CLINICAL INVESTIGATION

New developments in the treatment of ALK-driven malignancies

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ALK has been recognized as a therapeutic target in several neoplasias, including anaplastic large cell lymphoma, non-small-cell lung cancer, neuroblastoma and colorectal cancer. Both chromosomal rearrangements, leading to the expression of fusion kinases, and kinase-activating point mutations, have been found to trigger the oncogenic activation of ALK. ALK-positive cancers are highly dependent on ALK catalytic activity. Since the normal, wild-type *ALK* gene is expressed at low levels in a limited population of nervous tissue cells, the targeting of oncogenic ALK proteins has great therapeutic value. Hence, a large effort is ongoing worldwide to develop small-molecule inhibitors of ALK. One compound has been approved for the treatment of ALK-positive non-small-cell lung cancer and a number of second-generation compounds are undergoing clinical evaluation. Here, we review the molecular biology of normal and oncogenic ALK, its involvement in the pathogenesis of cancer and the current status of ALK inhibitors research, including preclinical and clinical development and acquired resistance to ALK inhibition. The results obtained so far in ALK-positive tumors emphasize the importance of a deep understanding of the genetic alterations that cause transformation, in order to achieve major advances in cancer therapy.

Keywords: ALK • crizotinib • lymphoma • non-small-cell lung cancer • targeted therapy • tyrosine kinase

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Molecular biology of ALK

ALK was first identified as the partner of *NPM* in the t(2;5) chromosomal translocation in a subset of CD30-positive large-cell non-Hodgkin lymphomas [1,2]. The sequence of the newly identified gene showed homology to the catalytic domain of tyrosine kinases, in particular to LTK. Indeed, the chimeric gene product was then shown to be hyper-phosphorylated [3], to possess kinase activity and to be able to transform NIH-3T3, Ba/F3 and Rat-1 cells [4]. The NPM–ALK fusion protein was shown to localize to the cytoplasm of anaplastic large cell lymphoma (ALCL) cells [4,5]. Interestingly, it was found that the NPM portion is necessary for the transforming ability of the fusion oncogene, as the mere overexpression of the isolated ALK catalytic domain was not sufficient to transform cells [4,6].

NPM is a ubiquitously expressed gene mainly localized in the nucleolus and normally involved in various cellular functions such as ribosome biogenesis, centrosome duplication, DN/A repair and response to stress [7]. The non-translocated, normal *ALK* gene encodes for an approximately 200-kDa transmembrane receptor tyrosine kinase and is mainly expressed in late embryonic and neonatal nervous tissue, suggesting a role in the development of the nervous system [8,9]. In addition to the central and peripheral nervous system, mouse *Alk*

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expression was also noted in the gut epithelium, eye, olfactory epithelium and tongue during embryonic life [10]. The specific function of ALK in vertebrates is still elusive, while some hints have come from lower organisms. In *Caenorhabditis elegans*, the ALK homologue T10H9.2 is highly expressed in motor neurons, where it is proposed to be involved in synapse formation [11]. *Drosophila* Alk was shown to be essential for the development of the visual system [12]. In the developing eye of flies, Alk is expressed on neurons of the optical ganglion and is activated by a ligand called Jelly belly (Jeb), which is secreted by photoreceptor cells of the retina. Alk signaling then mediates target selection of photoreceptor cell axons in the optic lobe. In addition, the Jeb/Alk system has been shown to guide the development of visceral muscles in *Drosophila* [13–16]. A secreted protein with Jeb-like motifs was found to integrate sensory signals and to regulate learning processes in *C. elegans* [17]. No Jeb homolog has been isolated so far in vertebrates. Therefore, ALK remained for a long time an orphan receptor, until it was shown to bind to at least two related neurotrophic factors, PTN and MK (interestingly, PTN expression is also restricted to perinatal brain tissue and immature neurons) [18,19]. The two identified ligands were shown to bind to a sequence, within the ALK extracellular region, that is homologous to MAM domains, which are normally involved in cell–cell adhesion. The MAM domain is conserved in *Drosophila* Alk [20]. However, other authors could not confirm agonist activity of PTN in human cells [21,22]. PTN is also known to signal through the receptor tyrosine phosphatase RPTPβ [23]. Lu *et al.* identified two different forms of PTN, one of which induced ALK-dependent proliferation, while the other one triggered RPTP-dependent effects, in glioblastoma cells [24]. Thus, whether PTN and MK are direct ALK ligands is still an open question. An alternative model for ALK activation involves PTN-mediated inhibition of RPTPβ; in the absence of ligand, RPTPβ keeps ALK in a dephosphorylated state, and upon PTN binding to RPTPβ, phosphatase activity is blocked and ALK is free to autophosphorylate. This model would suggest that ALK is activated independently of a direct interaction with PTN [25].

The intracellular portion, which is retained in the fusion genes, includes the kinase domain and a carboxy-terminal tail that is highly phosphorylated and serves as the docking site for downstream effectors. Autophosphorylation of the activation loop Tyr-1278 is necessary for full activation of the ALK domain as well as for the transforming ability of ALK fusions [26]. Downstream signaling activated by ALK has mainly been studied in cells expressing fused *ALK* genes, typically NPM–ALK, since the full-length receptor is barely detectable in normal adult cells. Downstream signals include the RAS/MAPK, JAK/STAT, and PI3K/AKT pathways. The RAS/RAF/MEK/ERK cascade is a typical mitogenic signal, activated by numerous stimuli. In fact, it is probably the most aberrantly activated signal in all cancers. ALK activates Ras via direct docking of SH2 or PTB domain-containing proteins, such as IRS1, SRC, SHC, and the SHP2/GAB2/GRB2 complex, to phosphotyosine residues of its intracellular domain [4,27]. In addition, phospho-ALK directly binds PLCγ, which in turn activates PKC, thus indirectly contributing to ERK activation [28]. The second key pathway triggered by ALK is represented by the JAK/STAT axis. In particular, STAT3 is strongly activated by NPM–ALK, either directly or through JAK3, and mediates the expression of several antiapoptotic factors [29–32]. In addition, JAK2/STAT5B have also been found to play a role in NPM–ALK-induced transformation [33]. Finally, ALK binds and activates the p85 PI3K subunit, thereby causing hyperactivation of AKT kinase and downstream mTOR pathway, which in turn contributes to uncontrolled growth and increased survival [34,35].

