Targeting TRK: A Fast Tracked Application of Precision Oncology and Future Directions

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Abstract

The NTRK genes encode the tropomyosin-related receptor tyrosine kinases TrkA, TrkB and TrkC. TRK receptors play a critical role in the development of nervous system tissues during embryogenesis and early life. Recurrent genomic alterations in NTRK genes, typically fusions involving the 3' region encoding the kinase domain juxtaposed to 5' sequences from numerous partner genes, occur at a low frequency in a wide diversity of adult and pediatric cancers. Larotrectinib and entrectinib are potent first-generation NTRK inhibitors with IC50 in the nanomolar range in cellular contexts. Clinical trials of both drugs demonstrated significant and durable responses in patients with tumors harboring NTRK alterations, leading to first of its kind cancer agnostic FDA approvals in the United States for drugs targeting a genomic alteration. Unfortunately, acquired resistance inevitably develops. The second-generation NTRK inhibitors selitrectinib and repotrectinib are designed to overcome known mechanisms of resistance.

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I. Overview of NTRK structure and function

The *NTRK* genes *NTRK1* , *NTRK2* and *NTRK3* encode the tropomyosin-related receptor tyrosine kinases TrkA, TrkB and TrkC, respectively.[1] The three paralogs share sequence and structural homology, consisting of extracellular, transmembrane and intracellular domains, and differ in their ligand specificity and tissue-specific expression.[2-5] TRK receptors are activated by high affinity binding of the extracellular domain to their cognate ligands resulting in receptor dimerization and subsequent autophosphorylation of tyrosine residues in the intracellular domain. As the majority of early studies on the functional role of TRK focused on neurodevelopmental processes, cognate ligands are called neurotrophins, namely nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4, also known as NT-5).[4,5]

TRK receptors regulate the proliferation, differentiation and survival of many neuronal and non-neuronal glial cells, playing a role in synaptic plasticity, and have been identified to be particularly critical in the development of pain-mediating sensory neurons.[5,6] NGF-TrkA signaling plays an important part in the development and function of nociceptive perception in the dorsal root ganglia, the establishment of neuronal circuits involved in thermoregulation via sweating, and ovulation.[7,8] The rare genetic disorder congenital insensitivity to pain with anhidrosis (CIPA) has been linked to polymorphisms in *NTRK1* and indeed*Ntrk1* double-knockout mice express features of CIPA.[9-12] BDNF-TrkB signaling regulates appetite and loss of function mutations in *BDNF* have been linked to obesity in genome wide association studies.[13] *NTRK2* and*NTRK3* appear to be more widely expressed in central and peripheral nervous system tissues; *Ntrk2 and Ntrk3*double-knockout mice lack particular populations of motor and sensory neurons affecting movement and posture, exhibit a decreased density of myocardial blood vessels, and in the case of *Ntrk3* knockout mice also demonstrate severe cardiac anomalies.[14] Knockout of any of the three *Ntrk* genes in mice results in early death, usually surviving no more than one month.

TrkA, encoded by *NTRK1,* binds with high affinity to NGF.*NTRK1* pre-mRNA is subject to extensive alternative splicing that can result in several different TrkA isoforms.[15] Human*NTRK1* is located on the long arm of chromosome 1 and spans a region of 66 kilobases (kb) (Figure 1).[16] TrkB preferentially binds BDNF, NT-4, and at a lower affinity NT-3. The*NTRK2* gene is relatively large, spanning more than 350 kb, and is located on the long arm of chromosome 9.[6,16] The TrkC receptor selectively pairs with NT-3, with significantly less affinity for other neurotrophins.[17] The *NTRK3* gene is located on the long arm of chromosome 15, spanning a region of close to 400 kb. Following post-translational modifications including glycosylation of several residues in the extracellular domains, the mature TRK receptors all have molecular weights in the 140-145 kDa range. As is the case with other receptor tyrosine kinases, activation of the kinase domain of TRK has pleiotropic effects on downstream signaling cascades (Figure 2). While the rat sarcoma/mitogen activated protein kinase (Ras-MAPK) pathway plays a prominent role in promoting cell proliferation, the phosphatidylinositol 3-kinase (PI3K) and phospholipase C gamma 1 (PLC-γ1) pathways are also activated by TRK.[18,19]

II. NTRK Alterations in Cancer

Fusions involving all three *NTRK* genes have been described in multiple cancer types, resulting from intra-chromosomal gene rearrangements or interchromosomal translocations, and almost universally involve the 3' region encoding the kinase domain of this proto-oncogene juxtaposed to 5' sequences from numerous partner genes, several but not all of which contain coiled-coil or zinc finger domains that promote oligomerization (Table 1).[14,19] Loss of the ligand binding extracellular domain, which normally inhibits the homo-polymerization of unbound TRK and prevents autophosphorylation, as well as possible structural alterations introduced by the 5' binding partner, result in constitutive activation of the kinase domain.[20,21] The oncoprotein that results from the fusion event is both aberrantly expressed and constitutively activated, promoting malignant cell proliferation and survival.[21]

