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Targeting the Tropomyosin Receptor Kinase (TRK) Family: Opportunities and Challenges for Development of Cancer Targeted Therapeutics

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Abstract: The use of kinase inhibitors in cancer has been heavily pursued since the discovery and development of imatinib. Annually, it is estimated that around ~20,000 new cases of TRK cancers are diagnosed, with the majority of cases exhibiting a TRKA mutation. In this perspective article, we will thoroughly discuss the opportunities and challenges for the development of TRK-targeted cancer therapeutics (1) The biological background and significance of the TRK kinase family, (2) A compilation of known pan-TRK and TRK selective inhibitors with emphasis on TRKA, (3) Analysis of TRK crystal structures as well as TRK/inhibitor co-crystal structures, (4) Insights into pan-TRK and TRKA selective inhibitor design, and (5) Future perspectives for drug discovery and development of TRK inhibitors.

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Introduction

The TRK (tropomyosin receptor kinase) family of enzymes are transmembrane, receptor tyrosine kinases (RTK) that regulate synaptic strength and plasticity in the mammalian nervous system.[1-7] In this role, the TRK family has the potential to regulate cell differentiation, proliferation, and survival.[8-20] There are three members of the TRK family: TRKA (encoded by *NTRK1* gene), TRKB (*NTRK2*), and TRKC (*NTRK3*), all of which have been implicated to drive initiation and progression of malignancies.[21-38] Similar to the BCR-ABL gene fusion product that drives chronic myelogenous leukemia (CML),[39] *NTRK* rearrangements and fusion gene products have been observed in roughly 19 different tumor types.[40] Unlike CML, however, the incidence of *NTRK* fusion genes in each specific tumor type, in general is rare. This generates profound difficulties for patient identification and for recruitment of patients for clinical experimentation. For instance, *NTRK2* gene fusions have been identified in 0.2% of lung adenocarcinoma,[41] which represents approximately 184 patients of 92,138 diagnosed in 2010 in USA.[42] On the other end of the spectrum, *NTRK3* fusion genes have been identified in virtually all secretory breast carcinomas and of mammary analogue secretory carcinomas (MASC), an extremely rare tumor of the salivary (in general, of the parotid) gland.[43] In fact, the defining characteristic of MASC, when compared to other salivary carcinomas, is an *NTRK* gene fusion.[43] In addition, *NTRK* fusions are found in about 50% of pediatric diffuse intrinsic pontine glioma and non-brainstem glioblastoma.[44] Finally, similar to RET (rearranged during transfection) (another receptor tyrosine kinase), *NTRK* fusions (particularly ETV6-NTRK3) are common in post-Chernobyl radiation-induced papillary thyroid carcinomas.[45-46]

When an *NTRK* gene fusion occurs, the translocation event generates a hybrid oncogene composed of the active TRK kinase domain linked to an unrelated gene. This event triggers constitutive activation or overexpression of the TRK protein, which has oncogenic potential.[47] In major cancer subgroups, *NTRK* fusions occur in 3.3% of lung cancers,[41, 48] 2.2% of colorectal cancers,[41, 48-51] 16.7% of thyroid cancers,[41, 52-53] 2.5% of glioblastomas, and 7.1% of pediatric gliomas.[40, 54] The majority of *NTRK* fusion genes have been identified through next generation sequencing techniques and are likely to be actionable oncogenes based on preclinical data.[40] Thus, targeting TRK oncogenes is an attractive therapeutic approach for a diverse set of cancers.

The primary method employed to target TRK oncogenes is the use of small molecule kinase inhibitors. Because gene fusion products are the major oncogenes observed in TRK-driven tumors, other targeting strategies, such as antibody therapy, will not be effective since transmembrane tyrosine kinase fusions can lack the extracellular domain (Figure 1).[55] In this

case, the fusion genes localize in the cytosol and are particularly susceptible to small molecule inhibition.[56-58] In general, small molecules are designed to target the adenosine triphosphate (ATP) binding site of the TRK kinase to block catalytic activity. This is based on the principle that protein kinases catalyze a rapid phosphoryl transfer to a downstream substrate, and only have micromolar affinity for ATP.[59] Therefore, since ATP turnover is expeditious and kinase affinity for ATP is nominal, small molecules can efficiently regulate catalytic activity of TRK kinases. Because of the high drugability of the TRK enzyme class, a number of attempts to target TRKs have been completed, with inhibitors developed for pan-TRK activity or specificity for a particular isoform. In this perspective article, we will thoroughly discuss the opportunities and challenges for the development of TRK-targeted cancer therapeutics (1) The biological background and significance of the TRK kinase family, (2) A compilation of known pan-TRK and TRK selective inhibitors with emphasis on TRKA, (3) Analysis of TRK crystal structures as well as TRK/inhibitor co-crystal structures, (4) Insights into pan-TRK and TRKA selective inhibitor design, and (5) Future perspectives for drug discovery and development of TRK inhibitors.

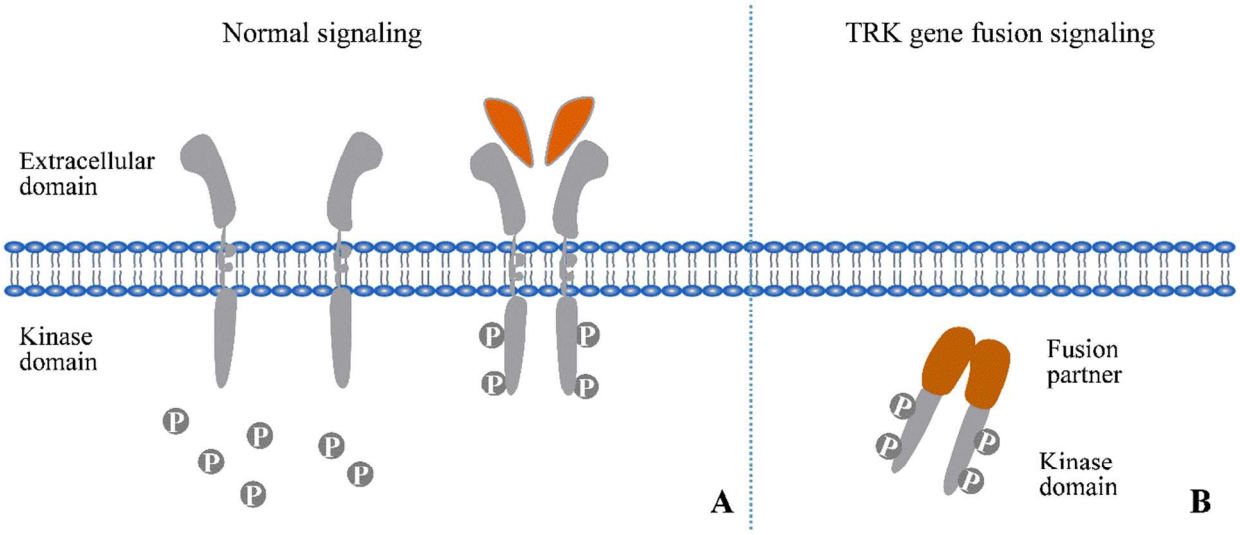

Figure 1. TRK signaling. (A) TRK proteins dimerize and activate after ligand binding under physiological conditions. (B) TRK fusion signaling, the kinase domain is fused to an unrelated gene, leading to constitutive activation and is resistant to TRK-directed antibodies due to the lack of an extracellular domain.

TRK Biology and Signaling

The TRK oncogene was initially discovered in colon cancer in which the cytoskeletal protein tropomyosin was fused to an unknown, catalytically active kinase domain.[49, 60] Further studies identified the kinase as a single-pass receptor tyrosine kinase (RTK) expressed in the

developing central nervous system and was given the name tropomyosin receptor kinase (TRK). In the extracellular region of TRK, there is a leucine rich motif, two cysteine-rich domains, and two immunoglobulin-like domains and all are essential for ligand recognition and binding.[61-63] Unlike typical RTKs, the TRK intracellular region is small and comprised of roughly 70 amino acids before and 15 amino acids after the kinase domain.[61-62] In comparison to other RTKs, TRK is most similar to the insulin receptor and has been implicated in insulin signaling.[64]

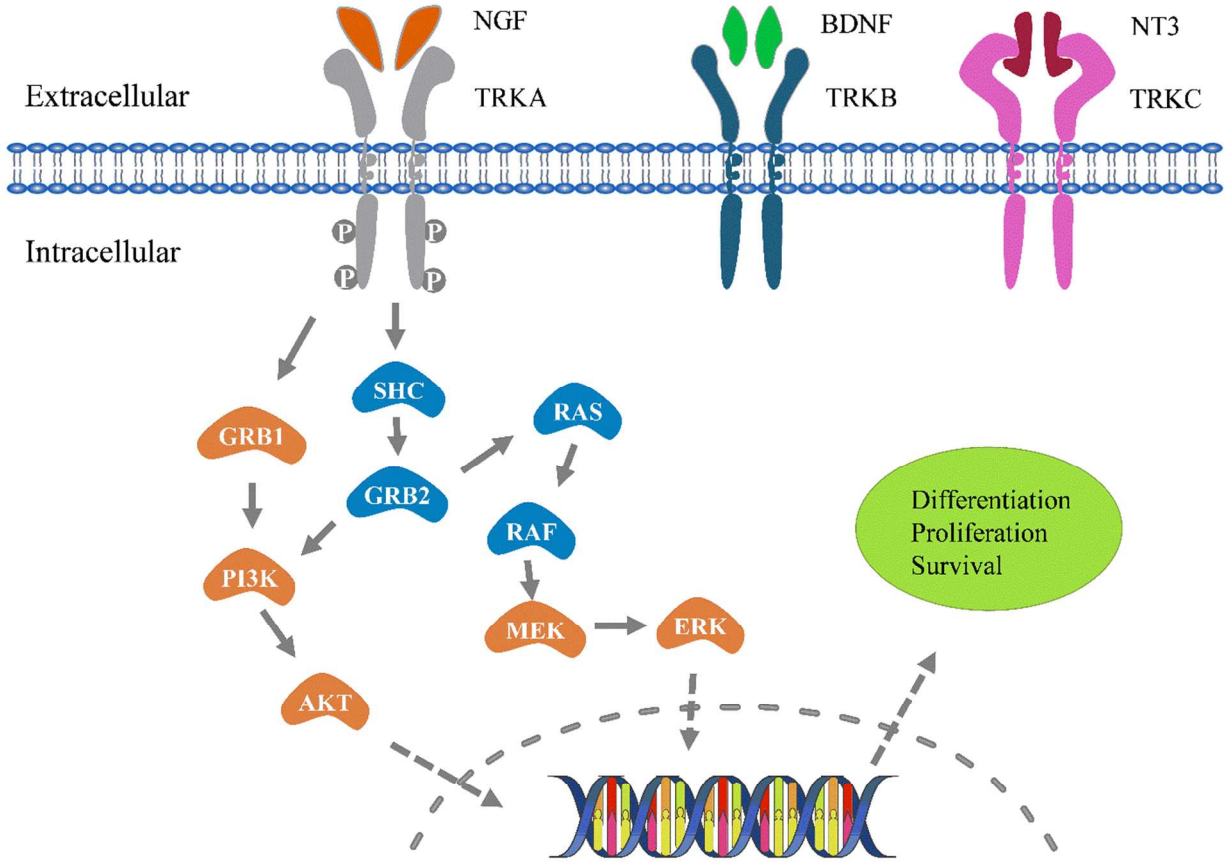

Figure 2. Schematic view of TRK receptor tyrosine kinases and major signal transduction pathways involved in cell differentiation, proliferation, and survival. TRKA is activated by nerve growth factor (NGF). TRKB is activated by brain-derived neurotrophic factor (BDNF). TRKC is activated by neurotrophin-3 (NT3). RAS, rat sarcoma oncogene; RAF, rapidly accelerated fibrosarcoma oncogene; MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GRB2, growth factor receptor-bound protein 2; SHC, SRC homology 2 domain containing; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, v-AKT murine thymoma

viral oncogene homologue; PLCγ, phospholipase C-γ; DAG, diacyl-glycerol; PKC, protein kinase C; IP3, inositol trisphosphate.

The TRK family is comprised of three distinct isoforms: TRKA, TRKB, and TRKC. A major difference between all three isoforms is the ligand that activates the receptor. TRKA is activated by nerve growth factor (NGF),[65-67] TRKB is activated by brain-derived neurotrophic factor (BDNF),[66-67] while TRKC is primary activated by neurotrophin 3 (NT3) (Figure 2).[68-69] Structurally, TRKA and TRKB are most similar, with approximately 88% conservation within the kinase domain.[70] This conservation, however, drops down to about 57% in the extracellular domain, which allows for specificity between NGF or BDNF growth factors.[70]

Similar to the RTK superfamily, TRKs dimerize in response to ligand binding (Figure 2).[71] After ligand binding, TRKs autophosporylate each monomer followed by rapid phosphorylation of the kinase activation loop.[72-74] These phosphorylation events enhance catalytic activity of the kinase. To generate attachment sites for adapter proteins, the NPXY motif (Y490 in TRKA) in the juxtamembrane domain and the YLDIG motif (Y785 in TRKA) in the carboxy terminus are phosphorylated.[75-78] These phosphorylation events create docking sites for SRC Homology Domain (SHC) and phospholipase C (PLC). After binding, SHC and PLC are activated through TRK-catalyzed phosphorylation.[79]

SHC was the first adaptor protein identified to bind to the phosphorylated NPXY motif of TRK, which results in the activation of the AKT and RAS canonical pathways.[76-78, 80] After SHC is activated, a secondary adaptor protein, growth factor receptor-bound protein 2 (GRB2),[81] is recruited and facilities GTP-loading of RAS via the guanine nucleotide exchange factor, SOS.[82] The activated, GTP-bound form of RAS activates the MAP kinase cascade, which includes activation of RAF, MEK, and ERK.[83] The ERK kinase translocates into the nuclear membrane where it activates transcription factors to express target genes involved in cell growth, survival, and proliferation.[84]

Activation of the AKT pathway occurs via recruitment of SHC and GRB2 to the NPXY motif, which signals through the intermediary molecule, GRB2-associated-binding protein 1 (GAB1). This simulates activity of phosphoinositide 3-kinase (PI3K) leading to phosphorylation of PI4,5 lipids at the 3' position.[85] On AKT, there is a conserved pleckstrin homology (PH) domain, which interacts with the 3' phosphorylated lipids leading to AKT activation.[86-89] Once AKT is activated, cell survival and proliferation genes are expressed,[90-93] which is a critical mechanism for TRK receptors to promote pro-survival phenotypes.[94]

TRK Implication in Cancer: TRKA Is Paramount

Genetic mutations in the TRK family have been reported in many cancers, namely carcinomas of the colon, thyroid, lung, ovary, breast (secretory breast carcinoma), salivary gland (mammary analogue secretory carcinoma), head/neck and pancreas, melanoma, spitzoid neoplasms, cholangiocarcinoma, stromal tumors (congenital fibrosarcoma, congenital mesoblastic nephroma, soft tissue sarcoma, gastrointestinal stromal tumor, inflammatory myofibroblastic tumor), brain tumors (pediatric glioma, astrocytoma and glioblastoma) and leukemia (Table 1 and Figure 3).[40, 95-96 46, 48, 50, 52, 97] Within the TRK family, TRKA is the most commonly identified oncogene, which is found at a rate of approximately 7.4% across multiple tumor types.[40] Following is TRKC and then TRKB, which are found at rates of 3.4% and 0.4%, respectively. The majority of TRKB mutations have a frequency of less than 0.5% and many TRKC mutations have a frequency of less than 1.0%.[48] Therefore, due to the rarity and low, sporadic frequency of cancers with TRKB and TRKC oncogenes, therapeutic efforts have been focused on TRKA cancers.

