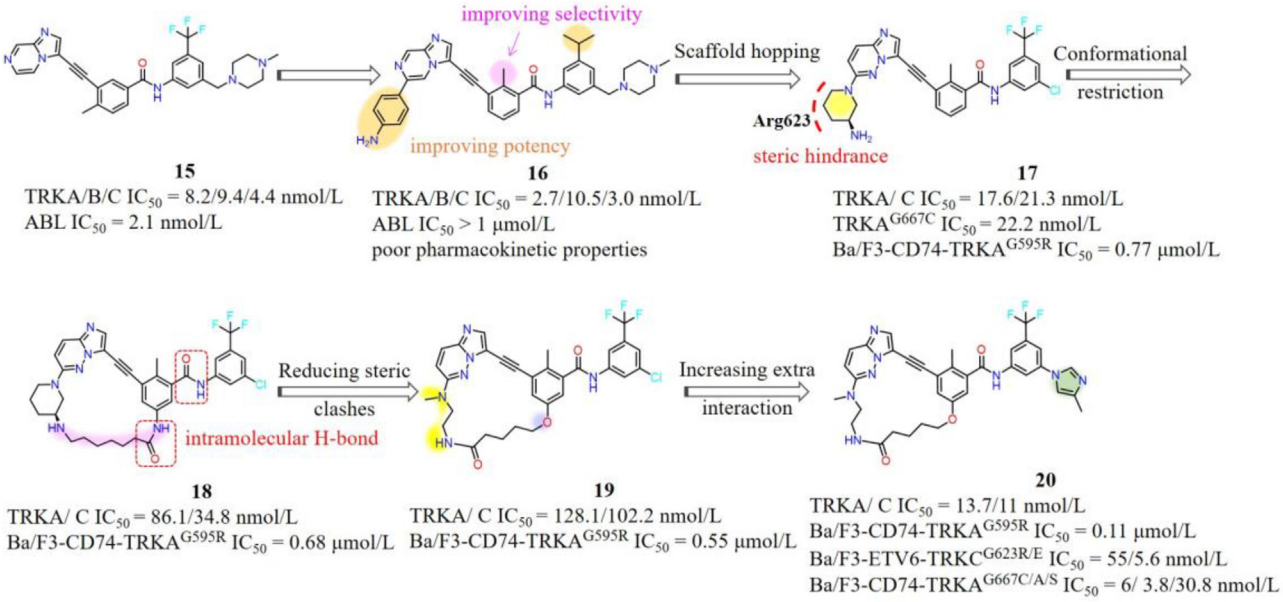

Figure 10 Optimization process of compound **20** and the optimization strategy employed to address the SF mutation.

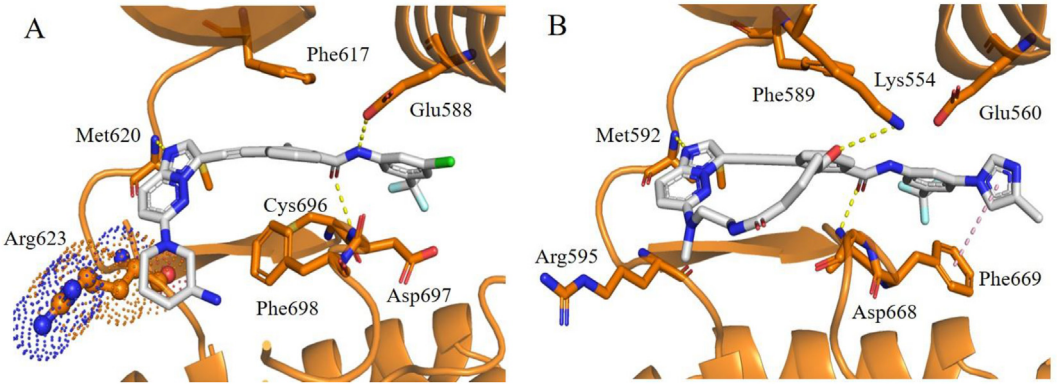

Figure 11 (A) The predicted binding mode of **17** with TRKC^{G623R} (generated from PDB code 6KZD). (B) The predicated binding mode of **20** with TRKA^{G595R} (generated from PDB code 7XAF). Steric clashes are highlighted in spheres. Compounds and key residues are showed in sticks, the hydrogen bonds are presented as yellow dashed lines, and $\pi-\pi$ interactions are presented as pink dashed lines.

first macrocycle-based type II TRK inhibitor shows extraordinary kinome selectivity among 373 wild-type kinases. Further drug-like properties optimizations for this series compounds are still in progress in our lab[98].

