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## Mini-review

## Anaplastic Lymphoma Kinase: Role in specific tumours, and development of small molecule inhibitors for cancer therapy

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## ARTICLE INFO

## Article history:

Received 2 July 2010

Received in revised form 27 August 2010

Accepted 1 September 2010

## Keywords:

ALK

Kinase inhibitor

PF-2341066

Neuroblastoma

NSCLC

ALCL

## ABSTRACT

The Anaplastic Lymphoma Kinase (ALK) is a receptor tyrosine kinase first identified as the product of a gene rearrangement in Anaplastic Large Cell Lymphoma. ALK has subsequently been found to be rearranged, mutated, or amplified in a further series of tumours including neuroblastoma, and Non-Small Cell Lung Cancer. There is strong preclinical evidence that ALK is a driving force for oncogenesis in these cases, and that inhibition of ALK kinase activity results in anti-tumoural efficacy. These observations have sparked the development of small molecule kinase inhibitors, the most advanced of which is currently in clinical testing and which has shown promising preliminary activity in the subset of lung cancer patients whose tumours harbour activated ALK. In this review, we describe the various oncogenic forms of ALK, relevant clinical settings, and give a detailed overview of current drug discovery efforts in the field.

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## 1. Introduction

Anaplastic Lymphoma Kinase (ALK) as a potential drug target in oncology has previously been the subject of several excellent reviews [1–4]: here we describe the receptor, its physiological function, genetic aberrations found in human cancers, consequent rationale as an oncology target and putative clinical settings, and we give an overview of chemical strategies that have been adopted in the search for small molecule inhibitors of ALK kinase activity. Finally, we review preliminary clinical findings observed to date with PF-2341066, the first selective ALK inhibitor to enter clinical testing, and we give our perspective of what future developments may hold in this exciting field.

## 2. ALK structure, expression and normal function

ALK is a receptor tyrosine kinase belonging to the Insulin Receptor superfamily. Based on overall homology,

it groups with Lymphocyte Tyrosine Kinase (LTK), forming a discrete subfamily. ALK was originally identified in 1994 as the product of a recurring chromosomal rearrangement, t(2;5)(p23;q35), in Anaplastic Large Cell Lymphoma (ALCL) patients [5,6]. The chimeric protein encoded by this hybrid gene consisted of the N-terminal portion of Nucleophosmin (NPM) fused to the cytoplasmic domain of a previously unknown tyrosine kinase. The full-length ALK gene was cloned in 1997 both from human and mouse genomes and possessed classical features of receptor tyrosine kinases, comprising an extracellular domain, an hydrophobic stretch corresponding to a single pass transmembrane region, and an intracellular kinase domain [7,8]. The human gene encodes a protein of 180 kDa which after post-translational modification, notably N-glycosylation, gives rise to a mature receptor of 220 kDa. The ALK kinase domain contains the three-tyrosine motif YxxxYY, which is in common with the other kinases of the same family. These tyrosine residues (Tyr1278, Tyr1282 and Tyr1283) are located in the activation loop and represent the major autophosphorylation sites, the sequential phosphorylation of this tyrosine triplet regulates kinase activity. Additional

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tyrosines in the juxtamembrane domain and in the C-terminal sequence have been identified as phosphorylation-dependent sites for binding of transducers [9,10]. The extracellular domain of human ALK is characterized by the presence of several motifs, including a MAM domain, suggesting possible involvement in cell–cell interaction, an ion-binding region and a ligand binding site. Recently, through screening of a phage display c-DNA library, pleiotrophin (PTN), a small heparin-binding growth factor, was identified as a putative ligand for ALK, and a second PTN-related molecule, Midkine, was subsequently found as an additional possible ligand [11–13].

Thorough evaluation of the distribution of ALK expression in normal tissues was performed by *in situ* hybridization in the mouse. These studies demonstrated that ALK expression is restricted to a specific area of mouse brain during development, with a strong signal detected from day 11 *pc* in thalamus, hypothalamus, mid-brain, dorsal root ganglia and olfactory bulb. ALK expression decreases after birth and becomes barely detectable in the adult mouse. Immunohistochemical analysis of adult human tissues revealed ALK expression only in rare scattered neural cells, endothelial cells and pericytes in brain, confirming a limited tissue distribution [7,14,15]. The restricted expression pattern of ALK mRNA in murine and human tissues suggests that this receptor tyrosine kinase might play an important role in development and function of the nervous system. Recent studies, for example, have provided evidence that ALK mediates PTN-stimulated neurite outgrowth in neurons during embryonic development as well as axonal regeneration of damaged motor neurons in the adult, and consequently the PTN–ALK axis has been suggested to be of possible therapeutic interest for conditions involving motor neuron/axon damage [16,17]. Phenotypic characterization of ALK knockout mice has provided further clues to possible physiological roles of this receptor in the nervous system: these mice develop normally, do not display any anatomical abnormalities and have a full life span, but intriguingly they do exhibit better performance relative to wild-type littermates in experimental models of clinical depression, such as behavioural despair tests [18]. Since many studies conducted in murine models have demonstrated that hippocampal neurogenesis is correlated with regulation of mood and is linked to learning and memory processes, it is interesting to note that ALK knockout mice exhibit an increase in basal hippocampal progenitor proliferation, similar to what is observed after chronic treatment with antidepressants [19,20]. Based on these observations, it has been suggested that treatment with ALK inhibitors might represent a possible new approach in therapeutic intervention for mood and cognitive disorders.

### 3. Role of alk in cancer

Following the initial observation of ALK gene rearrangement in ALCL, the role of ALK in cancer pathogenesis has also emerged in several additional clinical settings. A variety of mechanisms leading to aberrant kinase activation and constitutive phosphorylation of downstream pathway

components have been indentified, including missense mutation, gene amplification and chromosomal translocation. In the following sections we describe these various activation mechanisms, and the tumour types in which they have to date been described.

#### 3.1. Overexpression and point mutations of full-length ALK

Expression of full-length ALK has been observed in a variety of human cell lines and tumour specimens including rhabdomyosarcoma, glioblastoma and melanoma, but whether or not wild-type ALK plays a role in pathogenesis of these tumours still remains a matter of debate [21–24]. Full-length ALK cDNA was in fact originally cloned from a c-DNA library derived from the Rh30 rhabdomyosarcoma cell line, and expression of ALK was subsequently reported in a subset of rhabdomyosarcoma tumours [8,21–23]. Recently a genome-wide analysis identified ALK as target gene for PAX3-FKHR, the product of a recurring chromosomal translocation in alveolar rhabdomyosarcoma [25], suggesting that further exploration of ALK as a new therapeutic opportunity for this indication is warranted.