The most common activating TRKA mutation is a genomic rearrangement where *NTRK1* becomes fused to new unrelated gene.[52] Certain mutations in the extracellular domain of TRKA, namely P203A and C345S, have been identified as transforming under laboratory conditions but have yet to be identified in human tumor samples.[98-99] On the other hand, in-frame deletions (ΔTRKA) and splice variants (TRKAIII) of *NTRK1* have been functionally identified and characterized in human tumor samples.[51, 100-102] The ΔTRKA in-frame deletion, identified in acute myeloid leukemia (AML), results in a truncated extracellular domain that can transform both epithelial and fibroblast cells.[102] The TRKAIII splice variant, identified in neuroblastoma, has deletions in exons 6, 7, and 9, which results in the loss of Ig-like C2-type I (IG-C2) and glycosylation sites in the extracellular domain.[51, 101] TRKA activating mutations from either genomic rearrangements, point mutations, deletions, or splice variants all compromise structure and sequence at the extracellular domain. This suggests that a key attribute to the oncogenic potential of TRKA is the loss of regulatory domains in the extracellular region, which results in constitutive, ligand-independent activation of the TRKA kinase domain.

Table 1. Oncogenic TRK fusions are found across multiple tumor types[a]

[a] Only positive studies are listed, and thus the actual prevalence may be lower than reported.

Cancer Site	Estimated US Cases / yr	TRKA%	TRKB%	TRKC%
Lung Adenocarcinoma (NSCLC)	92,138[42]	3.3[48]	0.2[41]	/
Colorectal	135,430[103]	1.5[48-49, 51-50]	/	0.7[41]
Intrahepatic cholangiocarcinoma	2,970[104-105]	3.6[106]	/	/
Papillary thyroid cancer	45,496[103, 107]	12.3[52]	/	14.5[53, 108]
Glioblastoma	11,376[103, 109]	1.25[54, 110]	/	/
Head and neck squamous cell carcinoma	63,030[111]	/	0.2[41]	0.2[112]
Mammary analogue secretory carcinoma	151[103, 113]	/	/	100[114]
Ph-like acute lymphoblastic leukemia	1,192[115-116]	/	/	0.7[117]
Acute myeloid leukemia	15,976[116, 118]	/	/	0.0125[46, 119]
Skin cutaneous melanoma	87,110[116]	/	/	0.3[41]
Secretory breast carcinoma	252[116, 120]	/	/	92[121]
	Estimated Total Cases / yr	10,917	310	8,325
	Estimated Percent of Cases / yr	55.8%	1.6%	42.6%

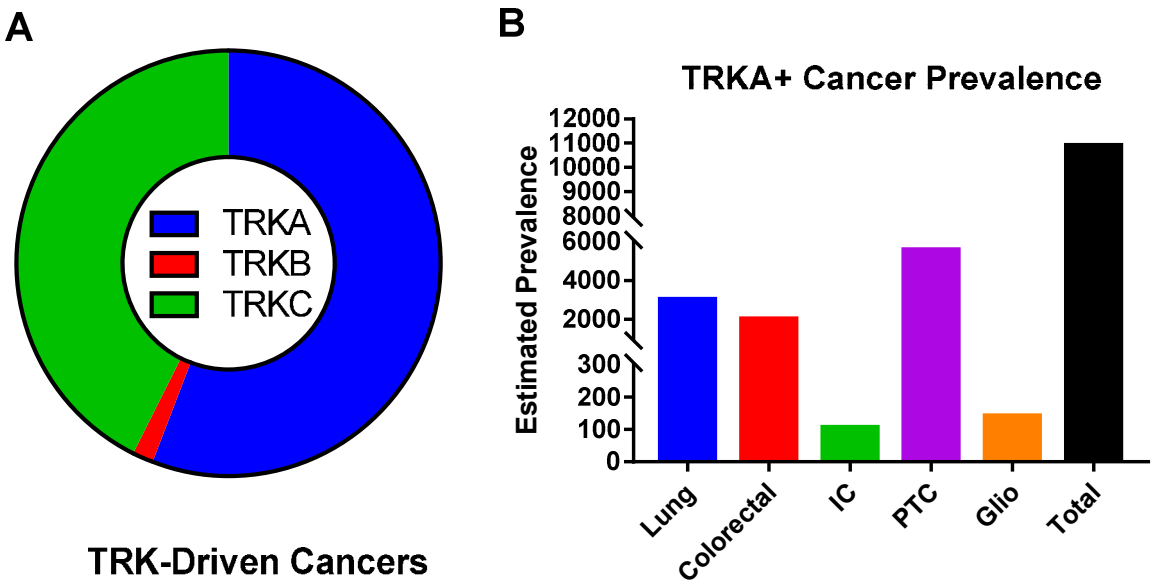

Figure 3: (A) Estimated contribution of TRKA/B/C mutations to all TRK-driven malignancies. Data is based on estimated incidence and prevalence of cancers at major sites and contribution of TRK mutations at each site. It is estimated that a total of 19,552 cancers are diagnosed each year that are driven by a TRK oncogene. Of the new cases, 55.8% (10,917) are $TRKA^+$, 42.6% are $TRKC^+$ (8,325) and 1.6% (310) are $TRKB^+$. Because of the limited sequencing data, the actual amount of TRK^+ tumors could be significantly greater or lower depending on robustness of sample size, sample selection, and data analysis. Also, TRK^+ tumors could be dependent on a separate pathway. (B) Breakdown of the estimated prevalence of TRKA mutations across

multiple tumor types. Lung (lung adenocarcinoma), IC (intrahepatic cholangiocarcinoma), PTC (papillary thyroid cancer), and Glio (glioblastoma).

In the tumor environment, TRKA oncogenes (genomic rearrangements, point mutations, deletions, or splice variants) can stimulate uninhibited signaling through the RAS/RAF and PI3K/AKT pathways since they still bind SHC and PLC adaptor proteins.[48, 122-124] The preferred signaling cascade is cell-specific, with dominance from the RAS/RAF pathway observed in both colorectal (KM12) and lung (CUTO-3) cancers. In certain cell types, TRKA oncogenic signaling also occurs through the PI3K/AKT and STAT3 signal transduction pathways [48], and in other cases the RAS/RAF and PI3K/AKT cascades are activated in concert.[125] Because of multifaceted pathway activation, TRKA oncogenes are potent and highly transformative by stimulating both antiapoptotic and proproliferative pathways.[126] Further, TRKA fusion oncogenes have been identified as important mediators to stimulate early tumor progression.[127] Taken together, inhibition of TRKA oncogenes can have chemotherapeutic and chemopreventive properties and, subsequently, has become a hotbed for therapeutic discovery efforts.

TRK Inhibitors: Emphasis on TRKA

The following section represents a comprehensive overview of known TRKA inhibitors and their corresponding discovery and developmental efforts. The inhibitors discussed all block TRK activity at the kinase domain and have typical kinase-inhibitor architecture.[128] Because most TRK-activating mutations alter or eliminate the extracellular domain,[56-58] antibodies directed at TRK or TRK growth factors will not be effective anticancer agents in TRK-driven tumors. Thus, all inhibitors reviewed are small-molecule kinase inhibitors that primarily target the TRK active site. The inhibitors are at various stages of development ranging from exploratory, pre-clinical research to in-human Phase II Clinical Trials.

Indole and indole derivatives

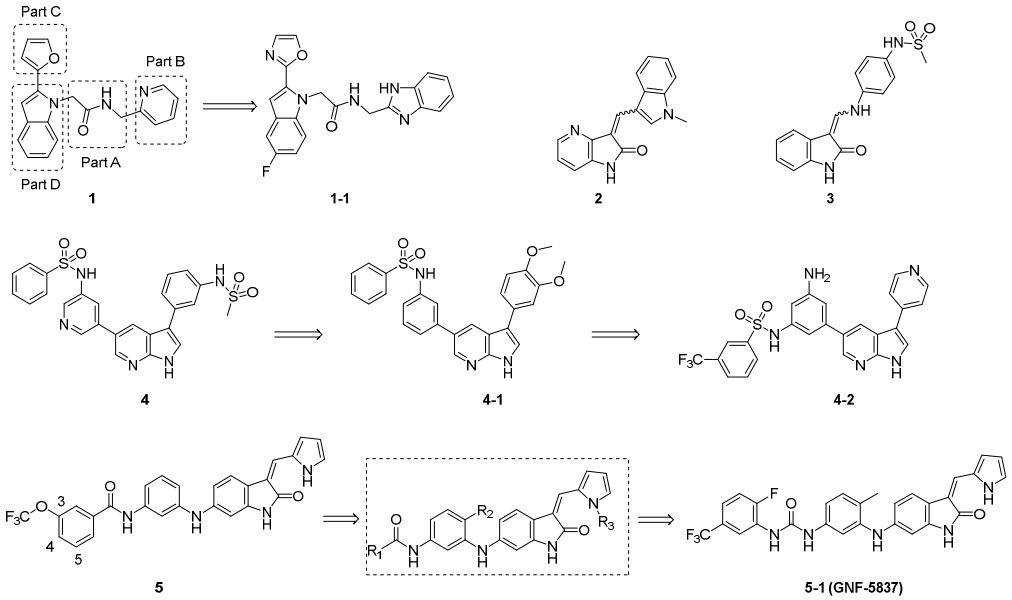

Figure 4. Chemical structures of representative indole and indole derivatives as TRKA inhibitors.

Using Automated Ligand Identification System (ALIS) screening technology, Hurzy *et al.* identified innovative substituted indoles as selective TRKA inhibitors (Figure 4).[129] The original hit **1** was determined to be a moderate TRKA inhibitor (IC_{50} = 0.607 μM), with 34-fold selectivity over TRKC. The main purpose and direction of their research was to identify molecular scaffolds with selectivity over TRKC, as the majority of all TRKA inhibitors exhibit pan-TRK activity. The linker in **1** (Part A) was found optimal for TRKA inhibition through SAR studies. Investigation of the linker consisted of shortening the linker, shifting the position of the amide, reducing of the carbonyl to methylene, and methylation of the –NH–, but all linker modifications resulted in a loss of TRK activity. On Part B of the scaffold, movement of the nitrogen to *meta*- and *para*-positions resulted in a 15- and 7-fold decrease in potency. Replacing the pyridine with phenyl resulted in a loss of activity and selectivity, indicating that a heteroatom is essential at the *ortho*-position. Further modification identified that benzimidazole was optimal for TRKA inhibition (IC_{50} = 0.07 μM) with greater than 1,000-fold selectivity over TRKC. To improve drug properties, the scaffold was further optimized at Parts C and D and led to the identification of compound **1-1**. Compound **1-1** had strong TRKA potency (IC_{50} = 0.113 μM) with 600-fold selectivity over TRKC. Importantly, **1-1** also displayed acceptable bioavailability (37%) and was moderate plasma bound (98.2%). With this type of selectivity profile, **1-1** is an effective molecular probe to further study TRKA biology without greater inhibition in the TRK superfamily.

Aza-oxindole and oxindoles were reported as potent TRK inhibitors by Wood *et al* (Figure 4).[130] Two representative compounds **2** and **3** exhibited an IC_{50} on TRK of 0.002 μM

and 0.006 μM, respectively. The two scaffolds obtained selectivity over a wide range of kinases but potently inhibited VEGFR2 (vascular endothelial growth factor receptor 2), likely due to structural similarity with known VEGFR2 inhibitors.

Hong *et al.* reported a series of 3,5-disubstituted-7-azaindoles as TRK Inhibitors (Figure 4).[131] Compound **4** was initially identified as a PI3Kα inhibitor with moderate TRK activity. Substituting the phenyl at the C5 position with pyridyl reverses selectivity (compound **4-1**, Kd = 2.3 μM for PI3Kα *vs* Kd = 0.091 μM for TRKA). Further modification uncovered compound **4-2**, which is a pan-TRK inhibitor with selectivity over 30 kinases. The activity of compound **4-2** against TRK isoforms was equal (pan-TRK IC_{50} = ~0.001 μM), but was 100-fold more selective against other kinases. Compound **4-2** also exhibited strong apoptotic and antiangiogenic effects by inhibiting HIF-1α and VEGF expression.

