ALK mutations conferring differential resistance to structurally diverse ALK inhibitors

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Translational Relevance

Our results show that previously described ALK resistance mutations as well as newly characterized mutations show a differential pattern of sensitivity to the ALK kinase inhibitors PF02341066 (currently in the clinic) and TAE684. Some mutations induce resistance to both kinase inhibitors, others only to one of the two inhibitors used in this study. Thus, the individual resistance mutation should influence the use of the appropriate ALK inhibitor to enhance tumor control and patient benefit in lung cancer, neuroblastoma and potentially other cancers with ALK aberrations.
Abstract

Purpose:

EML4-ALK fusions define a subset of lung cancers that can be effectively treated with ALK kinase inhibitors. Unfortunately, the duration of response is heterogeneous and acquired resistance limits their ultimate efficacy. Thus, a better understanding of resistance mechanisms will help to enhance tumor control in EML4-ALK positive tumors.

Experimental Design:

By applying orthogonal functional mutagenesis screening approaches we screened for mutations inducing resistance to the aminopyridine PF02341066 (crizotinib) and/or the diamino-pyrimidine TAE684.

Results:

Here, we show that the resistance mutation, L1196M, as well as other crizotinib resistance mutations (F1174L, G1269S), are highly sensitive to the structurally unrelated ALK kinase inhibitor TAE684. In addition, we identified two novel EML4-ALK resistance mutations (L1198P and D1203N) which unlike previously reported mutations induced resistance to both ALK kinase inhibitors. An independent resistance screen in ALK-mutant neuroblastoma cells yielded the same L1198P resistance mutation but defined two additional mutations conferring resistance to TAE684, but not to PF02341066.

Conclusions:

Our results demonstrate that different ALK resistance mutations as well as different ALK inhibitors impact the therapeutic efficacy in the setting of EML4-ALK fusions and ALK mutations.
Introduction

Lung cancers bearing EGFR mutations or EML4-ALK fusions can be effectively treated with EGFR and ALK inhibitors, respectively (1-2). Different oncogenic EML4-ALK fusion variants have been described, which induce ALK dependency (3-5). All of these variants contain the complete kinase domain of ALK, which is predominantly fused to the N-terminus of EML4 leading to constitutive activation of the kinase (6-7). In addition, activating ALK mutations have been described in neuroblastoma. These mutations are assumed to be driver mutations and may be amenable to therapeutic ALK inhibition (8-12). Unfortunately, all patients treated with targeted therapeutics will eventually relapse, in most cases due to the emergence of acquired genetic alterations conferring resistance (13-14). The knowledge about the actual resistance mechanism is, however, a prerequisite for development of secondary treatment strategies that can overcome resistance (15-16). The aminopyridine ALK kinase inhibitor, PF02341066 (crizotinib), is currently undergoing evaluation in Phase-III clinical trials (1). Until now, only two resistance mutations within the ALK kinase domain have been identified in a single EML4-ALK positive lung cancer patient with acquired crizotinib resistance as well as in a cell culture model, and another mutation was found in a patient with an inflammatory myofibroblastic tumor harboring a RANBP2-ALK translocation (13, 17-18). Structural modeling however suggests that diamino-pyrimidine scaffolds, such as TAE684 (19), should still be able to bind to the mutated kinase. We therefore tested the known PF02341066 resistance mutations for sensitivity to TAE684 and conducted orthogonal mutagenesis screens to identify novel PF02341066 and/or TAE684 resistance mutations that show differential sensitivity patterns to these ALK kinase inhibitors. This information may provide a mechanistic rationale for the development of second-generation ALK inhibitors.

Materials and Methods

cDNA and cell lines

pMA-3FLAG-EML4-ALK v1 plasmid was cloned into the retroviral pBabe puro backbone. pDONR-EML4-ALK v3a was cloned into the pBabe Gateway puro backbone. Full-length human wild-type ALK and ALKF1174L cDNAs were kindly provided by Rogier Versteeg (AMC, Amsterdam, Netherlands). Site-directed mutagenesis was performed as described
previously (20). Ba/F3 cell lines were established as described previously (20). SH-SY5Y neuroblastoma cells were cultured in DMEM supplemented with 8% serum, cells were validated by sequencing of ALK (data not shown). H3122 were cultured as described previously (21). Human cell lines have been tested by SNP-based genotyping.

**Compounds**

PF02341066 (racemic mixture) was purchased from Selleck Chemicals, TAE684 was purchased from AxonMedchem.