In glioblastoma, for example, ALK expression levels were found to correlate with those of its ligands, suggesting the possibility of an autocrine loop that putatively contributes to tumour cell proliferation. The 15 kDa truncated form of PTN and the MK were found to promote proliferation in a glioblastoma cell line, concomitantly with activation of ALK and downstream signalling, while combined targeting of ALK and PTN induced tumour growth inhibition in glioblastoma xenografts [12,13,26–28].

##### 3.1.1. Neuroblastoma

The involvement of full-length ALK in the pathogenesis of neuroblastoma is, on the other hand, well documented. Neuroblastoma is the most common solid tumour in childhood and originates from neural crest derived tissues, mainly at the level of adrenal glands. Whereas a few patients experience spontaneous regression, in the majority of cases the tumour progresses rapidly giving a metastatic phenotype and, despite aggressive therapeutic treatment, it is often fatal [29]. Initial studies identified ALK protein overexpression both in primary neuroblastoma and cell lines as a consequence of gene amplification [30]. Recent data published by four independent groups have further established the primary role of ALK as a critical oncogene in this paediatric malignancy [31–34]. To evaluate the possibility that, in addition to DNA amplification, other mechanisms could be responsible for ALK activation in neuroblastoma patients, Mossé and co-workers performed a genome-wide scan for linkage at ca. 6000 single nucleotide polymorphisms (SNPs) in twenty families in which two or more individuals are affected. This analysis led to the identification of three germline mutations in the tyrosine kinase domain of ALK. The R1275Q mutation was present in individuals from five families with an almost complete penetrance, and is localized in the kinase activation loop. Interestingly this mutation is adjacent to the corresponding L858R in EGFR which is the most common EGFR mutation in lung cancer. The R1192P mutation falls at the beginning of the  $\beta 4$  strand of the kinase domain.

The third mutation, G1128A, which occurs at the third Gly of the glycine-rich loop was detected only in one large pedigree and was associated with very low penetrance [31]. In addition to these germline mutations, sequence analysis of 167 cases of sporadic neuroblastoma specimens revealed the presence of ALK mutations in 8.4% of patients. The R1275Q mutation is the only one found both in familial and sporadic neuroblastomas, in all the other cases somatically acquired mutations were distinct from the ones identified as germline. In total Mossé et al. identified mutations at eight different codons (G1128A, M1166R, I1171N, F1174I, F1174L, R1192P, F1245C, F1245V, I1250T, and R1275Q). The most common somatic mutation is F1174L, identified in 4.3% of primary tumours [34], which occurs in a region of the kinase domain frequently mutated in EGFR and ERBB2. The expression of ALK F1174L as well as ALK R1275Q mutants in Ba/F3 cells, a murine interleukin-3 (IL-3) dependent pro-B cell line, was found to render these cells independent of IL-3 for growth, a widely established indication of kinase transforming potential. Expression of the F1174L mutant in Ba/F3 cells is associated with robust, constitutive autophosphorylation of ALK and consequent phosphorylation and activation of downstream transducers STAT3 and AKT. Analogously, the R1275Q mutant also induces constitutive activation of ALK kinase, though to a lower extent, and with activation of the downstream targets of ALK signalling ERK1/2 and AKT. On the other hand, neither T1151M nor A1234T mutants endow IL-3 independence to, nor do they exhibit ALK activation in Ba/F3 cells. These findings demonstrate that ALK mutant proteins F1174L and R1275Q are gain-of-function mutations. In addition to Ba/F3 transduced cells, a series of cell lines derived from neuroblastoma patients were also used as a tool to further investigate the role of ALK mutations/amplification and to evaluate effects on cells of currently available ALK inhibitors [34]. Genetic analysis of 27 cell lines derived from high risk patients revealed that 10 cell lines (35.7%) carried single-base ALK kinase domain point mutations, with mutants including F1174L and R1275Q. To evaluate the sensitivity of different ALK mutants to small molecule ALK inhibitors, both Ba/F3 cells expressing mutated ALK and neuroblastoma cell lines were treated with the highly potent ALK inhibitor NVP-TAE684 (Novartis) and with the dual c-Met/ALK inhibitor PF-2341066 (Pfizer), as will be discussed below, and were found to be sensitive to these small molecule inhibitors [35].

Passoni and co-workers have also recently described overexpression of wild-type ALK in sporadic primary neuroblastoma tumours and neuroblastoma cell lines, independently from kinase domain mutations or gene amplification [36]. Here, expression levels of wild-type ALK receptor appear to correlate with its activation status, since ALK tyrosine phosphorylation and kinase activity was detected in the IMR-32 cell line expressing high levels of wild-type receptor, but not in the NB-INT1 and NB5 cell lines, which respectively express low and undetectable levels of ALK. Treatment with the small molecule ALK kinase inhibitors CEP-14083 and CEP-14513 resulted in a dose-dependent inhibition of proliferation and increase in cell death in highly expressing cell lines, but not in lines with low or undetectable ALK expression.

Together, these data provide a strong indication that ALK gain-of-function mutations underlie most cases of hereditary neuroblastoma, though the possibility that secondary genetic events might contribute to tumour development is still under discussion. In addition, ALK mutations and amplification were proven to play a role in more than 10% of sporadic neuroblastoma patients. ALK therefore represents a valuable and innovative target in this paediatric malignancy and consequently, given the promising preclinical *in vitro* and *in vivo* results generated with PF-2341066, a clinical trial in paediatric neuroblastoma patients was initiated in autumn 2009 with this dual c-Met/ALK inhibitor (ClinicalTrials.gov #NCT00939770).