TRK inhibitors based on an oxindole-core were also disclosed by Albaugh *et al* (Figure 4). [132] Oxindole **5** inhibited Ba/F3-Tel-TRKA, TRKB, and TRKC with an IC_{50} less than 0.06 μM. Structure activity relationship (SAR) studies at the R^1 position showed that the phenyl ring is critical for TRK specific inhibitory activity. To improve drug properties, several basic groups were introduced at the 5-position of the 3-trifluoromethyl phenyl ring and were well tolerated and increased potency, but did not improve solubility. Exchanging the amide group for an amine resulted in a total loss of activity, indicating a location of a critical hydrogen bond with the TRK kinase. Replacement of the pyrrole with other groups (pyrrolidine, isooxazole, and cyclopentane) or methylation of the pyrrole nitrogen yielded significantly less potent compounds, suggesting that the acidic proton on pyrrole is involved in a key hydrogen bond at the hinge region. Replacement of the amide with urea generated compounds with increased potency and selectivity over VEGFR2. Further addition of a fluorine to the 2-position of the 3-trifluoromethyl phenyl ring led to compound **5-1 (GNF-5837)**, which exhibits pan-TRK inhibition (IC_{50}s = 0.011, 0.009, 0.007 μM for TRKA, TRKB, and TRKC, respectively) and about 300-fold selectivity over VEGFR2 (IC_{50} = 3.0 μM). In Balb/c mice and Sprague–Dawley rats, compound **5-1** demonstrated low drug clearance and moderate bioavailability. In mice bearing RIE xenografts expressing TRKA and NGF, compound **5-1** (100 mg/kg/d P.O.) significantly inhibited tumor growth.

Aminopyrimidine and aminopyrimidine derivatives

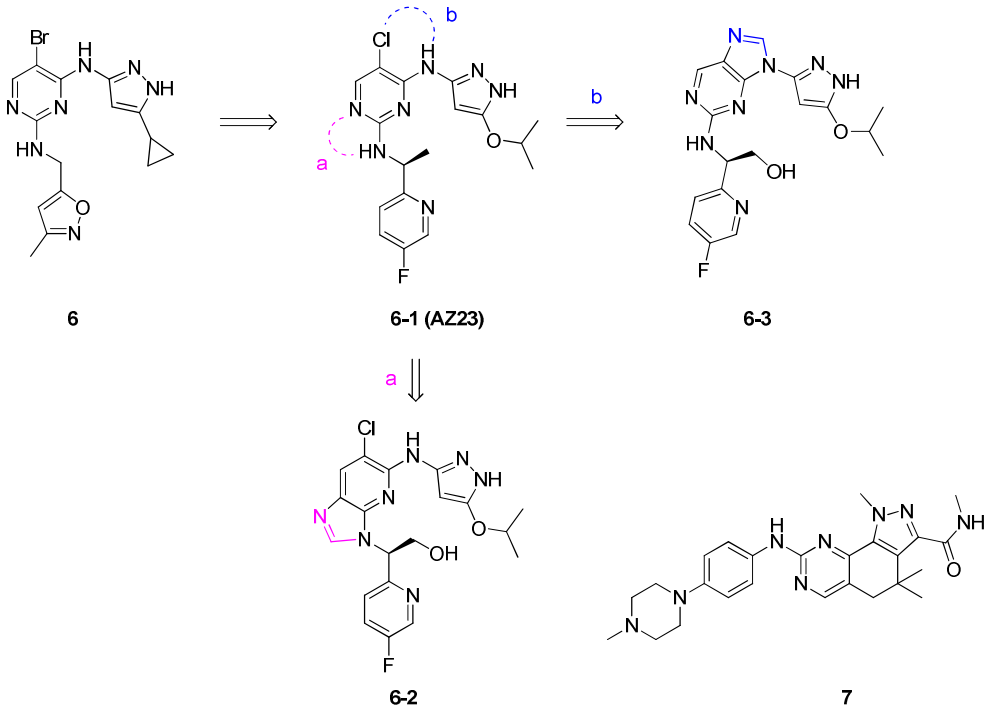

Figure 5. Chemical structures of representative aminopyrimidine and aminopyrimidine derivatives as TRKA inhibitors.

4-aminopyrimidines with TRK inhibitory activity have been reported by Wang *et al* (Figure 5).[133] The group first identified compound **6** and modified the structure with phenyl and then pyridine substitution to successfully improve the potency and solubility without increasing molecular weight. The (*S*)-enantiomer is preferred over the (*R*)-enantiomer (*vide infra*), suggesting conformation in the TRK kinase is paramount for activity. Cyclopropyl was replaced with O^{i}Pr or SCH_3, but reducing or increasing length or bulk decreases of potency. Further optimization furnished orally bioavailable compound **6-1** (**AZ23**). Compound **6-1** was active in TRKA and TRKB assays (IC_{50} = 0.002 μMM and 0.008 μM, respectively) and exhibited anticancer activity following oral dosing in a TRKA-driven allograft model and in a TRK-expressing xenograft model of neuroblastoma.[134] In 2012, a ring fusion study of this scaffold was reported by the same research group.[135] Two different ring fusion strategies were employed to generate imidazo[4,5-b]pyridine and purine derivatives. Representative compounds of the two scaffolds, **6-2** and **6-3**, exhibited potent TRK inhibition. Both compounds displayed IC_{50} values of 0.0005 μM against TRKA-dependent MCF10A cells (MCF10A-TRKA-Δ), and were also active in mice bearing 3T3-TRKA-Δ tumors.

Albanese *et al.* identified the dual CDK-TRK inhibitor compound **7** (CDK2/cyA IC_{50} = 0.045 μM, TrkA IC_{50} = 0.053 μM) (Figure 5).[136-137] *In vitro*, **7** was able to inhibit NGF-induced

phosphorylation of TRKA as well as downstream signaling in the DU-145 human prostate carcinoma line. *In vivo*, **7** was capable of inhibiting tumor growth in a human prostate DU-145 xenograft model in a dose dependent manner. Because of the inhibitory properties of **7**, the compound can be thought of as a paradigm shift for precision medicine. Instead of generating a 'magic-bullet' with high TRK kinase selectivity, Albanese *et al.* focused on validating and developing a 'smart-bomb' with activity on the oncogene lesion (TRK) as well as cell cycle inhibition (CDK).[138] Other research groups, such as Frett *et al.*, have also focused on integrating 'broad-specificity' into kinase inhibitor design.[139] The concept is called single-agent polypharmacology (SAP) and synergistic medicinal chemistry and may mitigate tumor resistant niches from the Darwinian-like selection pressures of targeted therapy.[138-139]

Thiazole and isothiazoles

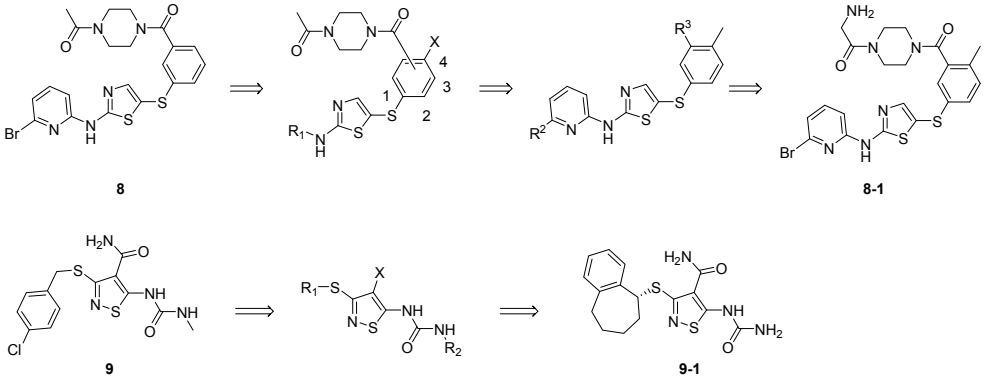

Figure 6. Chemical structures of representative thiazole and isothiazoles as TRKA inhibitors.

Through screening of an in-house kinase library, Kim *et al.* found 2-amino-5-(thioaryl)thiazole **8** as a promising TRKA inhibitor (Figure 6).[140] SAR studies indicated that 1,3-meta substitution in the thiophenyl ring was favored over other patterns, and a 50-fold increase in potency was observed by methylating X on the phenyl linker. Bromine, at C-6 of pyridine, was essential for TRK activity as removing this group or substituting with acetyl resulted in a total loss of activity. Exchanging bromine with methyl or altering its location decreased activity significantly, indicating critical interaction with TRKA at the C-6 position. SAR at R_3 was further explored and substituted piperazine was found to be optimal, thus generating compound **8-1** (TRKA IC_{50} = 0.0006 μM), which was selective over CDK, MET, IGF1R (insulin-like growth factor 1), and VEGFR (IC_{50} = 0.54 μM, >1 uM, 0.43 μM and NT, respectively).

Isothiazole derivatives have been identified as TRK inhibitors (Figure 6). From a high-throughput screening campaign directed at TRKA, Lippa *et al.* found compound **9**. Initial SAR studies at R^1 determined that substitution at the benzylic α-position produced remarkable

VEGFR2 selectivity. Orientation also played a key role: when (*R*)-ethyl was introduced, the resulting compound was 1300-fold more potent against TRKA over VEGFR2. Changing the X group to CN resulted in total loss of activity, indicating the amide is critical at this position. A diverse set of amino-heterocycles were tolerated at R_2 without any obvious differences in potency. Further optimization at R_1 identified that bicyclic moieties were important for TRK activity, especially the 7-membered ring systems. The resulting compound **9-1** had pico-molar kinase potency single-digit nanomolar potency in cells.

Pyrrolopyrimidine, imidazopyridazines, and pyrazolepyrimidines

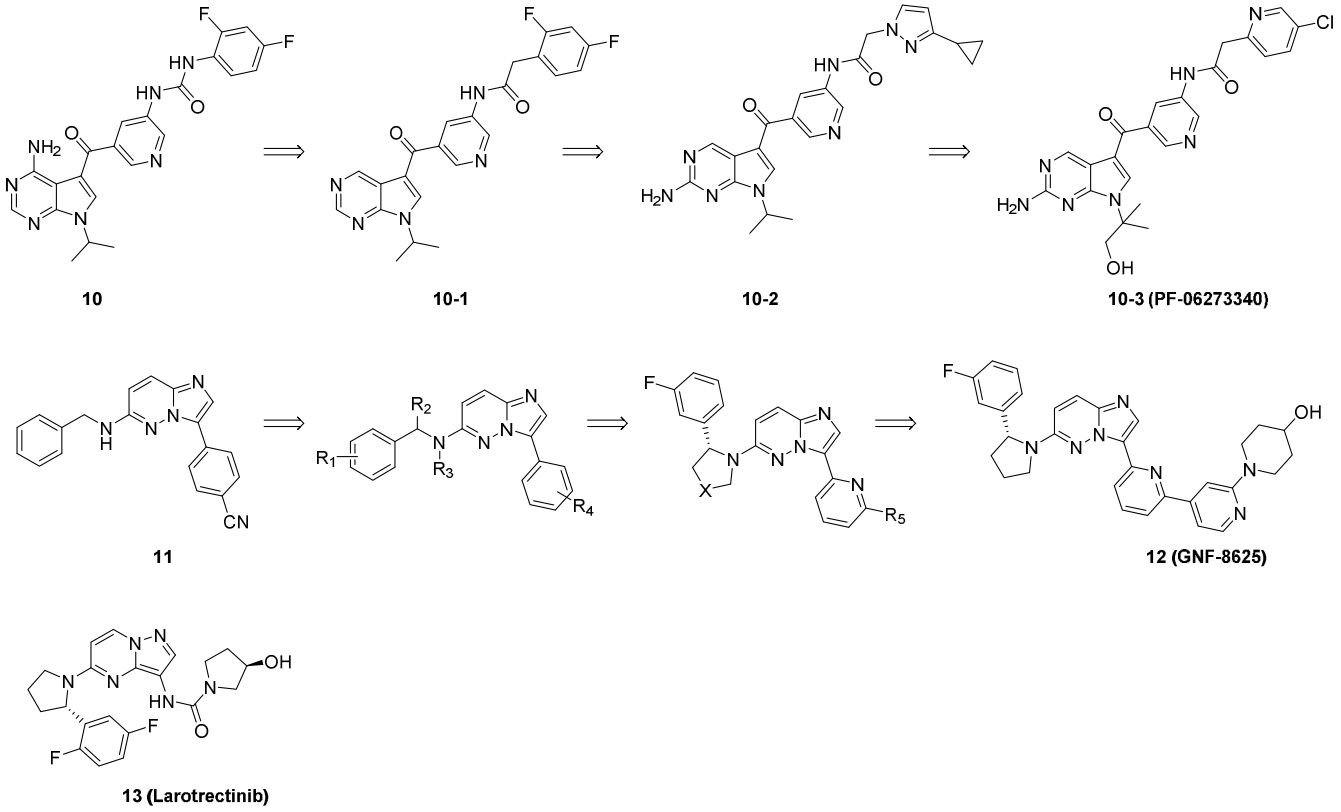

Figure 7. Chemical structures of representative pyrrolopyrimidine, imidazopyridazines, and pyrazolepyrimidines as TRKA inhibitors.

Skerratt *et al.* reported a series of pyrrolopyrimidine derivatives as pan-TRK inhibitors (Figure 7).[141] By employing a high-throughput TRKA/B cell screening assay, compound **10** was found to be a potent pan-TRK inhibitor in recombinant cellular assays (IC_{50}s for TRKA/B/C were 0.002 μM, 0.005 μM, and 0.004 μM, respectively) with strong kinome selectivity. To improve solubility and kinase selectivity, the urea group was replaced with an amide and the 4-NH_2 was

removed to generate compound **10-1**. It was determined that the scaffold was likely metabolized by aldehyde oxidase and inhibited hERG (human ether-a-go-go-related gene) potassium heart channels. In order to reduce metabolic liabilities and off target toxicities, an $-NH_2$ group was added at the 2-position furnishing compound **10-2**. To further improve physical-chemical properties and aqueous solubility, a hydrophilic hydroxymethylene group was added to N-*i*Pr motif and the terminal ring was replaced with pyridine to obtain compound **10-3**. Compound **10-3** was a potent pan-TRK inhibitor (IC_{50}s for TRKA/B/C were 0.006 μM, 0.004 μM, and 0.003 μM, respectively) and exhibited selectivity over a panel of 309 kinases.

