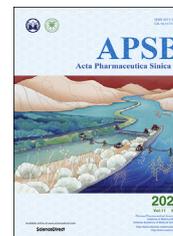




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REVIEW

Development of small-molecule tropomyosin receptor kinase (TRK) inhibitors for *NTRK* fusion cancers



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KEY WORDS

Tropomyosin receptor kinase;
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Abstract Tropomyosin receptor kinase A, B and C (TRKA, TRKB and TRKC), which are well-known members of the cell surface receptor tyrosine kinase (RTK) family, are encoded by the neurotrophic receptor tyrosine kinase 1, 2 and 3 (*NTRK1*, *NTRK2* and *NTRK3*) genes, respectively. TRKs can regulate cell proliferation, differentiation and even apoptosis through the RAS/MAPKs, PI3K/AKT and PLC γ

Abbreviations: AFAP1, actin filament-associated protein 1; AML, acute myeloid leukemia; ARHGEF2, Rho/Rac guanine nucleotide exchange factor 2; BCAN, brevican; BDNF, brain-derived neurotrophic factor; BTBD1, BTB (POZ) domain containing 1; CDK-2, cyclin-dependent kinase 2; CR, complete response; CRC, colorectal cancer; CTCs, sequencing of circulating tumor cells; DFG, Asp-Phe-Gly; DOR, durable objective responses; ETV6, ETS translocation variant 6; EWG, electron-withdrawing group; FDA, U.S. Food and Drug Administration; FISH, fluorescence *in situ* hybridization; GBM, glioblastoma multiforme; HNSCC, head and neck squamous cell carcinoma; HTS, high-throughput screening; ICC, intrahepatic cholangiocarcinoma; IG-C2, Ig-like C2 type I; LMNA, lamin A/C; MASC, mammary analogue secretory carcinoma; MPRIP, myosin phosphatase Rho interacting protein; NACC2, NACC family member 2; NCCN, National Comprehensive Cancer Network; NFASC, neurofascin; NGF, nerve growth factor; NGS, next-generation sequencing of tumor tissue; NSCLC, non-small cell lung cancer; NT3, neurotrophin-3; NTRK, neurotrophic receptor tyrosine kinase; OAK, osteoarthritis of the knee; ORR, overall response rate; PAN3, poly(A) nuclease 3; PPL, perioplakin; PROTAC, proteolysis targeting chimera, QKI, quaking I protein, RABGAP1L; RABGTPase activating protein 1-like, RFWD2; ring finger and WD repeat domain 2, E3 ubiquitin protein ligase; RTK, receptor tyrosine kinase; SAR, structure–activity relationship; SBC, secretory breast carcinoma; SCYL3, SCY1 like pseudokinase 3; SQSTM1, sequestosome 1; TFG, TRK-fused gene; TP53, tumor protein P53; TPM3, tropomyosin 3; TPR, translocated promoter region; TRIM24, tripartite motif containing 24; TRK, tropomyosin receptor kinase; VCL, vinculin; VEGFR2, vascular endothelial growth factor receptor 2.

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tyrosine kinase fusions;
Small-molecule inhibitor;
NTRK fusion cancer

pathways. Gene fusions involving *NTRK* act as oncogenic drivers of a broad diversity of adult and pediatric tumors, and TRKs have become promising antitumor targets. Therefore, achieving a comprehensive understanding of TRKs and relevant TRK inhibitors should be urgently pursued for the further development of novel TRK inhibitors for potential clinical applications. This review focuses on summarizing the biological functions of TRKs and *NTRK* fusion proteins, the development of small-molecule TRK inhibitors with different chemotypes and their activity and selectivity, and the potential therapeutic applications of these inhibitors for future cancer drug discovery efforts.

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1. Introduction

Tropomyosin receptor kinases A, B and C (TRKA, TRKB and TRKC), which belong to the cell surface receptor tyrosine kinase (RTK) family, are encoded by the neurotrophic receptor tyrosine kinase 1, 2 and 3 (*NTRK1*, *NTRK2* and *NTRK3*) genes, respectively¹. TRKs are transmembrane receptor proteins that contain an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain with kinase activity (Fig. 1A)². The

intracellular kinase domain of RTK is structurally conserved, and the kinase domains of TRKA, TRKB, and TRKC have a total sequence homology ranging from 71.9% to 78.3%, with TRKB and TRKC being the most similar homologs³. The main distinction between the three subtypes of the TRKs is the different ligands that activate the receptors. The nerve growth factors binding to TRKA are mainly nerve growth factor (NGF), neurotrophin-7, and neurotrophin-6^{2,4}, while TRKB binds nerve growth factors such as brain-derived neurotrophic factor (BDNF) and

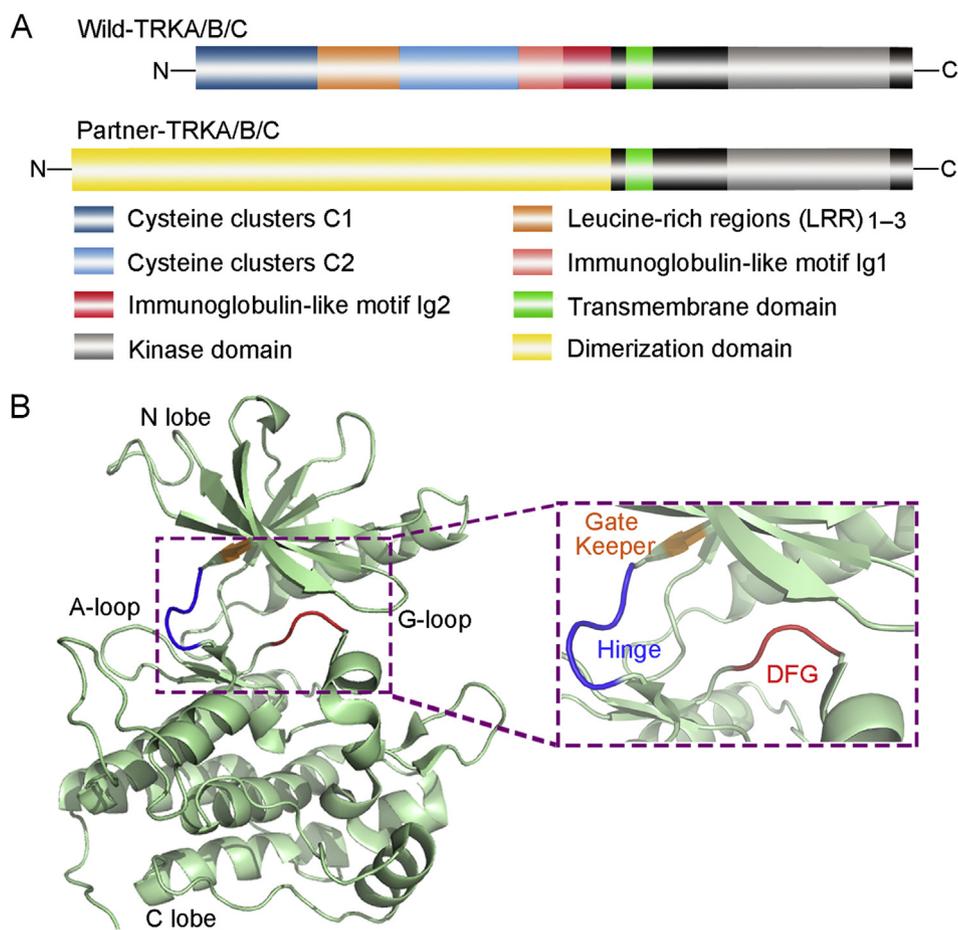


Figure 1 (A) The structure of TRKs and (B) apo crystal structure of TRKA protein (PDB ID: 6D22, 2.5 Å).

neurotrophin-4/5⁵, and TRKC specifically binds to neurotrophin-3 (NT3)⁶. Previous studies confirmed that as nerve growth factor receptors, these TRKs can modulate cell proliferation, differentiation, metabolism, and even apoptosis through the phosphorylation of their downstream matrix targets^{7–9}. TRKs are constitutively activated *via* various mechanisms in malignant human cancers. The most acknowledged mechanism of this is represented by the *NTRK* gene fusions, in which the 3' region of the *NTRK* gene is rearranged intrachromosomally or interchromosomally and then connects with the 5' sequence of the fusion partner gene, leading to a high risk of tumorigenesis¹⁰. Different *NTRK* fusion proteins, *e.g.*, tropomyosin 3-*NTRK1* (*TPM3-NTRK1*) in colorectal cancer (CRC)⁹ and ETS translocation variant 6-*NTRK3* (*ETV6-NTRK3*) in secreted mesoblastic congenital fibrosarcoma and in infantile fibrosarcoma^{11,12}, can mediate the same downstream pathway and increase the oncogenic risk of TRKs through the dimerization/oligomerization of the *NTRK* fusion proteins¹³. Other oncogenic forms of *NTRK*, *e.g.*, activation of the *NTRK1* gene, splice variants and overexpression of TRKs, have also been observed in multiple cancers, including human neuroblastoma¹⁴, acute myeloid leukemia specimens (AML)¹⁵, lung cancer, cylindrical tumor, breast cancer, and others^{16–19}. The activation of TRK receptors caused by *NTRK* fusions is considered as a pan-cancer carcinogenic factor. Because the transmembrane fusions of the TRKs regularly result in a lack of extracellular domains, common targeted therapies (*e.g.*, monoclonal antibody therapy) will not be efficient. Therefore, the main method of targeting *NTRK* fusion genes is the employment of TRK small molecule inhibitors.

Over the past decade, *NTRK* fusion cancers have attracted extensive attention from pharmaceutical companies and

pharmaceutical chemistry researchers, and TRKs have become important antitumor targets with promising research prospects. Consequently, substantial efforts have been made in the development of targeted therapies for this disease, primarily reflected in the emergence of a large number of TRK small-molecule inhibitors, including larotrectinib, entrectinib, belizatinib, AZ23, Cpd5n, PHA-E429, miliciclib, GNF-5837, cabozantinib, sitravatinib, and altiratinib. Among these, larotrectinib (Vitrakvi[®]) and entrectinib (Rozlytrek[®]) as anticancer drugs were approved by the U.S. Food and Drug Administration (FDA) in November 2018 and August 2019, respectively (Fig. 2)^{20,21}. Both of these drugs were approved based upon biomarkers rather than on being tumor-agnostic, which changes the traditional treatment strategy for the primary tumor site and reflects the progress in using biomarkers to guide drug development and more precise drug administration²². In addition, a number of other TRK inhibitors, such as XL184²³, MGCD516²⁴, DS-6051²⁵, TSR-011²⁶, DCC2701²⁷, LOXO-195^{28,29}, and TPX-0005³⁰ (Fig. 2), are currently being evaluated in various phases of clinical trials. Unfortunately, similar to other targeted therapies, acquired drug resistance toward entrectinib in an *NTRK1*-positive CRC patient was reported after four months of treatment, which limited the efficacy of the TRK small-molecule inhibitors^{28,31}. Encouragingly, more effective drugs have overcome the resistance of the first-generation TRK inhibitors. In particular, LOXO-195^{28,29} and TPX-0005³⁰ have shown *in vitro* activity in the low nanomolar range against multiple TRK mutants.