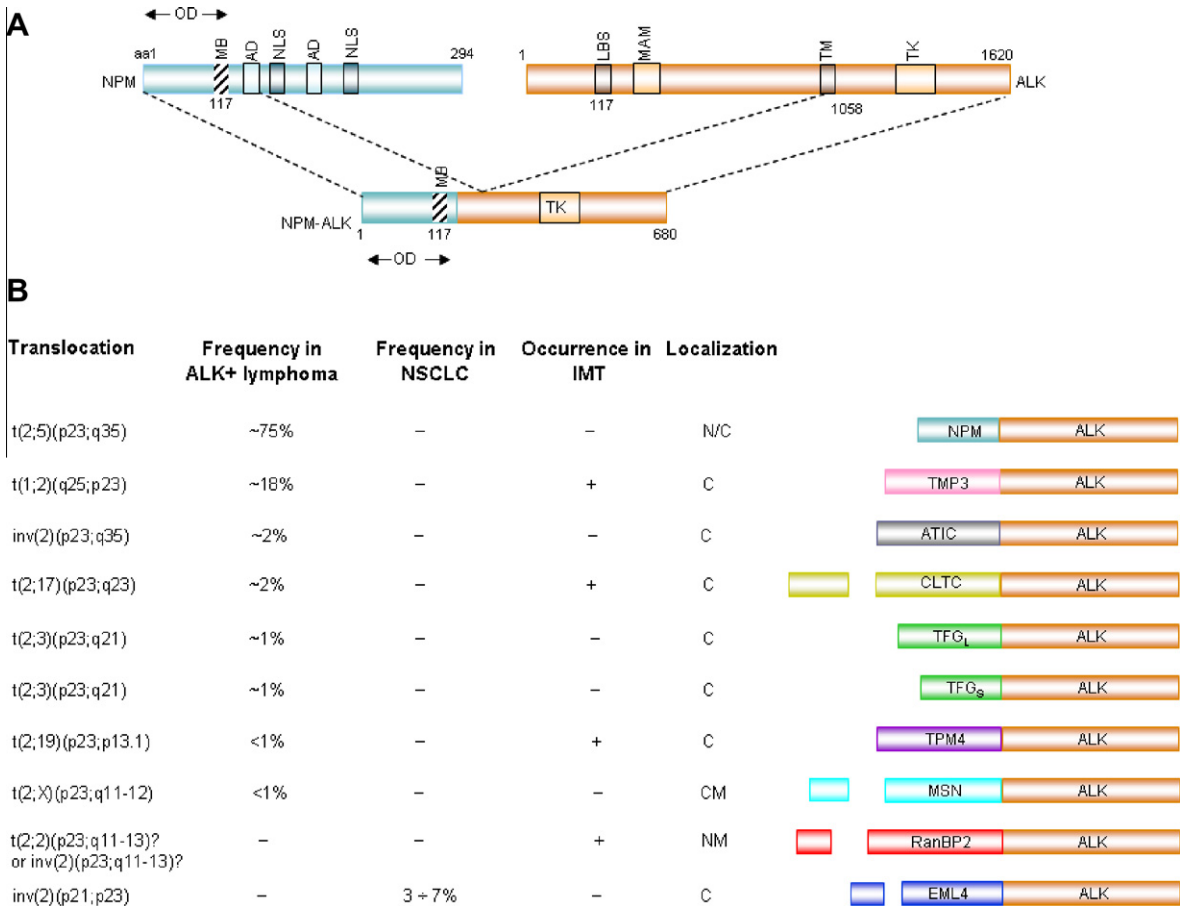
### 3.2. ALK fusion proteins in tumorigenesis

Notwithstanding the point mutation and gene amplification events described above, the most common ALK genetic alterations are chromosomal rearrangements. Various translocations or inversions have been described involving the 2p23 chromosomal locus where the ALK gene is located, leading to creation of fusion genes which encode the entire cytoplasmic domain of ALK at the 3'-end, fused to various 5'-end partners. Each of these rearrangements results in the expression of oncogenic chimeric proteins containing an activated ALK tyrosine kinase domain. As mentioned above, the first fusion protein identified was NPM-ALK in ALCL patients, but, more recently, several other ALK chimeras have been detected in additional tumour types (Fig. 1). Even though many different N-terminal partners have been identified, all these oncogenic fusion proteins share common features. The expression of the fusion protein is regulated by the promoter of the N-terminal partner, which is generally a protein widely expressed in normal tissues, and which thus leads to ectopic expression of ALK kinase domain. All the N-terminal fusion partners are characterized by the presence of oligomerisation domains, which are fundamental for oncogenic potential of the fusion protein: in physiological conditions wild-type full-length ALK, as for other RTKs, becomes activated only upon ligand-induced homo-dimerisation, which allows trans-phosphorylation of the corresponding intracellular kinase domains. This step is absolutely required for kinase activation and consequent downstream signalling. In contrast, the oligomerisation domains present in N-terminal fusion partners induces ligand-independent dimerisation of the ALK kinase domain, leading to constitutive kinase activation, aberrant activation of signal transduction pathways, and thus potential for malignant transformation [3,37–44].

#### 3.2.1. Anaplastic Large Cell Lymphoma (ALCL)

ALCL is a rare type of T-cell lymphoma comprising heterogeneous cellular entities, characterized by large cells with a variable shape (anaplastic pattern) but which invariably express the CD30 surface antigen [45–48]. Although ALCL arises from T-cell lymphocytes, expression of the T-cell receptor and several other T-cell specific markers is lost as the disease progresses.

ALCLs account for 2.5–5% of all human Non Hodgkin's lymphomas, although the frequency is higher in children



**Fig. 1.** (A) Molecular structure of Nucleophosmin, wild-type ALK, and of the NPM–ALK chimeric protein. The NPM–ALK fusion protein retains the oligomerisation domain with the metal binding region of NPM and the tyrosine kinase domain and the cytoplasmic tail of ALK. (B) Schematic representation of the most frequent ALK fusion proteins including chromosomal location, frequency in ALCL and in NSCLC, occurrence in IMT and sub-cellular localization. Aa: amino-acid, AD: acidic amino-acid domain, ALK: Anaplastic Lymphoma Kinase, ATIC: 5-aminoimidazole-4-carboxamideribonucleotide formyl transferase/IMP cyclohydrolase, C: cytoplasmic, CLTC: clathrin heavy chain, CM: cell membrane, EML: echinoderm microtubule-associated protein-like, LBS: ligand binding site, IMT: inflammatory myofibroblastic tumour, MAM: Mepripin/A5 protein tyrosine phosphatase Mu, MB: metal binding domain, MSN: moesin, N: nuclear, NLS: nuclear localization signal, NPM: nucleophosmin, NM: nuclear membrane, OD: oligomerisation domain, RanBP2: Ran-binding protein 2, TFG: TRK-fused gene, TK: tyrosine kinase domain, TM: transmembrane domain, TPM3: non-muscle tropomyosin.

and young adults, at ca. 10–15%. ALCL can be either systemic (involving the whole body) or cutaneous (involving only skin), is more frequent in males, and is frequently diagnosed at stage III or IV with a rapidly progressive clinical course. If untreated, ALCL is very aggressive, but response rate to therapy is high and long term survival is common, especially in patients bearing ALK gene rearrangements (see later). The most common treatment for ALCL is based on CHOP combination regimens (Cyclophosphamide, Doxorubicin, Vincristine, Prednisone), which cure 60–80% of ALK positive, but only 40% of ALK negative patients. Radiation therapy can also be used in combination with CHOP when large localized masses are present.

The vast majority of ALCL (60–80% of the whole population, but over 85% if only children are taken into account) are positive (as detected by FISH or RT-PCR) for the expression of a transgene derived from a genomic rearrangement involving the Anaplastic Lymphoma Kinase (ALK) gene [5].