Choi *et al.* developed a series of substituted imidazopyridazine derivatives as selective, pan-TRK inhibitors from the original compound **11** (TRKB IC_{50} = 0.083 μM) (Figure 7).[142] Methylation of the benzylic amine (R_3) led to no significant change in potency indicating that the acidic proton on the benzylic amine is not involved in a critical interaction with TRK. Cyclizing the benzyl amine moiety with the adjacent phenyl ring was performed to rigidify the structure and reduce rotatable bonds. The fused, five-membered ring system was optimal for TRK inhibition and the compounds exhibited a preference for the *R*-enantiomer. Derivatization at R_5 led to the discovery of the optimized compound **12** (**GNF-8625**). Compound **12** demonstrated potent antiproliferative activity against TRK transfected BaF3 and KM12 cell lines (IC_{50} = 0.001 μMM and 0.01 μM, respectively). In a KM12-derived tumor xenograft model, compound **12** demonstrated antitumor efficacy in a dose dependent manner, inducing 20% tumor regression at a dose of 50 mg/kg BID.

Compound **13** (Larotrectinib, LOXO-101) is a selective pan-TRK inhibitor with low nanomolar activity against the TRK family (Figure 7).[143] The compound can induce cell-cycle arrest in the G1 phase and apoptosis in KM12 cells.[48] Compound **13** is currently in a Phase 2, open-label study for patients with advanced solid tumors harboring a fusion of TRKA, TRKB, or TRKC.[44][NCT02576431]

Benzopyrazole and benzothiazoles

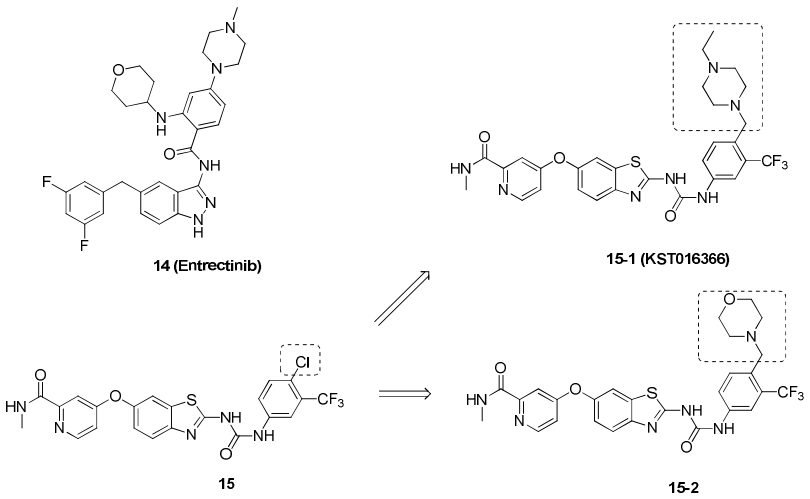

Figure 8. Chemical structures of representative benzopyrazole and benzothiazoles as TRK inhibitors.

Compound **14** (Entrectinib, RXDX-101) is a multi-targeted kinase inhibitor with pan-TRK activity (Figure 8). Recently, Menichincheri *et al.* disclosed research efforts to generate compound **14** for the ALK (anaplastic lymphoma kinase) kinase (IC_{50} = 0.012 μM), and serendipitously found this compound active against ROS1 (reactive oxygen species 1) (IC_{50} = 0.007 μM) and TRK (IC_{50}s for TRKA/B/C were 0.001 μM, 0.003 μM and 0.005 μM, respectively).[144-145] In antiproliferative studies, compound **14** was active against the colorectal cancer cell line KM12 (IC_{50} = 0.0017 μM) and also induced tumor stabilization (>90% TGI) when administered P.O. to mice bearing KM12 xenografts.[146] Compound **14** is currently in clinical trials for the treatment of patients with ALK-, ROS1- and TRK-dependent tumors and is exhibiting remarkable signs of efficacy.[144-145] In a Phase 2 Clinical Trial, **14** is being investigated for advanced or metastatic solid tumors that harbor TRKA/B/C, ROS1, or ALK gene rearrangements (NCT02568267).

El-Damasy *et al.* reported two benzothiazole derivatives as multi-targeted kinase inhibitors **15-1 (KST016366)** and **15-2** (Figure 8).[147] The scaffold was based on **15** and, to improve the physicochemical properties of the compound, the chlorine atom was replaced by (4-ethylpiperazin-1-yl)methyl **15-1** or (morpholin-1-yl)methyl **15-2**. Compound **15-1** was active on several kinases with IC_{50} values below 0.1 μM (including VEGFR2, ABL, TRKA, TRKB, and TIE2 *et al.*). Specifically, the IC_{50}s for TRKA and TRKB were 0.0038 μM and 0.0044 μM, respectively. In antiproliferative studies, compound **15-1** was active against the KM12 cell line with an IC_{50} of 0.019 μM.

Others

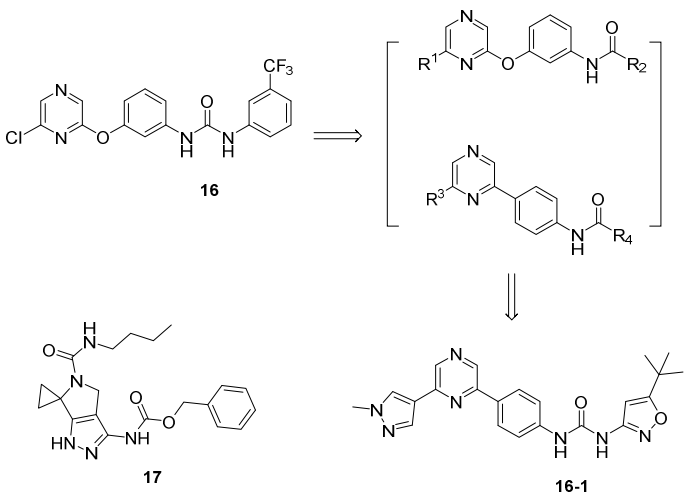

Figure 9. Chemical structures of other representative TRKA inhibitors

Pyrazine-based derivatives as inhibitors of TRKA were reported by Frett *et al* (Figure 9).[148] By utilizing a computational screening assay, compound **16** was found active against TRKA (IC_{50} was 3.5 μM). At the R_1 position, studies indicated a non-linear SAR, as moieties at R2 strongly influenced TRKA activity as well. At R_2, a lipophilic, aryl ring system that reaches into the DFG-out allosteric pocket is required to achieve activity. To lower the amount of conformational energy, compounds with a direct C–C bond were evaluated and were found to be far more potent than their ether-linked counterparts. Further modification led to the identification of compound **16-1**, which had a TRKA IC_{50} of 0.005 μM. Compound **16-1** is currently under pre-clinical development.

Choe *et al* reported a series of pyrrole[3,4-c]pyrazole compounds as TRKA inhibitors (Figure 9). The representative compound **17** inhibits TRKA with an IC_{50} value of 0.019 μM. SAR studies indicated that the cyclopropyl and benzyl carbamate group are essential for potency because removing either resulted in a loss of activity.[149]

Kinase Inhibitors with Fortuitous TRK Activity

As kinase inhibitors are discovered and developed activity profiles are generated that exhibit overlapping inhibitory properties. In many instances, drug discovery efforts have been focused on an unrelated kinase but have serendipitously been identified to function as TRK inhibitors. These compounds are summarized in Figure 10-13 and may have multiple therapeutic indications based on their multi-kinase inhibitory profiles. Although much efforts are placed on enhancing selectivity, it is important to note that some of the most successful kinase inhibitors, such as sunitinib (SUTENT®), dasatinib (SPRYCEL®), and sorafenib (NEXAVAR®), are multi-targeted kinase inhibitors. Therefore, although the following inhibitors were developed for an

unrelated kinase, the novel pharmacological properties could adequately target niche TRK-driven malignancies with unique mutations and gene expression profiles.

Aminopyrimidine and aminopyridines

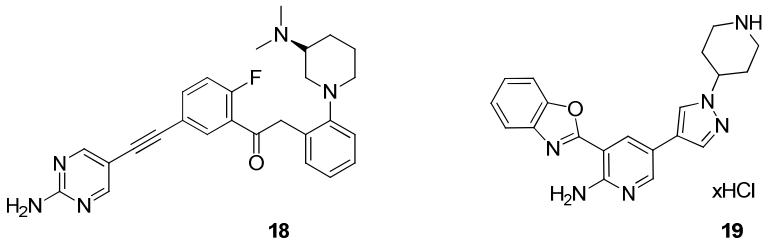

Figure 10. Chemical structures of representative aminopyrimidine and aminopyridines with TRKA activity.

Amino pyrimidine derivatives were developed as TIE-2 (tunica interna endothelial-2) inhibitors with compound **18** exhibiting TIE-2 inhibition (IC_{50} = 0.005 μM) but also exhibited TRKA inhibitory activity (IC_{50} = 0.008 μM).[150] Aminopyridines substituted with benzoxazole were found to have c-MET inhibitory activity, and Cho *et al.* disclosed the identification of compound **19** as a potent c-MET inhibitor (IC_{50} = 0.08 μM).[151] Compound **19** was later found to have multi-kinase inhibitory activity: IC_{50}s for RON (recepteur d'origine nantais), FLT3 (fms-like tyrosine kinase 3), and TRKA were 0.07 μM, 0.03 μM, and 0.039 μM, respectively, and was especially active on the Y1230D mutant c-MET kinase (IC_{50} = 0.003 μM).[152]

Dicarboxamides

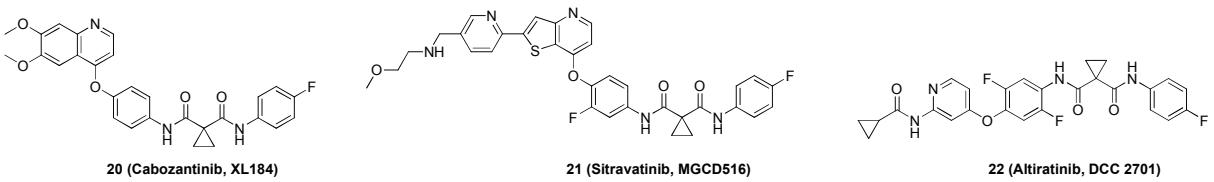

Figure 11. Chemical structures of representative dicarboxamides with TRKA activity.

Compound **20** is a c-MET/VEGFR2 dual inhibitor, also found to have a potent TRKB inhibitory activity (IC_{50} < 0.02 μM). Compound **20** is currently under Phase 2 Clinical Trials in patients with RET fusion-gene non-small cell lung cancer and those with other activating genes, such as ROS1, NTRK, MET or AXL. (NCT01639508).

Compound **21** was developed by Patwardhan *et al.* as a potent multi-kinase inhibitor, which had single digit IC_{50}s against several kinases (AXL: 0.0015 μM, VEGFR2: 0.005 μM, FLT3: 0.008 μM, c-KIT: 0.006 μM , TRKA: 0.005 μM and TRKB: 0.009 μM). Compound **21**

blocked phosphorylation of several RTKs and induced potent anticancer effects *in vitro*; the compound was also active *in vivo* in MPNST (neurosarcoma) and LS141 (hybridoma) mouse xenograft models.[153] Compound **21** is currently in Phase 1 Clinical Trial being evaluated in patients with advanced solid tumor malignancies. (NCT02219711).

Compound **22** was reported by Smith *et al.* as a multi-targeted kinase inhibitor with preferential activity on MET, TIE-2, and VEGFR2 (IC_{50}s were 0.0027 μM, 0.008 μM, and 0.0092 μM, respectively). Subsequently, **22** was identified to exhibit activity as a pan-TRK inhibitor (TRKA/B/C IC_{50}s were 0.00085 μM, 0.0046 μM, and 0.0083 μM, respectively) and FLT3 inhibitor (IC_{50} 0.0093 μM).[154] In cell antiproliferative assays, compound **35** exhibited IC_{50}s of 0.00069 μM in K562 cells, 0.0012 μM in SK-N-SH cells, and 0.0014 μM in KM-12 cells. Compound **22** inhibited tumor growth in the MET-amplified MKN-45 xenograft model in a dose-dependent manner. Further, compound **35** can actively penetrate the blood-brain-barrier, indicating its potential for the treatment of brain cancers, brain metastases, and cancer pain. Compound **22** is currently under Phase 1 clinical development for patients with advanced solid tumors (NCT02228811).

Pyrazolopyrimidine, pyrroloipyrimidine, and pyrrolotriazines

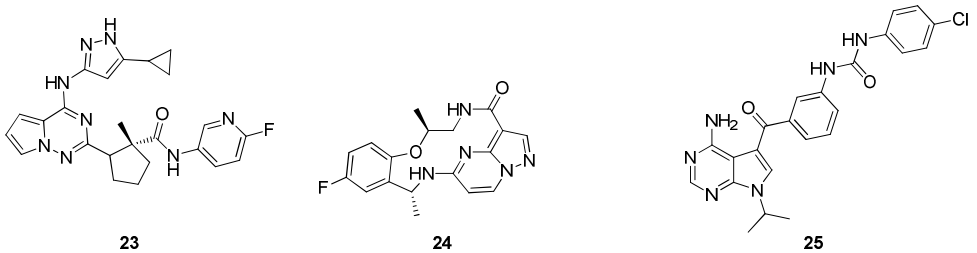

Figure 12. Chemical structures of representative pyrazolopyrimidine, pyrroloipyrimidine, and pyrrolotriazine with TRKA activity.

