



REVIEW ARTICLE

Targeting anaplastic lymphoma kinase in neuroblastoma

GANESH UMAPATHY, PATRICIA MENDOZA-GARCIA, BENGT HALLBERG and
RUTH H. PALMERDepartment of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy,
University of Gothenburg, Gothenburg, Sweden

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Over the last decade, anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase (RTK), has been identified as a fusion partner in a diverse variety of translocation events resulting in oncogenic signaling in many different cancer types. In tumors where the full-length ALK RTK itself is mutated, such as neuroblastoma, the picture regarding the role of ALK as an oncogenic driver is less clear. Neuroblastoma is a complex and heterogeneous tumor that arises from the neural crest derived peripheral nervous system. Although high-risk neuroblastoma is rare, it often relapses and becomes refractory to treatment. Thus, neuroblastoma accounts for 10–15% of all childhood cancer deaths. Since most cases are in children under the age of 2, understanding the role and regulation of ALK during neural crest development is an important goal in addressing neuroblastoma tumorigenesis. An impressive array of tyrosine kinase inhibitors (TKIs) that act to inhibit ALK have been FDA approved for use in ALK-driven cancers. ALK TKIs bind differently within the ATP-binding pocket of the ALK kinase domain and have been associated with different resistance mutations within ALK itself that arise in response to therapeutic use, particularly in ALK-fusion positive non-small cell lung cancer (NSCLC). This patient population has highlighted the importance of considering the relevant ALK TKI to be used for a given ALK mutant variant. In this review, we discuss ALK in neuroblastoma, as well as the use of ALK TKIs and other strategies to inhibit tumor growth. Current efforts combining novel approaches and increasing our understanding of the oncogenic role of ALK in neuroblastoma are aimed at improving the efficacy of ALK TKIs as precision medicine options in the clinic.

Key words: Anaplastic lymphoma kinase; ALKAL; TKIs; neuroblastoma; non-small cell lung cancer; ALK-positive tumours; signal transduction.

Bengt Hallberg and Ruth H. Palmer, Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg SE-405 30, Sweden. e-mails: bengt.hallberg@gu.se (BH); ruth.palmer@gu.se (RHP)

The ALK receptor tyrosine kinase can be activated in a wide range of human cancers by both chromosomal translocations leading to ALK-fusion proteins and mutation in the context of full-length ALK (Fig. 1). In addition to these two main mechanisms of ALK activation, ALK overexpression and activation in the absence of genetic aberration has also been described.

ALK-FUSION PROTEINS IN HUMAN CANCER – IDENTIFICATION OF CHROMOSOMAL TRANSLOCATIONS

Anaplastic lymphoma kinase was originally described as a fusion partner with nucleophosmin

(NPM) in anaplastic large cell lymphoma (ALCL) (1). Since then, almost 30 different ALK-fusion partners have been reported, identifying the ALK locus as a ‘hot spot’ for translocation events that occur in a wide range of cancers (2, 3). ALK-fusion proteins share common features, including: (i) regulation of expression by the promoter of the fusion partner, (ii) modulation of subcellular localization by the fusion partner and (iii) ALK-fusion dimerization/oligomerization by the fusion partner, leading to trans-autophosphorylation of the ALK kinase domain and subsequent signaling to downstream targets (4–7). Here, we briefly introduce ALK fusions in three of the more studied cancers: ALCL, inflammatory myofibroblastic tumors (IMTs) and non-small cell lung cancer (NSCLC).

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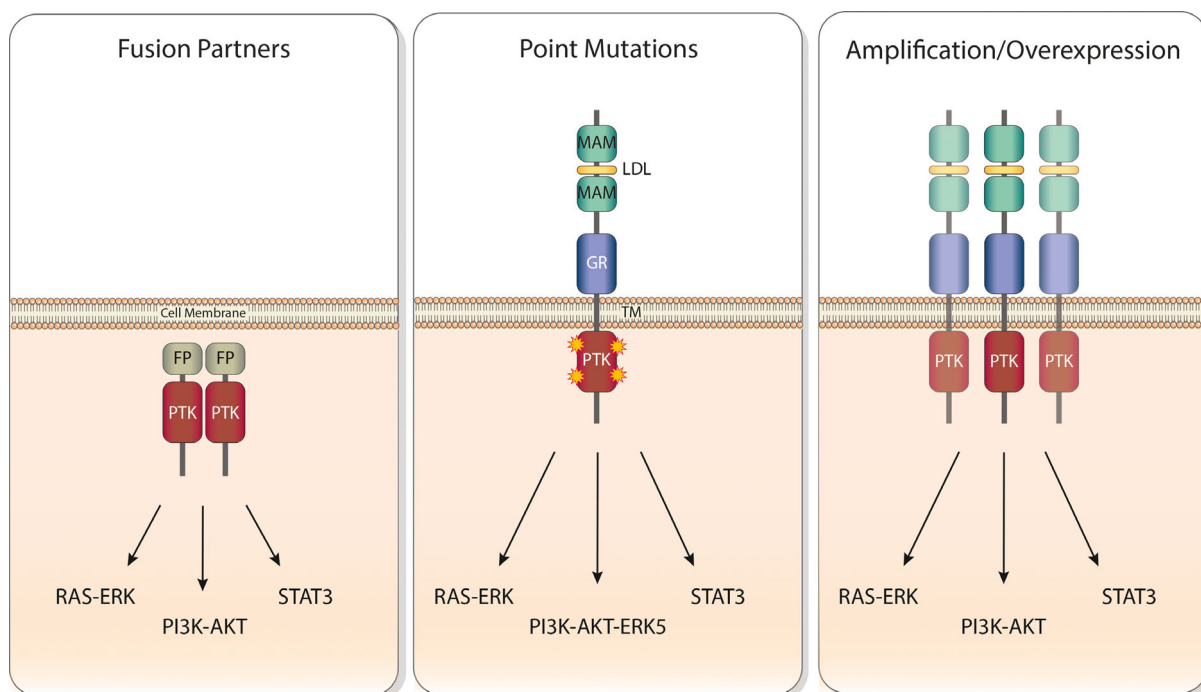