The first described, best studied, and also most frequent (75% of ALK-positive ALCL) ALK translocation (t(2;5)(p23;q35)) involves the Nucleophosmin (NPM) gene. NPM is an abundant, nucleolar phospho-protein that shuttles between nucleus and cytoplasm. It is involved in numerous cellular processes including ribonucleoproteins transport, centrosome duplication and control of genomic stability [49,50]. Another frequent (18%) ALK rearrangement involves the non-muscle Tropomyosin 3 (TPM3) gene at chromosome 1q25. Tropomyosins are actin-binding proteins, and are components of cytoskeletal microfilaments, providing stability to the actin filaments and regulating interactions with other actin-binding proteins.

Currently 15 different ALK fusion proteins have been identified, and the most frequent are reported in Fig. 1. Interestingly, seven of these fusion proteins have also been reported in Inflammatory Myoblastic Tumours (IMT), suggesting a preferential choice of ALK recombination

partners also in different tissues. The N-terminal partner determines the sub-cellular localization of the fusion protein and to date, the only ALK fusion protein detected both in the nucleus and cytoplasm is NPM-ALK, with all the others being cytoplasmic.

ALCL patients possessing any of these ALK rearrangements have a substantially good response to CHOP therapy, but the various fusion proteins produce subtle differences in tumour-related properties when transfected into murine 3T3 fibroblasts, and implanted as xenografts. The effect of the different ALK N-terminal partners was assessed by expressing five ALK fusion variants in 3T3 cells [51]: NPM-, TFG-, CLTL- and ATIC-ALK were found to increase proliferation and soft agar colony formation, while TPM3 had a stronger effect on invasion. TPM3-ALK was subsequently shown to co-immunoprecipitate with endogenous tropomyosin, further supporting an effect on cytoskeleton organization with consequent decrease in cell adhesion [52]. All the different ALK fusions expressed in NIH3T3 developed tumours in nude mice, but NPM-ALK and TFG-ALK transfected cells gave more rapidly growing tumours.

ALK fusion proteins activate the classical receptor tyrosine kinase signalling pathway, but several data suggest that the most relevant role in ALK mediated oncogenesis is played by STAT3 phosphorylation and activation [53–55].

The causative role of NPM-ALK in lymphoma development has been widely explored both using retroviral transducing systems and with transgenic models. Different studies report long latency induction of B-lineage large cell lymphoma, lymphoblastic lymphomas of T-cell type, plasmacytomas, plasmoblastic/anaplastic diffuse large B cell lymphomas upon retroviral transduction of NPM-ALK [56–58]. Transgenic mouse models have been generated expressing NPM-ALK under the control of the hematopoietic cell specific “Vav” promoter, or under the control of CD4 and LCK promoter, thus specifically targeting NPM-ALK expression to T-cells [59–61]. CD4-driven NPM-ALK transgenic mice develop short latency thymic lymphomas with a T-cell phenotype and frequent expression of CD30 antigen. Similarly, Lck-driven NPM-ALK transgenic mice develop large cell lymphoblastic lymphomas involving thymus and lymph nodes, with extra-nodal involvement within 8 weeks.

ALK was shown to be a valid therapeutic target for ALCL through several approaches, for example recent data of ALK silencing using shRNA demonstrated cell cycle arrest and apoptosis in ALCL cells, as well as tumour growth regression *in vivo* upon knock-down of cellular levels of the NPM-ALK fusion protein [62]. The final validation that ALK inhibition can revert ALK + ALCL tumour growth was provided by the studies with recently developed ALK kinase inhibitors, which very effectively block proliferation and *in vivo* tumour growth of ALK driven cellular models, as will be discussed below.

### 3.2.2. Non-Small Cell Lung Cancer (NSCLC)

Interest in ALK as a drug target in oncology was further heightened by the identification in 2007 of a new fusion gene in a small subset (ca. 6–7%) of NSCLC patients

[63,64]. In this case, ALK gene rearrangement involves an inversion within the short arm of chromosome 2 (between loci 2p21 and 2p23), leading to expression of an oncogenic protein containing the N-terminal portion of echinoderm microtubule associated protein like 4 (EML4) and the entire intracellular portion of ALK. Although EML4-ALK is to date by far the most frequent and best characterized ALK gene rearrangement in NSCLC patients, a translocation involving kinesin family member 5 B (KIF5B) and ALK has also recently been reported in two NSCLC cases [65], reinforcing the relevance of ALK as target in this disease.

With regards to EML4-ALK, although several different truncations of EML4 have been observed (occurring at exons 2, 6, 13, 14, 15, 18 and 20) the breakpoint in ALK is always in intron 20 of the gene, and all EML4-ALK fusion proteins contain the entire cytoplasmic domain of the receptor. The presence of the coiled-coil oligomerisation domain of EML4 mediates the constitutive dimerisation of the fusion protein and thus the deregulated activation of the kinase domain.

The oncogenic potential of the EML4-ALK chimeric protein was confirmed by expression in 3T3 fibroblasts, which acquired capacity to grow as transformed foci *in vitro* and to generate tumours in nude mice [63], both of which are classical properties of oncogenes. On the contrary, the EML4-ALK kinase inactive mutant (K589M) does not possess such transforming capacity, demonstrating that the catalytic activity of the kinase domain is fundamental. Similarly, further studies have shown that efficient dimerising capability of EML4 is required for maintaining oncogenic potential of the fusion protein [63,66,67].

To further assess the role of EML4-ALK in the pathogenesis of NSCLC, transgenic mice specifically expressing the fusion protein in lung alveolar epithelial cells were generated [68]. EML4-ALK transgenic mice were found to develop hundreds of adenocarcinoma nodules in both lungs with a very short latency period, and with 100% penetrance (i.e. in all mice bearing the transgene). Strong reduction of tumour burden was observed after oral treatment of transgenic mice with a potent ALK inhibitor (Novartis compd 1 reported in Example 3–39 of PCT WO2005016894, [69]), confirming that these tumours are dependent upon ALK tyrosine kinase activity for growth, and providing further experimental validation of the concept that ALK is a relevant target in the subset of lung cancers that harbour EML4-ALK.