Herein, we summarize the structural characteristics, biological functions, and aberrations of TRKs. In addition, this review aims to present an overview of *NTRK* fusion proteins and a summary of typical TRK inhibitors from the perspective of their activity,

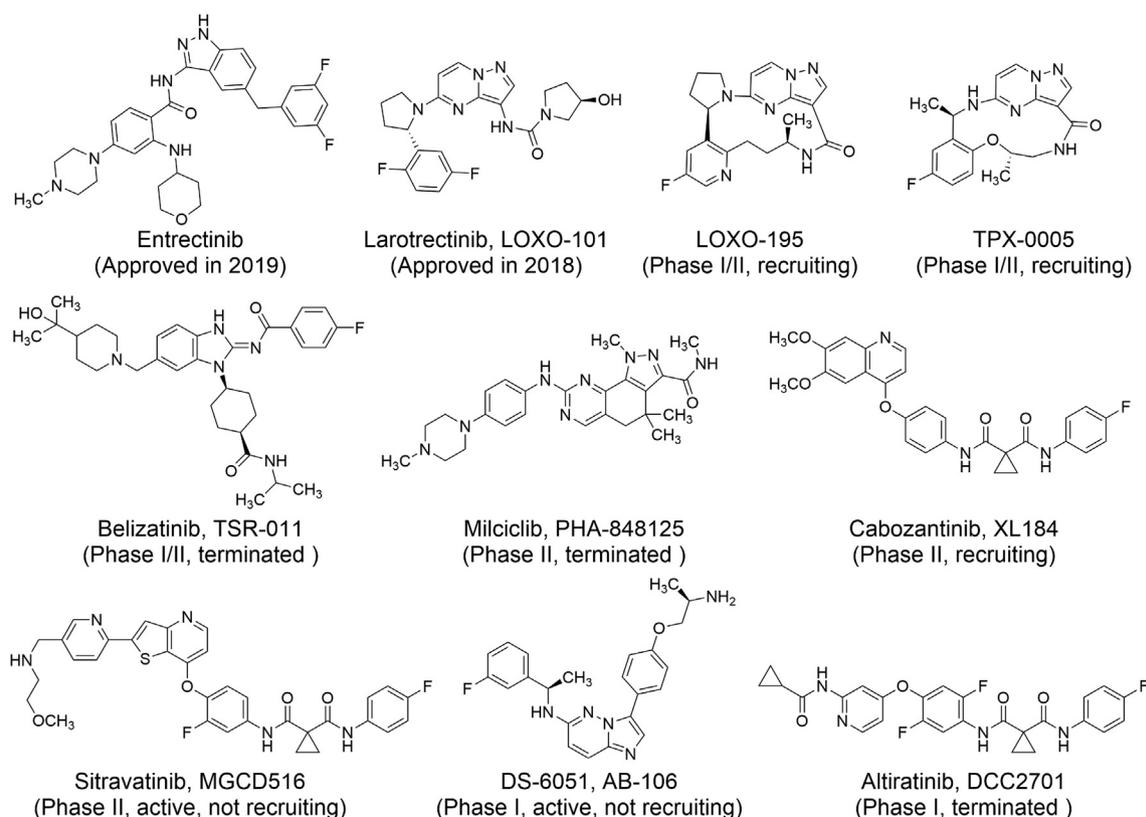


Figure 2 Representative TRK inhibitors in clinical trials and clinic.

selectivity, cocrystal structures and the structural basis of drug resistance, as well as the potential therapeutic strategies against acquired drug resistance.

2. Structures and downstream signaling pathways of TRKs

As summarized in our previous review, a kinase is a part of a signaling cascade, and its main function is to catalyze the diversion of a terminal phosphate group from ATP to a protein substrate³². The catalytic domain of TRKs consists of a small amino terminus (N-lobe) and a large carboxyl terminus (C-lobe) connected by a hinge region; an example of this is shown in Fig. 1B in the apo crystal structure of the TRKA protein. The proximal activation loop is labeled with a conserved Asp-Phe-Gly (DFG) sequence to regulate enzyme activity. This loop is completely ordered in the inactivated state of TRKs and occupies the active site, thereby preventing the binding of ATP³³. In the DFG-out conformation, the phenylalanine of the DFG motif is located in the hydrophobic pocket behind the gatekeeper phenylalanine of the active site. The DFG-in conformation is characterized by a rotation of the DFG residues out of the active site, in which ATP binds to the kinase and subsequently transfers its terminal phosphate group.

Similar to most tyrosine kinases, the receptor–ligand interaction leads to receptor dimerization, autophosphorylation, and the subsequent activation of TRK activity^{34,35}. As shown in Fig. 3, activated kinases trigger the transduction of downstream signaling pathways, leading to cell proliferation, differentiation, and survival³⁶. TRK proteins can activate multiple signaling pathways (Fig. 3). The combination of NGF and TRKA can inhibit apoptotic proteins, promote neuron differentiation and axon growth, and mediate allergic reactions by activating the PI3K/MAPK/PLC γ signaling pathways³⁷. BDNF binds to the TRKB receptor and triggers a variety of downstream signaling cascades, including the RAS/MAPK pathway, the PI3K/3–PDK1/AKT pathway, and the activated PLC γ pathway, causing nerve metabolic cell

proliferation and differentiation³⁸. TRKC mediates NT3 signaling through the PI3K/AKT pathway, thereby increasing cell survival and preventing apoptosis³⁹. However, the TRK signaling pathways have not been fully clarified. There are other ways to activate downstream signaling pathways. For instance, in colon cancer KM12 cells, the abnormal expression of the *TPM3-NTRK1* chimeric protein makes the downstream PI3K/AKT, RAS/MAPK, and PLC γ pathways also abnormally active¹⁶. The TRK fusion proteins will be continuously active, triggering a permanent signaling cascade that drives the proliferation and growth of the TRK fusion tumors.

3. *NTRK* gene expression in related diseases

3.1. *NTRK* mutations, splice variants, and TRK overexpression

It is now well established that TRKs are activated through *NTRK* fusions, mutations, splice variants, and TRK overexpression. These activated TRKs are no longer regulated and controlled by nerve growth factor ligands and have a risk of inducing carcinogenesis. Somatic *NTRK* mutations have been reported in different types of cancers, such as CRC⁹, melanoma⁴⁰, AML⁴¹, large cell neuron cancer and non-small cell lung cancer (NSCLC)^{42,43}. However, the potential role of *NTRK* mutations in promoting tumorigenesis and cancer progression has not been identified. In addition, splice variants that activate *NTRK1* have been detected and characterized. It is reported that the *NTRK1* splice variant TRKAI and the framework deletion mutant (Δ TRKA) have been detected to be carcinogenic in human neuroblastoma⁹ and AML specimens^{44–47}. Finally, the overexpression of TRKs in all kinds of adult and pediatric cancers, including breast cancer, intraepithelial carcinoma, malignant melanoma, lung cancer, and neuroblastoma^{16–19}, can affect the migration of cancer cells. In summary, TRK activated by *NTRK* mutations, splice variants, and TRK overexpression can disrupt the ability to regulate the kinase domains, suggesting that loss of

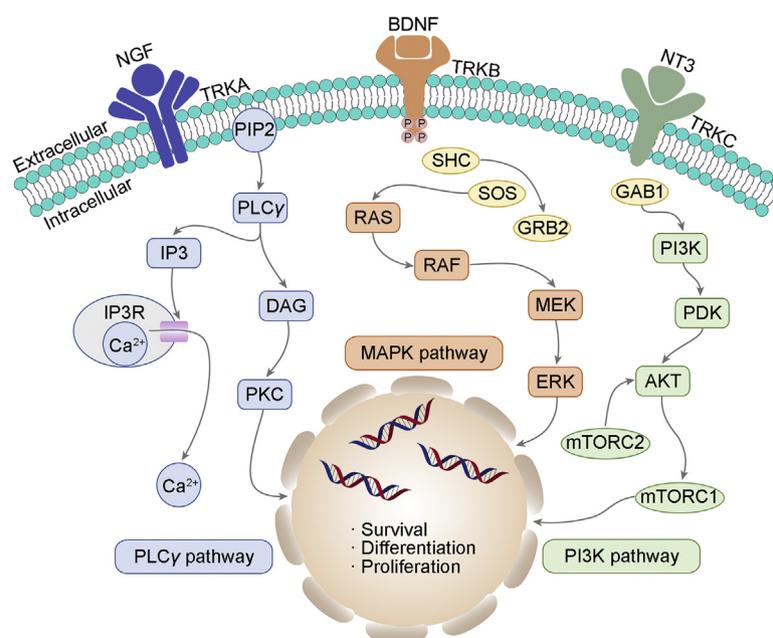


Figure 3 Schematic representation of TRK receptors signaling pathways.

the extracellular domain form or function is a key attribute in TRK's oncogenic potential.

3.2. *NTRK* fusions

Gene fusions involving *NTRK* represent the most common mechanism for TRK activation in malignant tumors⁵. These fusions stimulate the occurrence and development of tumors through the PLC γ , PI3K and RAS/RAF pathways to stimulate uncontrolled signal amplification and transmission^{48–51}. For example, the RAS/RAF pathway has been observed in both CRC (KM-12) and lung cancers (CUTO-3). *NTRK* fusions can be found in adult and pediatric patients with multiple tumor types, but the exact frequency in solid tumors is unknown^{45,52}. In common cancers, including NSCLC, CRC, and others, the incidence of *NTRK* fusion is relatively low, approximately 1%–3%^{45,52}, but in some rare cancers, such as infantile fibrosarcoma, similar to secretory breast cancer, congenital mesoderm nephroma (cell or mixed subtype), and others, the incidence of *NTRK* fusion can reach 90% or higher^{6,53–56}. In general, due to the promotion of tumor formation, *NTRK* fusion genes have been considered as an important mediator of early tumorigenesis and development. To date, approximately 25 *NTRK* fusion proteins have been reported to act as oncogenic drivers in various tumor types, among which *TPM3-NTRK1* and *ETV6-NTRK3* are the two most common variants⁴⁵. Table 1^{11,48,57–80} shows the *NTRK* fusion proteins and their associated cancer types reported to date.