Fig. 1. Oncogenic anaplastic lymphoma kinase (ALK) signaling. Schematic representation of different oncogenic forms of ALK (fusions, point mutations, amplification/overexpression) and downstream signaling pathways. FP, fusion protein; GR, glycine-rich domain; LDL, low-density lipoprotein class A domain; MAM, meprin, A-5 protein and receptor protein tyrosine phosphatase Mu domain; PTK, protein tyrosine kinase domain; TM, transmembrane region.

ALK FUSIONS IN ALCL, IMT AND NSCLC

Anaplastic large cell lymphoma

Anaplastic large cell lymphoma (ALCL) is a rare type of Non-Hodgkin lymphoma involving T-cell receptor rearrangement that commonly occurs in children and young adults (8). In ALCL, the predominant ALK translocation fusion partner is NPM-ALK, which occurs in approximately 80% of ALK-positive ALCL cases (Fig. 2) (9, 10). The molecular characterization of NPM-ALK was first reported in ALCL in 1994, with a number of other ALK translocation fusions since reported in ALCL, including MSN-ALK, ALO17-ALK, TFG-ALK, TPM3-ALK, TPM4-ALK, MYH9-ALK, ATIC-ALK, CLTC-ALK and TRAF1-ALK (3, 8).

Inflammatory myofibroblastic tumor

Inflammatory myofibroblastic tumors (IMTs) are rare mesenchymal neoplasms that frequently originate in the lung, abdomen and retroperitoneal region and mostly affect young adults (11). Almost 50% of IMT cases exhibit rearrangement of the *ALK* locus at 2p23, of which half are fusions with

TPM3 that result in the TPM3-ALK fusion protein (Fig. 2) (12, 13). ALK translocations in both ALCL and IMT are associated with better prognosis (14–16). Similar to ALCL, other ALK fusions, such as TPM4-ALK, SEC31A-ALK, PPFIBP1-ALK, RANBP2-ALK, CARS-ALK, ATIC-ALK, CLTC-ALK, TFG-ALK, EML4-ALK, PRKAR1A-ALK, LMNA-ALK, FN1-ALK and NUMA1-ALK, are also found (3, 17, 18).

Non-small cell lung cancer

Lung cancer is one of the leading causes of cancer death worldwide, which is classified into two subgroups: (i) small cell lung cancer (SCLC) and (ii) non-small cell lung cancer (NSCLC) (19, 20). Almost 80% of lung carcinoma belongs to the NSCLC subgroup. The EML4-ALK fusion protein accounts for around 2–9% of NSCLC adenocarcinoma cases, and ALK-positive NSCLCs therefore represent the largest ALK-positive patient group (2, 5, 21, 22). EML4-ALK is the product of an inversion event at chromosome 2p, which results in the fusion of N-terminal region containing coiled coil domain of the *EML4* gene with the tyrosine kinase domain of the *ALK* gene (5, 21). At least 15

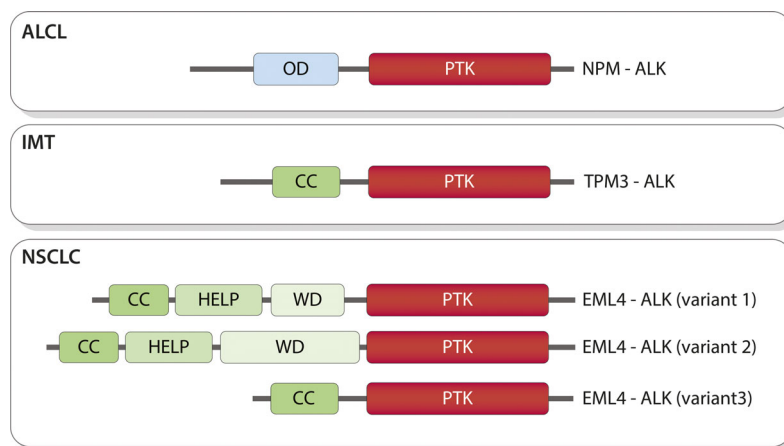


Fig. 2. Schematic depicting the domain structure of the most common anaplastic lymphoma kinase (ALK) fusions found in anaplastic large cell lymphoma (ALCL), inflammatory myofibroblastic tumors (IMTs) and non-small cell lung cancer (NSCLC). Fusion partners mediate dimerization of the ALK-fusion proteins resulting in constitutive activation of the ALK tyrosine kinase. Domains are highlighted as: oligomerization domain (OD, in blue), ALK protein tyrosine kinase (PTK, in red), coiled coil (CC, in green), HELP (lime green), WD40 (light green).

different EML4-ALK variants have been described to date, with variants 1, 2 and 3a and 3b being most common (Fig. 2) (23, 24). Almost all EML4-ALK variants contain exons 20–29 of *ALK* encoding the intracellular kinase domain; however, they contain different portions of *EML4*, which are thought to play a role in the stability or activity of the resulting fusion protein (7, 23–25). In addition to EML4-ALK, other translocations reported in NSCLC are HIP1-ALK, STRN-ALK, PTPN3-ALK, TFG-ALK, KLC1-ALK, KIF5B-ALK and TPR-ALK (3, 21, 26–31). ALK-targeted therapies are routinely employed clinically for ALK-positive NSCLC; however, understanding the resistance mechanisms that arise in response to ALK inhibitor therapy is currently a major clinical challenge (2, 32–35).