**Immunoblotting**

Immunoblotting was performed using standard procedures (22). The following antibodies were used: p-ERK, ERK1 and ERK2 from Santa Cruz Biotechnology. Antibodies against pALK Tyr1604, pALK Tyr1278/1282/1283 (23), p-AKT Ser473 and total AKT from Cell Signaling, Actin from MP Biomedical and total ALK from Cell Signaling and Bethyl Laboratories.

**Mutagenesis screens**

Saturation mutagenesis (24-25) was performed by propagation of EML4-ALK cDNA containing plasmids in the mismatch-repair-deficient E.coli strain XL1-Red (Stratagene). Bacteria were grown for 48 or 72 hours. The resulting plasmids were expanded in XL1-Blue bacteria and packaged in retroviruses followed by infection of Ba/F3 cells and subsequent selection of stable cell lines in the absence of IL-3 and presence of compound (750nM/1000nM/1500nM of PF02341066) to only allow proliferation of resistant clones. Mutant inserts were recovered from drug-resistant polyclonal lines, pooled and sequenced on a GS Flex instrument. The raw data was aligned and visualized by IGV. In an orthogonal chemical mutagenesis screen, EML4-ALK-expressing Ba/F3 cells were treated with N-ethyl-N-nitrosourea (ENU; 100μg/ml) over night and subsequently cultured in the presence of ALK inhibitor (750nM/1000nM/1500nM of PF02341066) to select for resistant clones. Inserts were PCR-amplified and sequenced as described above. For differentiation of the clonal origin of the two mutations found, dideoxy-sequencing was performed for each polyclonal resistant clone separately. For the PCR-based random mutagenesis screen, a NotI fragment containing the ALKF1174L open reading frame was cloned into pMX-IRES-blasticidin. To generate an ALKF1174L cDNA
library with random mutations restricted to the kinase domain, the $\text{ALK}^{F1174L}$ kinase domain was amplified by an error prone PCR using the following primer pair: $\text{ALK}^{\text{Kin-fwd}}$ GGCATCATGATTGTACCG, $\text{ALK}^{\text{Kin-rev}}$ TCTTTTTGTTGGTTTCTCTG. The PCR products were digested with the restriction enzymes BpI and BsiWI and cloned into the pMX-ALK backbone. To achieve a sufficient representation of random mutations of $\text{ALK}^{F1174L}$, we pooled $\sim 4 \times 10^5$ bacterial clones and isolated the pooled plasmid DNA. SH-SY5Y cells stably infected with the $\text{ALK}^{F1174L}$ mutant library were seeded at low density and incubated with TAE684 (100nM). Resistant colonies were pooled and genomic DNA was isolated using DNAzol (Invitrogen). The ALK kinase domain was recovered from the genomic DNA of the pooled TAE684 resistant colonies by PCR using the primers indicated above. The PCR products were cloned into the pMX-ALK backbone and sequenced. To identify recurring mutations, we sequenced 82 bacterial clones covering the entire region that was targeted for random mutagenesis.

**Colony formation assay**
For colony formation assays, SH-SY5Y cells were seeded at low density and treated with the various concentrations of the ALK inhibitors or left untreated. At reaching confluence, the cells were fixed with formaldehyde, stained with crystal violet and photographed.

**Viability assays**
Ba/F3 viability assays were performed as described previously (26) measuring cellular ATP content (Cell- Titer-Glo; Promega) after 96h of treatment.
SH-SY5Y cells were seeded at high density (4x10^3 per 384-well) and ALK inhibitors were added 24h after seeding. After 5 days, cell viability was determined with cell titer blue (Promega) according to the manufactures recommendations. Cell titer blue signals were detected with the EnVision Multilabel Reader (PerkinElmer).

$\text{EML4-ALK}$ expressing cells were treated for ALK kinase inhibitors for the indicated duration before staining with trypan blue and counting of negative (viable) and positive (dead) cells.

**Structural modeling**
The modeling of resistance mutations into ALK (PDB-codes: 2XP2 (crizotinib) and 2XB7 (TAE684) was performed using PyMol (Schroedinger).
Results