As mentioned above, the role of ALK in NSCLC was initially reported in 2007 by Soda and co-workers, who found the EML4-ALK fusion protein expressed in 5 out of 75 (6.7%) Japanese patients. Subsequently, many other cohorts of NSCLC patients were analyzed either by FISH or RT-PCR, confirming the presence of this gene rearrangement in a small subset of NSCLC patients in both Asian and Western populations. In general, the frequency of the rearrangement in the Western population (ca. 3%) appears to be lower than that in Asians (ca. 6%) but the various studies conducted to date have highlighted interesting common features [70]. For example, the incidence of ALK gene rearrangement appears restricted to patients with an adenocarcinoma subtype, of acinar histology and is prevalent in non- or light-smokers, and in young patients. In

the Asian population a clear prevalence of woman was found. Interestingly, ALK aberrations were also found to be mutually exclusive to EGFR mutations and K-RAS mutations [71–73]. Falini and co-workers have however questioned the oncogenic significance of EML4–ALK in NSCLC [74,75], since they were able to detect EML4–ALK transcripts by RT-PCR in non-neoplastic lung tissue from NSCLC patients, as well as in lymphoid tissues. Additionally, in RT-PCR-positive lung tumours and normal lung tissue, presence of the transgene by FISH analysis was limited to ca. 1–3% of the total cell population, and EML4–ALK protein was undetectable by IHC, Western Blotting, or immunoprecipitation. There is some degree of controversy concerning these findings [76], but as suggested by these authors themselves, it is likely that significance of EML4–ALK in NSCLC will ultimately be determined during ongoing clinical trials using selective ALK inhibitors.

Lung cancer is the leading cause of cancer-related death in the United States and worldwide, and despite recent advancements in treatment of the disease, the medical need remains very high, with an overall 5-years survival rate of 15% [77]. Clinical experience in NSCLC with EGFR inhibitors has demonstrated that treatment of selected patients bearing drug-sensitive mutations is associated with strong clinical benefit [78,79]. By analogy, and supported by the preclinical results described above, lung tumours harbouring constitutively activated ALK would be expected to be responsive to clinical treatment with selective ALK inhibitors. Although several small molecule inhibitors of ALK kinase activity are currently being characterized at the preclinical level, to date only the dual c-Met/ALK inhibitor PF-2341066 (Pfizer) has reached clinical development. Preliminary clinical responses observed with this agent in NSCLC patients bearing ALK rearrangement will be discussed below.

### 3.2.3. Inflammatory myofibroblastic tumour (IMT)

Chromosomal rearrangements involving the 2p23 locus were described over 20 years ago as recurrent events in IMT, and were subsequently found to encode ALK fusion proteins [80,81]. These tumours are of mesenchymal origin and are composed of neoplastic spindle cells mixed with a reactive inflammatory infiltrate of lymphocytes and plasma cells. IMTs are rare, with a frequency of 150–200 new cases per year in the United States. Surgical resection is usually the first treatment, but many cases develop a more aggressive phenotype with occurrence of metastases. IMTs are in general poorly responsive to standard chemotherapy. Approximately 50% of cases are characterized by the presence of chromosomal rearrangement involving the short arm of chromosome 2, where ALK is located. After the initial identification of TPM3–ALK and TPM4–ALK chimeric proteins in three IMT patients in 1999, a series of additional fusion proteins were detected including CARS–ALK, CLTC–ALK, ATIC–ALK, RANBP2–ALK, SEC31LB–ALK [39,82–84]. With the exception of RANBP2–ALK, which is localized to the nuclear membrane, all the other fusion proteins display a typical cytoplasmic staining. The expression of ALK was generally found in younger patients and correlated with local recurrence rather than with distant metastasis formation. These observations suggest that

ALK targeted therapy could be useful in patients with ALK positive, recurrent IMTs.

### 3.2.4. Other tumours with ALK gene rearrangement

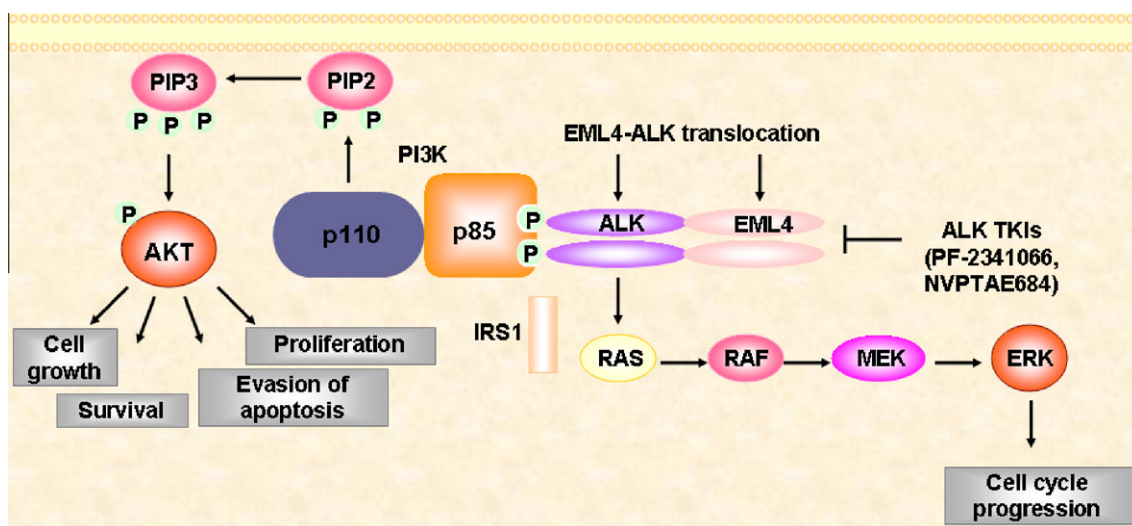
In 2003 several independent groups identified CLTC–ALK and NPM–ALK fusion proteins in a rare form of B-cell Non-Hodgkin Lymphoma [85–87]. This subset of lymphoma is characterized by an aggressive phenotype and poor prognosis. In the case of CLTC–ALK fusion protein, both RT-PCR and FISH analysis confirmed that the expression of the transgene is the consequence of the chromosomal rearrangement t(2;17)(p23;q23). Although demonstration of constitutive ALK kinase activation in this tumour type is still lacking, dimerisation of the fusion protein might be expected based on the presence of an oligomerisation domain in the CLTC N-terminal region. Thus, it can be hypothesized that ALK might represent a valuable target for therapy also in this clinical setting.

In 2006 the fusion protein TPM4–ALK was found expressed in oesophageal squamous cell carcinoma in an Iranian patient population [88], and although similar findings have subsequently been confirmed in a Chinese population [89], the frequency of the rearrangement and relevance for oesophageal squamous cell carcinoma requires further evaluation.

Finally, in 2008, ALK fusion proteins were detected in three cases of systemic histiocytosis, an hematopoietic neoplasm characterized by hepatosplenomegalia, anaemia and thrombocytopenia. Also in this case, additional validation data are required [90].