Compound **23** was developed by Carboni *et al.* as an IGF-1R and IR dual inhibitor (IC_{50} = 0.0018 μM and 0.0017 μM, respectively). Kinase profiling showed that compound **23** was also active on several other kinases (MET, RON, TRKA, TRKB, AURORA-A, and AURORA-B with IC_{50} values of 0.006 μM, 0.044 μM, 0.007 μM, 0.004 μM, 0.009 μM, and 0.025 μM, respectively).[156] Compound **24** is a multi-kinase inhibitor identified by Cui *et al.* and was specially designed to overcome drug resistance caused by kinase domain mutations.[155] In Ba/F3 cell proliferation assays, compound **24** potently inhibited ROS1-G2032R (IC_{50} = 0.0084 μM), TRKA-G595R (IC_{50} = 0.0004 μM), TRKB-G639R (IC_{50} = 0.0019 μM) and TRKC-G623R (IC_{50} = 0.0004 μM). **24** was also active in xenograft tumor models bearing WT and kinase

domain mutations of ALK, ROS1 and TRKA fusion genes. Arcari *et al.* reported a series of 4-aminopyrrolopyrimidine derivatives as TIE-2 inhibitors.[156] Medicinal chemistry efforts led to the identification of compound **25**, which was found to be a TIE-2/TRKA dual inhibitor (IC_{50} for TIE-2 and TRKA were 0.0037 μM and 0.004 μM, respectively).

Others

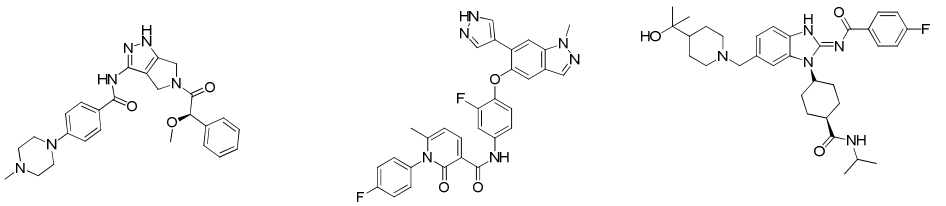

Figure 13. Chemical structures of other compounds with TRKA activity.

Fancelli *et al.* identified a series of 5-phenylacetyl 1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazole as AURORA kinase inhibitors. The representative compound **26** was found to have 2-fold less activity against TRKA (IC_{50} = 0.03 μM) vs AURORA A (IC_{50} = 0.013 μM).[116] Compound **27** was identified as a potent, pan kinase inhibitor with activity against c-MET (IC_{50} = 0.0047 μM) and MST1R (IC_{50} = 0.0012 μM) and exhibited anti-tumor activities in multiple mouse xenograft models.[157] Compound **27** was reported to have pan-TRK inhibitory activities with IC_{50}s ranging from 0.015-0.32 μM and also inhibited KM12 cellular proliferation (IC_{50} = 0.011 μM).[158] Compound **27** is currently under Phase 2 clinical development for patients with non-small cell lung cancer and solid tumors. (NCT02920996). Lewis *et al.* developed a class of 2-acyliminobenzimidazoles as potent ALK inhibitors.[159] Compound **28** exhibited high affinity for ALK (IC_{50} = 0.001 μM) with pan-TRK activity (IC_{50} for TRKA/B/C < 0.003 μM).[160] Compound **28** is now under a Phase 1/2 study in patients with advanced solid tumors and lymphomas. (NCT02048488). There are another three compounds **PLX7486** (NCT01804530), **DS-6051B** (NCT02279433), **F17752** (2013–003009–24) that are under clinical trials but whose structures have not been disclosed yet.

Overview of Substrate-Ligand Interactions with Kinase Inhibitors

Kinase inhibitors are classified depending on substrate interactions with the target protein.[161] In general, kinase inhibitors are broken down into five, distinct subtypes: (A) Type I, (B) Type II, (C) Type III, (D) Type IV, and (E) Type V.[128] (A) Type I kinase inhibitors are ATP competitive and bind directly to the highly conserved ATP-binding site. (B) Type II kinase inhibitors are typically ATP non-competitive and exhibit non-competitive or *pseudo*-competitive binding kinetics. The defining characteristic of Type-II inhibitors is the ability for a compound to displace

the conserved Asp-Phe-Gly (DFG) motif to gain access into an adjacent allosteric pocket. Only a subset of kinases permit such binding, which can be exploited to increase kinome selectivity. (C) Type III kinase inhibitors bind to the kinase domain outside of the active site. Type III inhibitors are unlike Type II inhibitors in that Type III inhibitors do not engage the ATP-binding site of the kinase. Therefore, Type III inhibitors are true allosteric inhibitors and can exploit unique kinase-specific functionality to generate highly-selective inhibitors. (D) Type IV kinase inhibitors bind to a region other than the kinase domain. The majority of kinases are expressed as multi-domain proteins with a catalytic domain and a regulatory domain. Type IV inhibitors can interrupt key protein-protein interactions or ligand interactions that limit activity of the kinase domain.[163] (E) Type V kinase inhibitors are bivalent that bind to the ATP active site and another site on the kinase, which are covalently linked through a synthetic linker.[164-165] Because Type V inhibitors exploit two sites on the kinase, the resulting inhibitors are typically much more selective in a similar fashion to Type II inhibitors.

For TRKA, all inhibitors currently being investigated in clinical trials are either Type I or Type II kinase inhibitors. Further, most FDA (food and drug administration) approved kinase inhibitors either possess Type I or Type II binding kinetics. Because of the high druggability of the ATP pocket, inhibitors can be developed that exhibit low nanomolar or even picomolar affinity for the target kinase. The ease to which high affinity binders at the ATP pocket can be generated provides an excellent avenue for therapeutic intervention of rouge kinases. Although kinase selectivity is a major issue in pre-clinical research to define new molecular pathways and identify novel biology, dirty kinase inhibitors have dominated and been surprisingly successful in the clinic.[162-164] In fact, selective kinase inhibitors in oncology are predisposed to fail because of the heterogeneous nature of tumor biology and formation of resistance (see below).[138] In the case of TRKA-driven tumors, it will be more clinically beneficial to develop precision medicine that blocks strategically paired molecular pathways and variations of the TRK oncogene. With this approach, precision medicine can be engineered to incorporate the multifaceted nature of tumor biology to extend suppression of malignant disease. In the following is an analysis of inhibitors co-cystalized with TRK to enumerate necessary molecular interactions for inhibition. It is expected that this comprehensive overview will illustrate key interactions and aid in the design of next generation TRK inhibitors.

Analysis of Co-Crystal Complexes of TRK with Inhibitors

There have been several reported co-crystal complexes of the TRK kinase with inhibitors that bind at the ATP active site. These complexes provided insight into the interactions between the TRK protein and inhibitors for the design of novel, enhanced TRK inhibitors. At the ATP active

site, the TRKA hinge is comprised of Glu590, Tyr591, and Met592. The 'gatekeeper' residue for TRKA is Phe589. At the DFG-loop, the residues are Asp668, Phe669, and Gly670. An important amino acid on the c-Helix of TRKA is Glu560. All of the aforementioned amino acid residues contribute to inhibitor recognition and binding for compounds that bind to the ATP active site of TRKA and will be mentioned frequently throughout analysis of co-crystal complexes.

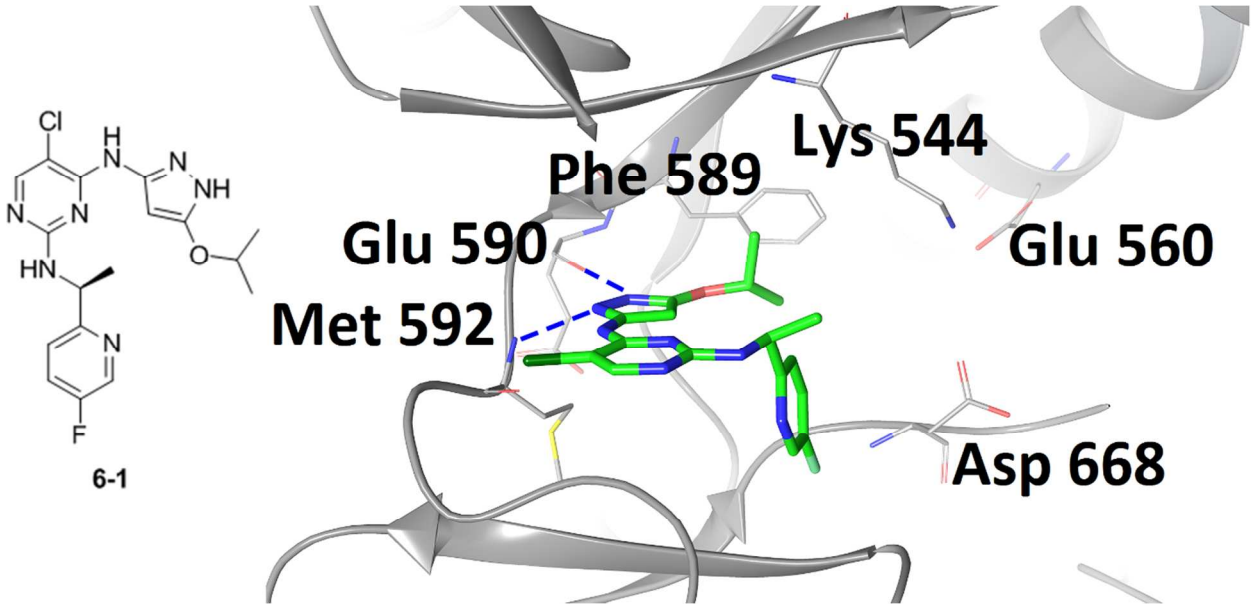

Figure 14. Co-crystal structure of compound **6-1** in the ATP binding pocket of TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines. (PDB ID: 4AOJ, 2.75 Å)

In the co-complex of compound **6-1** with TRKA (Figure 14), TRKA is forced into an inactive conformation with the C-helix pushed out into a non-catalytically active orientation. Compound **6-1** forms two hydrogen bonds at the hinge motif with the pyrazole-moiety, and interacts with the amide backbone of Glu590 and Met592. The isopropoxy group is oriented toward Phe589, which is the gatekeeper amino acid. The fluoropyridine ring engages in an interaction with Leu657, and the fluorine atom is in close proximity to Asn665 on the C-helix and Gly667. This region is frequently referred to as the ATP back-pocket region and is the same location where the conserved lysine is located.[165] Numerous TRK kinase inhibitors exploit this region to increase selectivity and potency. Additionally, on compound **6-1**, the pyrimidine forms a shared

water contact with Asp596 at a solvent exposed region.[135] Based on the crystal structure, compound **6-1** is a Type I kinase inhibitor.

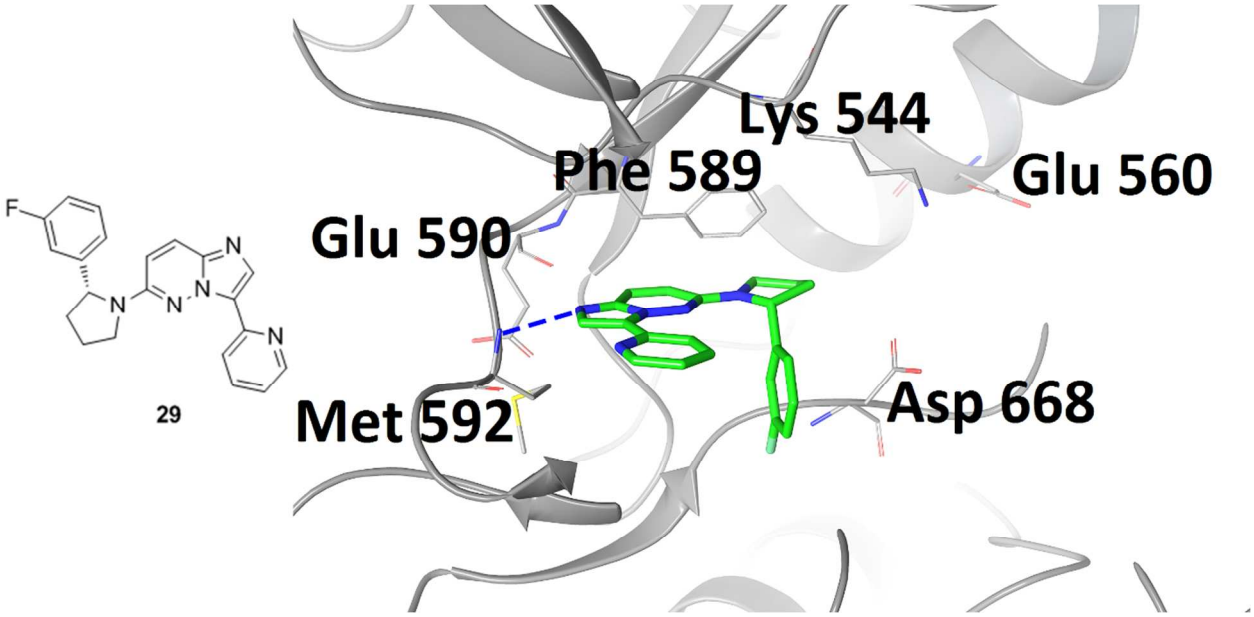

Figure 15. Co-crystal structure of compound **29** in the ATP binding pocket of TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines. (PDB ID: 4YNE, 2.02 Å)

Imidazopyridazines are known kinase inhibitors and expected to form a single hydrogen bond at the hinge region with the C-6 carbon oriented towards the solvent (Figure 15).[166] Unexpectedly, the co-crystal structure of **29** with TRKA revealed an unanticipated horizontal flip of the imidazopyridazine core while maintaining the same key hinge interaction. The (*R*)-enantiomer is the only active form because the 3-F-phenyl is optimally positioned in the hydrophobic pocket. The moiety fills a pocket originally occupied by Phe669 and provides excellent shape complementary, likely contributing significantly to potency improvement. Molecular modeling indicated that two distinct binding modes (i.e. core flipping) with this particular scaffold. It was concluded that the preferred binding mode likely depends on C-6 substitution. When the C-6 substitution was modified to (*R*)-phenylpyrrolidine, the "flipped" orientation was still preferred as the pyrrolidine anchors the phenyl group in the hydrophobic pocket.[142] The phenomenon of 'core flipping' could be employed to generate compounds with activity in multiple, distinct kinase families. Typically, kinase inhibitors have overlapping activity in the family that is targeted. In

the case of TRKA, scaffolds typically have overlapping activity in the tyrosine kinase (TK) family with activities on kinases such as AXL, VEGFR2, FLT3, c-KIT, and RET. 'Core flipping' could be employed to generate TRKA inhibitors with dual-activity on potential, contributing oncogenes outside of the TK family. Based on the crystal structure, compound **29** is a Type I kinase inhibitor.