Notably, recurrent *NTRK* fusions have been described in several rare cancer [sub]types, including secretory breast carcinoma, mammary analogue secretory carcinoma (MASC) of salivary glands, congenital infantile fibrosarcoma, and congenital mesoblastic nephroma, as well as various other pediatric cancers.[14,19] In fact, recent studies indicate that *NTRK* fusions are the pathognomonic genomic alteration in over 90% of infantile fibrosarcoma and breast secretory carcinoma, and are present in a similarly high percentage of MASC and mesoblastic nephroma.[14]*NTRK* fusions also occur at a low percentage across a wide spectrum of common adult cancers, although have likely been under-reported due to the inherent technical and bioinformatics challenges of gene fusion discovery in multi-omics studies. The most sensitive and accurate diagnostic modality to detect *NTRK* fusion events has not been defined but will be a question of utmost clinical importance, and each modality has strengths and weaknesses. Immunohistochemistry to detect membrane overexpression of TRK proteins may be an initial screening test for *NTRK* fusions but has not been validated as a surrogate biomarker to predict response to targeted inhibitors, unlike ALK overexpression in lung adenocarcinoma for example. Fluorescence in situ hybridization (FISH) using break apart probes for the 5' and 3' ends of the

three *NTRK* genes has high analytic sensitivity but might miss subtle intra-chromosomal fusions involving nearby genes and has all the normal limitations of FISH including significant tissue requirement and inability to multiplex at scale.[22] Reverse-transcriptase polymerase chain reaction (RT-PCR) targeted at specific panels of known gene fusions in cancer is the basis of multiple commercially available, CLIA certified assays.[23] Next generation short read sequencing (NGS) of either DNA or mRNA can detect *NTRK* fusions along with multiple other genomic alterations, but high depth sequencing can be cost prohibitive and is subject to bioinformatics optimization for accurate variant calling.[24]

In the study published by Gatalica et al, 11,502 tissue samples of different cancer types were analyzed for 53 gene fusions, excluding common germline variants.[1] Biotinylated RNA probes were used to capture exons from 592 genes, followed by targeted sequencing using a multiplexed NGS platform. In this study, only 31 cases (0.27% of the entire cohort) had detectable *NTRK* fusions, underscoring the rarity of this oncogenic event. The most common fusions identified involved the ETS-leukemia virus variant transcription factor 6 gene, *ETV6-NTRK3* (n = 10), and the tropomyosin 3 gene, *TPM3-NTRK1* (n = 6). Interestingly, *ETV6* is a partner in several well characterized oncogenic fusions in hematologic malignancies with *RUNX1* , *JAK2* , *FLT3* , or *SYK* , although it is unknown if its proclivity for involvement in fusions is due to regional chromosomal fragility at 12p13 or added function.[25] Gliomas had the highest number of *NTRK* fusions overall (14 of 982 tumors, 1.4%), most commonly involving *NTRK2* (n = 9). The 17 non-glioma cases with *NTRK* fusions included carcinomas of the lung, thyroid, breast, cervix, colon, nasal cavity, cancer of unknown primary, and soft tissue sarcomas.

Due to the rarity and variety of *NTRK* fusions, their functional effects are not fully characterized. In addition to enhancing native TRK signaling, neomorphic functions ,au also emerge to drive oncogenesis. The *ETV6-NTRK3* fusion is the best studied as it is identified as the dominant recurrent *NTRK* fusion event in several malignancies including secretory breast carcinoma, MASC of the salivary gland, infantile fibrosarcoma, congenital mesoblastic nephroma, acute myeloid leukemia (AML), and radiation-associated papillary thyroid cancer. Although it would be expected that most fusions would use the same downstream signaling cascades as ligand-activated TRK given the preservation of the kinase domain and the critical tyrosine docking sites, the *ETV6-NTRK3* fusion may be an exception as it loses the tyrosine docking site at residue 485.[26,27] Phosphorylated Y485 interacts with the important adapter protein SHC1 (Src homology 2 domain containing transforming protein 1), which then recruits the Grb2-SOS complex, a central feature in initiating downstream RAS-MAPK signaling that may be absent with the *ETV6-NTRK3* fusion. On the other hand, studies of *NTRK1* fusions in thyroid cancer have revealed that mutant TrkA binds to a number of different adaptor molecules, similar to full length wildtype TrkA, but is preferentially engaged in signaling through the RAS-MAPK pathway in lieu of PI3K or PLC-γ1 signaling.[21]