### 3.3. ALK signalling in cancer

The transforming potential of activated ALK is due to the aberrant phosphorylation of downstream substrates, which triggers deregulated intracellular signalling cascades. The critical pathways involved in ALK-mediated transformation are similar to those activated by other normal or oncogenic receptor tyrosine kinases. In cellular models in which ALK is activated through chromosomal rearrangement it has been demonstrated that the constitutive dimerisation of ALK-containing fusion proteins mediates the enhanced activation of three major pathways, the JAK–STAT3, PI3K–AKT and RAS–MAPK pathways, which control cell proliferation and survival [53,91,92]. Tissue context is also known to play a role, and different ALK rearrangements have been demonstrated to produce differential pathogenic signalling. In ALCL, an elegant set of *in vitro* and *in vivo* studies confirmed that all three pathways are strongly activated by NPM–ALK fusion protein and both an RNA interference approach and treatment with selective ALK inhibitors confirmed that these signalling cascades mediate cell growth and resistance of ALK positive cells to apoptosis induction. Nevertheless, there is some evidence that the transforming potential of NPM–ALK in ALCL is mediated mainly through STAT3 activation [53]. Direct or JAK3-mediated phosphorylation of STAT3 as a consequence of NPM–ALK dimerisation was demonstrated to be sufficient for stimulating proliferation and survival, while antisense oligonucleotides able to suppress STAT3 expression strongly impaired tumourigenesis



**Fig. 2.** ALK signalling. In NSCLC harbouring EML4–ALK rearrangement, the PI3K–AKT and RAS–MAPK pathways are strongly activated as consequence of the constitutive dimerisation and activation of the expressed chimeric protein.

*in vivo*. Moreover, gene expression profiles in ALCL models confirmed that STAT3 increases the expression of survival factors and cell cycle regulators. On the other hand, in NSCLC cellular models harbouring EML4–ALK rearrangement, the PI3K–AKT and RAS–MAPK pathways are strongly activated whereas STAT3 is unlikely to be a major transducer (Fig. 2) [67,93]. It has been postulated that the different tissue context and the different cellular localization of the two chimeric proteins can justify these differences [4].

#### 4. ALK small molecule inhibitors

The identification of constitutively activated forms of the ALK protein in different tumour types, both as activated fusion proteins derived from chromosomal rearrangements (such as NPM–ALK in ALCL and EML4–ALK in NSCLC) and as mutationally activated ALK proteins (such as the activating mutations in neuroblastoma) has fostered the discovery and development of new small molecules capable of blocking ALK dependent cancer cell growth. Since aberrantly active forms of ALK depend upon the intracellular kinase domain of ALK for their transforming activity, major effort is currently focused on the search for small molecule inhibitors of the kinase activity. This approach has been already proven to be efficacious in clinical settings with other Tyrosine Kinase Inhibitors (TKIs) [94] such as Gleevec (imatinib) in chronic myeloid leukaemia, where tumour cell growth is driven by the kinase activity of the fusion protein Bcr–Abl [95,96]. Analogously, a subset of NSCLCs harbouring activating mutations in the epidermal growth factor receptor (EGFR) gene, has been successfully treated with Iressa (gefitinib) and Tarceva (erlotinib), two small molecule inhibitors of the kinase activity of EGFR [78,79].

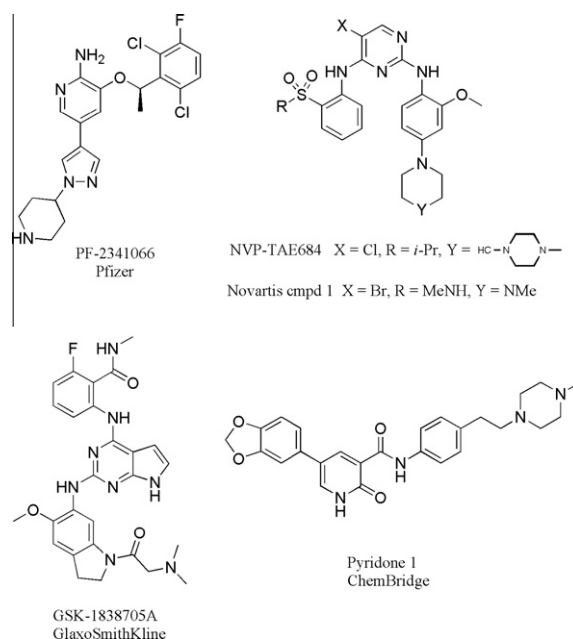
The most explored and successful approach for the design of small molecule kinase inhibitors is based on targeting the ATP binding site of the catalytic domain, which is highly conserved in kinases. The potential issue of selectiv-

ity has been addressed by targeting different kinase conformations, and enzyme specific lipophilic pockets, whose accessibility is dependent on the gatekeeper residue [97–99].

Despite the lack of published ALK structures, homology models of the kinase have been described and represent a valuable tool for inhibitor design [100].

##### 4.1. Chemical classes of ALK inhibitors

Among the well-known kinase inhibitors, the promiscuous compound staurosporine has been reported to block



**Fig. 3.** Chemical structure of Pfizer, Novartis Pharma, GlaxoSmithKline and ChemBridge ALK inhibitors.

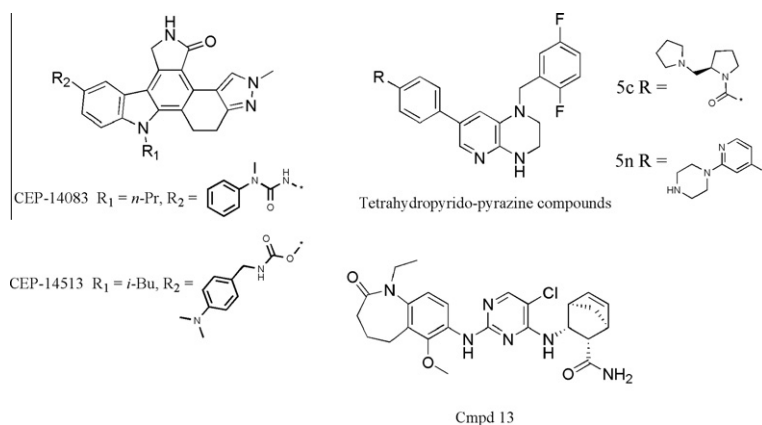


Fig. 4. Chemical structure of Cephalon ALK inhibitor.

ALK kinase activity in enzymatic assays with an ATP competitive mechanism [101].

However in the last 5 years more specific and potent ALK inhibitors have been discovered and described in the literature [1,3,102,103]. The major player in the field is currently represented by the Pfizer compound PF-2341066, which is in clinical development both for c-Met and ALK driven cancer indications, even though other interesting and potent small molecule ALK inhibitors have also emerged, as depicted in Figs. 3 and 4.

#### 4.1.1. Pfizer (PF-2341066)

The Pfizer compound PF-2341066 (Fig. 3) was originally discovered and optimized as an inhibitor of the c-Met-HGF signalling pathway [92].