ALK POINT MUTATIONS IN HUMAN CANCER

Activation of ALK, whether by ALKAL ligands or by mutation, leads to downstream signaling via MEKK2/3-MEK5-ERK5, PI3K-AKT-mTOR, RAS-MAPK and PLC- γ pathways (Fig. 3) (7, 36, 37). The signaling initiated by ALK varies depending on the cell or tumor type as well as the method of ALK activation, whether by ligand, fusion partner, overexpression or activating mutation. A number of cancers have been associated with activating point mutations in ALK, including anaplastic thyroid tumors (ATC), NSCLC and neuroblastoma (2, 7, 38–43). While the ALK point mutations ALK-L1198F and ALK-

G1201E were described as gain-of-function activating point mutations in anaplastic thyroid tumor (ATC) (43), a recent report has shown that neither is constitutively active, thus questioning the role of ALK as an oncogenic driver in ATC (44). We focus in the following sections on ALK variants in neuroblastoma and patients, predominantly with ALK-positive NSCLC, who have been treated with ALK TKIs.

NEUROBLASTOMA – A BRIEF OVERVIEW

Neuroblastoma is a childhood cancer that arises in the sympathetic nervous system. It accounts for 8–10% of all childhood cancer deaths and is the most commonly diagnosed cancer in infants under one year (45). Neuroblastoma is a complex and heterogeneous disease which affects very young children with a median age of 22 months at diagnosis (46, 47). Children can develop tumors at any point along the sympathetic chain; however, neuroblastoma most frequently originates in the area of the adrenal medulla, disseminating to tissues of the abdomen, chest, pelvis and neck region (45, 47–49). Neuroblastoma is classified in five clinical stages (stages 1–4 and 4S) according to the International Neuroblastoma Staging System (INSS) (50–52). In neuroblastoma, as in other pediatric cancers, the mutation load is low (53–55). In contrast, chromosomal aberrations are important for prognosis in neuroblastoma, with the most common genetic anomalies being deletions of parts of chromosome arms 1p and 11q, 17q gain, triploidy, as well as *MYCN* and *ALK* amplifications (47, 56–60). Amplification of *MYCN*

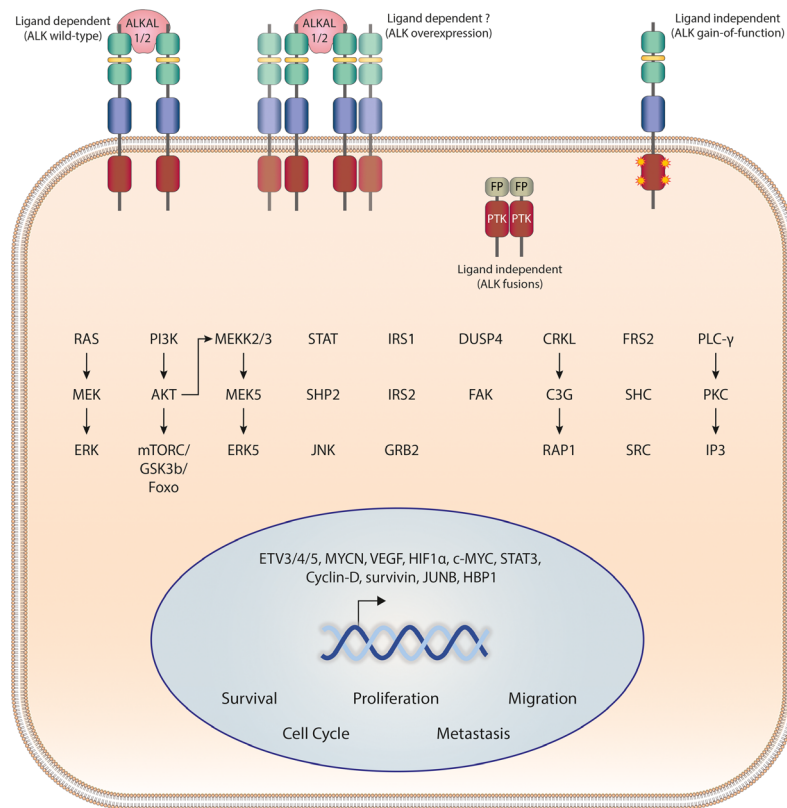


Fig. 3. General overview of anaplastic lymphoma kinase (ALK) downstream signaling. ALK signaling can be activated in a ligand-dependent (ALK wild-type) or a ligand-independent manner (ALK gain-of-function, ALK fusions, overexpression/amplification). ALK signals through multiple downstream pathways and stimulates the initiation of transcription to regulate specific cellular processes. The range of signaling pathways and cellular responses activated in response to ALK activation varies with cell type and ALK status (such as ALK fusion, overexpression or point mutation).