Our group also employed hybridization strategy to design a series of 6-(pyrrolidin-1-yl)imidazo[1,2-*b*]pyridazine-based type II TRK inhibitors[99]. Compound **21** is a potent type I TRK inhibitor developed by Novartis, which displays high potency towards WT TRKs but weak inhibitory activity against TRKA^{G667C} mutation (Fig. 12)[102]. Based on the recent studies that TRK xDFG mutations stabilize the DFG-out inactive conformation and sensitize these mutant kinases to type II inhibitors[28,29], we intended to switch this type I inhibitor **21** to a type II inhibitor to overcome xDFG resistances. Superimposition of the X-ray cocrystal structures of **21** and our reported type II inhibitor **22**[95] indicates that the C3 position of pyrrole ring in **21** is an optimal site to introduce a type II "tail" (Fig. 13A). Subsequently, the hybrid compound **23** was yielded by attaching the *N*-(3-((4-methyl-piperazin-1-yl)methyl)-5-(trifluoromethyl) phenyl)formamide "tail" of **22** and removing the fluorophenyl of **21** that is closed to the xDFG site to avoid steric hindrance[99]. Compound **23** exhibits potent kinase inhibitory activities against WT TRKs (IC_{50} ranging from 5.1 to 8.1 nmol/L) as well as TRKA^{G667C} mutation (IC_{50} = 16.5 nmol/L), but moderate cellular inhibitory activity against Ba/F3-*CD74*-TRKA cells (IC_{50} = 61.5 nmol/L). In order to enhance the cellular activities of **23**, different types of classical type II "tails" were employed to replace the initial "tail" of **23**. And the most potent compound **24** displays superior cellular inhibitory activities against xDFG mutations with IC_{50} values of 2.6 and 6.1 nmol/L against Ba/F3-*CD74-TRKA*^{G667C} and Ba/F3-*ETV6-TRKC*^{G696C} cells, respectively, compared to type I-based larotrectinib and selitrectinib. Encouragingly, **24** also shows potent antiproliferation inhibitory activities against SF alterations with IC_{50} values of 31.0 and 28.2 nmol/L towards Ba/F3-*ETV6-TRKC*^{G623R} and Ba/F3-*ETV6-TRKC*^{G623E} cells (Fig. 12). The docking model of **24** with TRKC

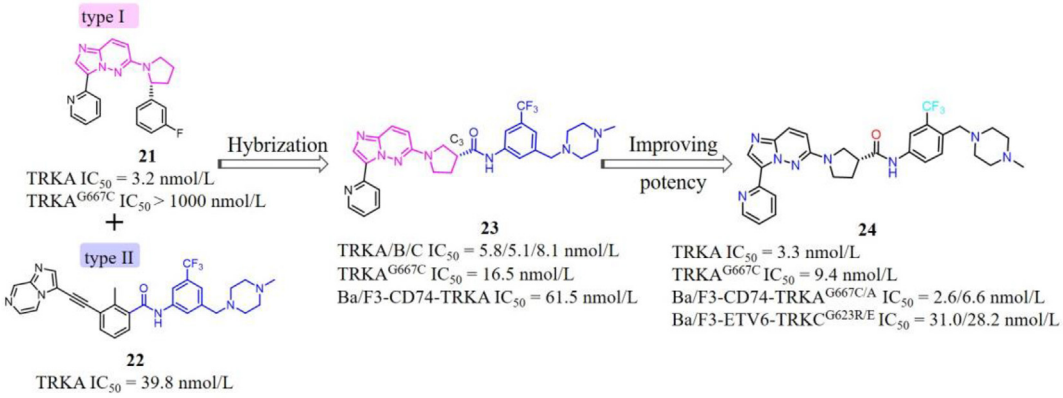

Figure 12 Hybridization of type I TRK inhibitor **21** and type II TRK inhibitor **22** resulted in **24**.