This compound proved to be a potent ATP-competitive inhibitor of recombinant human c-Met kinase activity [92,104]. When profiled on a panel of >120 kinases in biochemical assays, it was shown to be more than 100-fold selective for c-Met compared to most (>90%) of the tested kinases. However PF-2341066 displayed a potent antiproliferative activity in ALCL cell lines (Karpas-299 and SU-DHL-1, IC<sub>50</sub> 32 and 43 nM respectively), with a strong correlation with inhibition of NPM-ALK tyrosine phosphorylation. In addition, both cell lines displayed G1-S phase cell cycle arrest and apoptosis following treatment. When orally administered to Karpas-299 engrafted SCID-Beige mice, PF-2341066 induced a dose-dependent tumour growth inhibition with complete tumour regression within 15 days of treatment at the maximum tested dose (100 mg/kg/d). Again, a good dose-dependent correlation was also observed between tumour growth inhibition and target modulation in tumours. PF-2341066 was also shown to inhibit the proliferation of the ALK driven NCI-H3122 NSCLC cell line and of neuroblastoma cell lines [105]. In particular, PF-2341066 seems to be more potent against neuroblastoma cell lines bearing ALK gene amplification or the R1275Q mutation with respect to cells bearing the F1174L mutation [35,105].

The compound was characterized for its toxicological profile in preclinical studies, and was found to be safe upon chronic repeated administration to mice at up to 200 mg/

kg/d, for up to 30 days and at comparable dose levels in dogs and primates [104].

Currently this compound is under evaluation in clinical trials for several cancer indications, both c-Met and ALK dependent, and preliminary findings from these studies will be discussed below.

#### 4.1.2. Novartis Pharma (NVP-TAE684)

The compound NVP-TAE684 (Fig. 3) was identified through cellular screening of a kinase targeted library based on the evaluation of cytotoxic activity against NPM-ALK transformed Ba/F3 cells [106]. NVP-TAE684 inhibited proliferation of the NPM-ALK driven cancer cell lines Karpas-299 and SU-DHL-1 with an IC<sub>50</sub> range of 2–5 nM, with dose-dependent down-modulation of NPM-ALK autophosphorylation. When tested for selectivity on a panel of 35 Ba/F3 cells transformed by various tyrosine kinases, the compound proved to be 100- to 1000-fold selective for ALK-driven cells. Despite the strong sequence homology between ALK and insulin receptor (InsR) kinase, and the *in vitro* potency of NVP-TAE684 on recombinant InsR kinase (IC<sub>50</sub> 10–20 nM), no significant impairment of IR phosphorylation was detected in cellular models (IC<sub>50</sub> 1.2 μM).

The good pharmacokinetic properties of NVP-TAE684 allowed *in vivo* studies of the compound following oral administration. Thus, in a disseminated growth cancer model for ALCL, SCID mice were injected intravenously with luciferised Karpas-299 cells, allowing systemic tumour growth to be monitored using the Xenogen bioluminescence imaging system. Treatment with NVP-TAE684 induced a 1000-fold reduction in bioluminescent signal after 2 weeks dosing at 10 mg/kg, with *ex-vivo* target modulation. Despite these excellent data in animals, this compound is not currently being developed.

NVP-TAE684 showed a preferential activity in a small subset of NSCLC, neuroblastoma, and ALCL cell lines, when profiled on a panel of 602 cancer cell lines [105].

Another compound of the 2,4-pyrimidinediamine chemical series, Novartis cmpd 1 (reported in Example 3–39 of PCT WO2005016894 [69]) showed impressive results in the EML4-ALK transgenic mouse model [68]. Oral



daily dose of 10 mg/kg resulted in tumour disappearance in the treated animals (at day 11 and 25) compared to large tumour masses in the lungs of control animals.

#### 4.1.3. GlaxoSmithKline (GSK1838705A)

GSK1838705A (Fig. 3) is a potent, highly selective, ATP-competitive inhibitor of ALK, IGF-1R and InsR with low nanomolar activity in enzyme assays ( $IC_{50}$  0.5, 1.6 and 2, respectively) [107]. When tested against a panel of 224 protein kinases it was found to inhibit only seven additional kinases by >50% at 0.3  $\mu$ M. GSK1838705A inhibits proliferation of different tumour cell lines, displaying nanomolar  $IC_{50}$  values in ALK-dependent cell lines such as L-82, SUP-M2, SU-DHL-1, Karpas-299 and SR-786, with a dose-dependent down-modulation of NPM-ALK and downstream signalling pathway [107].

The compound also inhibited proliferation of the EML4-ALK NSCLC cell line NCI-H2228 with an  $IC_{50}$  of 191 nM, and EML4-ALK phosphorylation. The good pharmacokinetic properties and the excellent oral bioavailability of this compound ( $F = 98\%$ ) [108], allowed further *in vivo* investigation. Treatment of SCID mice bearing Karpas-299 tumours with GSK1838705A resulted in complete tumour regression at the well-tolerated dose of 60 mg/kg once daily (21 days treatment), with *ex-vivo* target modulation and induction of apoptosis. Despite the inhibitory activity of this compound on InsR, only minimal effects on glucose homeostasis were reported [107].

#### 4.1.4. Cephalon

The first ALK inhibitors identified at Cephalon were potent in biochemical and cell-based assays but displayed unfavourable physicochemical properties that precluded their use for *in vivo* studies (compounds CEP-14083 and CEP-14513, Fig. 4) [109].

Cephalon thus developed a second generation of ALK inhibitors, a series of tetrahydropyrido-pyrazine compounds, that exhibit enzymatic ALK  $IC_{50}$  values in the low nanomolar range and good cell-based ALK-inhibitory activity (see Fig. 4) [110]. Two representative compounds are 5c and 5n (Fig. 4) with  $IC_{50}$  values on ALK enzyme of 15 and 10 nM respectively. This series of analogs displayed a high degree of selectivity when tested at 1  $\mu$ M across a panel of 250–400 kinases.

The 2,4-diarylaminopyrimidine chemotype was also investigated and turned out to possess particularly favourable ALK-inhibitory properties, with hundreds of compounds yielding  $IC_{50}$  potencies <100 nM in enzyme assays [111]. In particular, Cmpd 13 (Fig. 4) revealed cellular  $IC_{50}$ s < 100 nM in ALK-positive ALCL cell lines. It is orally bioavailable and completely inhibits NPM-ALK tyrosine phosphorylation in ALCL tumours subcutaneously implanted in SCID mice at an oral dose of 55 mg/kg. This compound inhibited also EML4-ALK tyrosine phosphorylation and induced cytotoxicity in EML4-ALK positive NSCLC cell lines and in the NB-1 neuroblastoma cell line bearing ALK amplification [112,113].

#### 4.1.5. ChemBridge

The ChemBridge ALK inhibitor, Pyridone 1 (Fig. 3) inhibits ALK with an enzymatic  $IC_{50}$  of 380 nM and more than

10-fold selectivity over other members of the Insulin Receptor superfamily [114]. Cellular activity was however modest and unselective. To date, additional data on this series have not been reported.