on chromosome 2p24 is one of the main hallmarks of neuroblastoma, observed in 20–30% of all neuroblastoma cases and associated with poor survival (47, 51, 61). MYCN is involved in cell proliferation, apoptosis, survival and differentiation (62). Neuroblastoma models in which MYCN is overexpressed in the neural crest lead to neuroblastoma tumor development, that is accelerated by cooperation with other oncogenes and tumor suppressor genes, such as *ALK*, *NF1*, *TP53*, *LIN28B* and *LMO1*, driving increased penetrance and earlier onset of neuroblastoma (63–68). Other factors, which also contribute to neuroblastoma tumorigenesis, are loss of heterozygosity (LOH) for chromosome 14 (14q), loss of *NF1* and *CDKN2A*, amplification of *DDX1* and *MDM2*, aberrant expression of neurotrophin receptors, ganglioside GD2, polycomb complex protein Bmi-1, micro RNAs (miR-10b, miR-29a/b, miR-335), as well as mutations in *PHOX2B*, *ATRX*, *CHEK2* and *BARD1* (53, 69–77). In addition to protein coding genes, long noncoding RNAs, such as neuroblastoma associated transcript-1 (NBAT-1)

and Cancer Susceptibility 15 (CASC15), regulate neuroblastoma tumorigenesis via cell proliferation and neuronal differentiation (78, 79).

ALK MUTATIONS IN NEUROBLASTOMA

Initial reports of *ALK* gene amplification and ALK protein overexpression suggested a role of ALK in neuroblastoma (80, 81). This was firmly established with the identification of ALK point mutations in both familial and sporadic neuroblastoma (56, 57, 82–84). In addition, ALK activating deletions and translocations have been described (40, 85). The majority of the reported mutations are located within the ALK kinase domain and are present in 7–8% of all neuroblastoma cases (58, 86). While a large range of mutations are observed, the most frequently found ‘hotspot’ mutations are *ALK-F1174* (V, L, S, I, C), *ALK-F1245* (C, I, L, V) and *ALK-R1275* (L or Q) in the kinase domain, which account for around 85% of all ALK mutant cases

Table 1. Frequency of ALK mutations in neuroblastoma

	Bresler et al.	De Brouwer et al.
Number of neuroblastoma investigated	1596	709
Number of neuroblastoma with ALK mutations (%)	126 (8)	49 (6.9)
Number of ALK-F1174L/V/S/I/C (% of ALK-positive)	38 (30)	17 (34.7)
Number of ALK-F1245C/I/L/V (% of ALK-positive)	15 (12)	3 (6.1)
Number of ALK-R1275L/Q (% of ALK-positive)	54 (43)	24 (49)

ALK, anaplastic lymphoma kinase.

Percentage of ALK-F1174, ALK-F1245, ALK-R1275 mutations in the neuroblastoma patient population is shown. Data from (58, 86).

(Table 1 and Fig. 4A). ALK-F1174, ALK-F1245 and ALK-R1275 mutant variants are transforming when expressed in either nude mice or NIH3T3 cells (82, 83, 87). Furthermore, ALK drives the transcription of *MYCN* and ALK-F1174L has been shown to cooperate with *MYCN* to enhance the tumorigenic activity in neuroblastoma mouse models (3, 64, 88–90). Subsequent analyses of the remaining 15% of ALK mutations found in neuroblastoma patients have highlighted differential activity, and ligand-dependent/ligand-independent characteristics of mutant variants, many of which remain to be characterized fully in the context of neuroblastoma development (86, 90, 91). Mutations at residues ALK-G1128, -M1166, -I1170, -I1171, -R1192, -L1196 (gatekeeper), -L1240 and -Y1278 have also been shown to be activating neuroblastoma mutations (Fig. 4A). These mutations are in close proximity to important structures within the kinase domain and likely regulate the activation of ALK activity, such as the alpha-C-helix, and the activation loop (3, 58, 86). More recent analysis of relapsed neuroblastoma has highlighted an increased frequency of activating ALK point mutations in these patients (92–95).

Studies of ALK germline mutations in familial neuroblastoma have shown that neuroblastoma has an incomplete penetrance and the risk of developing the disease likely depends on other players, such as segmental chromosomal aberrations (57, 84, 96–98). As illustration, a recent study reported two siblings both carrying a germline *ALK-R1275Q* mutation that exhibited very different neuroblastoma aggressiveness and chemotherapy response. Genetic analysis identified several differing segmental chromosomal aberrations including the amplification of *MYCN* between the siblings that potentially impacted on the progression of their disease (98). Indeed, studies in model systems such as mice or zebrafish suggest that activating *ALK* mutations alone do not drive neuroblastoma, rather ALK works together with other oncogenes to promote neuroblastoma tumor development (64, 89, 99–102). A better understanding of the developmental processes that

regulate the penetrance of *ALK* germline mutations should aid in unraveling of the underlying mechanisms of oncogenesis of familial neuroblastoma.

TARGETING ALK

Since oncogenic ALK signaling is involved in several cancer forms, it stands to reason that targeting ALK and its downstream partners would be therapeutically beneficial in ALK-positive cancer patients. ALK downstream signaling involves multiple known pathways, such as MEKK2/3-MEK5-ERK5, PI3K-AKT-mTOR, RAS-MAPK and PLC- γ (Fig. 3) (7). NVP-TAE684 was one of the first ALK-specific inhibitors identified to target the ATP-binding site of ALK (103), and initial studies identified reduced cell proliferation in ALK-positive ALCL, NSCLC and neuroblastoma cell lines on treatment with NVP-TAE684 (87, 104). While NVP-TAE684 is not used therapeutically, a number of other ALK tyrosine kinase inhibitors (TKIs) have been developed and employed clinically in ALK-positive patient populations (2, 105, 106).