4. TRK inhibitors and their therapeutic implications

The prevalence of *NTRK* gene fusions is estimated to be 17%–20% in cancer^{24,76}. Blocking abnormal TRK signaling pathways through small-molecule inhibitors has been suggested to be a useful method for inhibiting the proliferation of *NTRK* fusion tumor cells, which has boosted the ever-increasing interest of the scientific community in developing drugs to inhibit the TRK domains. Therefore, tremendous efforts have been made over the past decade to develop TRK inhibitors with different chemotypes. TRK small-molecule inhibitors can be classified into three different types, namely type I, II and III, based on their site of binding to the TRKs. Small-molecule inhibitors that target the ATP-binding site of an activated kinase are known as type I kinase inhibitors. Inhibitors that target the kinase ATP-binding site and interact with the adjacent hydrophobic pockets are defined as type II kinase inhibitors. Type III kinase inhibitors are also called allosteric inhibitors. They target hydrophobic pockets distant from the ATP-binding site and regulate kinase activity by causing conformational changes in the ATP-binding pocket. Next, we will introduce the discovery, structure–activity relationship (SAR) and activity of existing inhibitors from the perspective of medicinal chemistry.

4.1. Type I TRK inhibitors

Type I kinase inhibitors with ATP competition can recognize the DFG-in conformation of the kinase. Owing to the highly conservative structure of the ATP-binding sites of all kinases, most of these inhibitors have inhibitory activity against other kinases in the same family. The loss of selectivity for the TRKs directly leads to side effects and the toxicity of these drugs. However, through

Table 1 Reported *NTRK* gene fusion partners and corresponding cancers.

Cancer type	NTRK1	NTRK2	NTRK3	Ref.
CRC	TPM3	–	–	11,57,58
	LMNA	–	–	59
	TPR	–	–	60
	SCYL3	–	–	61
NSCLC	CD74	–	–	48
	MPRIIP	–	–	48
	SQSTM1	–	–	62
	–	TRIM24	–	63
	–	–	–	63
GBM	ARHGEF2	–	–	64
	BCAN	–	–	65,66
	NFASC	–	–	66
	TPM3	–	–	63
	–	–	ETV6	63,64
Pilocytic astrocytoma	–	VCL	–	63
	–	–	BTBD1	63
	–	NACC2	–	67
	–	QKI	–	67
Spitzoid melanoma	TP53	–	–	68
	LMNA	–	–	68
Papillary thyroid cancer	TPM3	–	–	69
	TFG	–	–	70
	TPR	–	–	71
Thyroid carcinoma	PPL	–	–	62
	Large cell neuroendocrine tumor (lung)	RFWD2	–	72
ICC	RABGAP1L	–	–	73
MASC	–	–	ETV6	74,75
SBC	–	–	ETV6	76
Infantile fibrosarcoma	LMNA	–	–	77
	–	–	ETV6	78
HNSCC	–	PAN3	–	79
Mesoblastic	–	–	ETV6	80
Low-grade glioma	–	AFAP1	–	79

–Not applicable.

structural optimization, it is still possible to find potential drugs with a high selectivity for the TRKs.

4.1.1. Approved drugs targeting the TRKs

At present, two TRK inhibitors, larotrectinib and entrectinib, have been approved by the FDA for treating patients with solid tumors that have an *NTRK* fusion without a known acquired drug-resistant mutation. Metastatic or surgical resection may lead to serious morbidity without a satisfactory alternative treatment or progression after treatment^{22,79}. In 2008, by screening with a TRKB scintillation proximity assay, Choi and colleagues⁸¹ identified a TRKB inhibitor (IC₅₀ = 83 nmol/L) with a novel benzonitrile-substituted imidazopyridine core (compound **1**, Fig. 4A). Due to its poor pharmacokinetic properties, it was difficult to undertake clinical trials. Therefore, it was further optimized. According to the SAR analysis, the 3-substitution of the electron-withdrawing group (EWG) on the benzylamine phenyl ring resulted in a 10-folds increase in the titer (Fig. 4A), e.g., 3-F, while the benzene ring on the other side was substituted with a greater tolerance (compound **2**)⁸¹. The structurally rigid benzylamine moiety was then changed in a cyclic manner to reduce the conformational entropy, which further improved the potency. As a result, it was found that the dense five-membered ring and the *R*-configuration showed better efficacy (Fig. 4A, compound **3**)⁸¹. Compound **3**

occupied the front pocket of ATP in the DFG-in conformation and formed a key hydrogen bond with Met592 (Fig. 4B). Derivatization with a cyano group resulted in the report of compound **4** having an effective antiproliferative activity on TRK-transfected BA/F3 and KM-12 cell lines⁸¹. However, compound **4** was not entered into clinical trials due to its poor pharmacodynamics⁸¹. Compound **5** (larotrectinib) showed low nanomolar cell potency against TRKA/B/C with IC₅₀ values of 2–20 nmol/L (Fig. 4A). Moreover, the biochemical and cell experiments illustrated that the selectivity of compound **5** for TRKA/B/C was approximately 100-folds over a panel of 100 kinases derived from various families²¹. Currently, the discovery of compound **5** cocrystal structures with TRKs has not been reported. However, due to the structural similarity of compounds **5** and **4**, we can deduce that

compound **5** has an SAR and crystal-binding mode similar to **4**. Compound **5** (larotrectinib) was the first TRK inhibitor approved by the FDA. A large number of clinical tests, including phase I trials for adults (LOXO-TRK-14001, NCT02122913), phase I/II trials for pediatric patients (SCOUT, NCT02637687), and phase II trials for adults and adolescents (NAVIGATE, NCT02576431), showed that the overall response rate (ORR) based on investigator assessment was 81% (95% CI: 72–88, 95% confidence interval: 72–88), in which 63% of the patients had a partial disease response (PR), and 17% of the patients had a complete response (CR)⁴⁵. Overall, regardless of age or tumor type, larotrectinib has significant and long-lasting antitumor activity in patients with *NTRK* fusion cancers. Larotrectinib is recommended by the National Comprehensive Cancer Network (NCCN) guidelines as a

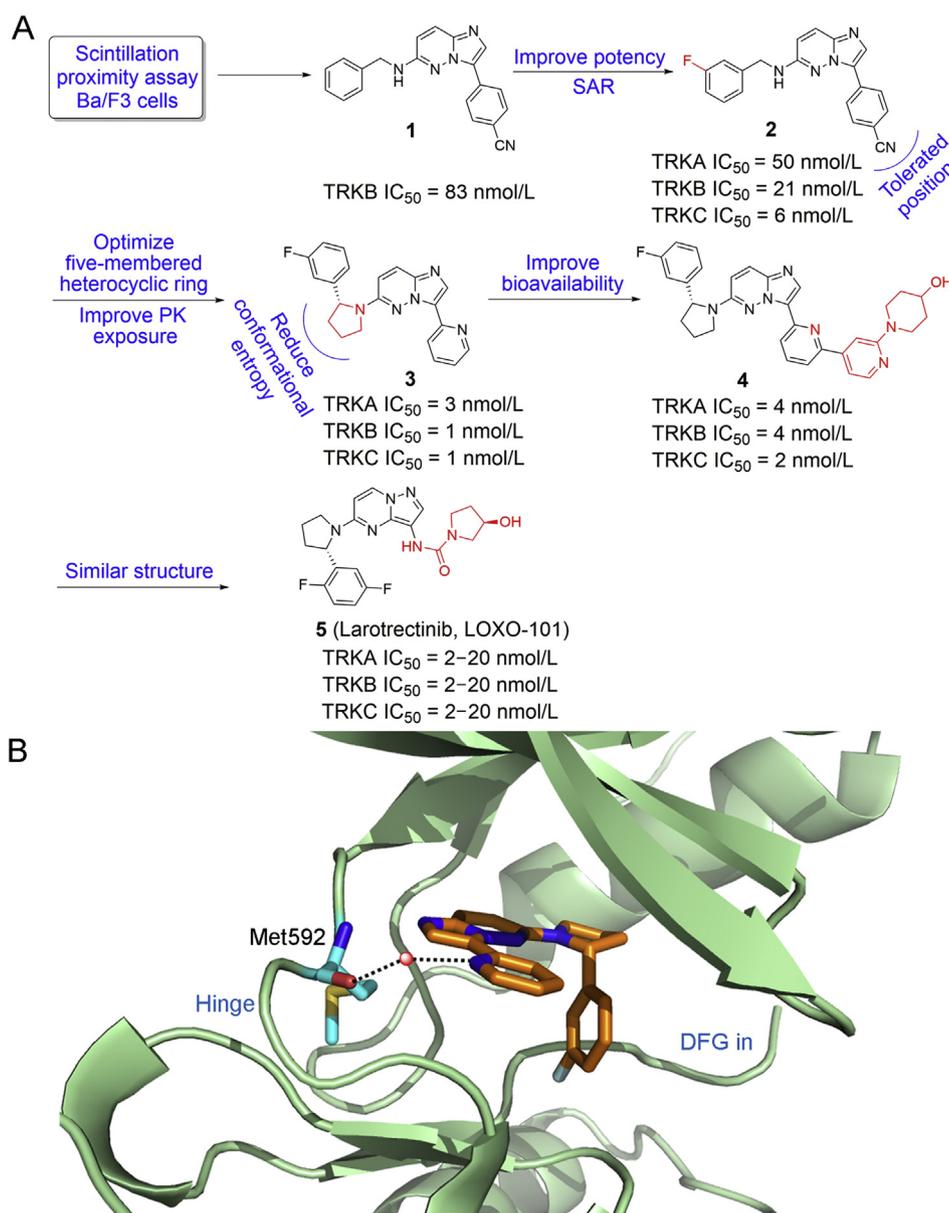


Figure 4 (A) Discovery and design procedure of the TRK inhibitor, larotrectinib, by combing Scintillation Proximity Assay and medicinal chemistry and (B) X-ray cocrystal structure of TRKA with the *R*-enantiomer of compound **3** (PDB ID: 4YNE, 2.0 Å). The kinase is illustrated with green ribbons, and the hydrogen bonds are depicted in black dashed lines. Compound atoms are in different colors: C, orange; N, blue; F, light cyan.

first-line therapy for those advanced NSCLC patients carrying *NTRK* fusions⁸² and as a new second-line therapy for patients with *NTRK* fusions in metastatic CRC⁸³.