The L1196M mutation has recently been reported to confer acquired resistance in an EML4-ALK positive patient treated with the ALK inhibitor Crizotinib (13). This mutation was also found in an experimental model of acquired resistance involving the EML4-ALK mutant lung cancer cell line H3122 (18). We first expressed the predominant crizotinib resistance mutations EML4-ALK1196M and EML4-ALKF1174L (13, 17-18) in the EML4-ALK positive NSCLC cell line H3122 (Supplementary Figure 1A) and determined the sensitivity of the resulting mutants to the structurally unrelated ALK kinase inhibitor TAE684. As expected, both mutations induced resistance to PF02341066, whereas EML4-ALKF1174L was sensitive to higher PF02341066 concentrations. However, confirming previous reports (18), the L1196M mutant retained high sensitivity to TAE684. Furthermore, H3122EML4-ALK F1174L cells were also found to be exceptionally sensitive to this structurally different compound (Supplementary Figure 1B). To validate these findings in an independent model, we expressed these mutations in Ba/F3 cells and treated them with both ALK inhibitors. Furthermore, we introduced a mutation at position G1269 (G1269S) adjacent to the DFG-motif in EML4-ALK, that we predicted to induce resistance to PF02341066 but not to TAE684 due to steric hindrance (Supplementary Figure 2). Of the resulting Ba/F3 mutants, EML4-ALKF1174L (17) and EML4-ALKG1269S showed an increase in ALK phosphorylation whereas the phosphorylation levels of EML4-ALK1196M (13) were comparable to those of the wild type kinase (Figure 1A). Confirming previous reports (13, 17-18) all mutations induced resistance to PF02341066, with F1174L leading to a slight increase in resistance and L1196M and G1269S leading to a high level of resistance (Figure 1B). Confirming and extending our results in the H3122 cells, the three mutants were highly sensitive to TAE684 with the two activating mutants EML4-ALKF1174L and EML4-ALKG1269S again being particularly sensitive to treatment with TAE684 compared to the original EML4-ALK variant (Figure 1C). Accordingly, phosphorylation of mutated EML4-ALK remained unchanged after treatment with up to 2.5μM of PF02341066 but disappeared under treatment with TAE684 at concentrations as low as 30nM in all EML4-ALK variants (Figure 1D).

Structural modeling of G1269S suggests a steric clash with PF02341066 but to a much lesser degree with TAE684 as the underlying mechanism of resistance (Supplementary Figure 2). Furthermore, the activating nature of this mutation should – by itself – reduce binding of PF02341066 because this compound binds the inactive
conformation of the kinase. We speculate that resistance to TAE684 might be induced by a larger amino acid than Ser at this position (Supplementary Figure 2). A similar mechanism of steric hindrance might be induced by L1196M, preventing the binding of PF02341066 (13) but allowing the binding of TAE684 (Figure 2A/B). The differential resistance for EML4-ALKF1174L has been discussed previously (17). Thus, resistance mediated by L1196M, F1174L and G1269S may be overcome by ALK inhibitors that circumvent interference with the side chains of certain amino acids of the ALK kinase and are also able to bind the active kinase confirmation (Figure 2A/B) (27).

In order to discover additional mutations conferring ALK inhibitor resistance, we conducted a saturation mutagenesis screen (24-25) to express randomly mutated versions of EML4-ALK in Ba/F3 cells and selected for cells that were resistant to ALK inhibition (Supplementary Figure 3A). This approach yielded two novel resistance mutations (L1198P, 49% and D1203N, 12%) at high prevalence (Figure 2A/B, Supplementary Figure 4). The L1198P mutation induced a similar degree of resistance to the ALK inhibitors PF02341066 (crizotinib) (GI50, 3396nM) and TAE684 (GI50, 624nM) (Figure 3A/B). Counting of viable cells following treatment confirmed this observation (Figure 3C). Resistance was reflected by an increase in basal kinase activity (Supplementary Figure 5) as well as sustained phosphorylation of ALK at concentrations up to 2.5μM of PF02341066 and 300nM of TAE684 in immunoblotting assays (Figure 3D). The second mutation, D1203N, induced resistance to PF02341066 (GI50, 3357nM) and to TAE684 (GI50, 604nM) (Figure 3A/B). This mutation led to a shift towards higher compound concentrations needed for inhibitor-mediated dephosphorylation as well, although this effect was less pronounced compared to the L1198P mutation and no basal kinase activation was observed (Supplementary Figure 6A/B).

As an orthogonal approach, we mutagenized Ba/F3 cells expressing EML4-ALK v3a using the chemical mutagen N-ethyl-N-nitrosourea (28) and selected for resistance mutations by subsequent culture in PF02341066 (Supplementary Figure 3B). Most of the resulting resistant polyclonal cell lines expressed the mutation L1198P and were highly resistant to ALK inhibition (Supplementary Figure 7).