In addition to fusions, other genomic alterations in *NTRK* have been described that could potentially be oncogenic drivers. An intragenic deletion in *NTRK1* resulting in a variant called *deltaTrkA* was first described in 2000 in a patient with AML, and encodes a receptor that lacks 75 amino acids in the N-terminus extracellular domain and four glycosylation sites adjacent to the transmembrane domain.[28] This loss of glycosylation sites and cysteine residues results in a conformational change in the receptor that apparently promotes dimerization and thus ligand-free activation of the intracellular kinase domain. The oncogenic properties were confirmed *in vitro* and *in vivo* using an AML cell line xenograft model that expressed *deltaTrkA* .[28]

Point [substitution] mutations in the kinase domain of *NTRK1* were reported in 4 of 188 cases of AML following high-depth resequencing of a panel of tyrosine kinase genes.[20] Recently, *NTRK1* kinase domain point mutations were also described in 3 of 159 patients with acute erythroid leukemia, with preclinical models demonstrating concurrent *TP53* loss of function mutations were requisite for leukemogenesis, with the combination resulting in high penetrance of erythroid leukemia in mice.[29] Other studies reported four novel point mutations in *NTRK2* and *NTRK3* in AML and two different point mutations in *NTRK3* in patients with B-cell lymphoma.[20] Whether these substitutions represent true oncogenic drivers and susceptibility to pharmacologic TRK inhibition remains to be determined; they may be sub-clonal events in some cases.

III. Preclinical studies

The recognition of *NTRK* fusions as recurrent oncogenic events and the ability to effectively inhibit kinase domain activity with small molecule inhibitors has fueled drug development efforts over the past decade. The impressive clinical results seen by targeting other receptor tyrosine kinases that are recurrently activated by gene fusions, such as ALK, ROS1 and the FGFRs serve as an important paradigm in the development of highly selective TRK inhibitors. The first two in class agents to proceed to clinical development were larotrectinib and entrectinib. Both demonstrated promising preclinical results, impressive proof of concept in early phase clinical trials, and ultimately fast tracked to regulatory approval through innovative clinical trial design.

Larotrectinib (LOXO-101) is a competitive inhibitor of the adenosine triphosphate (ATP)-binding site of the kinase domain of TrkA, TrkB, and TrkC and consequently interferes with autophosphorylation and subsequent downstream signaling.[21,25] It is a highly selective and potent inhibitor with IC_{50} levels in the low nanomolar (nM) range. Larotrectinib exhibited dose dependent activity in a panel of cancer cell lines harboring *NTRK* fusions including CUTO-3.29 (IC_{50}: 59 nM) developed from a patient with lung adenocarcinoma with *MPRIP-NTRK1* ; KM12 (IC_{50}: 3.5 nM) derived from a patient with colorectal adenocarcinoma harboring*TPM3-NTRK1* ; and MO-91 (IC_{50}: <10 nM) from a patient with AML with *ETV6-NTRK3.* [25] The*in vivo* activity of larotrectinib was confirmed in athymic nude mice xenografted with the KM12 cell line. Mice were treated with an oral gavage preparation of larotrectinib for two weeks. Dose dependency was again observed, with mice receiving a 200 mg/kg dose demonstrating significantly superior and sustained tumor growth inhibition in comparison to the 60 mg/kg dose group.[4,25]

Preclinical studies of entrectinib (RXDX-101) demonstrated similar results to larotrectinib. Entrectinib inhibits the enzymatic activity of all three TRK receptors at concentrations in the 1 to 5 nM range, but also potently inhibits ROS1 and ALK with IC_{50} of 12 and 7 nM in a radiometric kinase assay, respectively.[30]The selectivity of entrectinib was confirmed *in vitro* against a panel of 200 cancer cell lines following 72 hours of continuous exposure. Entrectinib demonstrated potent growth inhibition in only seven of these cell lines with IC_{50} values less than 100 nM; the strongest activity was against the KM12 cell line harboring*TPM3-NTRK1* (IC50: 17 nM), while cell lines with *ALK* and*FLT3* alterations exhibited IC_{50} values in the range of 20 to 81 nM.[30] The activity of entrectinib against recurrent alterations observed in patient tumors was further explored in the IL3-dependent murine B-cell line Ba/F3. These cells with ectopically expressed *ETV6-NTRK2* (IC_{50}: 2.9 nM),*ETV6-NTRK3* (IC_{50}: 3.3 nM), and *ETV6-ROS1*(IC_{50}: 5.3 nM) were all highly sensitive when treated with entrectinib, while parental Ba/F3 cells and those ectopically expressing alternative oncogenic tyrosine kinases such as *ABL* or*RET* were not ($IC_{50} > 1$ μM).[30]