Menichincheri and coworkers⁸⁴ reported a potential 3-amino-5-substituted indazole compound **6** (Fig. 5A). Compound **6** showed a good inhibitory activity against ALK ($IC_{50} = 73$ nmol/L) and a moderate antiproliferative activity in ALK-positive Karpas-299 cells ($IC_{50} = 253$ nmol/L)⁸⁴. Further optimization of the ring A substituent resulted in the identification of compound **7** (ALK $IC_{50} = 15$ nmol/L) and compound **8** (entrectinib, ALK $IC_{50} = 12$ nmol/L), of which compound **8** displayed a better cell activity (Fig. 5A). Due to the similar three-dimensional structure and catalytic mechanism of the phosphorylation catalytic region of ALK and TRK, compound **8**, as an ALK inhibitor, also presented a pan-TRK suppressive activity (TRKA/B/C $IC_{50} = 1, 3,$ and 5 nmol/L, respectively)⁸⁵. In the cocrystal complex of TRKA bound with **8** (Fig. 5B), compound **8** occupied the front pocket of ATP in the DFG-in conformation and formed two hydrogen bonds with Glu590 and Met592. Compared with compound **6**, the 4-amino tetrahydrofuran moiety was grafted onto ring A in the most optimal way to fill the sugar pocket of ATP and adopt a substantially orthogonal orientation relative to the scaffold. In the phase II STARTRK-2 trial of compound **8** (NCT02568267), the ORR was 56.9% in patients with *NTRK*

fusion solid tumors, and the intracranial ORR for patients with central nervous system metastases was 50%, with durable objective responses (DOR) of 10.4 months⁸⁶. In view of its potency, high selectivity for TRKs, and favorable pharmacokinetic properties, compound **8** was accelerated for approval by the FDA on August 15, 2019 as a treatment for solid tumors carrying *NTRK*, *ROS1* or *ALK* gene fusions⁸⁶. The launch of larotrectinib and entrectinib is another great example of a basket trial, an innovative clinical trial involving a precision medicine design.

4.1.2. Other type I TRK inhibitors

In 2012, Lewis et al.⁸⁷ employed high-throughput screening (HTS) to obtain a novel benzimidazole compound **9**, which exhibited strong biological efficacy for ALK ($IC_{50} = 3$ nmol/L, Fig. 6A). To enhance cellular viability, selectivity for ALK, and metabolic stability, compound **10** was discovered by further optimization of loop A (Fig. 6A). Compared to compound **9**, the selectivity of compound **10** for ALK was greatly improved. Fig. 6A illustrated that modification of ring B yielded a more metabolically stable compound **11** (belizatinib, TSR-011). As shown in Fig. 6B, the branch structure promoted the dual occupation of the ribocyst and the hydrophobic pocket II through the cyclohexyl and the benzamide motifs⁸⁷. Subsequently, Weiss et al.⁸⁸ found that compound **11** inhibited TRKA/B/C with an IC_{50}

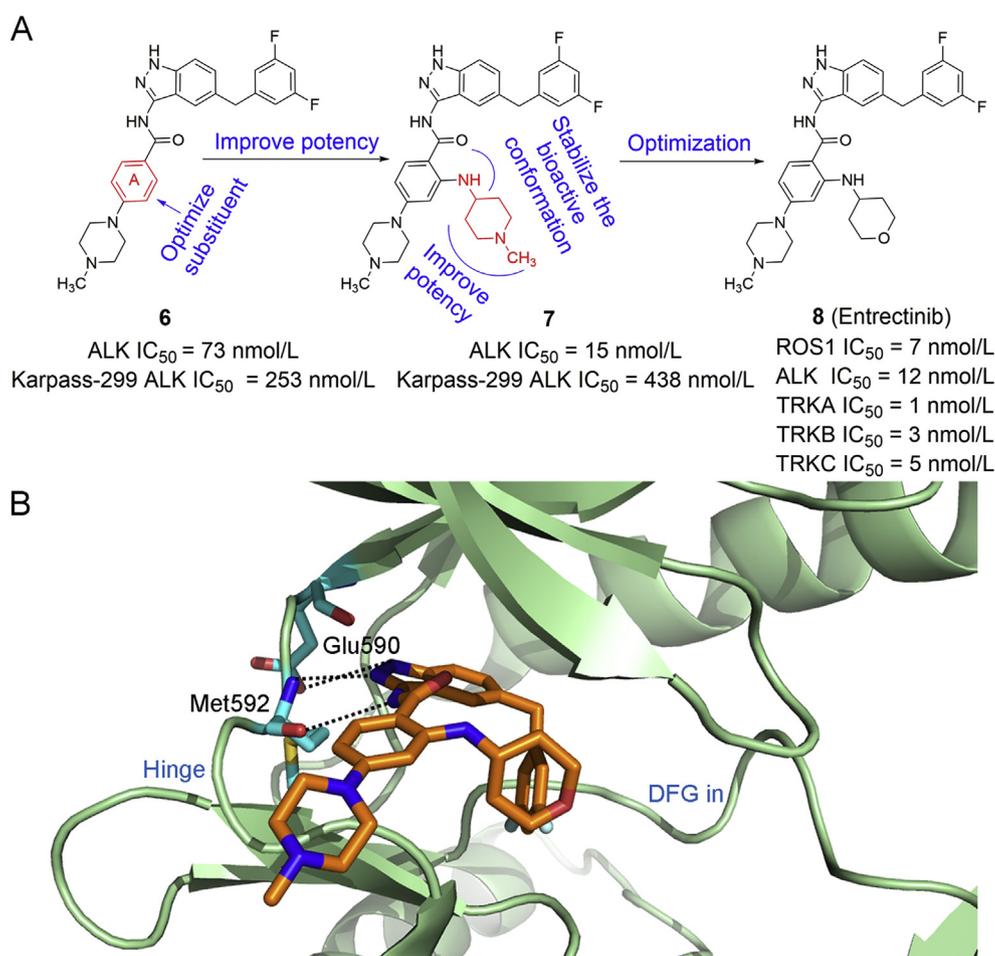


Figure 5 (A) Entrectinib was generated by optimizing the substituent of benzene A in compound **6** and (B) X-ray cocrystal structure of compound **8** in TRKA (PDB ID: 5KVT, 2.5 Å). The kinase is illustrated with green ribbons, and the hydrogen bonds are depicted in black dashed lines. Compound atoms are in different colors: C, orange; N, blue; O, red; F, light cyan.

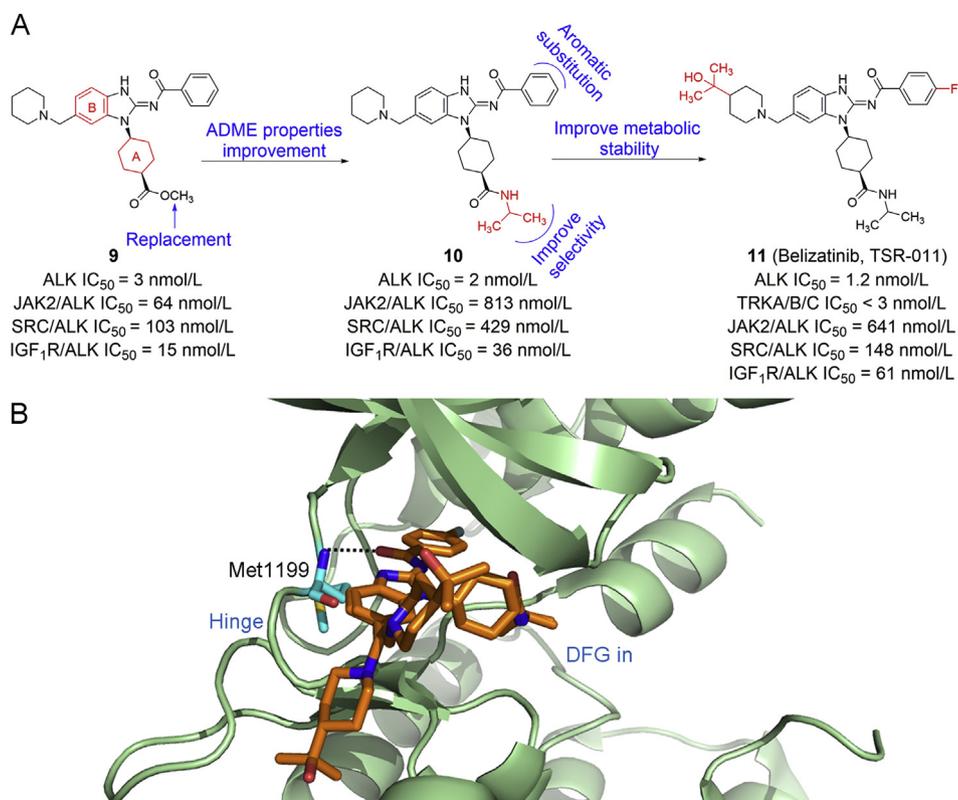


Figure 6 (A) Optimization of ring A and B in compound **9** resulted in the generation of TSR-011 and (B) X-ray cocrystal structure of compound **11** in ALK (PDB ID: 4FOD; 2.0 Å). The kinase is illustrated with green ribbons, and the hydrogen bonds are depicted in black dashed lines. Compound atoms are in different colors: C, orange; N, blue; O, red; F, light cyan.

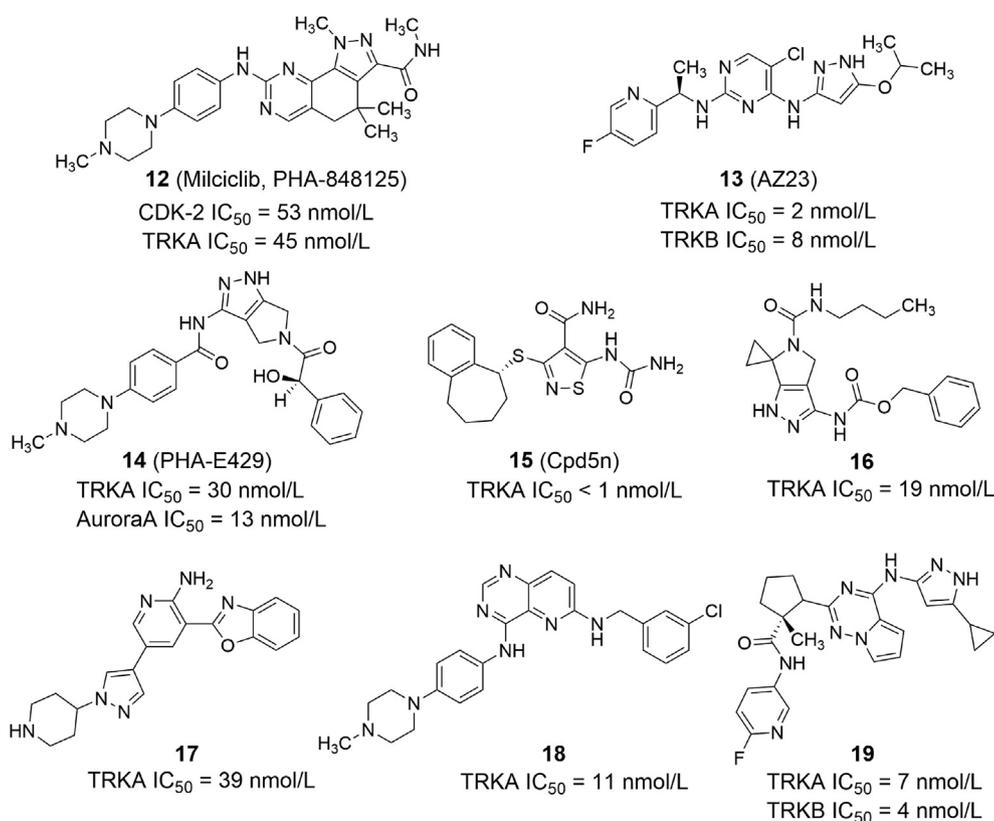


Figure 7 Chemical structures of reported other type I TRK inhibitors and their kinase activity.