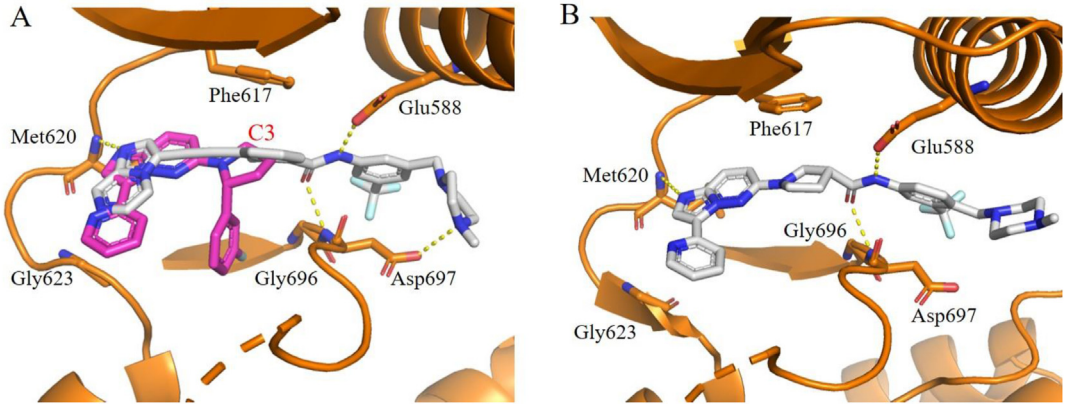

Figure 13 (A) Superimposition of the X-ray cocrystal of compound **21** with TRKA (PDB code 4YNE) and **22** with TRKC (PDB code 6KZC). Compounds **21** and **22** are shown in magenta and gray sticks, respectively. (B) The predicated binding mode of compound **24** complexed with TRKC (docking based on PDB code 6KZC). Compounds and key residues are showed in sticks and the hydrogen bonds are presented as yellow dashed lines.

suggests that it binds to TRKC in a type II mode with the imidazo[1,2-*b*]pyridazine core forming key hydrogen bond with Met620 in the hinge region, the amide group forming two hydrogen bonds with Glu588 in the α-C helix and Asp697 in the DFG motif, respectively (Fig. 13B)[99]. Compound **24** provides a potential novel scaffold for the discovery of potent and selective type II TRK inhibitors to overcome TRK clinical resistances.

Chugai Pharmaceutical Co., Ltd.[103] reported a series of potent type II TRK inhibitors with a unique tetracyclic scaffold. Hit compound **25** was obtained by screening a kinase-inhibitors library, which displayed single digital nanomolar inhibition against TRKs, but moderate antiproliferative activities towards NIH3T3 *MPRIP-NTRK1* cells (IC_{50} = 59 nmol/L) as well as a potential risk of CYP3A4 induction (Fig. 14A). Several reports demonstrated that the CYP3A4 induction is caused by the activation and increased expression of nuclear hormone receptors as well as stabilization interactions of mRNAs or proteins[104–106]. Therefore, they intended to conduct some structural modifications on compound **25** to destabilize interactions with mRNAs and/or proteins to attenuate the CYP3A4 induction. The 8-position morpholinoethoxy group extending to the solvent-accessible region was firstly replaced by different bulkier substituents to generate steric repulsion with mRNAs and/or proteins without losing interactions with TRK. Compound **26** with a morpholine substituent at the 8-position significantly reduces CYP3A4 induction. However, **26** also shows strong inhibitory activity against lymphocyte-specific protein kinase (LCK) (IC_{50} = 22 nmol/L) (Fig. 14A). Subsequently, they aimed to weaken favorable interactions with proteins to decrease the CYP3A4 induction by increasing the rigidification of **25**. Compound **27** with a methanesulfonamide group effectively avoids the CYP3A4 induction and maintains TRK inhibitory activity (IC_{50} = 3.9 nmol/L), selectivity as well as antiproliferation activity towards NIH3T3 *MPRIP-NTRK1* cells (IC_{50} = 75 nmol/L). In order to further improve the potency of **27**, the γ-position cyclopropylmethoxy substituent of pyridine was replaced by *tert*-butyl amide and a methyl was introduced to the α-position of pyridine. The target compound **28** (CH7057288) displays high TRKA inhibitory activity and antiproliferative activity with IC_{50} values of 1.2 nmol/L against TRKA and 8.4 nmol/L against NIH3T3 *MPRIP-NTRK1* cells. The oral administered CH7057288 once a day shows strong antitumor activity in the NTH3T3 *MPRIP-NTRK1*-fusion NSCLC xenograft model with the TGI value of 171%. More importantly, CH7057288 displays comparable antiproliferative inhibitory activity against NIH3T3 *MPRIP-NTRK1*^{G667C} cells (IC_{50} = 1.8 nmol/L) and less potent towards NIH3T3 *MPRIP-NTRK1*^{G595R} cells (IC_{50} = 3.0 μmol/L)[103,107]. The structural modeling of CH7057288 with TRKA^{G667C} indicated that CH7057288 binds to a DFG-out conformation of TRKA with the carbonyl oxygen and pyridine nitrogen forming hydrogen bonds with Met592 and Lys544, respectively. The mutant Cys667 forms favorable sulfur−π interaction with the pyridine moiety, while the