Several next generation TRK inhibitors have also demonstrating promising preclinical activity and have entered clinical trials. A study published in 2019 demonstrated that taletrectinib (DS-6051b), a selective ROS1 and TRK inhibitor, induced dramatic growth inhibition of *NTRK* -fusion positive tumors *in vivo* in a dose dependent manner, with almost complete growth inhibition achieved at doses [?]50 mg/kg in KM12 xenografts.[31] Intriguingly, this agent also demonstrated activity in xenografted tumors derived from Ba/F3 cells with ectopic expression of rearranged *ROS1* harboring a recurrent secondary drug resistance kinase domain mutation, G2032R. This secondary mutation renders cancers resistant to crizotinib, lorlatinib, and entrectinib, but potent growth inhibition was seen with taletrectinib at doses as low as 30 mg/kg and there is reason to believe that this agent may also overcome resistance in *NTRK* -fusion positive cancers.[31]

IV. Clinical trials

Preclinical studies consistently demonstrated the potent efficacy of larotrectinib and entrectinib in tumors harboring *NTRK* fusions and the relative selectivity of these agents compared to several other kinase inhibitors in clinical use suggested low potential for off-target toxicity. Subsequent clinical trials have demonstrated outstanding response rates in patients with *NTRK* fusion-positive cancers, leading to biomarker based regulatory approval and represent a remarkably fast timeline of only two decades from the widespread recognition of this important oncogenic event to the effective treatment of patients.

The safety and efficacy of larotrectinib were evaluated in three early-phase, cancer-type agnostic clinical trials enrolling 55 subjects, including eight patients in an adult phase I dose finding trial, 12 pediatric patients enrolled to the phase I/II basket trial SCOUT, and 35 patients, at least 12 years of age, enrolled to the phase II NAVIGATE trial.[32-34] Patients eligible for these studies had locally advanced or metastatic *NTRK* -positive solid tumors and were previously treated with standard of care therapy or would be unlikely to tolerate or have meaningful benefit from appropriate standard of care therapy. Measurable disease by RECIST criteria was required and central nervous system (CNS) metastases were allowed, although NAVIGATE excluded symptomatic CNS disease. In preclinical evaluations, there was lack of evidence of efficacy of larotrectinib in tumors harboring *NTRK* substitution mutations, and thus clinical trials were restricted to *NTRK* fusions. Other eligibility criteria included an ECOG performance status 0-2 in NAVIGATE and a Karnofsky or Lansky performance score of at least 50 in SCOUT and adequate hematologic parameters and major organ function. The median age of subjects enrolled to NAVIGATE was 45 years, with 77% being 15 years or older. Subjects enrolled in these trials had malignancies that originated from 17 different tissues, including salivary gland carcinoma (22%), infantile fibrosarcoma (13%), thyroid carcinoma (7%), colon cancer (7%), lung cancer (7%), and melanoma (7%). The specific*NTRK* fusion involved *NTRK3* in 53%, *NTRK1* in 45%, and *NTRK2* in only 2% of subjects.

The phase I studies evaluated six oral dose levels of larotrectinib ranging from 50 mg once daily to 200 mg twice daily and established 100 mg twice daily as the optimal recommended phase II dose. Peak plasma concentrations of larotrectinib were observed 30 to 60 minutes after dosing, consistent with rapid oral bioavailability and relatively short half-life.[35] Ninety-eight percent inhibition of TrkA, TrkB, and TrkC was achieved at all dose levels.[4]

The primary efficacy endpoint was the objective response rate (ORR) by RECIST, scored by independent review. The ORR was an impressive 75% across the three trials including a complete response rate of 13% and partial response rate of 62%, while 13% demonstrated stable disease and only 9% had progressive disease at first assessment; 4% could not be evaluated owing to early withdrawal for clinical deterioration.[32] These response rates are on par with the most potent EGFR and ALK inhibitors in the first line treatment of advanced *EGFR* mutated and *ALK*- rearranged lung cancers, respectively.[36,37] Two subjects with infantile fibrosarcoma were able to proceed with curative limb-sparing resection. Although studies were underpowered for subgroup analysis, responses were noted across tumor types, age, and specific *NTRK* fusion. Interestingly, responses were typically seen early, with the median time to response 1.8 months (range 0.9 to 6.4) and the median duration of response was 8.3 months. This duration of response is notably shorter than the 12 to 18 months with the aforementioned newer EGFR and ALK inhibitors in lung cancer. The median progression-free survival across all subjects was not reached at 9.9 months of median follow-up and the clinical benefit was durable with 55% of subjects remaining free of progression at 1 year of follow-up.