Thus, it appears that ALK fusions can elicit a global network of oncogenic signaling by recruiting at least three major cell growth/survival pathways. In line with this, pharmacological inhibition or genetic ablation of either SHP2, JAK2, JAK3, PI3K or mTOR, reduces proliferation and survival of ALCL cells [27,31,33,36–39]. Although most studies have been carried out in NPM–ALK-transformed cells, there are data available on the wild-type ALK signaling network. Stoica *et al.* showed that PTN- and MK-induced activation of ALK full-length receptor in ALK-transduced human cells resulted in the phosphorylation of SHC, IRS-1, PLCγ and PI3K [18,19]. In *Drosophila*, Jeb binding to Alk activates the MAPK pathway [16]. Stimulation of endogenous ALK in human neuroblastoma cells using an anti-extracellular ALK antibody leads to ERK activation [40]. Similar results were obtained in ALK-transfected HEK293 cells [22]. Moreover, engineered active transmembrane ALK proteins induced neuronal differentiation via the MAPK and PI3K pathways [22,41,42]. Finally, neuroblastoma-associated ALK point mutants with constitutive kinase activity show the full array of downstream phosphorylation, including phosphorylated STAT3, ERK and AKT [43]. It is interesting to note that full-length transmembrane ALK seems to predominantly activate the MAPK pathway, while ALK fusion proteins appear to signal through STAT3 in most cases. The reason for this difference may be related to the distinct subcellular

localization of the two ALK forms.

Another important aspect of ALK biology is the induction of cell morphology changes and migration. ALK has been shown to induce neurite outgrowth in neurons [40] and to alter cell shape in transfected fibroblasts [44]. More importantly, ALCL cells show a typical anaplastic morphology that is specifically induced by NPM–ALK activity, as inhibition by small-molecule inhibitors reverts this effect, restoring the typical round shape of a T lymphocyte [45]. The molecular pathways leading to ALK-mediated effects on the cell cytoskeleton involve the activation of CDC42 and Rac GTPases through the direct binding of guanine exchange factors of the Vav family to ALK phosphotyrosine residues [46,47]. Interestingly, inhibition or silencing of CDC42 not only reverts anaplastic morphology, but also causes growth arrest and apoptosis of ALCL cells [46], suggesting a significant degree of crosstalk between CDC42 and the aforementioned growth-related pathways.

Involvement of ALK in human cancer

ALCL was the first of a growing list of cancers that show ALK abnormality. Classical ALCL can be divided into two major clinical types: systemic or cutaneous. The latter is typically indolent and can be cured by surgical excision, unless it spreads to extracutaneous sites [48]. The systemic type presents as an aggressive disease with or without extra nodal sites and requires multi-agent chemotherapy [49]. ALCL represents approximately 2–8% of all non-Hodgkin lymphomas in adults, while it is more frequent in pediatric patients (30–40% of cases) [50]. The hallmark of ALCL cells is the expression of the CD30 antigen, which is shared with Hodgkin's lymphoma cells [51]. Depending on the method used, approximately 50–90% of ALCL cases have been found to express ALK [5,51,52]. ALK-positivity does not correlate with any specific histological subtype of ALCL. In fact, the characterization of ALK-positive lymphomas has led to the definition of a new clinical entity, with distinct features; 'ALKomas' have been shown to occur in younger patients and to have a better prognosis compared with ALK-negative lymphomas [53]. Thus, previous classifications of ALCL (e.g., pleomorphic, common type, lymphohistiocytic or small cell) were to be considered as morphologic variants of the same disease [54]. This represented one of the first examples of molecular classification replacing morphologic classification.

The initial ALCL-transformed cell is, in most cases, a T cell, as shown by TCR rearrangement. However, ALCL cells often show a null immunophenotype (lacking surface expression of typical T-cell molecules) such as the TCR itself and TCR-associated proteins (e.g., CD3 and ZAP70) [55]. NPM–ALK was shown to directly cause the downregulation of these molecules via an epigenetic mechanism [56]. Rare B-cell cases that were initially classified as ALCL are now considered as a different clinical entity. In fact, a small proportion of large B-cell lymphomas are now recognized to be caused by ALK through a variety of different rearrangements, most often with the *CLTC* gene (see below).

Approximately 80% of ALK-positive ALCL express the t(2;5) rearrangement. Less frequently (10–20% of cases) *ALK* is fused to the TPM3 locus on chromosome 1, leading to the TPM3–ALK fusion protein. Rare translocations have been reported occasionally in ALCL patients, involving different 5′ fusion partner genes, including *TFG*, *ATIC*, *CLTC1*, and a few more [57–63]. Importantly, in most cases, the 5′ fusion partner provides a relatively high expression of the chimeric gene, and in all cases constitutive dimerization of the ALK catalytic domain is present, leading to unregulated activation of ALK.

ALK translocations, by causing high inappropriate expression of the ALK, also offer a very good diagnostic tool to the pathologist, since ALK is not detected in normal lymphocytes. Thus, a simple immunochemistry staining can reveal the presence of an ALKoma. In some instances, the intracellular distribution of the ALK signal can even help understand the type of fusion, because the localization of the fusion protein will depend on the amino-terminal partner. NPM–ALK is distributed throughout the nucleus and the cytoplasm, whereas TPM3–ALK, TFG–ALK and ATIC–ALK only localize to the cytoplasm. CLTC1–ALK localization resembles that of clathrin-coated vesicles, while MSN–ALK is associated to the cellular membranes and RANBP2–ALK shows a nuclear membrane-specific ALK staining. Despite differences in cellular localization, all ALK fusion oncoproteins are thought to drive ALCL tumor growth through constitutive kinase activity. The role of NPM–ALK as a tumor driver has been formally demonstrated. First of all, expression of NPM–ALK in the bone marrow causes lymphoid malignancy in mice [64–66]; secondly, siRNA-mediated silencing of NPM–ALK in human ALCL cells leads to cell cycle arrest and apoptosis *in vitro* and tumor regression *in vivo* [67].

Aberrant expression of ALK in non-ALCL cancer was first documented by Delsol *et al.* in large B-cell lymphomas, although at that time no rearrangement was observed [68]. Later, both classical NPM–ALK as well as other ALK translocations were found in diffuse large B-cell lymphoma. The most frequent fusion is CLTC1–ALK, representing approximately 75% of

ALK-positive diffuse large B-cell lymphoma cases reported in the literature [69]. These tumors are usually CD30-negative and show IgH locus rearrangements typical of committed B cells. However, they are mostly CD20-negative. Another recent case of B-cell lineage ALK-positive tumor was reported in a patient affected by extramedullary plasmacytoma expressing the CLTC–ALK fusion [70]. Three cases of RANBP2–ALK-positive myeloproliferative disorder/myeloid leukemia were recently described, expanding the role of ALK in hematological neoplasia [71].