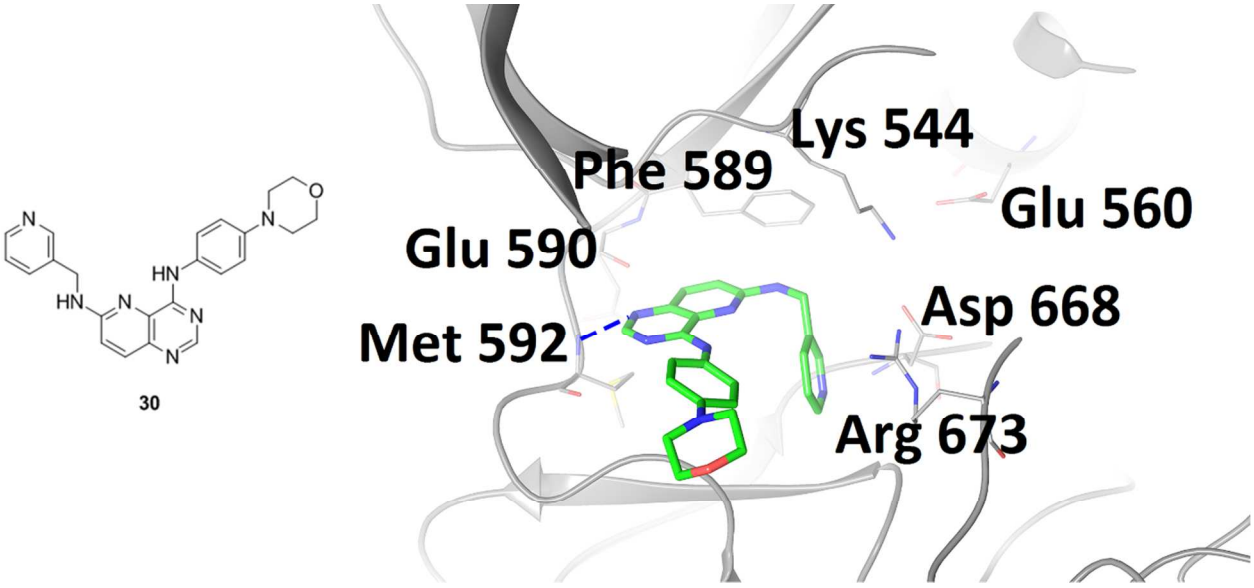

Figure 16. Co-crystal structure of compound **30** in the ATP binding pocket of TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines. (PDB ID: 4PMT, 2.1 Å)

Stachel *et al.* reported the co-crystal structures of four TrkA inhibitors.[167] In the co-crystal structure of compound **30**, the pyridopyrimidine was found to bind at the hinge and behaves like a Type I inhibitor (Figure 16). The main hydrogen bonding interactions between the enzyme and inhibitor **30** occur through a direct hydrogen bond between the pyridopyrimidine N-1 nitrogen and the amide backbone of Met592. The benzylic pyridine portion of the molecule is buried in the front pocket and formed a π-cation interaction with Arg673. The morpholine extended into the solvent exposed region, which is consistent with other kinase inhibitors that employ solubilizing groups at this region.

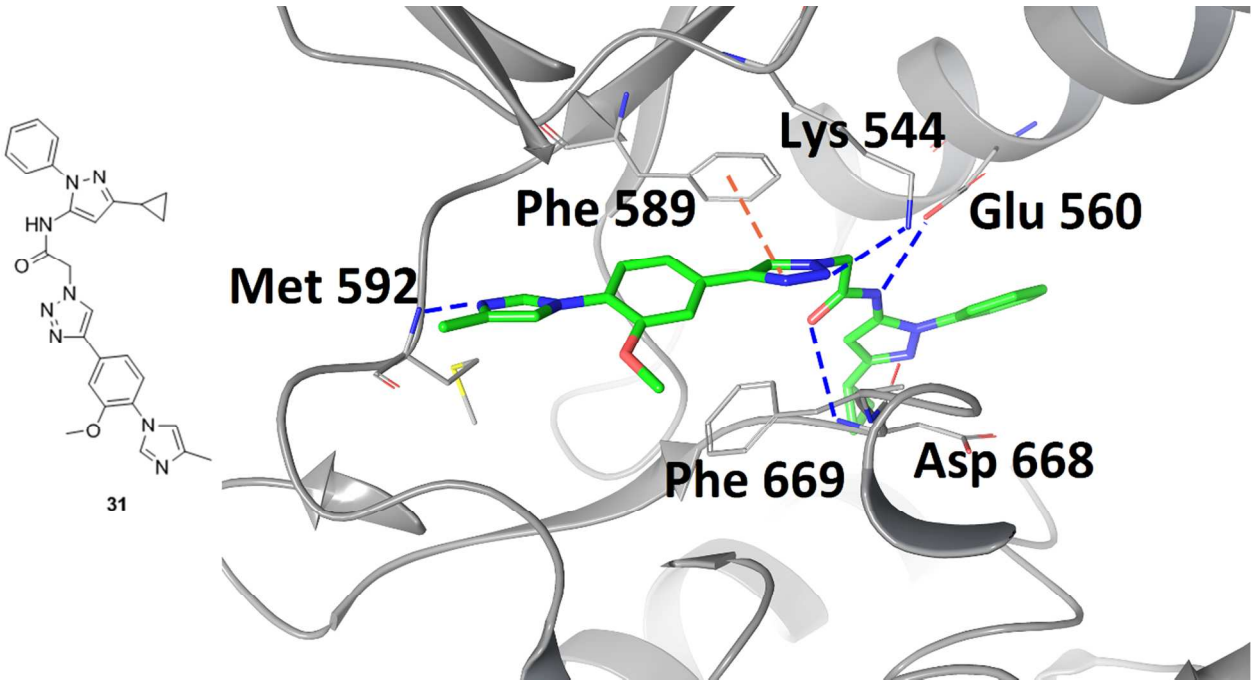

Figure 17. Co-crystal structure of compound **31** and its Type II binding mode in TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines and the π- π stacking interactions in orange. (PDB ID: 4PMM, 2.0 Å)

Compound **31** bound to TRK kinase in the DFG-out conformation (Figure 17), which is a prototypical feature of Type II kinase inhibitors.[168] At the hinge region, the imidazole warhead binds to the amide backbone of Met592. The central triazole ring forms an interaction with the gatekeeper residue, serving as a hydrophobic anchor for the inhibitor in the active site. The amide carbonyl engages Asp668 from the DFG motif and Glu560 from the C-helix. The N-phenylpyrazole occupies a selectivity pocket normally occupied by Phe669 in the DFG-in conformation. The nitrogen on the pyrazole forms a water-mediated hydrogen bond to the carboxylate of Asp668. Based on the inhibitor/substrate binding interactions of **31**, the inhibitor is highly unique because of the interesting triazole linker imidazole warhead.

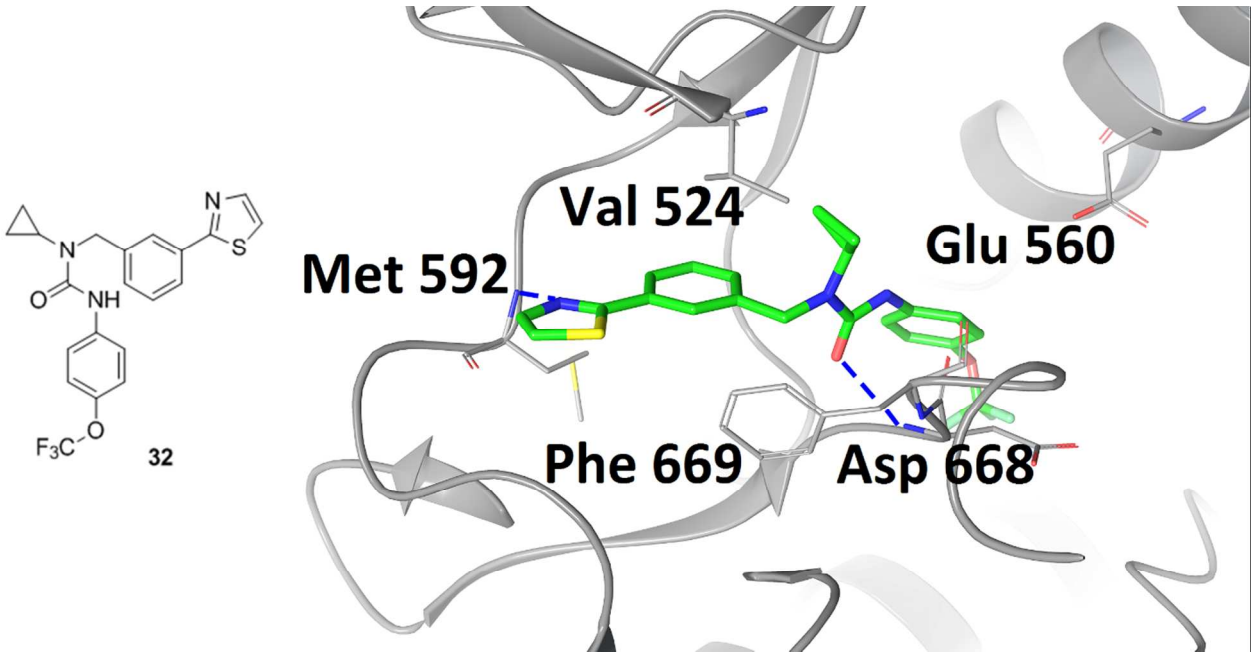

Figure 18. Co-crystal structure of compound **32** and its binding mode in TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines and the π- π stacking interactions in orange. (PDB ID: 4PMP, 1.8 Å)

Similar to **31**, compound **32** also bound to the kinase in the DFG-out conformation (Figure 18), which is typical of amide- and urea-linked kinase inhibitors. However, **32** is unlike most Type II kinase inhibitors, because of its low molecular weight and simple architecture. At the ATP-binding site, the thiazole heterocycle forms a very weak hydrogen bond with the amide backbone of Met592. The benzylic ring is involved in hydrophobic interactions with the gatekeeper residue, Phe589. The para-trifluoromethoxyphenyl group occupies the hydrophobic pocket normally occupied by Phe669 in the DFG-in conformation. The amide carbonyl forms a hydrogen bond with Asp668 as is typical with most Type II kinase inhibitors. A highly unique attribute to **32** is the cyclopropyl group, which occupies a hydrophobic cleft and is tucked neatly against Val524. This is a very unique attribute, as most kinase inhibitors are unsubstituted at this position and hydrogen bond to Glu560 on the c-Helix. One interpretation of the cyclopropyl SAR is that a critical hydrophobic mass is required to fill a small pocket near Val524 and Phe589. The placement of a hydrophobic group at this region is hypothesized to displace water, which would otherwise occupy the area.

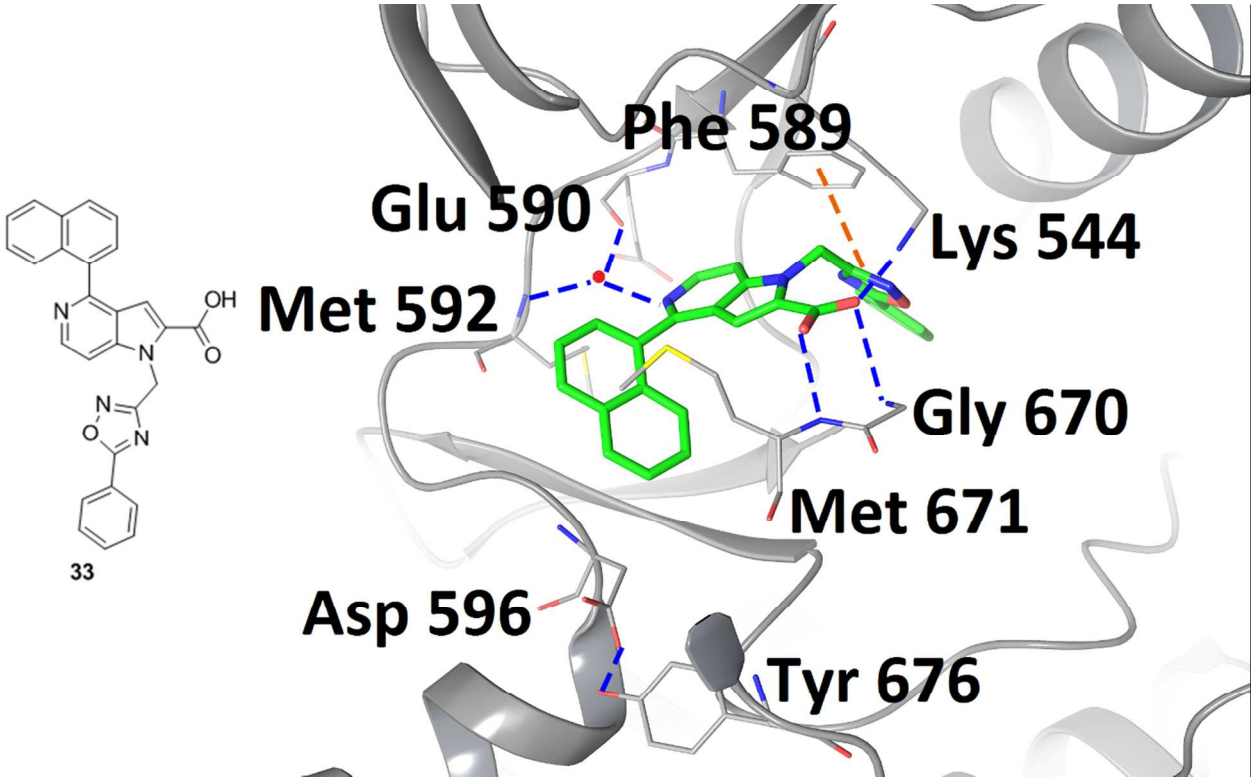

Figure 19. Co-crystal structure of compound **33** and its binding mode in TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines and the π- π stacking interactions in orange. (PDB ID: 4PMS, 2.8 Å)

Compound **33** also bound to the DFG-out conformation of TRKA (Figure 19). The co-crystal structure revealed several unusual binding features. First, no direct interaction between hinge backbone and the inhibitor was found. Instead, the N5 nitrogen of the azaindole was shown to participate in a water-mediated hydrogen bond to the hinge region. The naphthalene moiety was buried in the hydrophobic cleft at the front of the hinge flanked by the activation loop residue Met671. One of the more unusual binding interactions was that of the indole carboxylic acid interacted with two backbone NHs in the activation loop. The unique interactions at the hinge and the activation loop regions anchor **33** to the ATP binding site. A third interaction was also evident between the carboxylic acid and Lys544 on the roof of the ATP binding pocket. Compound **33** exhibited phosphorylation-dependent binding, which was due to the unusual conformation between the indole carboxylic acid and the activation loop. In the co-crystal structure, Tyr676 was involved in a hydrogen-bond interaction with Asp596. Since Tyr676 is a known phosphorylation site on TRKA, phospho-Tyr676 would disrupt this interaction with Asp596, forcing the activation loop away from the active site and stabilizing the active

conformation. As such, the phosphorylated form of the enzyme is not able to bind compound **33**.