Importantly, larotrectinib was found to be well tolerated in this patient population, despite the fact that many were heavily pretreated including 35% who had received three or more prior chemotherapies.[32] Only 15% of subjects required dose reduction and no subject with an objective response required drug discontinuation due to an adverse event. The most common adverse events grade 3 or higher, regardless of attribution to drug, were anemia (11%), neutropenia (7%), increased alanine aminotransferase or aspartate aminotransferase level (7%), and weight gain (7%). There were no treatment-related grade 4 or 5 events reported. Overall, it was concluded that larotrectinib is highly effective and well tolerated in patients with *NTRK* fusion-positive solid tumors, regardless of cancer type and age, resulting in accelerated approval by the Food and Drug Administration (FDA) in the United States in November 2018.[4,32] Larotrectinib represents the first ever antineoplastic agent targeting a genomic alteration with a cancer type agnostic approval label. While no specific companion diagnostic was linked to the approval, the FoundationOne CDx assay is approved for use in this setting.

The safety and efficacy of entrectinib were first evaluated in the phase I Alka-372-001 and the phase I/IIA STARTRK-1 studies that enrolled patients with advanced solid tumors harboring genomic alterations in any of the *NTRK* genes, *ROS1* , or*ALK* .[38,39] The median age of enrolled subjects was 55 years (range, 18–80).

All subjects had a histologically or cytologically confirmed diagnosis of relapsed or refractory advanced or metastatic solid tumor that did not respond to standard therapy or for which standard therapy was considered unsuitable or intolerable. Additional inclusion criteria included ECOG performance status [?]2 with the vast majority (n = 114/119) having an ECOG of 0 or 1, an anticipated life expectancy of [?]3 months, and adequate hematologic parameters and major organ function. The majority of subjects had received three or more prior lines of treatment (83%), including prior ALK/ROS1 inhibitors (27%), chemotherapy, and immunotherapy regimens. Given the inclusion of tumors with *ALK* and *ROS1* alterations, the predominant tumor types were non-small cell lung cancer (NSCLC: 60%) and tumors of the gastrointestinal tract (15%). Of the 119 subjects in the trials, 60 had tumors harboring a rearrangement in *ROS1* , *ALK* , or *NTRK1/3/2* , in order of frequency. Of the remaining 59 subjects, 53 had other genomic alterations in these same genes on central review, broadly categorized as point mutations, copy number gains, or insertions/deletions, while six subjects were enrolled to only the phase I component without a known genomic alteration in any of these five genes.

Subjects were enrolled in two groups, with the first cohort of 54 treated on the following dosing schedules of entrectinib: Schedule A, n=19, drug taken on empty stomach, four days on followed by three days off, for 21 of 28 days each cycle; Schedule B, n=29, drug taken with food, continuous daily dosing for 28 of 28 days; and Schedule C, n=6, drug taken with food, four days on followed by three days off, for 28 of 28 days.[38-40] Entrectinib was provided in capsule form with starting doses of 100 mg, 200 mg, 400 mg, 800 mg, 1200 mg, or 1600 mg in ALKA-372–001 and 100 mg, 200 mg, 400 mg, 600 mg, or 800 mg in STARTRK-1. The expansion cohort enrolled 65 subjects, all receiving entrectinib 600 mg once daily continuous dosing. Treatment was stopped if there was evidence of radiographic progression, severe toxicity, or withdrawal of consent. A subgroup of n=24 subjects with cancer harboring one of the five gene fusions of interest and who were tyrosine kinase inhibitor (TKI)-naïve, and whose dosing achieved therapeutic exposures consistent with entrectinib 600 mg daily were defined as a "Phase II-eligible population". Subjects with *NTRK* fusions previously treated with crizotinib were considered TKI-naïve in this context due to the poor potency of crizotinib at inhibiting TRK activity (IC_{50} >500nM). The efficacy of entrectinib is further being prospectively evaluated in subjects meeting these criteria in the ongoing STARTRK-2 phase II trial.

Entrectinib demonstrated strong antitumor activity in the Phase II-eligible subgroup with an objective response rate of 83% (n=20/24), including responses in all four subjects with cancers harboring *NTRK* fusions, and a complete response rate of 8.3%.[38,39] Similar to the larotrectinib data, the majority of responses occurred early – first observed during the first two cycles, and several subjects continued treatment beyond a year, with the longest response approaching 2.5 years at time of data cutoff. Clinical benefit was observed across a broad range of solid tumors regardless of histology, including NSCLC, MASC of the salivary gland, melanoma, gliomas, colorectal cancer, and renal cell carcinoma. Alternatively, objective responses were exceedingly rare in subjects whose tumors harbored non-fusion genomic alterations involving *NTRK1/2/3, ROS1* , or *ALK* or those previously treated with a TKI. The one exception was a patient with neuroblastoma with an *ALK* p.F1245V point mutation for whom a confirmed partial response lasted 8.3 months; this patient in fact remained on study treatment for more than 3.5 years due to clinical benefit. No responses were observed in 25 subjects with *ALK or ROS1* rearrangements who had previously received crizotinib, ceritinib, or alectinib.