The first description of ALK rearrangement in nonhematological tumors was reported in 1999 by Griffin and colleagues [72]. They found recurrent chromosomal alteration at 2p23 in inflammatory myofibroblastic tumors (IMTs) using a probe flanking the *ALK* gene. This finding was then confirmed by several other groups [73,74]. IMT is a rare soft tissue tumor characterized by spindle cell appearance and a prominent inflammatory infiltrate [75]. Approximately 50% of IMTs carry *ALK* translocations. Several fusion partners have been identified, such as TPM3, TPM4, ATIC, but not NPM. Other fusions (e.g., RANBP2–ALK, CARS–ALK) are unique to IMT.

In 2008, five papers described the identification of oncogenic point mutations of *ALK* in neuroblastoma, a pediatric neoplasm arising from neural crest-derived tissues with a peak incidence at <4 years of age [43,76–79]. Mutations are found in >90% of familial neuroblastomas and approximately 10% of sporadic cases. Another 5% of patients show *ALK* gene amplification [80]. Although the various mutations span the entire kinase domain, substitutions at two residues (F1174 and R1275) represent over 70% of the cases [81]. Most neuroblastoma-associated mutant ALK variants show constitutive activity, increased autophosphorylation and transforming ability. An exception is represented by the rare *T1151M* and *A1234T* alleles, which did not transform cells [76]. Specific silencing of mutated ALK has a dramatic effect on growth and survival of neuroblastoma cells [43,76–78].

The involvement of *ALK* as an oncogene in adult brain tumors is less well defined. Both ALK and its ligand PTN were found overexpressed in glioblastoma compared with normal brain tissue, and ribozyme-mediated downregulation of ALK caused tumor growth inhibition [82]. Enhanced antitumor effects were obtained by double targeting of PTN and ALK [83]. However, the oncogenic relevance of this autocrine loop remains to be further verified, because in many cases the mere overexpression of a wild-type receptor/ligand simply reflects the transformed phenotype, and thus it is a consequence rather than a cause of the tumor. In addition, PTN can also bind other receptors (e.g., RPTPβ) and ALK can be activated by other ligands (e.g., MK). Nonetheless, the data suggest a possible therapeutic option in cases of ALK hyperexpression in glioblastoma multiforme. At the 2011 American Association for Cancer Research (AACR)–National Cancer Institute (NCI)–European Organisation for Research and Treatment of Cancer (EORTC) meeting, *ALK* gene amplification by fluorescence *in situ* hybridization was reported in 60% of glioblastoma cases [84].

Although ALK was already known as a cancer driver in ALCL and IMT, it was put in the spotlight of cancer research with the discovery of ALK fusions in a small subset (~5%) of non-small-cell lung cancer (NSCLC) [85,86]. NSCLC is the leading cause of cancer-related deaths worldwide, with an estimated incidence of approximately 1.4 million new cases each year [201], which makes approximately 70,000 new ALK-positive NSCLC cases, compared with less than 5000 new ALK-positive ALCLs. The group of EML4–ALK-positive NSCLCs is distinct from EGFR-or KRAS-mutated lung cancers [87]. Most frequently, EML4–ALK-positive NSCLCs are adenocarcinomas in patients who are non- or light-smokers. Molecular analysis showed that the fusion product can dimerize via the N-terminal basic domain of EML4 and is a constitutively active tyrosine kinase. As expected, EML4–ALK confers tumorigenic potential to NIH-3T3 cells. Mice expressing the *Eml4–Alk* chimeric gene under the control of a lung epithelial cell promoter develop multiple lung adenocarcinoma tumors [88]. The same rearrangement was later found in approximately 2% of breast and colorectal carcinoma [89]. More recently, novel rare fusions have been described in NSCLC and colon cancer [90–93].

Two independent reports described expression of the TPM4–ALK fusion in oesophageal squamous cell carcinoma [94,95]. Both studies employed a proteomic approach to identify proteins expressed differentially between tumors and normal tissues. Although no functional data were reported in either paper, the finding is interesting and potentially identifies a distinct molecular subgroup of this highly lethal cancer.

Direct sequencing of the ALK domain in thyroid neoplasms revealed two novel point mutations in anaplastic thyroid carcinoma specimens [96], with a prevalence of approximately 11%. The two mutant kinases showed increased activation of downstream signaling pathways and conferred anchorage-independent growth and invasive properties to the cells. No mutation was found in well-differentiated carcinomas.

The last addition to the list of ALK-positive cancers comes from a case of sickle cell trait-associated renal medullary carcinoma in which the ALK domain was fused to the cytoskeletal protein vinculin [97]. Interestingly, although no homodimerization domain is retained in the fusion product, VCL–ALK co-immunoprecipitates with the membrane-associated protein talin. Therefore, the authors suggested that transphosphorylation between two neighboring VCL–ALK molecules occurs on a talin scaffold.

The recent introduction of genome sequencing has led to the detailed description of cancer genomic landscapes [98]. Analysis of whole-genome sequencing data revealed the presence of tumor-associated point mutations of ALK in several cancers, such as breast, colon, lung and ovary carcinomas and melanoma [99–102]. A summary can be downloaded from the COSMIC database [202]. Obviously, the simple observation of a mutated gene does not detail its role and significance as a cancer driver oncogene until biological data are available. However, it is becoming increasingly clear that ALK is potentially involved in many more tumors than previously thought.

The recognition of *ALK* as a driver oncogene will revolutionize the management of ALK-positive tumors. Prior to crizotinib, the therapeutic options offered to patients with ALK-positive systemic ALCL were limited. In general, multi-agent chemotherapy was the preferred treatment [49,103]. Bone marrow transplantation is considered when possible in relapsed patients [104]. As ALCL cells express CD30, anti-CD30 monoclonal antibodies have been tested in clinical trials, but the results have not been encouraging [105]. A recent alternative is represented by brentuximab vedotin, an anti-CD30 antibody conjugated to a cytotoxic drug [106]. This novel CD30-targeted agent was approved by the US FDA in 2011.