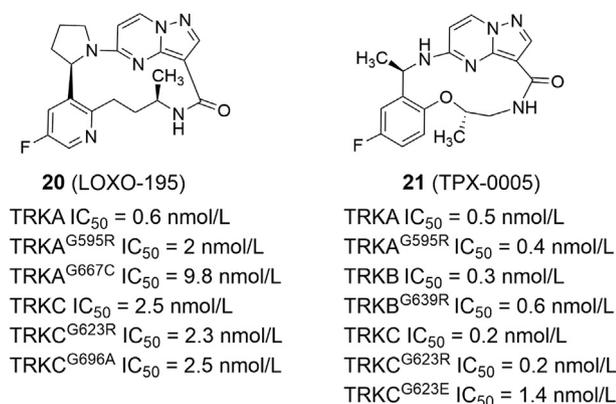


Figure 8 The reported chemical structures of two second-generation type I TRK inhibitors in clinical and their inhibitory activity against TRK mutations.

value below 3 nmol/L. A phase I/II clinical trial (NCT02048488) of belizatinib for the therapy of patients with advanced solid tumors and lymphomas was completed. Due to limited clinical activity, the further development of TSR-011 as an ALK inhibitor was ceased.

Albanese's group^{89–91} reported compound **12** (milciclib, PHA-848125) as a dual inhibitor of cyclin-dependent kinase (CDK-2)/TRKA (Fig. 7), which can effectively inhibit NGF-induced TRKA phosphorylation and downstream signaling in DU-145 human prostate cancer cells with IC₅₀ values of 53 and 45 nmol/L, respectively. Phase II clinical trials (NCT01011439 and NCT01301391) of milciclib for therapy for malignant thymoma were terminated. Thress's research group⁹² discovered a series of 4-aminopyrimidine compounds as highly selective and potent TRKA/B inhibitors. Representative compound **13** (AZ-23, Fig. 7) effectively inhibited TRKA and TRKB with IC₅₀ values of 2 and 8 nmol/L, respectively⁹². Through screening, Fancelli et al.⁹³ identified a series of 5-phenyl acetyl 1,4,5,6-tetrahydropyrrolo [3,4-*c*]pyrazoles, which are dual inhibitors of the TRKA/cell cycle kinases (Aurora). The IC₅₀ values of representative compound **14** against TRKA/Aurora were 30 and 13 nmol/L, respectively (Fig. 7). In Fig. 7, compound **15** is an isothiazole derivative screened by HTS, which has picomolar activity against kinases (TRKA IC₅₀ < 1 nmol/L) and nanomolar activity against cell growth driven by TRKs (IC₅₀ = 7 nmol/L)⁹⁴. Compound **16** is a type I TRK inhibitor, and its IC₅₀ value was 19 nmol/L. SAR studies have shown that benzyl cyclopropyl carbamate and benzyl carbamate are crucial for the potency of these compounds⁹⁵. Han et al.⁹⁶ reported benzoxazole-substituted aminopyridine nuclear compound **17** as a c-Met inhibitor. Kinase profile analysis showed that compound **17** inhibited TRKA with an IC₅₀ of 39 nmol/L (Fig. 7). Stachel et al.⁹⁷ devoted themselves to designing valid TRK inhibitors on the basis of a pyrido [3,2-*d*]pyrimidine core scaffold, of which compound **18** (Fig. 7) exhibited a favorable biochemical activity (TRKA IC₅₀ = 11 nmol/L). Compound **19** (Fig. 7), developed by Carboni⁹⁸, is a dual inhibitor of IGF₁R/IR. It was subsequently identified as active against TRKA/B (IC₅₀ = 7 and 4 nmol/L). All of these type I kinase inhibitors have nanomolar-level kinase inhibitory activity. Due to their low selectivity within the same family of kinases, their use for specific treatment is limited. However, their pan-inhibitory activity also promotes their treatment of broad-spectrum tumors and provides an impetus for their development.

4.1.3. Second-generation type I TRK inhibitors

Similar to many other metastatic cancers, the cancers caused by *NTRK* fusions will relapse repeatedly, leading to acquired resistance, which necessitates the choice of new targeted therapies. For example, drug tolerance to larotrectinib is caused by three diverse mutations, including solvent frontal mutations (*NTRK1* p. G595R, *NTRK3* p. G623R), a gatekeeper mutation (*NTRK1* p. F589L), and 3-*x*-DFG (*NTRK1* p. G667S, *NTRK3* p. G696A) mutations¹⁷. The structural model predicted that each mutation directly interfered spatially with the binding of larotrectinib and other TRK inhibitors, thereby reducing their inhibitory abilities^{11,28}. It is encouraging that the second-generation TRK inhibitors **20** and **21** (LOXO-195 and TPX-0005) are undergoing clinical studies to overcome these recurrent resistance mutations (Fig. 8). Both compounds were designed as fused macrocyclics to decrease the superficial area in contact with the active site, thereby reducing unnecessary interaction with the binding pocket^{28,29}. Compound **20** achieved low nanomolar inhibitory activity against TRKA^{G595R}, TRKA^{G667C}, TRKC^{G696A} and TRKC^{G623R} (IC₅₀ = 2, 9.8, 2.3 and 2.5 nmol/L, respectively)²⁸. Compound **21** can effectively inhibit ROS1^{G2032R} (IC₅₀ = 8.4 nmol/L), TRKA^{G595R} (IC₅₀ = 0.4 nmol/L), TRKB^{G639R} (IC₅₀ = 0.6 nmol/L), TRKC^{G623R} (IC₅₀ = 0.2 nmol/L) and TRKC^{G623E} (IC₅₀ = 1.4 nmol/L)²⁹ (Fig. 8). Undoubtedly, both drugs also had potent inhibitory activity against the wild type protein and had little selectivity between wild type and mutant TRKs.

Blue medicines disclosed a series of pyrazolopyrimidine compounds in the patents WO201703554 and WO2017087778, wherein compounds **22** and **23** (Fig. 9) exhibited inhibitory activity on TRKA and TRKA^{G595R} in the nanomolar range^{99,100}. The pyrazolopyrimidine compound **24** (Fig. 9) reported by Chia Tai Tianqing Pharmaceutical¹⁰¹ was very similar to larotrectinib, and it demonstrated effective inhibitory activity against TRKA/B/C (TRKA/B/C IC₅₀ < 1 nmol/L), as well as TRKA^{G667C} mutant. Fochon Pharmaceuticals¹⁰² also covered a series of pyrazolopyrimidine compounds in patent WO2019174598. The representative compound **25** (Fig. 9) indicated nanomolar inhibition against TRKA^{G595R} and TRKC^{G623R} in cellular assay¹⁰². Hitgen¹⁰³ disclosed imidazopyridazine compound **26** in patent WO2019120267. Compound **26** was similar to LOXO-195 and TPX-0005, all of which were macrocyclic compounds. Compound **26** showed good inhibitory activity in the assay of mutant cell activities of TRKA^{G595R}, TRKA^{F589L} and TRKC^{G623R} (IC₅₀ = 1, 2.3, and 2.4 nmol/L, Fig. 9)¹⁰³. In view of second-generation type I inhibitors reported in recent years, most of them have effective inhibitory effects on TRKA mutants. However, compared with first-generation type I inhibitors, their structures are relatively simple and lack of diversification. Therefore, the second-generation inhibitors still have a broad development prospect.

4.2. Type II TRK inhibitors

Type II inhibitors can bind to the DFG-out conformation of the kinase and thereby stabilize it. Similar to type I inhibitors, type II inhibitors not only bind to the ATP-binding site in the kinase but also extend the interaction to an allosteric site. The DFG motif can be rotated to an "outward" state to access its allosteric site, thereby opening the adjacent hydrophobic pockets. These inhibitors with heterocyclic or aromatic heterocyclic structures can form one or two hydrogen bonds with the hinge region, and its extended structures can occupy exposed allosteric sites. Type II TRK inhibitors have a higher safety and selectivity for the TRKs

than the type I inhibitors, due to the non-conservative allosteric sites.

4.2.1. Type II TRK inhibitors in clinical trials

Albaugh et al.¹⁰⁴ screened a promising oxindole compound **27** (Fig. 10A), which showed excellent inhibitory activity against B3/F3-Tel-TRKA/B/C with an IC₅₀ value below 60 nmol/L. Compound **28**, obtained by replacing the amide with urea, had a higher cell potency and selectivity for the TRKs (Fig. 10A)¹⁰⁴. The methyl group and fluorine were introduced into the middle benzene ring and the terminal benzene of compound **28** to form compound **29** (GNF-5837). Fig. 10A illustrates that compound **29** had pan-TRK inhibitory activity (IC₅₀ values towards TRKA/B/C were 11, 9, and 7 nmol/L, respectively), and showed good selectivity in the human kinase¹⁰⁴. The cocrystal complex of **28** in TRKC was obtained and is shown in Fig. 10B. Compound **28** was immobilized to the hinge region *via* two key hydrogen bonds between the pyrrole nitrogen and the backbone of residues Tyr619 and Met620. The urea functional group engaged in a strong hydrophobic interaction through Asp697 of the DFG motif¹⁰⁴.

