**Differential Protein Stability and ALK Inhibitor Sensitivity of EML4-ALK Fusion Variants**

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**Abstract**

**Purpose:** ALK rearrangement–positive lung cancers can be effectively treated with ALK inhibitors. However, the magnitude and duration of response is heterogeneous. In addition, acquired resistance limits the efficacy of ALK inhibitors, with most upfront resistance mechanisms being unknown.

**Experimental Design:** By making use of the Ba/F3 cell line model, we analyzed the cytotoxic efficacy of ALK kinase inhibitors as a function of different EML4-ALK fusion variants v1, v2, v3a, and v3b as well as of three artificially designed EML4-ALK deletion constructs and the ALK fusion genes KIF5b-ALK and NPM1-ALK. In addition, the intracellular localization, the sensitivity to HSP90 inhibition and the protein stability of ALK fusion proteins were studied.

**Results:** Different ALK fusion genes and EML4-ALK variants exhibited differential sensitivity to the structurally diverse ALK kinase inhibitors crizotinib and TAE684. In addition, differential sensitivity correlated with differences in protein stability in EML4-ALK–expressing cells. Furthermore, the sensitivity to HSP90 inhibition also varied depending on the ALK fusion partner but differed from ALK inhibitor sensitivity patterns. Finally, combining inhibitors of ALK and HSP90 resulted in synergistic cytotoxicity.

**Conclusions:** Our results might explain some of the heterogeneous responses of ALK-positive tumors to ALK kinase inhibition observed in the clinic. Thus, targeted therapy of ALK-positive lung cancer should take into account the precise ALK genotype. Furthermore, combining ALK and HSP90 inhibitors might enhance tumor shrinkage in EML4-ALK–driven tumors. *Clin Cancer Res; 18(17); 1–9. ©2012 AACR.*

**Introduction**

ALK gene fusions occur in 2% to 7% of lung adenocarcinomas (1, 2). These fusions are oncogenic in vitro and in vivo and cause oncogene dependency by constitutive kinase activation of the ALK tyrosine kinase (1, 3). All preclinical models show that ALK fusion–positive tumor cells are exquisitely sensitive to ALK kinase inhibition (1, 4, 5). Furthermore, a phase I/II study in advanced, ALK–positive, non–small cell lung cancer showed dramatic radiographic responses using the ALK tyrosine kinase inhibitor crizotinib (PFO2341066; ref. 2). However, more recent studies suggest more heterogeneous responses to this inhibitor (6).

ALK was first discovered in a gene fusion with nucleophosmin (NPM1) in anaplastic large-cell lymphoma, giving this gene its name (anaplastic lymphoma kinase; ref. 7). The predominant 5′-partner in ALK fusion–positive lung cancer involves echinoderm microtubule–associated protein like-4 (EML4), with few reported cases harboring an ALK fusion with kinesin family member 5B (KIF5b; ref. 8). Several different EML4-ALK variants have been described, with variant 1 (v1, 33%), variant 1 (v2, 10%), and variants 3 a/b (v3a/v3b, 29%) being the most frequent fusions (9, 10). All variants contain exons 20–29 of ALK (which encode the entire ALK tyrosine kinase domain) fused to a varying proportion of EML4 (3). EML4s are believed to represent a class of microtubule destabilizers, even though their exact function remains unknown (11). The proportion of EML4 fused to the kinase domain of ALK varies depending on the respective fusion variant, whereas the coiled–coiled domain of EML4 seems to be essential for homodimerization and kinase activation (1).

Importantly, all EML4-ALK fusion variants studied so far are oncogenic and all induce ALK dependency (3, 5).

Despite the initial success of ALK inhibitors in ALK fusion–positive lung cancer, it is presently unclear whether...
Translational Relevance
Lung cancers with ALK rearrangements are sensitive to ALK inhibitors but responses are heterogeneous. Here, we show that EML4-ALK variants show differential sensitivity to ALK and HSP90 inhibitors. These phenotypes were independent of inhibitor scaffold and binding mode. Furthermore, a combination of ALK and HSP90 inhibitors led to synergistic cytotoxicity in ALK fusion gene-expressing cells. Thus, the application of ALK and HSP90 inhibitors might have to be adapted to the precise variant of ALK fusion to enhance tumor control and optimize patient benefit in lung cancer.

the different variants impact the therapeutic efficacy of ALK inhibition (10). In light of the differential sensitivity of various EGF receptor (EGFR) mutants to EGFR inhibition (12–14), we sought to determine the responsiveness of various EML4-ALK fusion variants to structurally diverse ALK inhibitors. Furthermore, we aimed at providing mechanistic insight into any potential differences in inhibitor sensitivity.