Recently the activity profile of another interesting compound, CRL151104A, developed by ChemBridge Research Laboratories and St Jude Children's Research Hospital was reported in the literature [3].

#### 4.1.6. Ariad pharmaceuticals AP26113

Another interesting ALK inhibitor of undisclosed structure is the Ariad compound AP26113 [115–117]. It is reported to inhibit ALK with an  $IC_{50}$  of 0.53 nM with good selectivity against IR and IGF-1R, and to cause growth inhibition of Karpas-299, SU-DHL-1, and SUP-M2 cell lines with  $IC_{50}$  respectively of 10, 9, and 15 nM. Antiproliferative activity in the low nanomolar range was reported for cell lines bearing the EML4-ALK translocation, namely for the NCI-H3122 and NCI-H2228 cell lines. Good selectivity against ALK-negative cell lines was obtained.

The compound, when administered to Karpas-299 and NCI-H3122 xenograft bearing mice (daily oral dosing of 50 mg/kg), caused almost complete tumour regression in both cases with dose-dependent down-modulation of ALK phosphorylation. The Ariad compound is reported to be orally bioavailable across multiple species and tolerated above the predicted efficacious plasma levels. However the most interesting data on AP26113, were related to its activity on a series of EML4-ALK mutated forms reported to be resistant to PF-2341066 (as discussed below).

## 4.2. Clinical advances

A wealth of clinical data demonstrates that genetic aberrations of ALK are recurrent in specific tumour subtypes, and compelling data generated in preclinical models indicate that tumours harbouring ALK gene amplifications, translocations, or activating point mutations are partially or fully dependent upon ALK kinase activity for proliferation and survival. Importantly, many studies have demonstrated that inhibition of ALK signalling using small molecule kinase inhibitors yields potent antitumour efficacy in various preclinical models which closely recapitulate features of ALK-expressing tumours in humans, thus providing a sound rationale for clinical development of such inhibitors. A definitive proof of concept for this approach, however, has been provided by very recent preliminary data emerging from the first clinical study conducted with the Pfizer dual MET/ALK inhibitor PF-2341066 (ClinicalTrials.gov #NCT00585195), to date the only declared ALK inhibitor in clinical testing. Amongst other indications, PF-2341066 was tested as a single agent in refractory, heavily pre-treated, NSCLC patients with tumours harbouring the EML4-ALK rearrangement. Currently available data indicates that among 76 lung cancer patients enrolled, the overall response rate was 64%, with a disease control rate at 8 weeks of 87% [118,119]. Although further confirmation through extended, randomized clinical studies is required, such results are remarkable in this notoriously intractable disease. Adverse events reported to date were in general mild or moderate,

including gastrointestinal effects and disturbance of vision. Treatment-related severe toxicity (elevated liver transaminases) was infrequent and reversible. On the basis of these results, a Phase III study of PF-2341066 in ALK-positive lung cancer patients compared to standard chemotherapy has been initiated (ClinicalTrials.gov #NCT00932893). Given the similarly convincing preclinical data supporting the rationale for ALK being a valuable therapeutic target for treatment of neuroblastoma and ALCL patients bearing ALK mutations/rearrangements, a paediatric Phase I/II trial in these indications with PF-2341066 has also very recently started enrolment of patients (ClinicalTrials.gov #NCT00939770).

#### 4.3. Future perspectives

“Oncogene addiction” is a term used to describe the phenomenon whereby tumours appear to be exquisitely dependent upon a single mutated or aberrantly expressed gene [120]. Besides from ALK, other known examples of oncogene addiction include the kinases Abl in chronic myelogenous leukaemia, EGFR in a subset of lung cancer, c-Kit in gastrointestinal stromal tumour (GIST), B-Raf in melanoma, Flt3 in a subset of acute myelogenous leukaemias, and JAK2 in myeloproliferative syndromes [97,121]. Among these, ALK appears to be rather remarkable in terms of the multiplicity of mechanisms by which it acquires oncogenic potential (as reflected in the relatively vast array of fusion partners), and the diverse tumour tissues in which it appears to be a driver of oncogenesis. Indeed, it is tempting to speculate that there may be additional, as yet unidentified, tumour subsets that are driven by constitutively activated ALK.

Clinical experience with inhibitors which target kinases to which tumours are apparently “addicted” has revealed that despite the sometimes spectacular antitumour activity obtained, drug resistance will eventually arise in response to treatment, and that this is often due to secondary mutational events in the kinase domain which compromise inhibitor activity. This phenomenon has been observed for Bcr–Abl in CML following therapy with imatinib, for EGFR in NSCLC following gefitinib or erlotinib therapy, and for c-Kit in GIST following therapy with imatinib and sunitinib [122–125].

It is then likely that for ALK, such resistance will also occur with first generation, efficacious inhibitors such as PF-2341066, and this represents a potential window of opportunity for development of second-generation inhibitors. Cephalon, for example, has already attempted to address this possibility by assessing the activity of different inhibitor scaffolds against “synthetic” ALK variants mutated at the amino-acids positions corresponding to some of the most commonly mutated residues implicated in drug resistance in other kinases: the phosphate anchor and the gatekeeper residues [126]. When two representative compounds, the pyrrolocarbazole CEP-14513 and the diaminopyrimidine Cmpd 13 (Fig. 4), were tested for inhibition of tyrosine phosphorylation and cell growth in transformed Ba/F3 cells, CEP-14513 retained activity against NPM–ALK L182 M and L182 V mutants in the phosphate anchor residue comparable to that for NPM–ALK WT,

while being less potent against the NPM–ALK L256 M gatekeeper residue mutant. Cmpd 13 was instead much less effective in the inhibition of both tyrosine phosphorylation and cell growth of Ba/F3 transformed with all the three mutants compared to NPM–ALK WT cells. These data are suggestive of the crucial impact of the chemical template on inhibitor activity against mutated forms of the target protein, and the difficulty of targeting ALK mutation in the gate-keeper region.

Ariad addressed the same issue with an experimental approach that was successfully used to predict specific mutations that confer clinical resistance to known kinase inhibitors, i.e. for Bcr–Abl inhibitors in CML patients [116]. This resistance profiling method led to the identification of multiple mutants that confer resistance to PF-2341066 and subsequent experimental studies demonstrated that the Ariad ALK inhibitor AP-26113 could be able to overcome resistance to this first generation compound. Although still preliminary in scope, and with no data supporting the relevance of these mutations in treated patients, such efforts are laudable, and represent the probable future direction of drug development efforts aimed at targeting this important kinase.

#### Conflicts of Interest

All authors are current employees of Nerviano Medical Sciences.

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