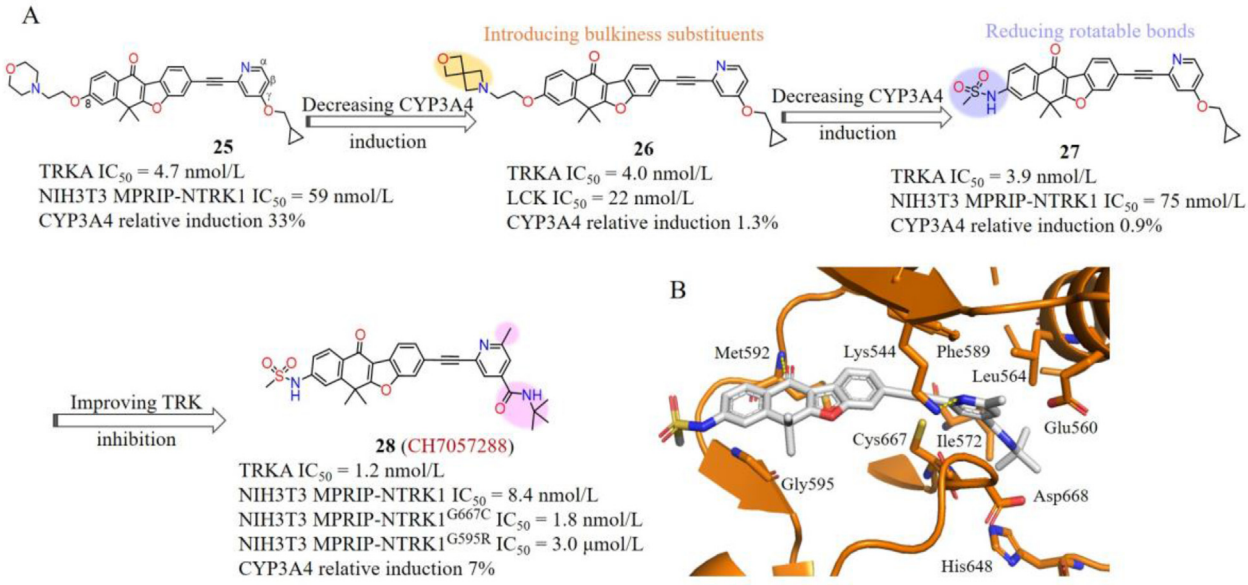

Figure 14 (A) The discovery of CH7057288 and optimization strategy employed to address the CYP3A4 induction. (B) The predicated binding mode of CH7057288 with TRKA^{G667C} (generated from PDB code 5WR7). CH7057288 and key residues are showed in sticks and the hydrogen bonds are presented as yellow dashed lines.

tetracyclic core is very closed to the Gly595, which explains the high potency of CH7057288 against TRKA^{G667C} mutation and weak activity to TRK^{G595R} mutation (Fig. 14B)[103,107].