Cisplatin-based doublets represent the current standard treatment for metastatic NSCLC [107]. For elderly patients or patients with a bad performance status, monotherapy is usually advised. Gemcitabine, vinorelbine and taxanes have been evaluated as monotherapies with comparable results. Docetaxel and pemetrexed are often used as second-line therapy. In general, median survival in advanced disease remains poor (<1 year). A major recent advance in NSCLC treatment was achieved with anti-EGFR therapy in patients carrying EGFR-activating mutations [108].

Small-molecule inhibitors of ALK

The involvement of ALK in various tumors and a convincing functional validation as a cancer-driving oncogene, together with the fact that the wild-type *ALK* gene is not expressed in adult cells, makes ALK a very attractive therapeutic target. Therefore, many groups as well as pharmaceutical companies have started programs to develop ALK-specific inhibitors.

Initial studies

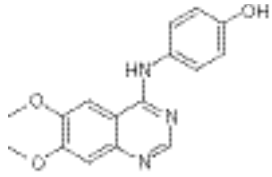
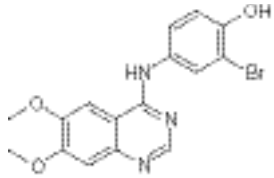
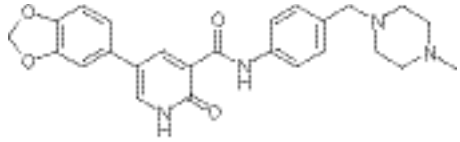
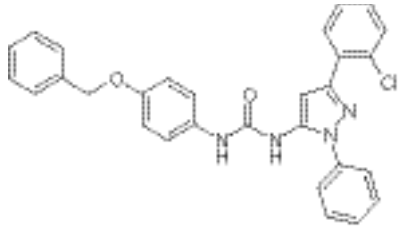
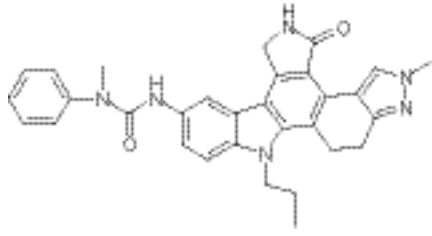
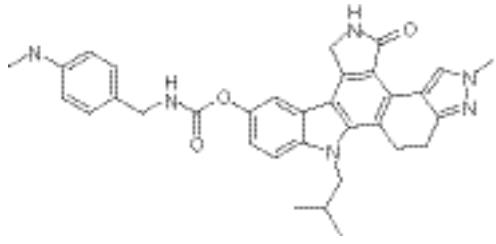
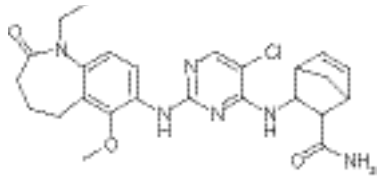
In 2001, a small Phase I trial with the protein kinase inhibitor UCN-01 yielded a prolonged stable disease in a patient with ALK-positive ALCL, suggesting that UCN-01 might be an ALK inhibitor [109]. However, subsequent analysis indicated that this compound is a poor and nonspecific inhibitor of ALK [110]. The HSP90 inhibitors herbimycin A and 17-AAG were also tested in ALK-positive ALCL cells, on the basis that NPM–ALK, similarly to many other kinases, is an HSP90-client protein [111,112]. These compounds blocked NPM–ALK expression, resulting in cell cycle arrest and apoptosis. Although the treatment is not ALK-specific, these studies corroborated the idea that pharmacologic inhibition of ALK-dependent signaling is a viable option for ALCL therapy.

Preclinical research

The first attempt to directly interfere with ALK activity was reported in 2005 by Marzec *et al.* [32]. The authors described two related compounds (WHI-P131 and WHI-P154) showing ALK phosphorylation inhibition and cell growth inhibition at micromolar concentrations in ALCL-derived cell lines (Table 1). The two molecules were already known as JAK3 inhibitors and have not been further developed for ALK inhibition. Around the same time, a rational *de novo* design of an ALK inhibitor was published by researchers at ChemBridge [113]. The authors followed a classic lead discovery program, with an initial compound library screening followed by medicinal chemistry-driven template modification. The series included compounds with submicromolar activity on the enzyme and 1-log selectivity versus IRK and four other kinases. Nonselective ALCL cell growth inhibition was obtained at micromolar doses.

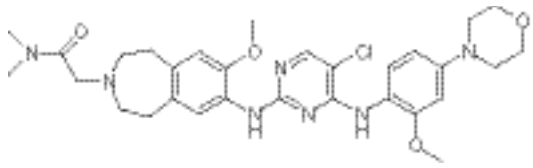
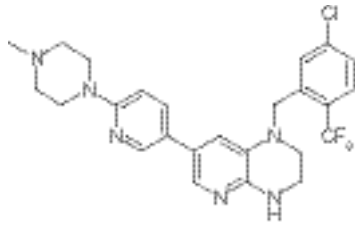
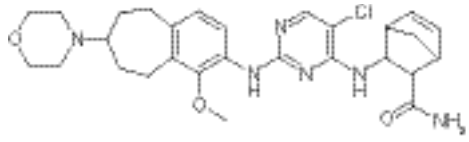
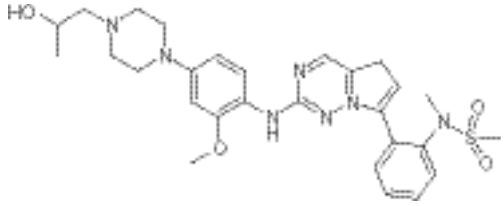
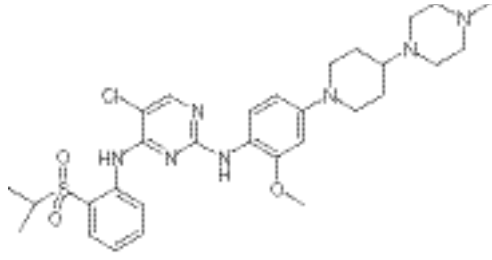
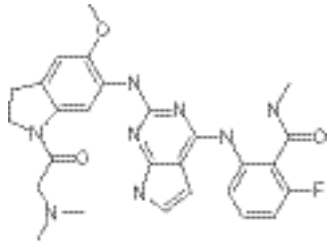
Naturally, knowing the 3D structure of a target protein helps the design of a specific inhibitor greatly. In absence of such information, Gunby *et al.* decided to investigate the available space within the ATP binding pocket of ALK by homology modeling, mutagenesis and analysis of inhibitor affinity [114]. They were able to render ALK sensitive to the ABL inhibitor imatinib by changing one key residue, termed the gatekeeper, that controls access to the ATP pocket in tyrosine kinases. Agreement between virtual data and experimental results indicated that the model was suitable for inhibitor design. Indeed, this molecular model then

Table 1. Small-molecule inhibitors of ALK reported in literature.