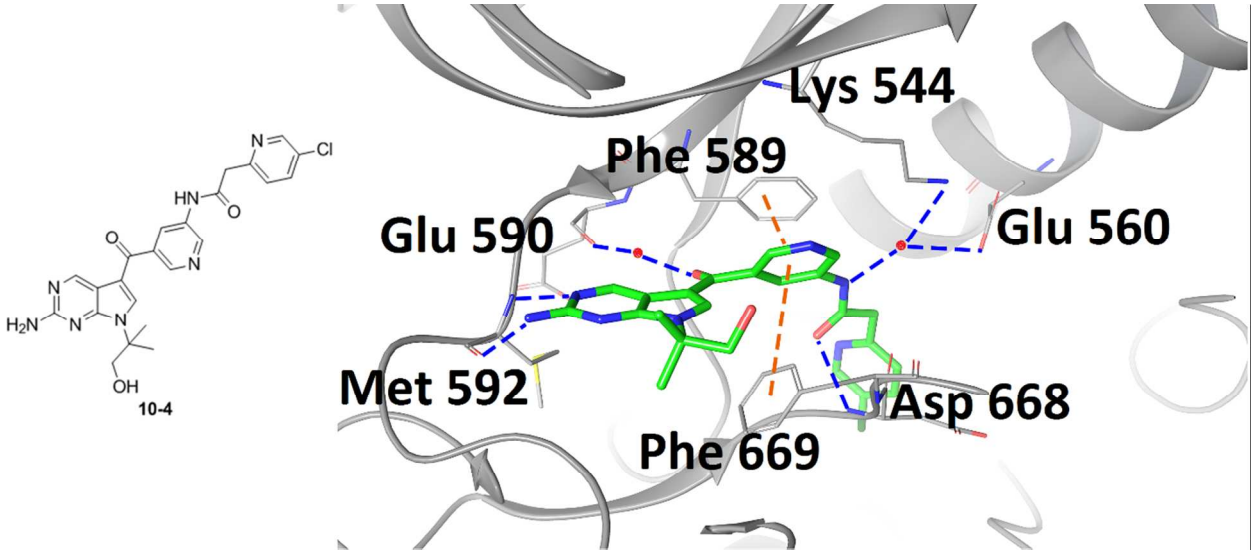

Figure 20. Co-crystal structure of compound **10-4** and its Type II binding mode in TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines and the π- π stacking interactions in orange. (PDB ID: 5JFX, 1.63 Å)

Figure 20 shows the co-crystal structure of **10-4** bound to TRKA and highlights key protein−ligand interactions. Compound **10-4** adopts a DFG-out binding mode, with the 2-aminopyrrolopyrimidine forming hydrogen bonds at the hinge and the ketone binding to Glu590 through a water contact. Most kinase inhibitors engage in one or two interactions at the hinge, while **10-4** is found to engage in three. The central pyridine group of **10-4** engages in a double π-π stacking interaction with Phe589 (gatekeeper residue) and Phe669 (DFG motif). The carbonyl oxygen forms a hydrogen bond with the amide backbone of Asp668 (DFG motif) and the amide engages TRKA through a water contact between Lys544 and Glu560 from the c-Helix. The lipophilic back pocket accommodates the chloropyridine group.[141] Based on the binding characteristics of **10-4**, the inhibitor can be considered Type II.

Co-crystal complex of TRKA with ligands at JM site: Type IV Inhibitors

TRKA/B/C share significant sequence homology at the kinase domain, which will make it extremely difficult to develop inhibitors selective for a single isoform. A method to circumvent selectivity issues is to develop allosteric inhibitors that do not bind to the highly conserved ATP

active site. Instead, a region that is highly varied within the TRK family is the juxtamembrane (JM) domain. The JM domain is located between the kinase domain and the transmembrane domain. Su *et al.* and Furuya *et al.* found that some TRKA inhibitors also bind to the JM region, and these inhibitors exhibit selectivity over TRKB and TRKC.[169-170] These findings provide the research community with a novel, exploitable region to generate TRK selective small molecule inhibitors.

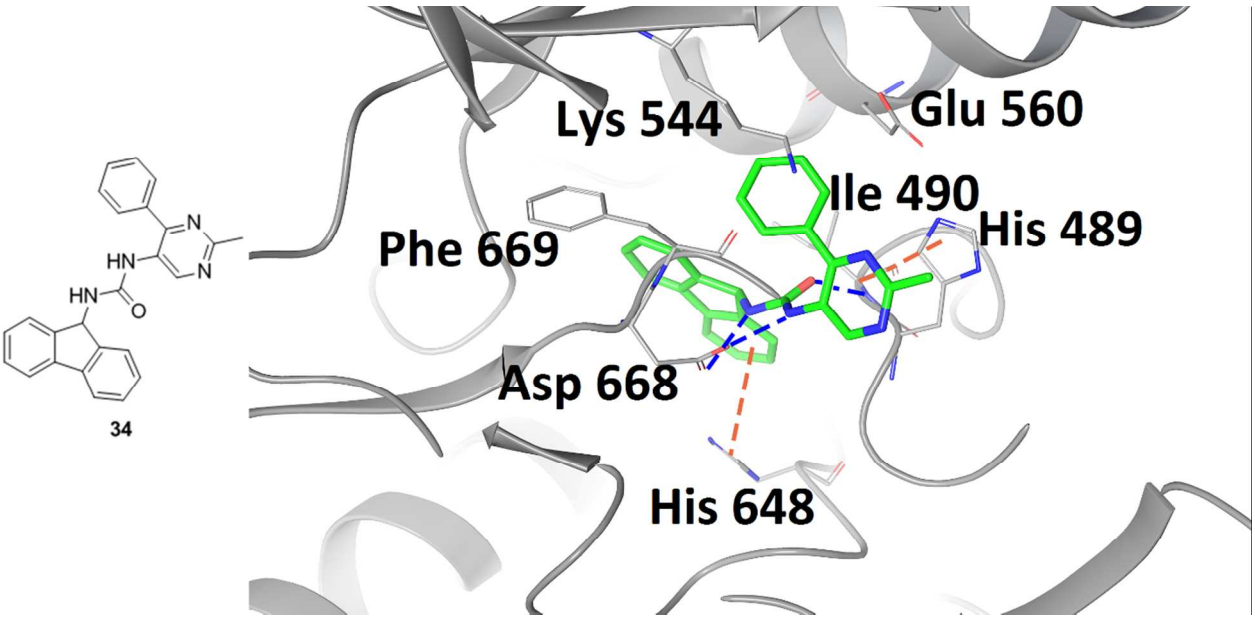

Figure 21. Co-crystal structure of compound **34** binding in the allosteric pocket of TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines and the π- π stacking interactions in orange. (PDB ID: 5KMI, 1.87 Å)

Compound **34** is a selective TRKA inhibitor (IC_{50}s for TRKA/B/C were 0.099 μM, > 81 μM and 25 μM, respectively).[169] In the co-crystal structure of **34** and TRKA (Figure 21), compound **34** bound behind the DFG motif opposite of the kinase active site. The DFG motif was found in a DFG-out, inactive conformation, with Phe669 pointed toward the active site. Asp668, which coordinates to the phosphate groups on ATP, is away from the active site in the structure. The central urea of compound **34** makes two hydrogen bonds with Asp668. Asp668 is part of the DFG motif, and binding to compound **34** requires the DFG motif to be in the 'out' conformation. The fluorine moiety of compound **34** occupies a relatively hydrophobic pocket formed primarily by aliphatic amino acids. The structure clearly reveals interactions at the JM region. Ile490, within the JM, sits on top of the fluorine moiety, aiding to the formation of the hydrophobic pocket. Similar to other TRKA co-crystal kinase structures, there is a shift in

Phe646, which creates a pocket to accommodate the fluorine. The oxygen of the central urea in compound **34** forms a hydrogen bond with the amide nitrogen of Ile490. The phenyl moiety occupies a position between Lys544 of the β3 strand and Glu560 of c-Helix.

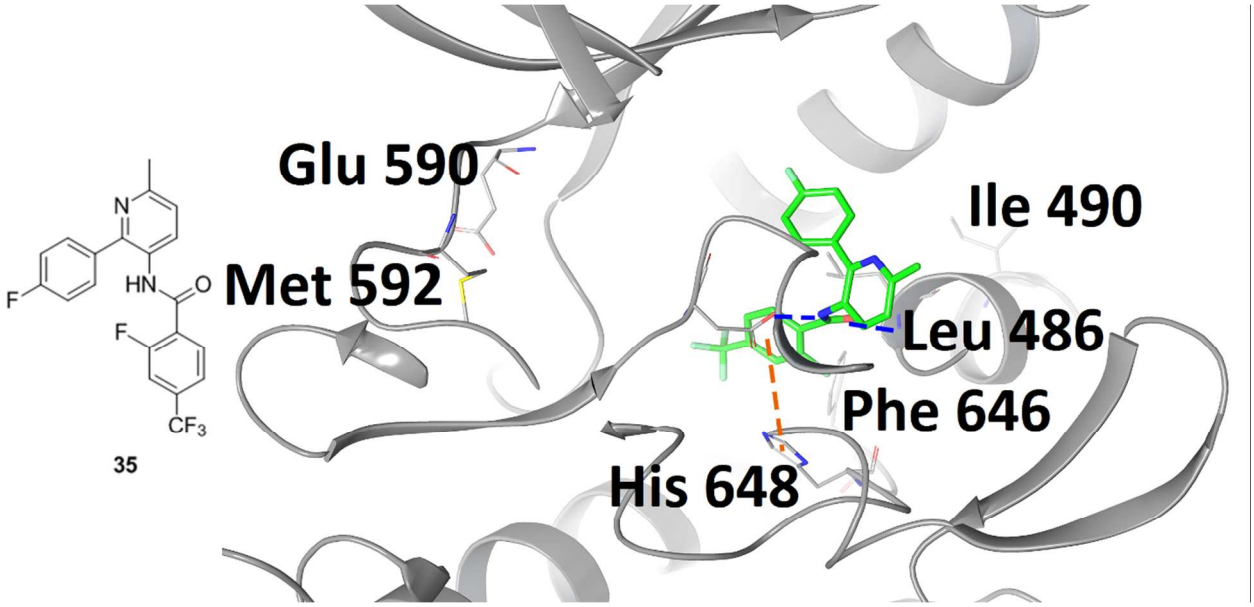

Figure 22. Co-crystal structure of compound **35** binding in the allosteric pocket of TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines and the π- π stacking interactions in orange. (PDB ID: 5KMK, 1.65 Å)

Compound **35** is another selective TRKA inhibitor disclosed by the same author (IC_{50}s for TRKA/B/C were 3.3 μM, >81.0 μM, and 27 μM, respectively).[169] The co-crystal structure of compound **35** revealed the compound bound to the same pocket as **34**, which occurs behind the DFG motif (Figure 22). The trifluorophenyl moiety of **35** sits in the pocket occupied by the fluorine of compound **34**. The central amide nitrogen is positioned close to the carboxylic acid of Asp668. The kinase site is quite similar between the two structures except for the following differences: (A) For compound **34**, Ile490 packs above the phenyl ring, but Leu486 packs above the phenyl ring for **35**; (B) for **34**, Phe646 was displaced by the bulky moiety in the hydrophobic pocket, but for **35**, the smaller moiety accommodates Phe646 in a position closer to the active conformation.