We also performed a PCR-based random mutagenesis screen on the ALKF1174L kinase domain, followed by ectopic expression of mutated ALK in the ALKF1174L-mutant neuroblastoma cell line SH-SY5Y (Supplementary Figure 8A). The F1174L mutation
increases the basal activation of ALK (Figure 1A), thereby reducing the binding of 
PF02341066 which tends to bind to the inactive conformation of ALK (29). Nevertheless, 
SH-SY5Y cells are still responsive to higher concentrations of PF02341066, consistent 
with our previous findings in EML4-ALKF1174L (Figure 1B). By contrast, SH-SY5Y cells are 
highly sensitive to TAE684 (30). To screen for resistance, SH-SY5Y cells stably expressing 
mutagenized ALK were treated with TAE684 and surviving clones were analyzed by PCR-
based cloning and sequencing (Supplementary Figure 8B). We identified ~2 mutations 
per clone with G1123S, G1123D and L1198P as single mutations, and Y1278H mutations 
only in the context of G1123S or G1123D (data not shown). We next expressed ALKF1174L, 
ALKF1174L/L1198P, ALKF1174L/G1123S and ALKF1174L/G1123D in the original SH-SY5Y cell line. All 
three mutations induced a high level of resistance to TAE684 in colony formation assays 
(Figure 4A left) and short-term viability assays revealed an intermediate level of 
resistance to TAE684 in those cells expressing G1123S or G1123D, whereas the 
expression of L1198P induced strong resistance (Figure 4B left). Immunoblotting 
showed that all three mutations prevented inhibition of ERK and AKT by TAE684 (Figure 
5A). Interestingly, only the L1198P mutation, but not G1123S/D, induced cross-resistance 
to PF02341066, as shown by colony formation (Figure 4A right), viability assays (Figure 
4B right) and immunoblotting (Figure 5B).

We next applied structural modeling to identify the mechanism of resistance 
induced by the L1198P mutation that was the most prominent resistance mutation across 
our saturation mutagenesis screens. Most in-cis resistance mechanisms show direct 
interactions with the inhibitor or shift the kinase equilibrium towards a more active 
conformation (14). The L1198P mutation is localized in the hinge region of the kinase 
domain at a position where both inhibitors form key hydrogen bonds to the backbone of 
the protein to mimic ATP binding (Figure 2A/B). The L1198P mutation is located next to 
E1197, whose side chain forms hydrogen bonds to K1267 and R1181 (Supplementary 
Figure 9). Such a polar interaction was recently described as a “molecular brake” to keep 
the kinase domain in an inactive state and proposed to be a common regulatory 
mechanism in receptor tyrosine kinases (31). Although P1198 is unlikely to directly 
participate in this inhibitory network, we speculate that it perturbs its function by 
restricting backbone conformations of neighboring amino acids (32), thereby shifting the 
kinase towards an active conformation. This notion is compatible with our observation of 
increased basal EML4-ALK phosphorylation in this mutant (Supplementary Figure 5)
and may explain resistance to PF02341066, an inhibitor that is known to bind to and stabilize inactive kinase conformations (29). ALK crystal structures suggest the methoxy group of TAE684 to bind in a small cavity of the hinge region (19, 23). Our modeling studies show that the L1198P mutation decreases the space occupied by this amino acid and results in suboptimal TAE684 binding. In general, L1198P seems to prevent ALK inhibition by all ATP-competitive analogs with different scaffolds.

By contrast, the D1203N mutation is in close proximity to both compounds at the lip of the ATP pocket (Figure 2A/B) but the charged side chain points away from the inhibitor cores towards the solvent. Due to the fact that the mutation did not lead to increased basal kinase phosphorylation, the underlying resistance mechanism remains unclear at this point.

The two mutations that were only found in the neuroblastoma resistance screen (G1123S/D) are located in the glycine-rich loop which is known to be crucial for ATP and ligand binding (33) and are the first mutations described that induce resistance to TAE684, but not to PF02341066. While PF02341066 does not directly interact with the glycine-rich loop, the sulfonated aniline moiety of TAE684 makes hydrophobic interactions to the Gly1123-His1124 segment (23). Our modeling studies indicate that mutations in this part of the protein are a) likely to sterically impede ATP binding and/or b) alter the dynamics of the glycine-rich loop and thus perturb interactions with inhibitors which require a particular conformation of the loop for binding (Figure 2A/B).

Discussion

Here, we show that the recently described PF02341066 resistance mutants, L1196M and F1174L, retain exquisite sensitivity to a structurally different ALK inhibitor, TAE684. Thus, compounds developed based on these structural considerations may have the potential to overcome resistance to PF02341066 when caused by these mutations.