Compound (developer)	Structure	IC_{50} (μM)	Stage of development	Ref.
WHI-P131 (University of Pennsylvania)		10†	Preclinical	[32]
WHI-P154 (University of Pennsylvania)		5†	Preclinical	[32]
9c (ChemBridge)		0.9	Preclinical	[113]
17 (ProKinase Consortium)		0.61	Preclinical	[115]
CEP-14083 (Cephalon)		0.002	Preclinical	[116]
CEP-14513 (Cephalon)		0.004	Preclinical	[116]
15 (Cephalon)		0.014	Preclinical	[120]

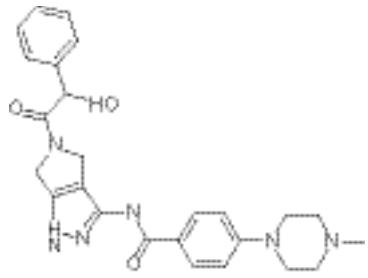
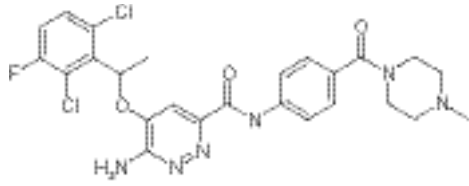
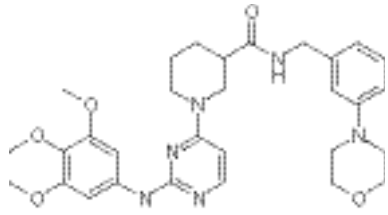
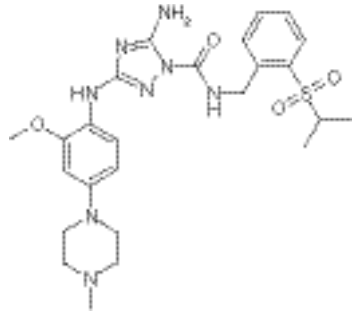
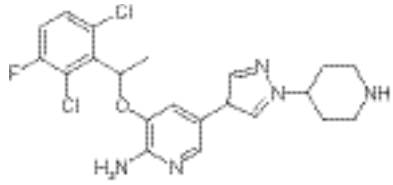
†The IC_{50} value refers to ALK inhibition in cells.

Table 1. Small-molecule inhibitors of ALK reported in literature (cont.).

Compound (developer)	Structure	IC_{50} (μM)	Stage of development	Ref.
18 (Cephalon)		0.004	Preclinical	[117]
5e (Cephalon)		0.010	Preclinical	[118]
CEP-28122 (Cephalon)		0.002	Preclinical	[121]
30 (Cephalon)		0.010	Preclinical	[119]
NVP-TAE684 (Novartis)		0.003†	Preclinical	[122]
GSK1838705A (GlaxoSmithKline)		0.0005	Preclinical	[130]

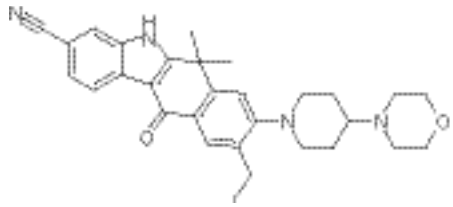
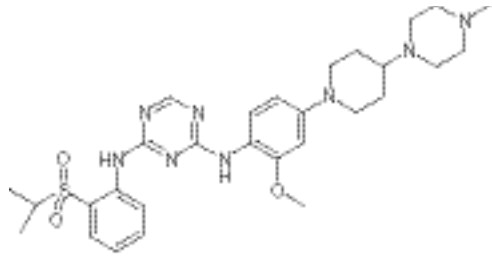
†The IC_{50} value refers to ALK inhibition in cells.

Table 1. Small-molecule inhibitors of ALK reported in literature (cont.).

Compound (developer)	Structure	IC_{50} (μM)	Stage of development	Ref.
PHA-E429 (Nerviano Medical Sciences)		0.091	Preclinical	[125]
NMS-E628 (Nerviano Medical Sciences)	Undisclosed	N/A	Preclinical	[132,133,169]
X-376 (Xcovery)		0.0006	Preclinical	[134]
11v (Amgen–Tesaro)		0.031	Preclinical	[135]
15a (Dana-Farber Cancer Institute)		0.07†	Preclinical	[137]
CRL151104A (ChemBridge)	Undisclosed	0.010	Preclinical	[139]
WZ-5–126 (Dana-Farber Cancer Institute)	Undisclosed	0.003	Preclinical	[123]
PF-02341066/crizotinib/ Xalkori™ (Pfizer)		<0.001	Approved	[141,170]

†The IC_{50} value refers to ALK inhibition in cells.

Table 1. Small-molecule inhibitors of ALK reported in literature (cont.).

Compound (developer)	Structure	IC_{50} (μM)	Stage of development	Ref.
CH5424802/AF802 (Chugai–Roche)		0.002	Phase I/II	[158]
AP26113 (Ariad)	Undisclosed	0.006	Phase I/II	[124]
LDK378 (Novartis)	Undisclosed	N/A	Phase I	[161]
ASP3026 (Astellas)		N/A	Phase I	[162,163]

†The IC_{50} value refers to ALK inhibition in cells.

proved useful in guiding screening and optimization of ALK inhibitors based on a urea core [115] or a completely new scaffold [Dattoma J, Bisson W, Mologni M, Gambacorti-Passerini C, Scapozza L, Goekjian P. Synthesis of ALK-L1196M mutant-selective aminothiazole inhibitors (2012), Manuscript in preparation].