Entrectinib demonstrated a comparable safety profile to larotrectinib with mostly grade 1 or 2 adverse events and dose reduction required by only 15% of all subjects.[39] The most commonly reported treatment-related adverse events of any grade included fatigue/asthenia (46%), dysgeusia (42%), paresthesias (29%), nausea (28%) and myalgias (23%); no grade 3 adverse event occurred in more than 5% of subjects. Potential on-target toxicity related to impaired physiologic TRK signaling appears to be generally mild with both larotrectinib and entrectinib, including mostly grade 2 paresthesias in 29%, dizziness in up to 25%, and weight gain in 10%. A single grade 4, potentially treatment-related adverse event was reported in a patient who developed eosinophilic myocarditis. There were no significant differences in the toxicity profile for the intermittent versus continuous dosing schedules.

Importantly, entrectinib is a small liposoluble molecule with evidence of significant blood brain barrier penetrance, with CSF concentrations estimated to be about 25% of plasma concentrations[41], and there is preliminary evidence for significant clinical activity against both primary and metastatic brain tumors from these trials. This is particularly relevant as *NTRK, ROS1,* and *ALK*rearrangements do occur in primary adult and pediatric brain tumors, including low grade glioma and glioblastoma, where systemic treatment options are limited, as well as in lung cancer and malignant melanoma that have a proclivity for CNS metastasis.[14,39]Entrectinib garnered accelerated approval by the Food and Drug Administration (FDA) in the United States in August 2019 for the treatment of advanced solid tumor malignancies harboring *NTRK*fusions and *ROS1* -rearranged NSCLC.

V. Acquired resistance to first-generation TRK inhibitors

Despite the impressive responses with larotrectinib and entrectinib realized in most patients with cancers harboring *NTRK* fusions, as with other targeted antineoplastics, resistance appears to inevitably emerge. The median duration of response to larotrectinib is 8.3 months[32] and to entrectinib 10.5 months for the subgroup with *NTRK* fusions[38] according to a pooled analysis of early phase trials. Subsequent targeted sequencing at time of progression in patients with an initial response demonstrated that up to 90% had secondary mutations in the kinase domain that are predicted to result in drug resistance.[4,39] The majority of the reported mutations involved amino acid substitutions in the solvent-front (*NTRK1* p.G595R, *NTRK3* p.G623R),gatekeeper residues (*NTRK1* p.F589L,), or the activation loop X-aspartate-phenylalanine-glycine, "xDFG" motif (*NTRK1* p.G667C,*NTRK3* p.G696A) (Table 2). Computational modeling and X-ray crystallography suggests that the majority of these mutations result in steric clashes between the charged, bulky side chains of involved amino acids (e.g. arginine) and the hydroxypyrrolidine or difluorophenyl groups of first-generation TRK inhibitors.[33] Some of these mutations are also predicted to increase the ATP affinity of the kinase domain. Several of the identified mutations are paralogous to recurrent solvent-front and gatekeeper drug resistance mutations reported in *ALK* and *ROS1* -rearranged cancers.

In addition to the reported secondary *NTRK* mutations, new alterations of other receptor tyrosine kinases or downstream signaling pathways were also identified at the time of acquired resistance, and are postulated to be potential mechanisms of drug resistance in some tumors. These included RAS-MAPK alterations – *BRAF* p.V600E and*KRAS* p.G12D, and *MET* amplification.[14,42]

VI. Second-generation TRK inhibitors in development

Overcoming resistance mechanisms is the focus of development for the second-generation of TRK inhibitors. So far, two second-generation inhibitors have entered clinical development (Table 3), selitrectinib (LOXO-195) and repotrectinib (TPX-0005), while ONO-5390556 and taletrectinib have undergone significant preclinical characterization. Both selitrectinib and repotrectinib are lower molecular weight compounds than the first-generation inhibitors, which allows them to interact with the ATP-binding site while avoiding steric clash with solvent-front mutations and competing substrates (Figure 3).[33,43,44] This property may also enhance blood brain barrier penetrance, but this is yet to be established.