Compound **30** (cabozantinib, XL184), with preferential activity for c-Met/vascular endothelial growth factor receptor 2 (VEGFR2), was subsequently identified as an effective TRKB inhibitor (IC₅₀ value was below 20 nmol/L)¹⁰⁵. Currently, compound **30** is undergoing multiple phase II trials (NCT01639508), including for NSCLC with the RET fusion and for cancers with other gene fusions (including *ROS1*, *NTRK*, *MET*, or *AXL*, Fig. 11). Patwardhan et al.²⁴ discovered a dicarboxamide derivative compound **31** (sitravatinib, MGCD516), which was initially identified as a multiple kinase inhibitor for UFO, VEGFR2, FLT3, and c-KIT. Subsequently, it was demonstrated that the IC₅₀ values for TRKA/B were 5 and 9 nmol/L (Fig. 11). Furthermore, compound **31** is currently in a phase I clinical study for treating

advanced solid tumor malignancies (NCT02219711). Compound **32** (altiratinib, DCC2701), identified by Smith and coworkers²⁷ in 2015, showed a high potency against TRKA/B/C, with IC₅₀ values of 0.85, 4.6, and 0.83 nmol/L, respectively (Fig. 11). Notably, compound **32** can cross the blood–brain barrier, which enables the capacity for treating brain cancer and brain metastatic cancer. A phase I study of compound **32** for the therapy of locally advanced tumors and metastatic solid tumors was terminated (Fig. 11), but the results have not been announced. Compound **33** (GZ-389988), a novel, potent, selective, and locally delivered TRKA inhibitor, effectively suppressed TRKA/B/C with IC₅₀ values of 0.3, 0.1, and 0.5 nmol/L, respectively¹⁰⁶ (Fig. 11). Additionally, a phase II study of GZ-389988 for treating osteoarthritis (NCT02845271) was completed. The results showed that GZ-389988 can reduce pain significantly and has an acceptable safety profile. Skerratt et al.¹⁰⁷ reported the pan-TRK inhibitor **34** (PF-06273340) through HTS of TRKA/B cells and SAR (IC₅₀ values towards TRKA/B/C were 6, 4, and 3 nmol/L, respectively). A phase I clinical trial was completed for assessment of the analgesic effects of PF-06273340 in healthy volunteers using evoked pain endpoints. The result demonstrated for the first time that the use of pan-TRK inhibitors translated non-clinical effects into humans in the pharmacodynamic endpoint of inflammatory analgesia.

4.2.2. Other type II TRK inhibitors

Compound **35**, according to the 4-aminopyrrolopyrimidine core scaffold, was initially reported as an effective oral bioavailable c-Met inhibitor by Vaishnavi et al.⁴⁸ (Fig. 12). Enzyme- and cell-based assays indicated that compound **35** displayed potent inhibition of TRKA/B/C, ROS1 and AXL. Compound **36** (CE-245677) was a dual inhibitor of TIE-2 and TRKA, with IC₅₀ values of 3.7 and 4 nmol/L, respectively (Fig. 12). Nevertheless,

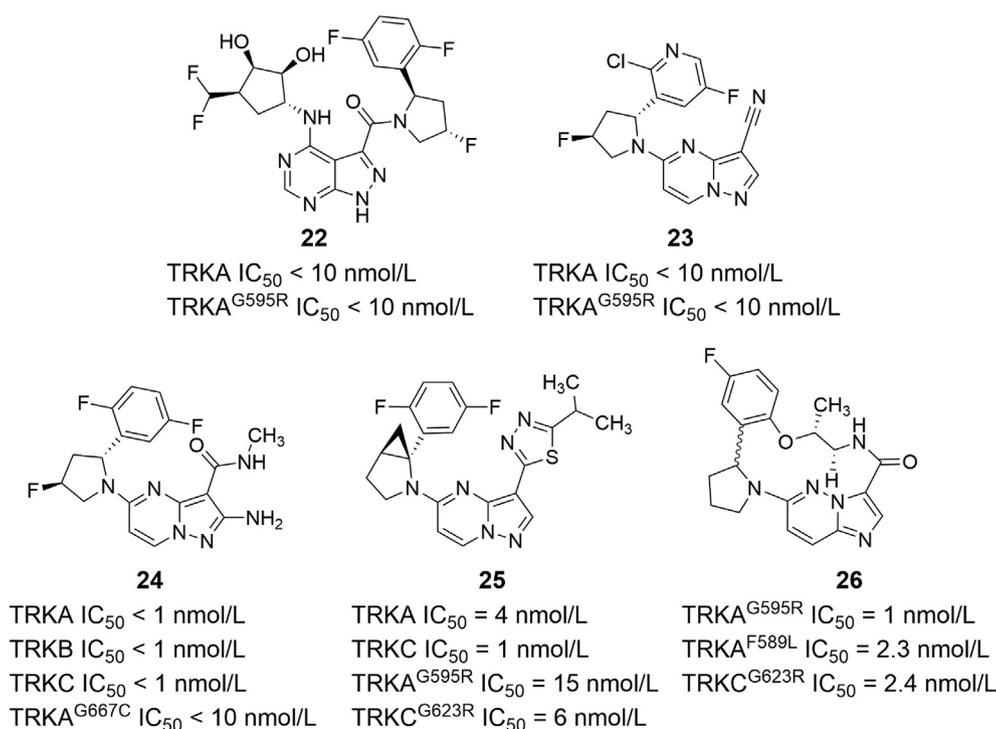


Figure 9 Chemical structures of reported other second-generation type I TRK inhibitors and their kinase activity.

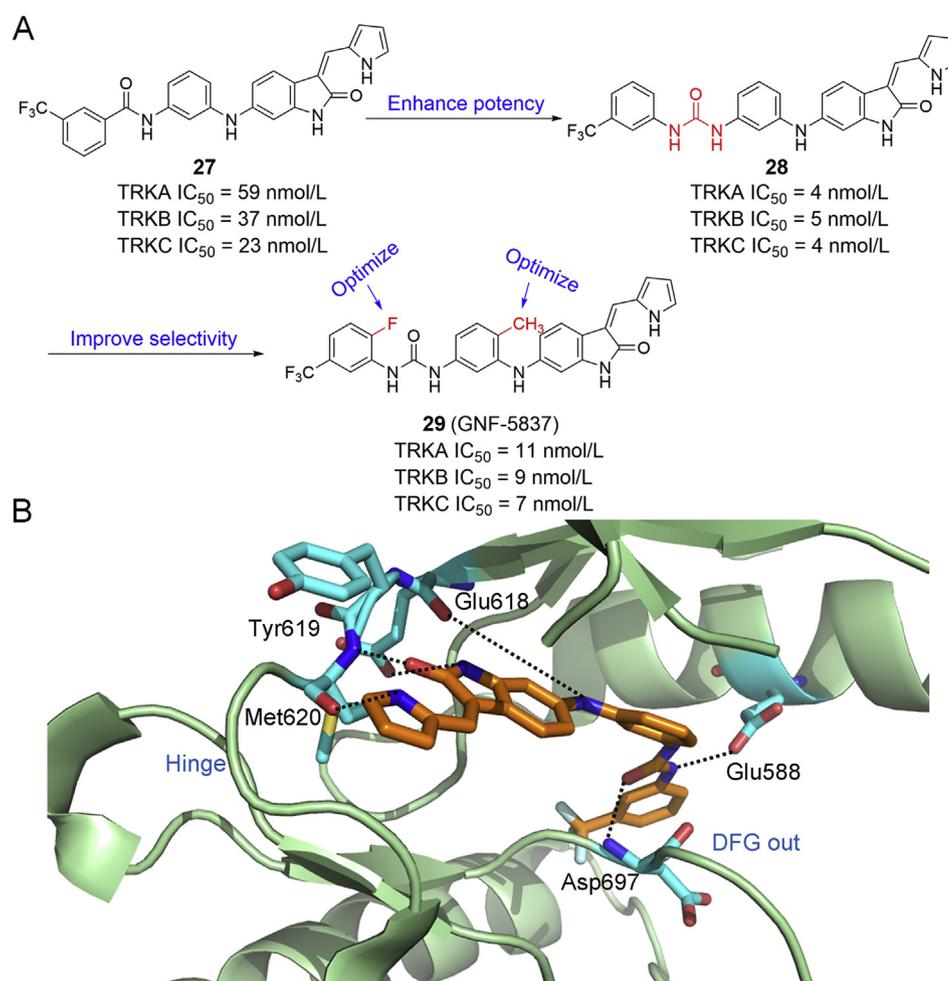


Figure 10 (A) Discovery and optimization procedure of compound **27** to **29** and (B) cocrystal structure of compound **28** in TRKC (PDB ID: 3V5Q; 2.2 Å). The kinase is illustrated with green ribbons, and the hydrogen bonds are depicted in black dashed lines. Compound atoms are in different colors: C, orange; N, blue; O, red; F, light cyan.

further research on compound **36** was unfortunately prohibited in a phase I clinical trial because of the severe central nervous system adverse events^{107,108}. EI Zein's group¹⁰⁹ developed a benzothiazole derivative **37** as a multiple kinase inhibitor, which displayed a good inhibitory activity against VEGFR2, ABL, and TRKA/B. The IC₅₀ values of TRKA/B/C were 1 nmol/L (Fig. 12). Compound **38** with a novel core scaffold, shown in Fig. 12, was obtained by HTS from a compound database⁹⁷. Of particular note, compound **38** (Fig. 12) showed a low nanomolar IC₅₀ value against TRKA in both enzyme activity and cell viability assays (the TRKA enzymatic IC₅₀ value was 9 nmol/L and the cellular IC₅₀ value was 7 nmol/L). Compound **38** bound to multiple sites across TRKA in a unique pattern, in which the imidazole formed a key hydrogen bond with the hinge, and the pyrazole ring occupied the hydrophobic pocket. The same binding mode was observed in compound **39** reported by Wang¹¹⁰. It exhibited excellent efficacy in enzyme- and cellular-based assays, with a TRKA enzymatic IC₅₀ value of 662 nmol/L and a cellular-based IC₅₀ value of 47 nmol/L (Fig. 12)¹¹⁰. Hong and colleagues¹¹¹ screened the promising 3,5-disubstituted-7-azaindole compound **40**, which demonstrated favorable biochemical viability against TRKA/B (IC₅₀ values of TRKA/B were 3.8 and 4.4 nmol/L, respectively, Fig. 12).

Aminopyrimidine derivative **41** was obtained through medicinal chemistry efforts (Fig. 12). It is a dual inhibitor for TIE-2 and TRKA (the IC₅₀ values of TIE-2 and TRKA were 5 and 8 nmol/L, respectively)¹¹². Through HTS, Stachel et al.¹¹² identified the TRKA inhibitor **42** (TRKA kinase IC₅₀ = 11 nmol/L, cellular IC₅₀ = 3 nmol/L, Fig. 12). Unlike typical type II inhibitors, the azaindole ring of compound **42** formed weaker hydrogen bonds with the hinge region. In addition, the most important interactions occurred in adjacent allosteric pockets.