In 2006, Cephalon (currently Teva Pharmaceuticals) described two staurosporine derivatives (CEP14083 and CEP14513) with low nanomolar inhibitory activity against ALK and ALK-positive ALCL cell lines [116]. The authors demonstrated that blocking ALK activity leads to apoptosis in ALCL cells. The compounds showed little effect on a BCR/ABL-dependent cell line, suggesting selectivity for ALK. However, no real selectivity profile was provided and no *in vivo* study could be performed due to unfavorable physical properties of the two molecules. Later, the Cephalon discovery program yielded several additional compounds, some of which were extensively profiled by KINOMEscan™ technology and demonstrated good activity *in vivo* [117–121]. In particular, CEP-28122 showed sustained complete regression in ALK-positive ALCL, NSCLC and neuroblastoma tumor xenografts, when given orally at 100 mg/kg twice daily. The company currently has a compound (CEP-37440) in late preclinical development.

In 2007, Novartis disclosed NVP-TAE684, a potent and selective ALK inhibitor [122]. NVP-TAE684 selectively inhibits proliferation and survival of NPM–ALK-dependent cell lines *in vitro* and *in vivo*. Dose-dependent inhibition of ALK phosphorylation and downstream signaling was demonstrated. The compound showed comparable activity against INSR and IGF1R in enzymatic assays, but not in cells. Notably, among 35 kinase-transformed BaF3 cell lines, NMP–ALK-transformed BaF3 cells showed a selectivity factor of 100 over the second most sensitive line. When a large panel of approximately 600 human cancer cell lines were screened for sensitivity to NVP-TAE684, only cells harboring genomic ALK alterations were significantly affected by the treatment, with the sole exception of a neuroblastoma cell line that carries no obvious ALK mutation or rearrangement, but is sensitive to IGF1R inhibition [123]. This result indicates that NVP-TAE684 is highly selective and ALK inhibition has a large therapeutic window, because it has minimal impact on ALK-negative cells. Interestingly, NVP-TAE684 is able to inhibit the L1196M gatekeeper mutant of ALK that confers resistance to the clinical inhibitor crizotinib [124] [Mologni L, Gambacorti-Passerini C, Unpublished Data]. The 3D structure of ALK in complex with NVP-TAE684 was recently reported, providing valuable information on the kinase conformation and inhibitor binding [125]. These data allowed mechanistic interpretation of cancer-associated mutations and their oncogenic properties. In addition, x-ray data can be used to fine tune the activity and selectivity of an ALK inhibitor; indeed, the authors were able to refine their lead compound, PHA-E429, guided by structural information, and they now have a clinical candidate (NMS-E628; see below) that will soon enter Phase I clinical studies.

Owing to its potency and specificity, NVP-TAE684, along with crizotinib, is still the most widely used ALK inhibitor in preclinical research [126–129]. Unfortunately, clinical development of the compound was stopped due to systemic toxicity. It is possible that inhibition of INSR and IGF1R contributes to the observed toxicity [122]. Along the same lines, chronic administration of GSK1838705A, a potent inhibitor of INSR, IGF1R and ALK from GlaxoSmithKline, caused hyperinsulinemia in mice [130]. Novartis is currently developing novel, safer analogs [131]. One of these derivatives showed efficacy in a transgenic mouse model of EML4–ALK-positive lung cancer [88].

Characterization of compound NMS-E628 from Nerviano Medical Sciences was presented at the AACR-NCI-EORTC International Conference in 2009 and 2011 [132,133]. The inhibitor was highly active on wild-type as well as crizotinib-resistant mutant ALK *in vitro* an *in vivo* and showed favorable pharmacokinetic and toxicological properties.

Two additional compounds were described by Vanderbilt University and Xcovery, Inc. These molecules (X-376 and X-396) showed higher potency against ALK and similar activity against MET, when compared with crizotinib. Moreover, they were able to inhibit the L1196M gatekeeper mutant at nontoxic doses, although they still have reduced activity against this mutant compared with the wild-type oncogene [134].

The medicinal chemistry program at Amgen identified a series of piperidine carboxamide compounds that bind in an intermediate conformation of ALK, allowing exploitation of a large hydrophobic pocket. This feature led to improved selectivity over related kinases [135]. The lead compound was then licensed to Tesaro for further development [136].

A novel series of triazole ureas with potent anti-proliferative activity in ALK-transformed cells was described by Deng and colleagues [137]. The authors identified a series of leads exemplified by compound 15a. These molecules inhibited a number of ALK mutants (including L1196M ALK), but showed broad activity across a panel of off-target kinases. Further development of this family of compounds is needed.

Recent data were presented at the 2012 Annual Meeting of the AACR describing the preclinical activity of two novel series of inhibitors from AstraZeneca [138]. The compounds showed excellent *in vitro* and *in vivo* activities and pharmacokinetics, and inhibited the gatekeeper L1196M mutant. However, it is not clear to what extent these molecules will be developed.

Finally, two ALK inhibitors (CRL151104A and WZ-5–126) have been mentioned in the literature but very little information is available [123,139].

Clinical research

Crizotinib

Crizotinib (PF-02341066; Xalkori™) was developed by Pfizer as a selective c-MET inhibitor [140]. Indeed, the compound caused regression of large c-MET-dependent xenografts, in part via its antiangiogenic effects. When tested on a large panel of 120 kinases, only one additional target showed equivalent sensitivity; that was ALK. Therefore, the next step was to assess its anti-ALK potential [141]. Crizotinib potently inhibited ALK phosphorylation and induced apoptosis in ALCL cells. In addition, administration of PF-2341066 to animals bearing ALCL tumor xenografts resulted in complete regression of all tumors at the dose of 100 mg/kg daily.

At this point, crizotinib entered the clinical stage. A Phase I dose-finding trial was run in patients with solid tumors (NCT00585195; Table 2) [142,203]. Crizotinib was administered daily or twice daily in dose-escalating cohorts. Dose-limiting toxicities were observed in three patients: one had a grade 3 increase in alanine aminotransferase and two patients reported grade 3 fatigue. The most common adverse events were nausea, emesis, fatigue, visual disturbances and diarrhea. However, side effects were generally mild and reversed upon drug discontinuation. The maximum tolerated dose was 250 mg twice daily. Mean area under the curve and C_{max} increased dose dependently. The median terminal half-life was 46 h, which means that steady state is reached only after 4 days of treatment. In the continuation trial, an expanded cohort of 82 patients with ALK-positive NSCLC was treated with 250 mg twice daily [143]. Reversible grade 1 nausea, diarrhea and visual disturbances were the most frequent side effects. Rare grade 3 increase in liver enzymes were reported. One patient achieved a complete response and 46 had partial responses, with an overall response rate of 57%; in addition, 27 patients had a durable stable disease. Thus, over 90% of the patients benefited from the treatment. No median progression-free survival had been reached when the data were published after a median 6.4 months of treatment. These results are in stark contrast with historical data in NSCLC patients, where response rates are generally approximately 10–40% and overall survival (OS) less than 10 months [107,108]. Data at 1 and 2 years showed 74 and 54% OS, respectively [144]. Interestingly, in a subset of patients with advanced disease who were receiving crizotinib as their second-/third-line therapy, a striking 70% (1 year) and 55% (2 years) OS was observed,

Table 2. Current clinical trials evaluating ALK inhibitors in cancer.