Selitrectinib is a selective and potent pan-TRK inhibitor being developed by Bayer. It was screened against a panel of transfected cell lines harboring dual *NTRK* -fusions and secondary resistance mutations identified in patients treated with larotrectinib or entrectinib and demonstrated potent IC_{50}'s against the majority.[33] Combined preliminary safety and efficacy data of selitrectinib from 31 patients treated on a compassionate access program or as part of a phase I trial of selitrectinib, all with progression or intolerance to at least one first-generation agent, was reported at AACR 2019.[33,45,46] Ten of 29 evaluable patients (34%) had a confirmed complete or partial response, including responses in nine out of 20 patients (45%) who developed secondary treatment-emergent *NTRK* mutations on the first-generation agent. The majority of these mutations [70%] were solvent front substitutions. On the other hand, patients with bypass mutations or with an unidentified mechanism of resistance to the first-generation agent generally did not respond to selitrectinib, an observation that mimics the experience with next generation EGFR and ALK inhibitors in lung cancer. Selitrectinib was well tolerated at lower doses up to 100 mg twice daily, but the maximum tolerated or optimal dose and

schedule has not yet been established.[4,33,45]

Repotrectinib is another second-generation pan-TRK inhibitor, being developed by Turning Point Therapeutics, which like entrectinib also demonstrates considerable preclinical activity against *ALK* and*ROS1* -rearranged tumors.[47] In an ongoing phase I/II trial, early reporting includes confirmed responses to repotrectinib in subjects with cancers harboring *ROS1* and*NTRK3* fusions who had relapsed on earlier generation TKIs.[47] Even though safety, dosing and clinical efficacy are still being established, repotrectinib appears to be a promising treatment option for *ROS1* and *NTRK* fusion positive malignancies including those with acquired resistance mutations.

ONO-5390556 is a compound being developed by Ono pharmaceutical in Japan as a potent second-generation TRK inhibitor. Preclinical studies demonstrated that ONO-5390556 is a highly potent and selective pan-TRK inhibitor that demonstrates antitumor activity against *NTRK*fusion positive tumors, including those expressing acquired drug resistance mutations, comparable to selitrectinib and repotrectinib.[48]

Finally, several multi-kinase inhibitors that potently but not selectively inhibit TRK, including cabozantinib, lestaurtinib, foretinib, merestinib, and sitravatinib demonstrate preclinical efficacy against secondary mutations and may overcome bypass mechanisms of resistance.[14] However, toxicity at pharmacodynamically relevant doses is a major concern and the overall rarity of *NTRK*alterations makes it an unappealing area for clinical development for these drugs.

VII. Discussion

The discovery of *NTRK* fusions as recurrent genomic alterations across a wide spectrum of adult and pediatric cancers, including as pathognomonic events in four rare cancer types, led to their rapid preclinical validation as powerful oncogenic drivers. This would then spur fast paced clinical development of highly selective small molecule TRK inhibitors in well-designed early phase basket trials, leading to regulatory approval at breakneck speed. Moreover, the approval of entrectinib and larotrectinib are potentially paradigm-shifting in precision oncology efforts as they represent the first molecularly targeted drugs to gain cancer type agnostic approval. Indeed, these approvals open the door for the development of several other classes of drugs targeting low frequency driver events that are not unique to any one cancer type and perhaps ultimately a taxonomy system in clinical oncology that is not histology dependent at all.

Several important questions remain. In addition to the advanced or metastatic setting, is there a role for TRK inhibitors in the adjuvant or perioperative setting? Will next generation TRK inhibitors significantly overcome or delay resistance, and potentially supplant larotrectinib and entrectinib in the first line setting, analogous to EGFR inhibitors like osimertinib and ALK inhibitors like alectinib, ceritinib and brigatinib? Can TRK inhibitors be safely combined with immunotherapy or chemotherapy and can this strategy potentiate responses? Will the development of improved clinical genomic platforms aimed at detecting gene fusions increase the number of candidates who will benefit from TRK inhibitors? Perhaps most importantly, given the excellent tolerability profile of these drugs, how can we maximize the number of patients who can benefit? Specifically, are there tumors dependent on TRK signaling in the absence of direct genomic alteration, such as activation through epigenetic events or sustained post-translational modification?