Crizotinib

Crizotinib was the first ALK-targeted TKI to enter the clinic (Fig. 5) (22). In 2011, the FDA approved crizotinib for the treatment of ALK-fusion positive NSCLC patients based on the results from phase I/II clinical studies (2, 22, 107). In subsequent clinical studies, crizotinib was shown to be superior to conventional chemotherapy in advanced ALK-fusion positive NSCLC (2, 108). The efficacy of crizotinib has been tested in other ALK-fusion positive cancer forms, including pediatric and adult ALCL with good responses (109–111). However, responses in patients with ALK-positive neuroblastoma and IMT were less encouraging (110). Response to crizotinib in ALK-fusion positive NSCLC is transient due to the acquisition of secondary mutations in the kinase domain of the ALK fusions themselves (Fig. 4B) or by ALK copy number gain or

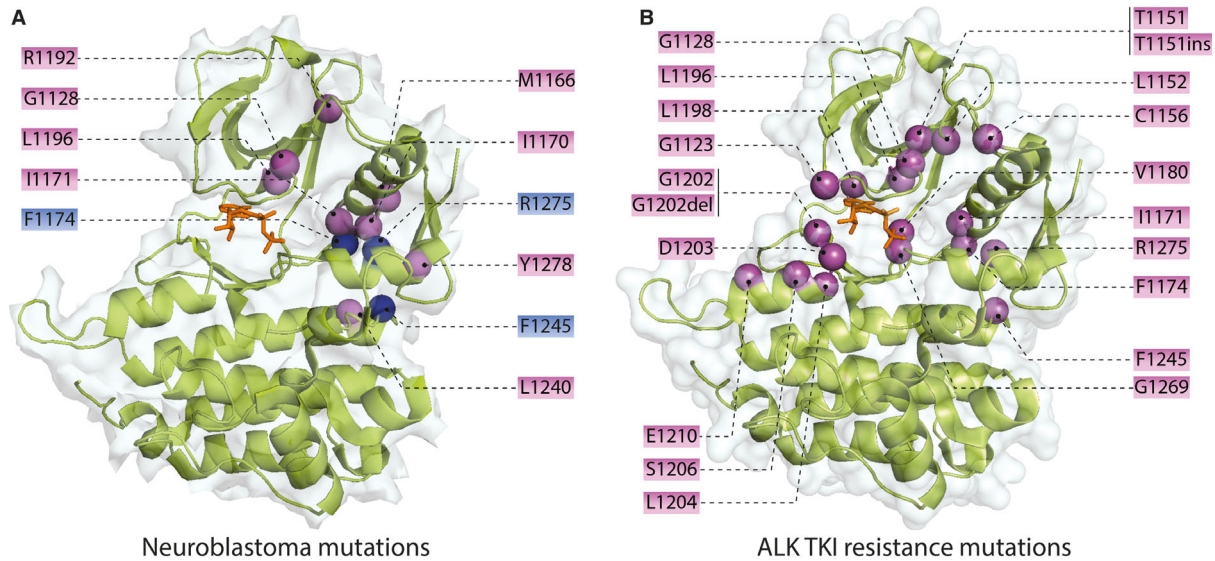


Fig. 4. Anaplastic lymphoma kinase (ALK) kinase domain point mutations in neuroblastoma and resistance mutations from patients treated with ALK tyrosine kinase inhibitors (TKIs). (A) ALK kinase domain showing activating mutations reported in neuroblastoma patients (*purple spheres*). The three hotspot mutations (F1174, F1245 and R1275, *blue spheres*) are indicated. (B) ALK tyrosine kinase domain resistance mutations from patients treated with ALK TKIs are shown (*purple spheres*). ATP is shown in the ATP-binding site of the kinase domain (*orange*).

bypass survival signaling via alternative oncogenes (2, 32, 34). Crizotinib is also less effective on brain metastases in ALK-fusion positive NSCLC patients (112). Next-generation ALK TKIs have been developed that address activity in the brain and secondary resistance mutations.

Ceritinib

In 2014, the FDA approved the second-generation ALK TKI ceritinib for crizotinib resistance ALK-fusion positive NSCLC patients (32, 113, 114). Like crizotinib, ceritinib is an ATP competitive inhibitor which binds in the ALK ATP-binding pocket (Fig. 5). Ceritinib is a derivative of NVP-TAE684 and in addition to inhibiting ALK is effective against insulin-like growth factor receptor-1 (IGF-1R), STK22D and INSR (115, 116). Ceritinib is able to overcome both ALK-crizotinib resistance mutations (G1269A, L1196M, I1171T/N and S1206C/Y) and ALK-alectinib resistance mutations (I1171T/N/S and V1180L) (Fig. 4B) (116, 117). Ceritinib is also effective in the treatment of ALK-rearranged ALCL (118). The median progression-free survival (PFS) with ceritinib in ALK-fusion positive NSCLC is 7–8 months, after which ALK secondary mutations arise and the response to ceritinib significantly decreases (119). While few reports exist in ALK-positive neuroblastoma, one patient with a complete response to ceritinib has been described (120).