Materials and Methods

cDNA and cell lines
pMA-3FLAG-EML4-ALK v1/v2/v3b, BCR-ABL, NPM1-ALK, and KIF5b-ALK cDNAs were cloned into the retroviral pBabe puro backbone. Site-directed mutagenesis was carried out as described previously to generate pBabe puro EML4-ALK v3a from pBabe puro EML4-ALK v3b, pBabe puro EML4-ALK del223 from pBabe puro EML4-ALK v3a, pBabe puro EML4-ALK del346, and 702 from pBabe puro EML4-ALK v2 (15). Ba/F3 and NIH3T3 cell lines were established as described previously (15, 16).

Compounds
Compounds were purchased from commercial suppliers or extracted from tablets and diluted in dimethyl sulfoxide (DMSO). Bortezomib was a kind gift from Nina Reina, Fingerle-Rowson Lab, Klinik I für Innere Medizin, Uniklinik Köln, Germany.

Immunoblotting
Immunoblotting was done using standard procedures (17). The following antibodies were used: pALK (Tyr1604), pAKT (Ser473), AKT, pERK, and ERK from Cell Signaling, actin from MP Biomedical, and total ALK from Bethyl Laboratories. Signal intensities were measured using ImageJ (1.42q).

Immunohistochemistry
NIH3T3 cells expressing the respective transgene were seeded on 22-mm diameter glass slides. After 24 hours of incubation, slides were fixed in 4% PFA for 15 minutes before incubation with PBS + 0.2% TritonX for 5 minutes. Primary antibody incubation was carried out in a humidified chamber for 1 hour at room temperature. Fluorescein isothiocyanate (FITC)-labeled secondary antibody plus 4', 6-diamidino-2-phenylindole (DAPI) staining was carried out for 45 minutes in a dark humidified chamber before covering slides on coverslips with SlowFade Gold (Invitrogen). Pictures were taken on an Aristoplan machine (Leica/Leitz Microsystems).

Viability assays
Ba/F3 viability assays were carried out as described previously (18) measuring cellular ATP content (Cell-Titer-Glo; Promega) after 72 hours or 96 hours of treatment.

Domain search
Protein domains were searched using SimpleModular-ArchitectureResearchTool (http://smart.embl-heidelberg.de/)

Results
To test whether different EML4-ALK fusion variants exhibit differential sensitivity to ALK inhibition in a defined cellular background, we made use of the Ba/F3 cell line model (15). All 4 different EML4-ALK variants studied (v1, v2, v3a, and v3b) rendered Ba/F3 cells independent of addition of exogenous interleukin 3 (IL3) and showed similar proliferation rates (Supplementary Fig. S1). EML4-ALK variants v1 and v3b showed no significant differences in the half-maximal growth inhibitory concentration (\( \text{GI}_{50} \) values) when treated with the aminopyridine ALK inhibitor crizotinib (PF02341066; \( \text{GI}_{50} \) values 470 nmol/L, Fig. 1A). Surprisingly, Ba/F3 cells expressing v2 and v3a showed considerable differences in sensitivity to ALK kinase inhibition (\( \text{GI}_{50} \) 150 and 1,000 nmol/L, respectively; Fig. 1A). The same pattern of sensitivity was observed in cells treated with the structurally different diaminopyrimidine ALK kinase inhibitor TAE-684 with \( \text{GI}_{50} \) values ranging from 0.3 nmol/L for variant 2 to 24 nmol/L for variant 3a (Fig. 1A), indicating that the differences in sensitivity were independent of the binding mode of the inhibitor (19). To determine whether the accessibility or the binding affinity of the inhibitors to the ATP-binding pocket differs in these variants, we carried out immunoblotting to monitor ALK phosphorylation of variant 2 and variant 3a after 1 hour of ALK kinase inhibitor treatment. As has been shown for another ALK kinase inhibitor, both variants, expressed in Ba/F3 cells, showed the same pattern of ALK phosphorylation with kinase inhibition at crizotinib concentrations as low as 300 nmol/L (Fig. 1B; ref. 20). To verify these results in a different cell type, variants 2 and 3a were expressed in NIH3T3 cells. After 1 hour of treatment, variant 2 and variant 3a again showed no differences in ALK phosphorylation after crizotinib treatment (Fig. 1C, Supplementary Fig. S2A). As has already been shown for EML4-ALK–expressing NIH3T3 cells, in both variants ALK kinase inhibition led to a dose-dependent dephosphorylation of ERK, whereas levels of phosphorylated AKT remained almost unchanged after treatment (Supplementary Fig.
S2A; ref. 21). In line with these findings, a comparison of variant 1 and variant 3a yielded the same results (Supplementary Fig. S2B). These results suggested that inhibitor binding to the kinase domain is similar in all EML4-ALK variants tested.