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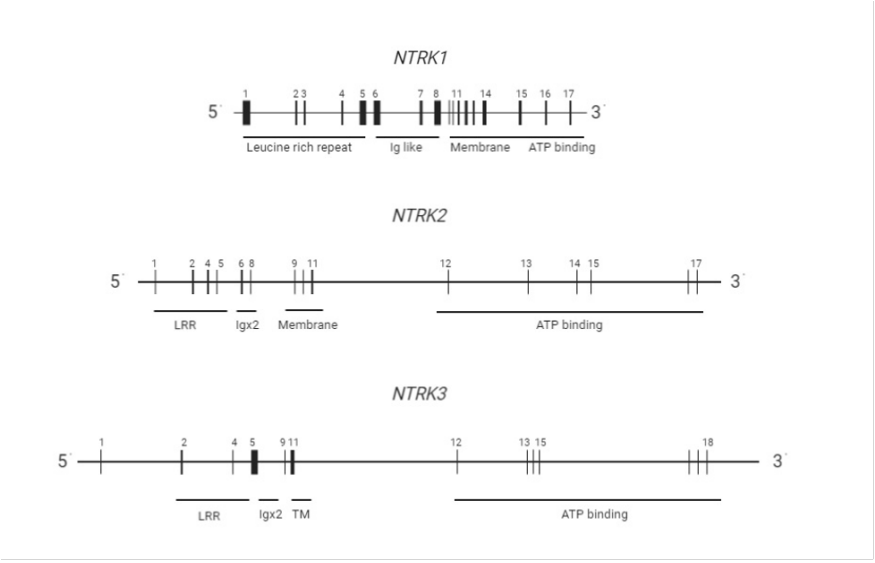

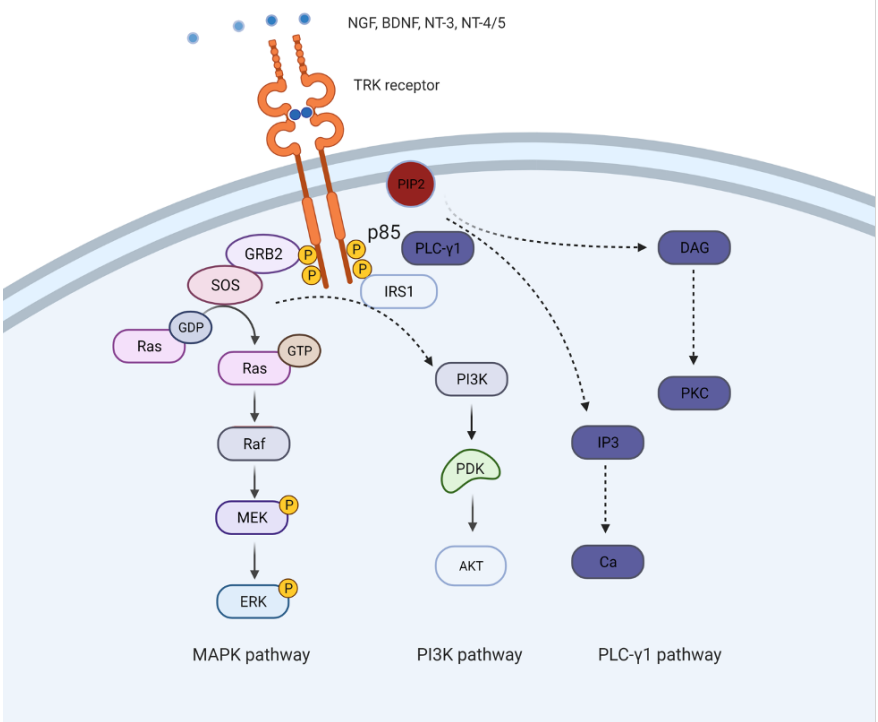

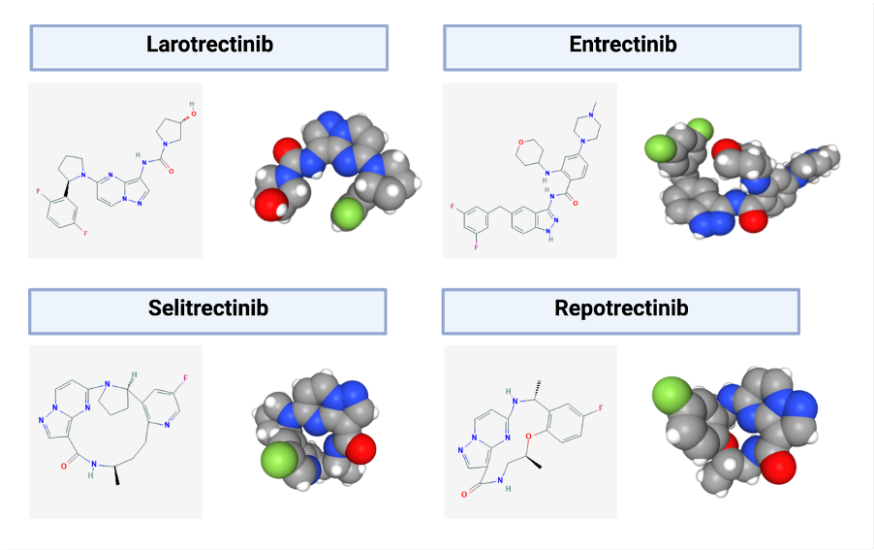

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