Alectinib

Alectinib is a potent ALK TKI which displays activity toward crizotinib resistance mutations including L1196M, F1174L, R1275Q and C1156Y (Fig. 5) (32, 121). A phase I/II trial in Japanese patients with ALK-rearranged NSCLC led to the approval of alectinib in Japan and in 2015 the FDA granted breakthrough therapy designation for ALK-fusion positive NSCLC patients who have progressed with crizotinib (122, 123). In a recent phase III clinical study of untreated advanced ALK-fusion positive NSCLC, treatment with alectinib showed superior efficacy, with a 12-month survival of 66.4%, and lower toxicity when compared with crizotinib (124). Further, alectinib demonstrated superior central nervous system (CNS) activity and delayed CNS progression in ALK-fusion positive NSCLC relative to crizotinib (125). Alectinib is an effective inhibitor of the gatekeeper mutation ALK-L1196M as well as the ALK-R1275Q and ALK-F1174L neuroblastoma hotspot mutations (121). Similar to other ALK TKIs, alectinib treatment leads to resistance with the ALK-G1202R, ALK-V1180L and ALK-I1171T mutations reported as well as other ALK-independent mechanisms (117, 126). Alectinib has been studied preclinically in neuroblastoma and has been employed in one heavily pretreated, refractory, metastatic ALK-F1245C neuroblastoma case, where a partial clinical response was observed (127, 128).

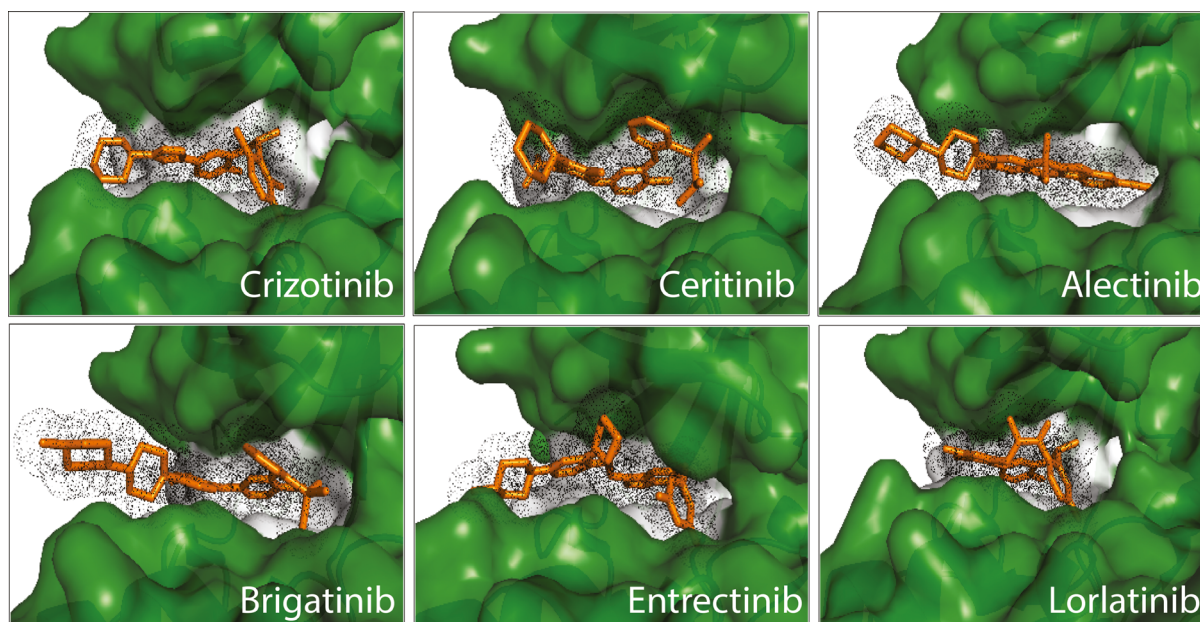


Fig. 5. A selection of FDA approved anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKIs) in the ATP-binding site of the ALK kinase domain. The different ALK TKIs (orange) bind in the ATP-binding pocket with varying contact sites resulting in unique profiles of inhibition of the ALK mutant variants observed in neuroblastoma patients as well as in ALK TKI resistant non-small cell lung cancer (NSCLC) and inflammatory myofibroblastic tumor (IMT) patients.

Brigatinib

Brigatinib, FDA approved in 2017, is a potent inhibitor of ALK that is also effective against epidermal growth factor receptor (EGFR) and ROS Proto-Oncogene 1, RTK (ROS1), and is effective against a range of ALK resistance mutations (Fig. 5) (129). In phase I/II trials in crizotinib resistance ALK-positive NSCLC patients, brigatinib showed 72% overall response with a median PFS of 11–13 months (32). A recently published phase III trial showed a superior efficacy of brigatinib as compared with crizotinib in the treatment-naïve ALK-fusion positive NSCLC, with an estimated 12-month event-free survival of 67% (130). The predominant resistance mutation seen in response to alectinib therapy is ALK-G1202R which is resistant to most ALK TKIs, with the exception of lorlatinib (see below). Sequential treatment with crizotinib and brigatinib has been reported to result in dual mutations such as ALK-E1210K+ALK-S1206C as well as ALK-E1210K+ALK-D1203N (2, 131). Brigatinib has been explored in preclinical neuroblastoma models, where it has been shown to inhibit ALK more effectively than crizotinib (132).

Entrectinib

Entrectinib, FDA approved as breakthrough therapy 2017, is a potent inhibitor of ALK, NTRK and ROS1 (Fig. 5), that is currently being evaluated in phase I/II

trials for patients with ALK, ROS1, NTRK alterations (32, 133). A recent report identified entrectinib as effective in the reduction of neuroblastoma cell proliferation and tumor growth (134). Entrectinib has orphan drug designation for treating neuroblastoma patients as well as for NTRK, ALK, ROS1 alterations in NSCLC and metastatic colorectal cancer. Entrectinib has been studied in preclinical models, where it has been shown to have activity toward the ALK-G1202R mutant (135).