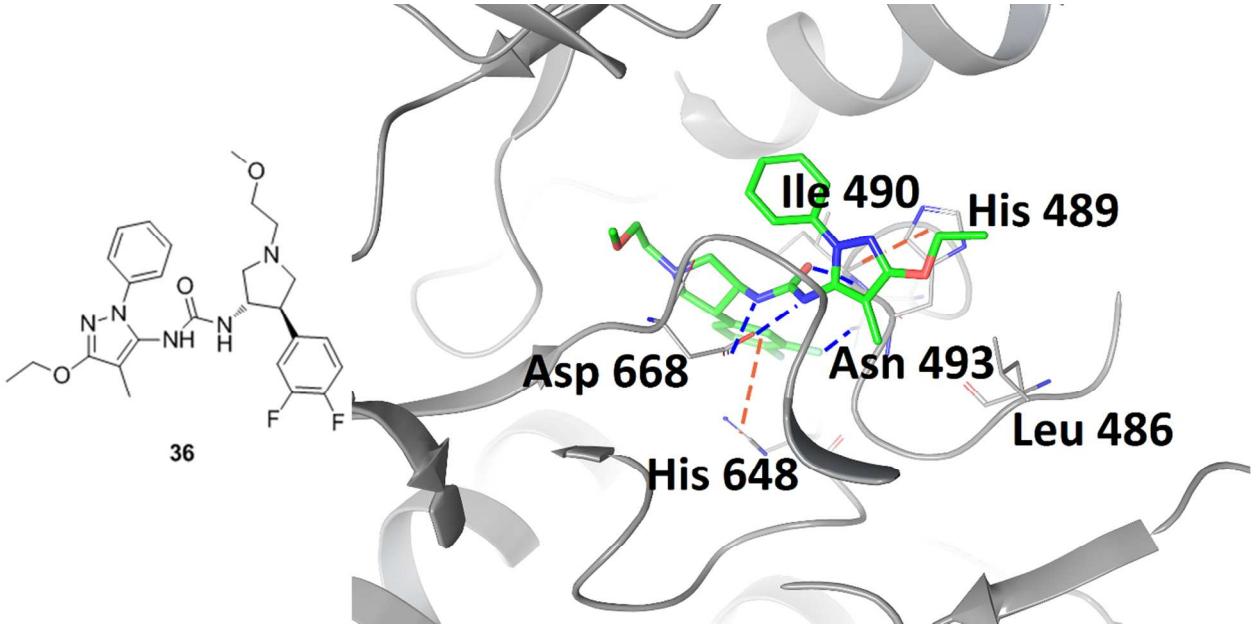

Figure 23. Co-crystal structure of compound **36** binding in the allosteric pocket of TRKA. The kinase is depicted in grey ribbons. The hydrogen bonds are illustrated in blue dashed lines and the π- π stacking interactions in orange. (PDB ID: 5H3Q, 2.1 Å)

Compound **36** showed potent inhibitory activity against TRKA (IC_{50} = 0.0027 μM) but was selective against TRKB and TRKC (IC_{50}s were 1.3 μM and 2.5 μM, respectively). Figure 23 illustrated the binding mode of **36** occupied the JM region. The configuration of the kinase domain and A loop is very similar to that observed for Apo TRKA. **36** binds to the deep pocket assembled from the DFG region of the A loop, glycine-rich loop (G loop), C-helix, and JM region. This pocket is completely separate from the ATP site. There are four key interactions that **36** creates at the binding region: (1) Asp668 of the DFG motif forms two hydrogen binds with the urea moiety; (2) His489 interacts with the pyrazole ring via π-π interactions and with the ethoxy group via CH-π interactions; (3) Ile490 forms a hydrogen bond with the urea moiety and interacts with the difluorobenzene group of **36** via CH-π interactions; (4) His648 interacts with the difluorobenzene moiety through a π-π stacking interaction. In addition to these, Leu486 is also involved in weak van der Waal interactions with the methyl and ethoxyl groups of S12. Interestingly, these amino acid residues are not conserved in TRKB and TRKC and is integral for high binding affinity and selectivity.[170] Further exploitation of these TRKA specific amino acids could furnish numerous allosteric scaffolds with high TRKA selectivity.

Conclusion and Future Perspective for TRK Inhibitors

The use of kinase inhibitors in cancer has been heavily pursued since the discovery and development of imatinib.[39] Kinases have since emerged as one of the most intensely pursued drug targets in current therapeutic research due to a highly druggable active site and their critical roles in cellular signaling. However, there has yet to be a clinically approved inhibitor for the TRK-receptor tyrosine kinase despite its intimate involvement in tumor pathology and disease. Annually, it is estimated that around ~20,000 new cases of TRK cancers are diagnosed, with the majority of cases exhibiting a TRKA mutation. The vast majority of kinase inhibitors developed for TRK exhibit limited selectivity against any of the three isoforms (TRKA/B/C) and are active in the greater kinome. These types of inhibitors will have limited utility as tools to further study TRK biology but might be important clinically to simultaneously target multiple drivers of malignant disease.

Clinically advanced TRK inhibitors include **14**, a pan-TRK, ALK and ROS inhibitor, and **13**, a specific pan-TRK inhibitor (see below). Both **14** and **13** have achieved orphan designation from regulatory authorities.[44] A recent report of the clinical activity of **13** in 55 TRK mutant patients, showed a 80% overall response rate and 71% of long-lasting responses (> 12-months).[96] An aspect that needs to be considered in the development of TRK inhibitors is, given their role in the nervous system, the possibility they may potentially exert adverse neuropsychiatric effects. One parameter that may influence this property is the panel of kinases they simultaneously inhibit; another one is their ability to cross the blood-brain barrier. Noteworthy, new generation TRK inhibitors currently in clinical development (such as **13** and **14**) showed limited neurotoxicity, probably due to their target specificity, although they are able to penetrate the blood-brain barrier and exert therapeutic effects against brain metastases.[44]

A key aspect to therapeutic development of TRK inhibitors is to engineer the inhibitor for activity on additional TRK mutations that can mediate secondary resistance. This type of profile is observed in compound **24** and should become the gold-standard for therapeutic development of precision medicine in oncology.[171] Lessons learned from previous inhibitors suggest that resistance to precision medicine is inevitable and predicting targetable resistance mechanisms or pathways upfront is paramount for sustained treatment efficacy. It is therefore necessary to understand the evolution of TRK^{+} cancers and determine various mechanisms of resistance to strategically target. From clinical work, obtaining inhibition on key, single-point mutations in the TRKA active site is crucial for sustained remission.[172] Therefore, inhibitors that do not exhibit activity on known mechanisms of resistance will likely have limited clinical utility. In fact, the clinical development of **14** followed the typical kinase inhibitor storyline where first, the inhibiter exhibited a remarkable response in the TRK-driven cancers, followed by the emergence of

resistant disease.[172] Every kinase inhibitor to date follows a similar plot, where there is profound, upfront efficacy immediately proceeded with resistant disease.[173-176] Specifically, secondary mutations have been found in the TRK kinase domain after treatment with **13** or **14**.[177] These mutations include TRKA G595R (and its paralogue TRKC G623R) in the solvent front of the nucleotide-binding loop of the kinase domain, TRKA G667C (and its paralogue TRKC G696A) adjacent to the DFG (xDFG) motif of the kinase domain, and TRKA F589L at the gatekeeper position.[43, 95, 171-172, 178] TRKA G595R and G667C are analogous to ALK G1202R and G1269A, respectively.[171] In TRKA, these sites are important for the accommodation of **13** and **14** and their substitutions create steric clashes with the drug. In addition, TRKA G595R also increases the ATP affinity of the kinase.[171] LOXO-195 has been rationally designed to overcome these resistance mechanisms and an innovative first-in-human clinical trial has demonstrated its efficacy in 2 patients who had developed acquired resistance to **13** mediated by secondary TRKA G595R or G623R mutations.[171]

As such, a new paradigm for precision medicine should focus on developing therapies engineered to withstand multiple, resistance mechanisms to prolong the advent of disease progression. It is naive to believe tumors will not evolve resistance to targeted therapies as this contradicts the Darwinian nature of tumor biology.[138] Akin to antibiotic resistance, as long as we continue to treat malignant disease, we will place selection pressures on the tumor for resistance. In the case of TRK inhibitors, the key to producing high-value, effective medicine is to engineer TRK-directed therapies with strategic properties that preemptively target resistant disease. Instead of focusing efforts on developing 'magic-bullets' precision medicine efforts for oncology should focus on identifying 'smart-bombs' tailored to the unique pathology of the tumor.[138] When targeting TRK-driven malignancies, this will involve developing inhibitors that are active on TRK kinase mutations or additional pathways that circumvent treatment. Emerging strategies for precision medicine is achieving this with single agent polypharmacology through synergistic medicinal chemistry optimization techniques that balance activity profiles to multiple, synergistic targets.[139, 179-182] Indeed, as targeted therapy moves beyond 'one-drug, one-target' a new field may surface appropriately called *synergistic medicine*.

Biographies

Wei Yan received his Ph.D. from East China University of Science and Technology, Shanghai, China, and worked collaboratively in Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Following, he worked at WuXi AppTec as a process chemistry scientist. He is currently a postdoctoral researcher at the University of Arkansas for Medical Sciences. His expertise is in medicinal chemistry and process chemistry for pilot plant manufacture of APIs.

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Massimo Santoro is Professor of General Pathology at the University of Naples Federico II, Dept. of Molecular Medicine and Medical Biotechnology. He graduated in Medicine and Surgery

at the University of Naples Federico II and then achieved the PhD degree in Molecular Pathology. He has been fellow at NCI, NIH. His main interest has been molecular genetics of thyroid cancer. He participated to the identification of RET gene fusions in papillary thyroid cancer and activating point mutations in medullary thyroid cancer.

Neil McDonald is currently a Senior Group Leader at the Francis Crick Institute. He is also Professor in Structural Biology at Birkbeck College, London. He received his Ph.D. degree from the University of London, and did his postdoctoral training at Columbia University, NY. He previously worked at Cancer Research UK Labs in London where he focused on the structural biology of oncology targets. His current research interests are neurotrophic factor receptors and their downstream signalling pathways, in particular Rho-GTPase activated protein kinases.

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Abbreviations used

TRK, tropomyosin receptor kinase; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT3, neurotrophin-3; CML, chronic myelogenous leukemia; RAS, rat sarcoma oncogene; RAF, rapidly accelerated fibrosarcoma oncogene; MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GRB2, growth factor receptor-bound protein 2; SHC, Src homology 2 domain containing; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, v-

akt murine thymoma viral oncogene homologue; PLCγ, phospholipase C-γ; DAG, diacyl-glycerol; PKC, protein kinase C; IP3, inositol trisphosphate; ATP, adenosine triphosphate; RTK, transmembrane, receptor tyrosine kinases; AML, acute myeloid leukemia; IC, intrahepatic cholangiocarcinoma, PTC, papillary thyroid cancer; Glio, glioblastoma; ALIS, automated ligand identification system. VEGFR2, vascular endothelial growth factor receptor 2; ALK, anaplastic lymphoma kinase; SAR, structure activity relationship; CDK, cyclin-dependent kinase; IGF1R, insulin-like growth factor 1; hERG, human ether-a-go-go-related gene; ROS1, reactive oxygen species 1; TIE-2 ,tunica interna endothelial-2; RETm rearranged during transfection; Ron, recepteur d'origine nantais; FLT3, fms-like tyrosine kinase 3; FDA, food and drug administration; PH, pleckstrin homology; mammary analogue secretory carcinomas (MASC)

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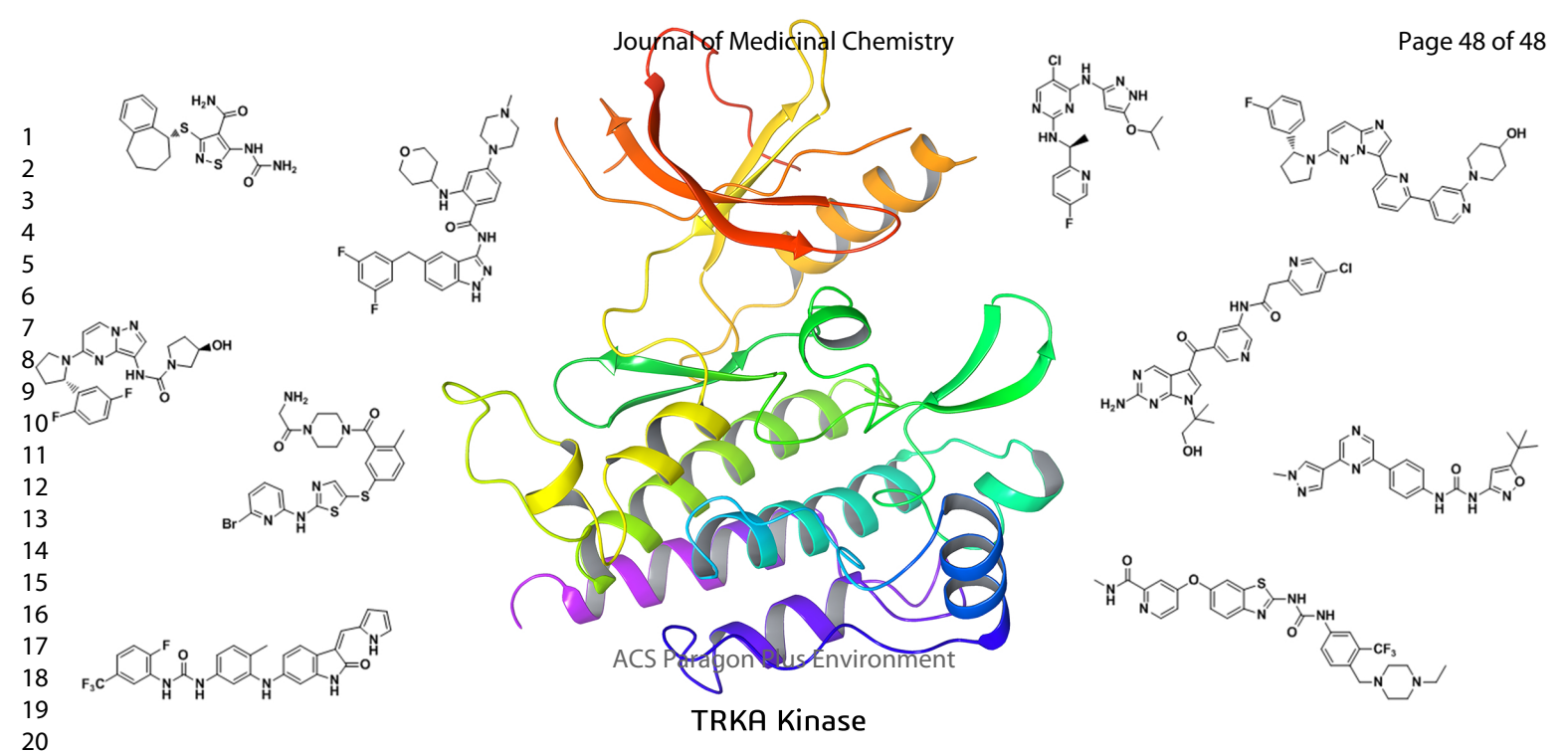
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