7. Conclusions and perspectives

Development of new generation TRK inhibitors that can overcome acquired resistance of larotrectinib and entrectinib becomes a research hotspot and unmet clinical need. Large numerous of second-generation macrocyclic inhibitors have been developed and entered in clinical trials. Among them, the representative selitrectinib and repotrectinib successfully combat SF mutations and GK mutations relapsed from the treatment of larotrectinib or entrectinib in patients, but TRK-dependent and TRK-independent resistance still emerge. As TRK-dependent resistance, acquired xDFG mutations are a factor in significant resistance to the second-generation TRK inhibitors. The xDFG mutation can not only cause steric hindrances with the fluoropyrimidine of selitrectinib and the fluorophenyl groups of repotrectinib, but also stable the mutant TRK in a DFG-out inactive conformation, thus limiting the sensitivity of current type I second-generation inhibitors and preferring to type II TRK inhibitors.

Type II TRK inhibitors can be divided into two categories: multi-targeted inhibitors and selective inhibitors. Although several multi-target type II TRK inhibitors can potently combat xDFG variants, side effects caused by substantial off-target inhibition may impede their future development. Currently, a series of selective type II TRK inhibitors with different scaffolds present potent inhibitory activities towards the xDFG mutations *in vitro* and *in vivo*. Among them, compound **14** and the first macrocycle-based type II TRK inhibitor **20** display significant inhibitory activities against both xDFG and SF mutations, presenting promising agents for drug discovery and deserving further investigation.

Despite these significant progresses, several limitations and challenges remain to be addressed. First, there are no second generation TRK inhibitor approved for the treatment of acquired resistance of larotrectinib and entrectinib. Although several type II selective TRK inhibitors have been reported, they are all in early biological investigation for cancer therapy. Further exploitation of potent type II TRK inhibitors with druggability becomes a major research focus. Second, most selective type II TRK inhibitors only overcame xDFG mutations and still showed weak efficacy against SF mutations, such as compounds **11** and CH7057288. In addition, compound mutations also occur in *cis*-form in patients who relapse after the treatment with selitrectinib, but there are no reported inhibitors designed to overcome these mutations. Therefore, there is an urgent need to develop novel TRK inhibitors that can simultaneously overcome major mutations in SF, xDFG and GK. Given that these point mutations are located around the ATP binding pocket, it is suggested that TRK allosteric inhibitors may have the potential to combat existing mutations due to no direct interactions with ATP-binding pocket[108,109]. On the other hand, it is revealed that proteolysis targeting chimeras (PROTAC) is iterative and less susceptible to target overexpression or mutations, which have been widely used in Bruton's tyrosine kinase (BTK) and Bcr-Abl, etc.[110–112]. Currently, a series of TRK allosteric inhibitors have emerged in attempt to develop TRKA selective inhibitors for the treatment of chronic pain based on the NGF-TRKA in pain signaling[113–117]. Several TRK degraders were also disclosed with potent degradation efficacy[118–120]. However, resistant associated activities of these TRK allosteric inhibitors and degraders were not presented currently. It is necessary to launch further studies to evaluate their potency in overcoming TRK clinical acquisition resistant mutations. Lastly, besides on-target resistances, bypass signaling resistances also cannot be addressed by new generation TRK inhibitors and the strategies to solve these off-target resistances also present a greater unmet need.

Currently, the general strategies for overcoming the TRK point mutations include avoiding steric hindrance or increasing additional interactions, *e.g.*, hydrogen bonds or hydrophobic interactions. Forming a covalent bond with a conserved cysteine or arginine is also an effective way to overcome point mutations in

$EGFR^{T790M}$ resistance[121], $KRAS^{G12C}$[122] and $KRAS^{G12R}$[123] mutations. Thus, covalent targeting the mutant residues Cys667 or Arg595 in TRKA may be a novel approach to increase the binding affinity of compounds with mutant kinase. Moreover, it is suggested that the use of computer-aided drug design and the emerging artificial intelligence (AI) generative models may help accelerate the discovery of TRK inhibitors to combat drug resistance. For example, Wang et al.[124,125] utilized a KinaFrag web platform to conduct fragment virtual screening and fragment growing and developed some highly potent TRK inhibitors that effectively overcome resistance mediated by point mutations. To date, the cocrystal structures of TRK xDFG mutations with TRK type II inhibitors have not been resolved, therefore more researches are still needed to disclose the detailed mechanisms of the type II inhibitors to overcome drug resistance.

Acknowledgments

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Author contributions

Xiaoyun Lu conceived the project and supervised the project. Shuang Xiang summed up the literature and drafted the manuscript. All authors approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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