4.2.3. Second-generation type II TRK inhibitors

In the patent WO2017073706, the Chugai Pharmaceutical Co.¹¹³ disclosed a novel pan-TRK inhibitor **43** (CH7057288, Fig. 13A) derived from dihydronaphthobenzofuran. The cocrystal structure of TRKA in complex with compound **43** revealed that compound **43** bonded to the DFG-out of TRKA, where picoline entered the allosteric pocket and interacted with Lys544 (Fig. 13B)¹¹³. Compound **43** possessed an excellent inhibitory effect on TRKA^{G667C} mutation resistant to entrectinib, while also remaining a favorable inhibitory activity against TRKA/B/C¹¹⁴. Furthermore, compound **43** showed strong *in vivo* growth inhibition in a

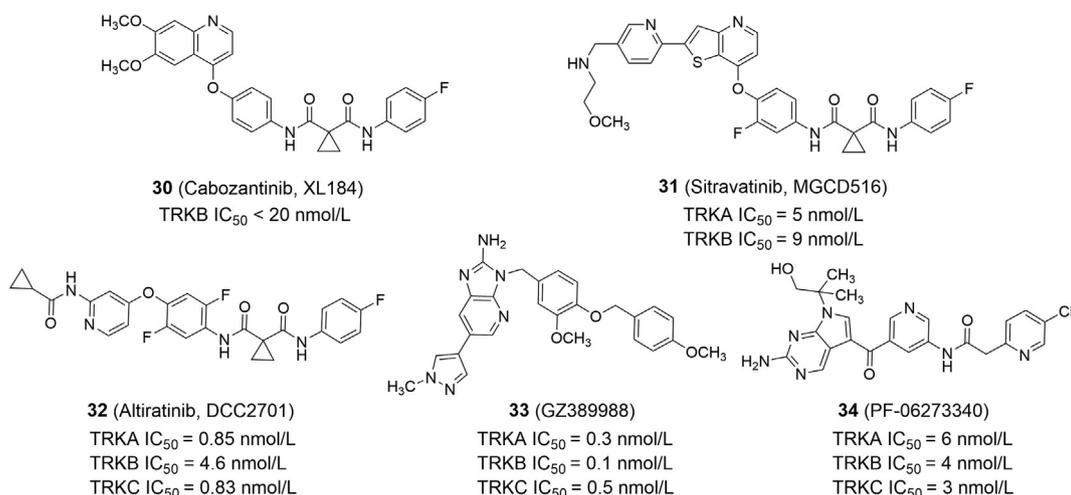


Figure 11 Type II TRK inhibitors in clinical trials and their kinase activity.

mouse xenograft tumor model¹¹⁴. Taken together, compound **43** has potential for further development.

4.3. Type III TRK inhibitors

The type III inhibitors bind to the allosteric site of the kinase and regulate the binding between the ATP and kinase, maintaining its

inactive state by inducing the conformational change. This type of molecule binds to a specific site on the kinase, *i.e.*, the near-membrane region. The near-membrane region of TRKA is shorter than in TRKB/C, and there are many different amino acid residues in the region where the ligand interacts with the protein. Therefore, this area provides new ideas for the development of selective TRKA inhibitors.

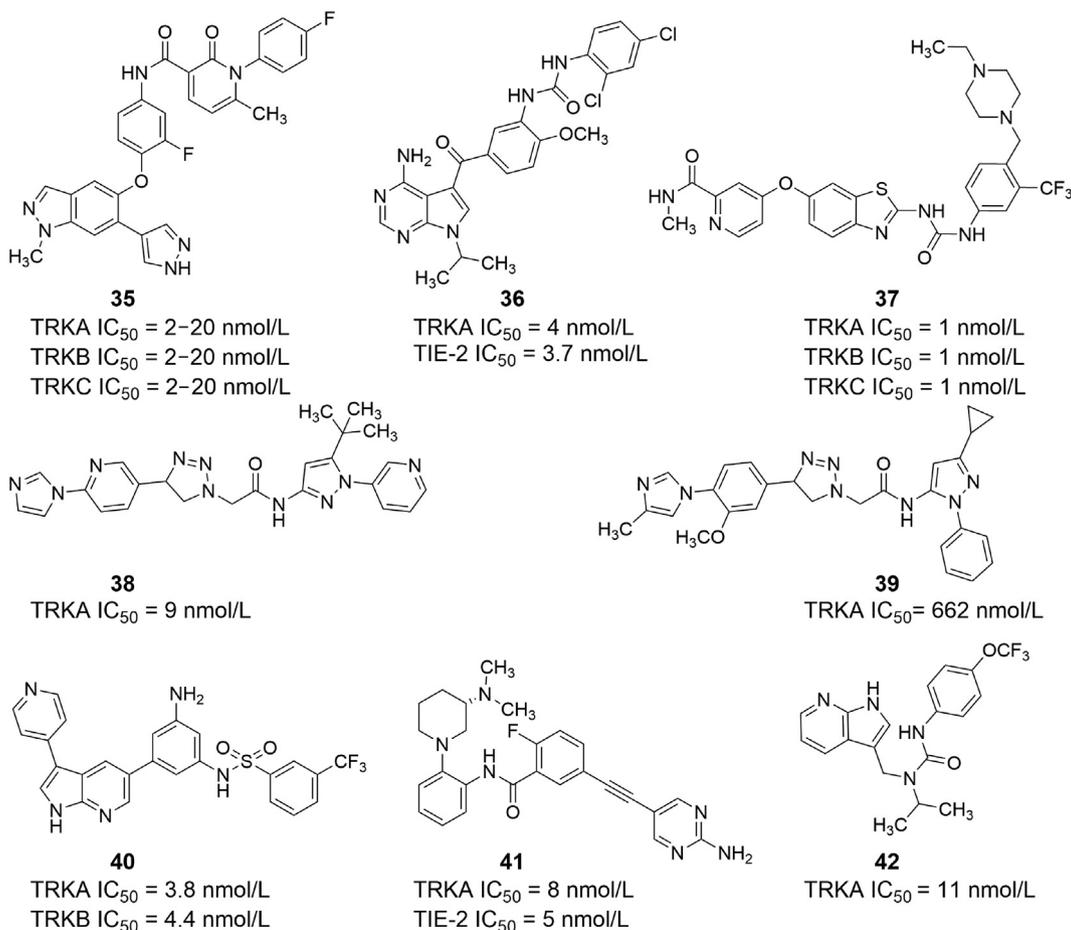


Figure 12 Other type II TRK inhibitors in preclinical trials and their kinase activity.

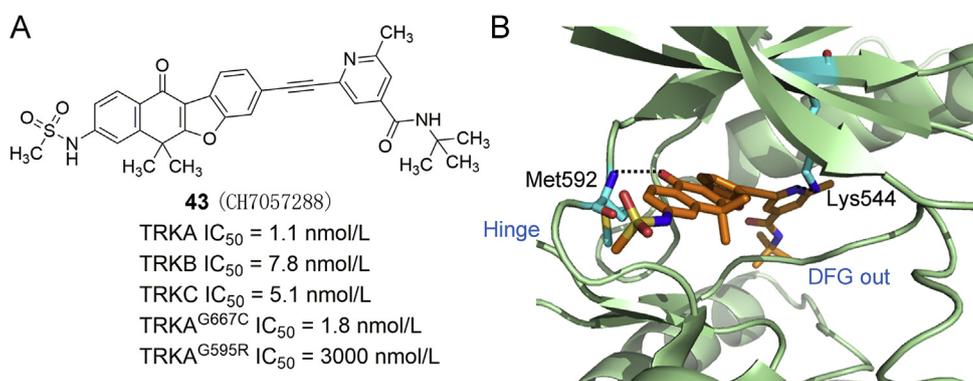


Figure 13 (A) Chemical structure of compound **43** and (B) cocrystal structure of compound **43** in TRKA (PDB ID:5WR7; 2.8 Å). The kinase is illustrated with green ribbons, and the hydrogen bonds are depicted in black dashed lines. Compound atoms are in different colors: C, orange; N, blue; O, red.

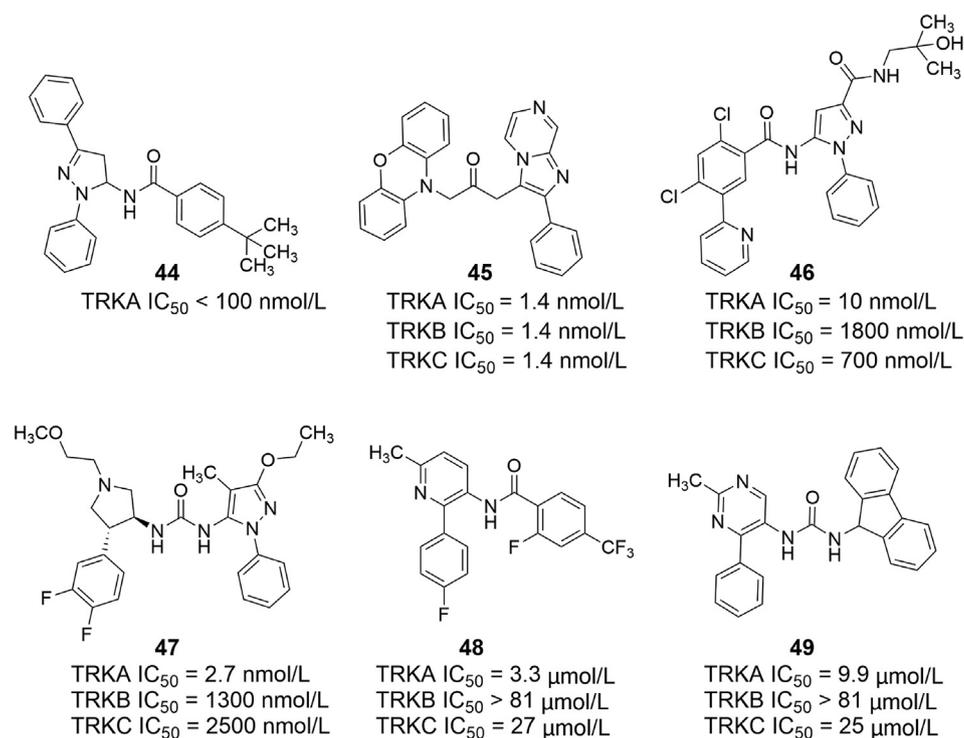


Figure 14 Chemical structures of other type III TRK inhibitors and their kinase activity.