Patient population	Drug(s)	Phase	Status	Identifier
Healthy	Crizotinib + esomeprazole	I	Not yet recruiting	NCT01549574
Healthy	Crizotinib	I	Completed	NCT01297595
Healthy	Crizotinib	I	Completed	NCT00939731
Healthy	Crizotinib	I	Completed	NCT01168934
Healthy	Crizotinib + ketoconazole	I	Completed	NCT01149785
Healthy	Crizotinib + rifampin	I	Completed	NCT01147055
Healthy	Crizotinib	I	Completed	NCT01154218
Healthy	Crizotinib	I	Completed	NCT01250730
Healthy	Crizotinib	I	Completed	NCT01082380
Healthy	Crizotinib	I	Completed	NCT01125904
Renal impairment	Crizotinib	I	Recruiting	NCT01419041
ALK-positive tumors except NSCLC	Crizotinib	I	Recruiting	NCT01121588
RCC, glioblastoma, HCC	Crizotinib + VEGFR inhibitors	I	Withdrawn	NCT01441388
ALK- or MET-sensitive tumors	Crizotinib ± ketoconazole or rifampin	I	Recruiting	NCT00585195
ALCL, IMT, other	Crizotinib	II	Not yet recruiting	NCT01524926
Brain tumors, ALCL	Crizotinib	I/II	Recruiting	NCT00939770
ALCL, NSCLC	Crizotinib	II	Not yet recruiting	NCT01500824
NSCLC	Crizotinib + erlotinib	I/II	Recruiting	NCT00965731
NSCLC	Crizotinib + PF-00299804	I	Recruiting	NCT01121575
NSCLC	Crizotinib + PF-00299804	I	Recruiting	NCT01441128
NSCLC	Crizotinib vs pemetrexed or docetaxel	III	Recruiting	NCT00932893
NSCLC	Crizotinib	II	Recruiting	NCT00932451
NSCLC	Resistance to crizotinib	-	Recruiting	NCT01300429
Advanced cancer	Crizotinib	I	Not yet recruiting	NCT01576406
Non-squamous lung cancer	Crizotinib vs pemetrexed/cisplatin or pemetrexed/carboplatin	III	Recruiting	NCT01154140
Advanced cancer	Crizotinib + pazopanib + pemetrexed	I	Not yet recruiting	NCT01548144
ALK-positive tumors	LDK378	I	Recruiting	NCT01283516
ALK-positive or ROS-positive tumors	ASP3026	I	Recruiting	NCT01284192
ALK-positive tumors	AP26113	I/II	Recruiting	NCT01449461
NSCLC	CH5424802/AF802	I/II	Recruiting	JapicCTI-101264

ALCL: Anaplastic large cell lymphoma; HCC: Hepatocellular carcinoma; IMT: Inflammatory myofibroblastic tumor; NSCLC: Non-small-cell lung cancer; RCC: Renal cell carcinoma.
Data taken from [203].

compared with only 44 and 12%, respectively, in a matched ALK-positive historical control group that received standard chemotherapy. Median OS was not reached at 2 years in the crizotinib group, while a median OS of 6 months was reported for the chemotherapy group. The retrospective analysis showed that survival data in ALK-positive patients treated with crizotinib did not differ significantly from EGFR-positive patients treated with EGFR inhibitors.

A Phase II study is ongoing in patients that failed more than two regimens (A8081005, PROFILE 1005, NCT00932451) [203]. Preliminary data indicate that 83% of patients had tumor shrinkage after a median 9 weeks of treatment, with mild adverse events [145].

Two papers reported activity of crizotinib in non-NSCLC patients. Butrynski *et al.* described a RANBP2–ALK-positive IMT patient who achieved a partial response that lasted over 6 months [146]. After some lesions started to grow again, the patient

underwent surgical debulking and restarted on crizotinib, achieving a complete remission. Only grade 1 side effects were reported. Activity in ALCL patients was first described by Gambacorti-Passerini *et al.* [147]. Two relapsed NPM–ALK-positive ALCL patients were treated at the recommended dose (250 mg twice daily) and showed rapid, complete regression of all lesions. The patients were still in complete remission at the time of report (after 6 and 5 months, respectively). Grade 1 dizziness and visual disturbances were the only adverse events observed.

Currently, a large randomized Phase III study is recruiting ALK-positive NSCLC patients to compare crizotinib versus standard chemotherapy (pemetrexed/docetaxel; NCT00932893) [203]. Another Phase I trial is recruiting non-NSCLC ALK-positive patients (e.g., ALK-positive ALCL and neuroblastoma patients; NCT01121588) [203].

Although efficacy seems to be impressive, 10 years of experience with tyrosine kinase inhibitors in chronic myeloid leukemia patients led the scientific community to ask whether resistance to crizotinib may arise. Indeed, both *in vitro* and clinical studies showed that resistance is an issue that needs to be confronted. The first report of a patient relapsed on crizotinib described two mutations within the kinase domain of ALK in the resistant disease: C1156Y and L1196M in 50 and 15% of the clones, respectively [148]. Both mutations conferred resistance to the drug when re-introduced in Ba/F3 cells *in vitro*. Structurally and biologically, the substitution at Leu1196 corresponds to the T315I mutation of BCR–ABL, which has emerged as the most frequent drug-resistant mutant in chronic myeloid leukemia patients. This key residue, called the 'gatekeeper' because it controls access to the ATP pocket by type II small-molecule inhibitors [149], is currently the biggest challenge in tyrosine kinase inhibitor research. The same type of mutation in EGFR (T790M), c-KIT (T670I), RET (V804M) and PDGFR (T674M/I) confers resistance to their respective inhibitors [150–153]. Another article found a F1174L mutation in an IMT patient who relapsed on crizotinib [154]. A third paper identified a L1152R mutation in a crizotinib-resistant NSCLC patient [155]. A cell line established from the pleural effusion of the patient harbored the L1152R mutation, but also showed EGFR and c-MET activation. Therefore, although still partially dependent on ALK activity, the cells were synergistically killed by a combination of crizotinib and EGFR inhibitors. Doebele *et al.* described 11 NSCLC patients who progressed under crizotinib [156]. Two patients had a *G1269A* mutation and two showed the known L1196M substitution. Interestingly, two patients had a wild-type EML4–ALK sequence but developed a KRAS mutation, and finally one lesion from a relapsed patient had lost the ALK translocation but carried an $EGFR^{L858R}$ mutant instead, suggesting that alternative mechanisms of resistance can be encountered. Finally, Katayama and colleagues presented a series of lung cancer patients with acquired resistance to crizotinib [157]. Approximately one fourth of relapsed patients carried mutations of ALK, including two novel substitutions at the hinge region (G1202R and S1206Y). The remaining patients had no mutation, but showed either *ALK* gene amplification or increased activation of alternative tyrosine kinases, such as EGFR and c-KIT.