By using complementary mutagenesis approaches, we have furthermore identified novel resistance mutations in EML4-ALK and ALK that induce a high level of resistance to both structurally unrelated ALK inhibitors as well as to one of the two inhibitors only. We have also provided mechanistic evidence for these observations, which are based on structural models of compound binding to the kinase and on biochemical analyses of kinase activity. Therefore, we predict that some patients with acquired in cis crizotinib
resistance mutations will respond to diamino-pyrimidine based ALK inhibitors. Others, depending on the respective resistance mutation, will not. Thus, further development is required to develop compounds that are capable of overcoming resistance mediated by these novel mutations. Furthermore, the individual resistance mutation may dictate the use of the appropriate ALK inhibitor.

In summary, we have shown that structurally diverse ALK inhibitors can elicit strikingly different cytotoxic potency in genotypically defined EML4-ALK and ALK mutants. This observation highlights that the development and application of ALK inhibitors should take into account individual resistance mutations to enhance tumor control and patient benefit in lung cancer, neuroblastoma and potentially other cancers with ALK aberrations.

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References

Legends to figures

Figure 1 PF02341066 resistance mutations show differential kinase activity and sensitivity to structurally diverse ALK inhibitors.
A Cellular extracts of Ba/F3 cells expressing the indicated EML4-ALK cDNAs were prepared and analyzed for pALK, ALK and Actin protein levels by immunoblotting.
B EML4-ALK expressing Ba/F3 cells were treated with PF02341066 with the indicated concentrations. After 48h of treatment, trypan blue negative (viable) and positive (dead) cells were counted in triplicate.
C EML4-ALK expressing Ba/F3 cells were treated with TAE684 with the indicated concentrations. After 48h of treatment, trypan blue negative (viable) and positive (dead) cells were counted in triplicate.
D Ba/F3 cells expressing the indicated ALK mutations were treated with PF02341066 or TAE684. Levels of ALK phosphorylation were determined by immunoblotting after 6h of ALK inhibitor treatment.

Figure 2 ALK resistance mutations that confer resistance PF02341066 and/or TAE684
A Crystal structures showing PF02341066 bound to ALK. Amino acids that confer resistance to ALK inhibitors if mutated (G1123S, F1174L, L1196M, L1198P, D1203N, G1269S) are indicated.
B Crystal structures showing TAE684 bound to ALK. Amino acids that confer resistance to ALK inhibitors if mutated (G1123S, F1174L, L1196M, L1198P, D1203N, G1269S) are indicated.

Figure 3 Novel ALK resistance mutations confer resistance PF02341066 and TAE684
A Polyclonal Ba/F3 cultures resistant to PF02341066 were treated with increasing concentrations of PF02341066. Viability was determined after 96 hours by measurements of cellular ATP content and expressed as a function of compound dose relative to DMSO-treated controls.
B Polyclonal Ba/F3 cultures resistant to PF02341066 were treated with increasing concentrations of TAE684. Viability was determined and depicted as in A.
C Ba/F3 cells stably expressing EML4-ALK<sup>wt</sup> or EML4-ALK<sup>L1198P</sup> were treated with 1μM of PF02341066 or 500nM of TAE684. Cells were stained with trypan blue and the fraction of viable cells was counted after 48h of treatment.

D Whole cell lysates of Ba/F3 stably expressing EML4-ALK<sup>wt</sup> or EML4-ALK<sup>L1198P</sup> were treated with different concentrations of ALK inhibitors. Levels of ALK phosphorylation were monitored by immunoblotting. Actin was used as loading control.

**Figure 4** G1123S/D and L1198P mutations induce distinct resistance to ALK inhibitors in ALK<sup>F1174L</sup> expressing SH-SY5Y cells.

A SH-SY5Y cells stably expressing the indicated resistance mutations were treated with the indicated doses of TAE684 (left) or PF02341066 (right). After two weeks of treatment dishes with cells were stained with crystal violet and photographed.

B SH-SY5Y cells expressing the indicated mutations were treated with TAE684 (left) or PF02341066 (right). Viability was determined by resazurin to resoruvin conversion after five days of treatment. Viability is shown as a function of compound dose and expressed as values relative to untreated controls.

**Figure 5** G1123S/D and L1198P mutations induce a distinct resistance phenotype to ALK inhibitors.

A SH-SY5Y cells expressing the indicated mutations were treated with TAE684 for 8 hours or left untreated. Whole cell lysates were analyzed for levels of ALK, pERK, ERK, pAKT and AKT by immunoblotting.

B SH-SY5Y cells expressing the indicated mutations were treated with PF02341066 for 8 hours or left untreated. Whole cell lysates were analyzed for levels of ALK, pERK, ERK, pAKT and AKT by immunoblotting.
Figure 5
# ALK mutations conferring differential resistance to structurally diverse ALK inhibitors

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