Lorlatinib

Lorlatinib, FDA accelerated approval in 2018, is a novel, highly potent ALK/ROS1 inhibitor that can pass the blood–brain barrier (Fig. 5). Lorlatinib overcomes almost all known ALK resistance mutations observed with other ALK TKIs, including the ALK-G1202 mutation (2, 136, 137). In both *in vitro* and *in vivo* systems, lorlatinib is more potent than other ALK TKIs (138). It has been shown that lorlatinib exhibits superior potency toward ALK in preclinical neuroblastoma tumor models (139, 140). Lorlatinib is currently being investigated in trials for ALK/ROS1-positive NSCLC as well as in neuroblastoma, where it shows strong antitumor and CNS activity both in treatment-naïve and previously ALK TKI treatment ALK-positive NSCLC patients (32, 141). Due to its high efficacy, lorlatinib may serve as a useful partner for combinatorial treatments to

overcome the emergence of resistance clones in ALK-positive cancers.

ALK KINASE DOMAIN RESISTANCE MUTATIONS IN RESPONSE TO ALK TKI TREATMENT

Based on *in vitro* drug screens, *in vivo* models and patient data, ALK TKI resistance mechanisms can be classified into two major groups (2, 34, 142). The first group is ALK dependent, which includes ALK secondary resistance mutations (Fig. 4B). The ALK gatekeeper mutations L1196M and C1156Y were the first reported resistance mutations in ALK-fusion positive NSCLC (39). The other common resistance mutations seen in NSCLC are I1151Tins, F1174C/L/V, L1152P/R, G1202R, G1269A/S, D1203N, S1206C/Y (2, 34, 39, 116, 117) (Fig. 4B). In most cases, ALK secondary mutations can be overcome by second- and third-generation ALK TKIs. However, consecutive treatment with ALK TKIs in patients can lead to dual mutational loads (such as E1210K/D1203N, C1156Y/L1198F and C1156Y/I1171N) which confer resistance to third-generation ALK TKIs (2, 131). Much effort is being expended to match the individual ALK resistance mutations with the most effective inhibitor in ALK-fusion positive NSCLC (2, 33, 35). Individual patient treatments can become complex involving therapeutic approaches that can include multiple ALK TKI treatments, illustrated by a report of therapeutic use of lorlatinib in a patient with crizotinib-resistant ALK-fusion positive NSCLC that led to the appearance of the ALK-L1198F+C1156Y resistance mutation (143). In the same study, it was shown that this lorlatinib resistance mutation is sensitive to crizotinib treatment (143), highlighting the importance of understanding the dynamics of resistance mutations that arise in response to ALK TKIs. Interestingly, secondary resistance mutations have not yet been reported in neuroblastoma, where mutations in ALK are already present as primary mutations. The second group of resistance mutations is ALK independent and includes the activation of alternative oncogenes (such as EGFR, IGFR, MET, KIT) and lineage alterations described in NSCLC (2, 34, 142). In neuroblastoma, activation of alternative oncogenic drivers Axl and ErbB4 has been reported to lead to ALK TKI resistance in preclinical analyses (144, 145).

TARGETING ALK IN NEUROBLASTOMA

The identification of ALK mutations in neuroblastoma, both at initial diagnosis and at increased

frequency in relapsed neuroblastoma cases, has driven efforts to effectively employ ALK TKIs clinically. A phase I trial looking at the safety and activity of crizotinib included 11 neuroblastoma patients with identified ALK mutant variant status, including the three hotspot mutations (F1174, R1245 and R1275), as well as one patient with an *ALK-Y1278S* mutation (110). Only one complete response was observed in a patient harboring a germline *ALK-R1275* mutation. Unfortunately, little is known about other somatic or companion mutations carried by these patients. A number of studies have investigated the effect of ALK TKIs in a neuroblastoma setting (86, 87, 90, 120, 132, 139, 140, 146, 147). Table 2 shows a selection of FDA approved ALK TKIs, brigatinib, ceritinib, lorlatinib and crizotinib (120, 132, 140), that vary in their efficacy to inhibit the different ALK mutant variants observed in neuroblastoma in pre-clinical models. Although not performed side-by-side, the accumulated results illustrate potential differences, important as the different ALK TKIs bind differentially within the ATP-binding pocket of the ALK kinase domain (Fig. 5). Since ALK mutations found in both initial and in relapsed neuroblastoma cases are located mostly in the alpha-C-helix and the activation loop, some overlap exists with the reported NSCLC resistance mutations (Fig. 4) (7). Comparing inhibition of the hotspot mutations F1174, F1245 and R1275 in *in vitro* assays shows that ceritinib is generally two-fold more effective than crizotinib, which inhibits the hotspot mutations in the range of 25–35 nM (Table 2). Brigatinib and lorlatinib abrogate the activity of ALK neuroblastoma hotspot mutations within a single digit nanomolar (nM) range (Table 2). The ALK-I1171N mutant variant presents challenges for many ALK TKIs, and both ceritinib and brigatinib are less efficient at inhibiting this variant than lorlatinib (Table 2). This likely reflects sensitivity to steric effects of the ALK-I1171N mutation on the binding surfaces in the ATP-binding pocket. A recent molecular dynamics study shows dislocation of the ALK TKI alectinib triggered by the ALK-I1171N mutation, which induces conformational changes at the inhibitor binding site that impact on the interaction between alectinib and the ALK-I1171N mutant (148). The third-generation ALK TKI lorlatinib shows strong activity toward all tested ALK neuroblastoma mutant variants, supporting the current clinical testing of lorlatinib in neuroblastoma (139, 140). Based on the efficacy in pre-clinical systems, both lorlatinib and brigatinib appear to be good options for the targeting of ALK in neuroblastoma.