Other compounds in clinical development

Researchers at Chugai Pharmaceutical identified a selective ALK inhibitor (CH5424802) that is able to block the two most frequent neuroblastoma-associated mutants as well as the gatekeeper L1196M crizotinib-resistant mutant [158]. The compound shows an excellent selectivity profile, with 2-log selectivity versus INSR and >3-log versus all other tested kinases. *In vivo*, CH5424802 induced tumor regression of both wild-type and L1196M ALK-driven xenografts. Structural analysis elucidated the reason for gatekeeper mutant inhibition. The drug (also called AF802) is codeveloped with Roche and is currently in Phase I/II.

ARIAD has published data on its second-generation ALK inhibitor [124,159]. AP26113 has approximately 1-log higher potency on ALK-positive cells compared with crizotinib and 2-log selectivity versus ALK-negative cells. In addition, it induces regression of tumors carrying crizotinib-resistant mutations of ALK. Moreover, 1-µM inhibitor prevented the outgrowth of any resistant mutant in an *in vitro* resistance screen, while crizotinib under the same conditions allowed the emergence of several mutants, which were then overcome by AP26113. In fact, AP26113 is a dual ALK/EGFR inhibitor, including the T790M gatekeeper mutant of EGFR [160]. Therefore, the compound will potentially benefit both ALK- and EGFR-mutated NSCLC patients, which altogether comprise approximately 15% of cases. A Phase I/II clinical trial of AP26113 started in September 2011.

After the description of NVP-TAE684, Novartis filed a series of patents covering various modifications of the same structure [131]. One of these molecules named LDK378 was presented at AACR-NCI-EORTC 2011 [161] and has shown to induce marked regression of both wild-type and L1196M-mutant EML4–ALK-positive xenografts with low toxicity. The drug has recently entered Phase I investigation.

Another compound named ASP3026, developed

by Astellas Pharma, is currently in Phase I trial. The preclinical characterization of ASP3026 was presented at the recent Annual Meeting of the AACR [162,163]. The compound was more selective than crizotinib when profiled in a tyrosine kinase panel. ASP3026 was challenged against the L1196M mutant *in vivo* and demonstrated potent antitumor activity, with no impact on animal body weights.

In conclusion, all second-generation ALK inhibitors are predicted to be active in crizotinib-resistant patients.

Future perspective

Imatinib opened a new era in cancer treatment [164,165]. At the time when imatinib was first described, cancer research was still tuned on cytotoxic drugs. After approximately 15 years, the concept of rational targeting is now widely accepted and research is more and more focused on the comprehension of cancer-driving forces and the development of drugs that are specifically targeted to the tumor-initiating genetic lesions. Tumors (and therapies they are likely to respond to) are now starting to be classified based on their genetics rather than histology. This attitude will certainly represent the future of cancer medicine, possibly to the point where each patient will be characterized in terms of oncogenic defects and addressed to a corresponding therapy. ALK-positive tumors are pioneering this field. While traditional registration strategies by drug companies focused on a selected tumor type, the advent of genomic medicine and the availability of genome sequencing will certainly result in more cancers being described as containing ALK alterations. An example of this trend is represented by the Pfizer protocol A8081013 in which patients are enrolled on the basis of the presence of an *ALK* gene alteration, and not of a certain tumor type or anatomical site (NCT01121588; Table 2) [203]. Although the advent of genomic medicine in cancer treatment is more complex than initially expected [166], the path toward a more rational, tumor-specific and hopefully effective treatment of human cancer, is set.

Drug resistance is another important issue. The fact that crizotinib-treated ALK-positive cancers most often relapse because they are able to reactivate ALK signal is an indication that we are hitting the right target. New inhibitors that overcome resistance to first-line drugs are needed and indeed second-generation ALK inhibitors are already under development and will likely be available within a couple of years. Similarly to a Darwinian host–pathogen competition, ALK-positive tumors will evolve to escape ALK inhibition and we will develop new weapons to counteract this adaptation. In addition, combination therapies might be an excellent future option. For example, following recent findings on spontaneous immune response to ALK in ALK-positive ALCL patients [167], a combination of ALK inhibitors and anti-ALK vaccination could be envisioned. Along the same lines, anti-ALK monoclonal antibodies were recently shown to potentiate the effects of crizotinib on neuroblastoma cells growth via antibody-dependent cellular cytotoxicity [168]. These combined approaches hold the promise of overcoming the problem posed by tumor heterogeneity.

Executive summary

Molecular biology of ALK

- ALK is a membrane-associated receptor tyrosine kinase expressed in nervous tissue with a possible role in nervous system development. Downstream signaling triggered by ALK activation is described in this review.

Involvement of ALK in human cancer

- The *ALK* gene is translocated, mutated or amplified in a number of tumors, most frequently lymphoma, lung cancer, neuroblastoma and myofibroblastic tumors.

Small-molecule inhibitors of ALK

- Several inhibitors of ALK have been described in the literature and a few have reached clinical evaluation. Preclinical and clinical results are discussed in this review. Crizotinib has recently been approved for the treatment of ALK-positive lung cancer and has shown impressive responses in ALK-positive lymphomas. Clinical data obtained thus far are thoroughly presented in this review.

Financial & competing interests disclosure

C Gambacorti-Passerini is Principal Investigator in the Pfizer protocol A8081013 at the Investigational Site, Monza, Italy. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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