4.3.1. Type III TRK inhibitors in clinical trials

Allosteric inhibitors may alleviate the challenge of acquired resistance on account that their unique binding pattern can circumvent obstructive kinase domain mutations. To date, some allosteric TRKA specific inhibitors have entered clinical trials, but their structures have not yet been disclosed. VM-902A participated in the short phase IIa clinical trial (NCT02847702) as an oral analgesic for treatment of patients with osteoarthritis of the knee (OAK), but the trial was terminated three months later¹¹⁵. VMD-928 was a dual allosteric and irreversible TRKA selective inhibitor. It is currently in a phase I clinical trial (NCT03556228) for the treatment of solid tumors or lymphomas and patients

driven by *NTRK1* and its drug-resistant mutants¹¹⁶. The allosteric TRKA inhibitor AK1830 (ARRY-954) developed by Array BioPharma is currently in phase I clinical trials for the inflammation therapy¹¹⁵.

4.3.2. Other type III TRK inhibitors

Subramanian et al.¹¹⁷ screened a promising benzamide derivative **44** from the ZINC database by HTRF analysis and then further improved its poor physical properties (such as solubility, Fig. 14). Compound **45** (TRKA/B/C IC_{50} = 1.4 nmol/L) was obtained through scaffold hopping and further SAR analysis (Fig. 14)¹¹⁸. Compound **45** bound in the conformation of DFG-out at the

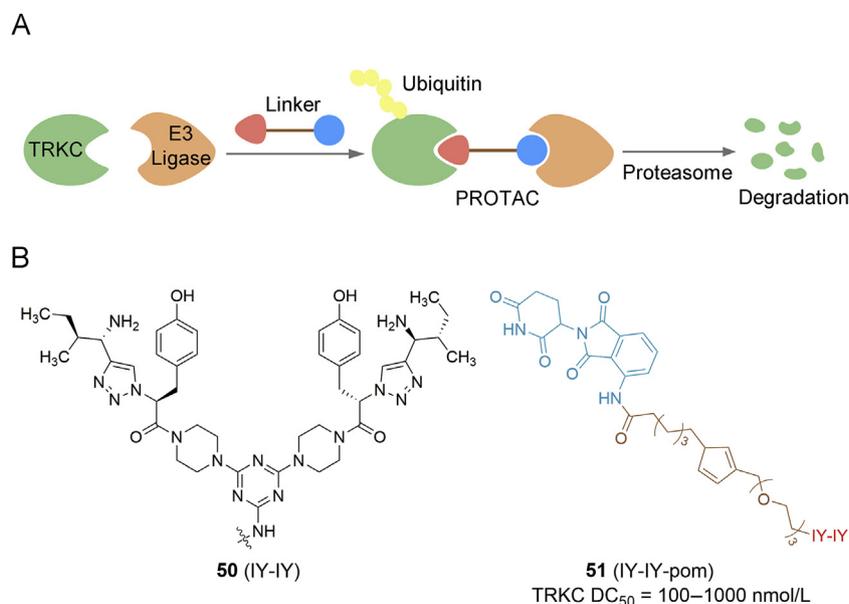


Figure 15 (A) The strategy of TRKC degradation and (B) chemical structures of the reported TRK PROTAC.

allosteric site and away from the ATP-binding site. The cocrystal structure of compound **45** and TRKA showed that the amide NH of compound **45** generated a key hydrogen bond interaction with D668 of the activation ring. In addition, the carbonyl group also had direct hydrogen bonding with I490 near the membrane region. Currently, Skerratt et al.¹⁰⁷ have discovered an *N*-phenyl-pyrazole derivative **46**, a highly selective TRKA inhibitor (Fig. 14). Compound **46** exhibited excellent TRKA inhibitory activity (the TRKA IC₅₀ value was 10 nmol/L), and TRKA had a nearly 100-folds selectivity over TRKB and TRKC (the IC₅₀ values of TRKB and TRKC were 1800 and 700 nmol/L, respectively)¹⁰⁷. Allen¹¹⁹ disclosed a novel TRKA inhibitor **47** in US2012158413A2. Compound **47** (Fig. 14) had a certain selectivity for different TRK isoforms and showed a favorable TRKA inhibitory activity (the IC₅₀ values of TRKA/B/C were 2.7, 1300, and 2500 nmol/L, respectively)¹¹⁹. Su et al.¹²⁰ employed an affinity ligand recognition mass spectrometry (ALIS) strategy to screen the promising benzamide derivative **48** (Fig. 14), which has a certain

selectivity for TRKA (TRKA IC₅₀ = 3.3 μmol/L, TRKB IC₅₀ > 81.0 μmol/L, TRKC IC₅₀ > 27.0 μmol/L). Compound **49**, another highly selective TRKA inhibitor (Fig. 14), was screened by Su et al.¹²⁰ via the same strategy. Compared with TRKB and TRKC, its selectivity for TRKA was more than 2500-folds (TRKA IC₅₀ = 9.9 μmol/L; 8182-folds and 2525-folds selectivity over TRKB/C, respectively).

4.4. Proteolysis targeting chimeras (PROTACs) of TRKs

At present, PROTAC technology has been extensively used in drug development^{121–123}, but TRK PROTACs have been rarely reported. The strategy of TRKC degradation is shown in Fig. 15A. In 2019, Zhao et al.¹²⁴ developed a TRK PROTAC (compound **51**, IY-IY-pom) by conjugating the small-molecule fragment **50** (IY-IY) to TRKC with the CRBN ligand pomalidomide (Fig. 15B). Compound **51** can induce effective TRKC degradation at 1–10 μmol/L, with a DC₅₀ value (50% concentration of protein

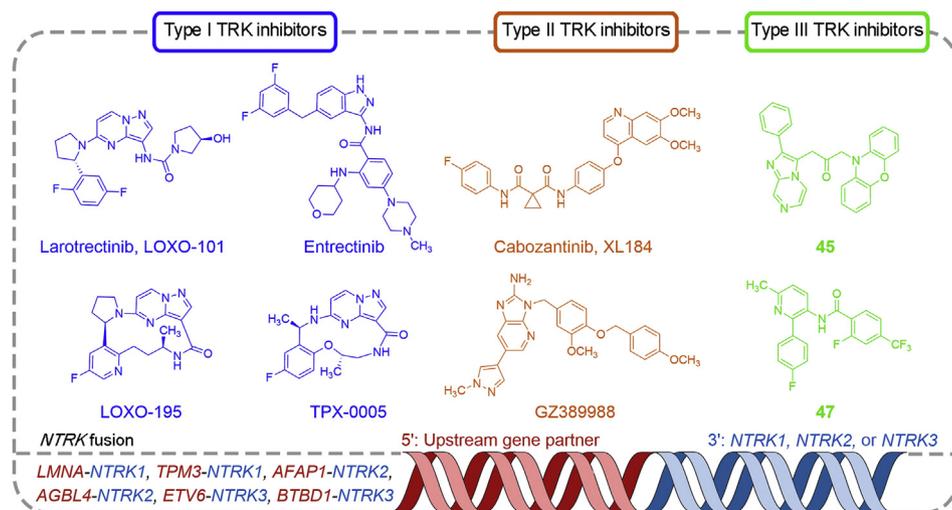


Figure 16 Representative small-molecule inhibitors targeting TRKs for the treatment of *NTRK* fusion cancers.

degradation) of approximately 100–1000 nmol/L¹²⁴. Although compounds that induce the degradation of TRKs need further exploration, TRK PROTACs have apparently provided a new strategy for targeted TRK therapies.

5. Concluding remarks and future perspectives

Of note, *NTRK* fusions may act as drivers in a variety of adult and pediatric cancers, which are inhibited by small-molecule inhibitors of TRKs (Fig. 16). Several small-molecule compounds targeting TRKs are currently used in preclinical and clinical trials (Fig. 16). Most of the kinase inhibitors developed for TRKs display their remarkable therapeutic potential in clinical trials but exhibit an inferior selectivity toward any of the three subtypes (TRKA, TRKB, and TRKC). In contrast, inhibitors with a high selectivity for a single TRK isoform can be further investigated on TRK biological functions. Moreover, it would be beneficial to utilize highly selective inhibitors with a single TRK isoform to determine the importance of subtypes and to elucidate interactions in preclinical TRK models. At present, the first-generation TRK inhibitors, larotrectinib and entrectinib, have demonstrated their remarkable antitumor activities in patients of different ages and with different cancer tissues but having *NTRK* fusion mutations.

However, similar to other targeted treatments, the occurrence of acquired (secondary) resistance limits the potency of TRK inhibitors. Although targeted resistance mediated by mutations in the TRK domains can be eliminated using the second-generation TRK inhibitors, the treatment of acquired resistance with TRK inhibitors remains an ongoing challenge. Inspired by the resistance of other kinases, dual-blocking TRKs and other kinases have been demonstrated to be a new alternative for a drug-resistant treatment approach. In addition, the combination of TRK inhibitors with chemotherapy or immunotherapy is yet another promising therapeutic method to combat drug resistance, but it is worth noting that additional toxicity should be avoided. In particular, the PROTAC technology has also been applied to develop TRK drugs. Compounds promoting degradation with a similar mechanism to PROTAC are also an active and quickly advancing research direction, which opens a new window for targeted TRK therapy.

At present, more accurate and efficient detection technologies, such as fluorescence *in situ* hybridization (FISH), next-generation sequencing of tumor tissue (NGS), and sequencing of circulating tumor cells (CTCs), may provide more accurate methods for detecting *NTRK* fusions and serve as complementary strategies to optimize the current treatments. With the development of computational biology technology, computer-aided drug design has been able to participate in the identification of protein residues in drug development and to gradually clarify the binding characteristics of existing drugs¹²⁵. Additionally, it is beneficial to develop novel TRK drugs and explore drug-resistance mechanisms based upon the sequence, structure and kinetic methods. In addition, a comprehensive understanding of the functions of TRKs in cancer therapies and more accurate detection means for *NTRK* fusions are critical for improving clinical treatments. We believe that more TRK inhibitors would enter clinical trials and become therapeutic applications, and the issue of drug resistance would be addressed soon with a further understanding of TRK intracellular signaling pathways and drug-resistance mechanisms.

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Author contributions

Liang Ouyang and Yi Chen conceived the project and supervised the project. Tingting Jiang and Guan Wang summed up the literature and drafted the manuscript. Yao Liu was involved in drawing the figures. Lu Feng and Meng Wang collected and organized the inhibitors. Guan Wang and Jie Liu proofread the structures and figures. Liang Ouyang and Yi Chen revised the manuscript. All authors approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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