COMBINATORIAL TREATMENTS IN NEUROBLASTOMA

Given the complex pattern of ALK resistance mutations that arise in response to ALK TKI treatment, combinatorial targeting of downstream targets or other bypass pathway components could offer therapeutic benefit for ALK-positive neuroblastoma patients and also hinder the development of resistance. Choosing the right target for polytherapy is complicated, considering the issue of toxicity and the fact that negative feedback signaling events may lead to the development of resistance (149, 150). Successful combinatorial treatments should not only show efficacy superior to mono-treatment but also be tolerable at effective

doses in patients. Several combinations of ALK TKI with chemotherapy agents, immunotherapy agents and downstream target agents have been evaluated (Table 3). These include the use of ERK5, mTOR, CDK4/6 and RET inhibitors together with ALK for improved inhibition of tumor growth in preclinical models (146, 147, 151–153). Recent proteomics-based studies have also identified additional targets, such as IGF-1R/INSR, identifying combined ALK and IGFR inhibition as effective in reducing the proliferation of ALK-positive neuroblastoma cell lines (154, 155). Efforts are currently being concentrated on understanding how ALK TKIs can be employed therapeutically in combination so in the future patients are treated accordingly.

Table 2. IC50 values for inhibition of ALK Y1604 phosphorylation in the context of full-length ALK expressed in PC12 cells by either brigatinib, lorlatinib, ceritinib or crizotinib

ALK mutation	ALK TKI IC50s				
	Brigatinib	Lorlatinib	Ceritinib	Crizotinib*	Crizo SD*
Wildtype	2.60	0.80	5.30	16.40	4.17
G1128A	2.00	1.20	19.80	33.53	10.15
I1171N	10.30	3.20	46.60	97.53	50.00
I1171T	7.50	6.80	17.40	193.00	
F1174L	1.50	0.70	17.00	23.16	5.8
R1192P	2.50	0.90	26.10	42.50	19.95
F1245V	6.60	0.90	24.70	32.02	6.78
G1269A	3.40	6.40	29.80	130.13	24.11
R1275Q	4.20	0.80	10.80	30.63	1.72
Y1278S	4.60	2.30	20.50	53.7	29.91

ALK, anaplastic lymphoma kinase; TKI, tyrosine kinase inhibitor.

The table is compiled from three independent articles, all of which have employed investigated crizotinib for comparison with other ALK TKIs (120, 132, 140). Crizotinib* indicates average of crizotinib treatment based on these studies and crizo* SD indicates standard deviation of these values. Results for ALK-I1171T are taken from (120).

Table 3. Different combinatorial targets in ALK-positive cancers

Molecular targets	Pre-clinical experimental model	Combinatorial effects
ALK + MEK	EML4-ALK (v1) positive NSCLC ALK-positive neuroblastoma	Inhibition of NSCLC cell proliferation and tumor growth Increased AKT activity
ALK + mTORC1/C2	NPM-ALK-positive ALCL (mTORC1) ALK-positive neuroblastoma (mTORC1/C2) ALK-positive neuroblastoma (mTORC1)	Inhibition of ALCL cell proliferation and tumor growth Inhibition of neuroblastoma cell proliferation and tumor growth Increased activation of AKT via Rictor
ALK + ERK5	ALK-positive neuroblastoma	Inhibition of neuroblastoma cell proliferation and tumor growth
ALK + HSP90	EML4-ALK-positive NSCLC	Inhibition of NSCLC cell proliferation and tumor growth
ALK + CDK4/6	ALK-positive neuroblastoma	Inhibition of neuroblastoma cell proliferation and tumor growth
ALK + IGF-1R	EML4-ALK-positive NSCLC ALK-positive neuroblastoma	Inhibition of NSCLC cell proliferation and tumor growth Inhibition of neuroblastoma cell proliferation

ALCL, anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; IGF-1R, insulin-like growth factor receptor-1; NSCLC, non-small cell lung cancer.

The table represents combinatorial effects of different combinatorial molecular targets in ALK-positive cancers. Choosing an appropriate molecular target for combinatorial treatment is important to avoid feedback mechanisms. Table refers to results from (146, 147, 150, 151, 155–159).

CONCLUDING REMARKS

Much current activity in the field of ALK inhibition in neuroblastoma centers around understanding how, when and if, the impressive arsenal of ALK TKIs can be usefully employed therapeutically in this patient population. This is an important challenge for the research field to tackle since current therapeutic regimes for high-risk neuroblastoma come with significant side-effects and morbidity. Those neuroblastoma cases that have shown responses to ALK TKI treatment would indicate that there is a window of opportunity to be better clarified. It is clear that tumor complexity in terms of heterogeneity and genetic background plays significant roles in neuroblastoma and that there is much to be learned. Improved understanding of the underlying biology of ALK in the neural crest during development and how that function is perturbed in neuroblastoma will be important. The combination of novel techniques now available, including single cell-based approaches, offers unique opportunities to define heterogeneity, immune cell tumor infiltration and tumor microenvironments, ranging from tumor models in simple model systems to patient samples. These advanced analyses offer the power to address many challenging questions in the coming years, leading to improved treatments with precision medicine.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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