Due to the fact that all EML4-ALK variants harbor the complete kinase domain of ALK (exon 20 to exon 29), we hypothesized that the observed differences in inhibitor sensitivity might be mediated by the proportion of EML4 that is fused to ALK (Supplementary Fig. S3). In addition to the coiled–coiled domain, which is essential for the oncogenic capacity of EML4-ALK (1), EML4 contains one HELP domain as well as 9 WD40 domains. The HELP domain of EML4 has been reported to mediate tubulin binding (11), whereas WD40 domains are known to mediate a variety of protein–protein interactions that are difficult to predict (22). To determine whether any of these domains mediate differences in intracellular localization and thereby could explain the observed phenotypes, we stained for ALK in EML4-ALK–expressing NIH3T3 cells by immunohistochemistry. EML4-ALK variants 1 and 2 were abundant in the cytoplasm, with only minimal staining in the nucleus (Fig. 2A). However, v3a was equally distributed throughout the cytoplasm and the nucleus, suggesting that the HELP domain (present in v1 and v2, but not in v3a) retains the fusion protein in the cytoplasm (Fig. 2A, Supplementary Fig. S3; ref. 11). Because EML4-ALK variant 1 and variant 2 showed no differences in intracellular distribution but showed considerable differences in kinase inhibitor sensitivity, the impact of the intracellular distribution is likely of minor relevance to ALK kinase inhibitor sensitivity.

In addition to short-term kinase inhibitor treatment, we carried out immunoblotting of ALK in whole-cell lysates of EML4-ALK–expressing NIH3T3 cells after 24 hours of treatment to analyze the effects of longer kinase inhibition. Strikingly, total ALK levels were considerably decreased in variant 2 at 1 and 3 μmol/L of crizotinib (Fig. 2B). By contrast, the total amount of variant 3a was not reduced at concentrations of 1 μmol/L of crizotinib, with reduced total protein levels appearing only at 3 μmol/L of treatment (Fig. 2B). This dose-dependent effect of total ALK degradation was also present in Ba/F3 cells and was again much more pronounced in variant 2 compared with variant 3a (Fig. 2C). High-dose treatment with TAE684 for 24 hours recapitulated this observation (Supplementary Fig. S4). To analyze overall ALK protein stability in

Figure 1. ALK inhibitor sensitivity of EML4-ALK variants. A, Ba/F3 cells expressing the indicated EML4-ALK variants were treated with increasing concentrations of crizotinib (left) or TAE684 (right). Viability was determined after 96 hours by measurements of cellular ATP content and expressed as a function of compound dose relative to the viability of the DMSO-treated controls. Each data point represents the mean of 3 independent triplicate measurements, error bars indicate SEM. B, Ba/F3 cells expressing the indicated EML4-ALK cDNAs were treated with crizotinib for 1 hour. Whole-cell lysates were prepared and analyzed for pALK, ALK, and actin protein levels by immunoblotting. C, whole-cell lysates of NIH3T3 cells stably expressing EML4-ALK v2 and v3a were treated with different concentrations of crizotinib for 1 hour. Levels of ALK phosphorylation were monitored by immunoblotting. Actin was used as loading control.

Figure 2. Intracellular distribution of EML4-ALK variants and crizotinib-induced ALK protein degradation. A, NIH3T3 cells expressing the indicated EML4-ALK variants were fixed on glass slides and stained for total ALK (FITC, green) and nuclei (DAPI, blue). Pictures were taken on an Aristoplan machine (Leica/Leitz Microsystems) at 400-fold magnification. B, NIH3T3 cells expressing the indicated EML4-ALK cDNAs were treated with increasing concentrations of crizotinib. After 24 hours of treatment, lysates were prepared and analyzed for pALK, ALK, and actin protein levels by immunoblotting. C, Ba/F3 cells stably expressing EML4-ALK v2 and v3a were treated with different concentrations of crizotinib for 24 hours. Levels of ALK phosphorylation and of total ALK were determined by immunoblotting. Actin was used as loading control.
more detail, we treated EML4-ALK–expressing Ba/F3 and NIH3T3 cells with 50 or 100 μg/mL of cycloheximide for 24 hours. Immunoblotting of ALK revealed remarkable differences in the amount of total ALK protein when comparing cycloheximide and untreated cell lysates. EML4-ALK variant 2–expressing cells showed the most pronounced differences in the amount of total ALK protein between untreated and treated cells, whereas cells expressing EML4-ALK variants 3a showed the least differences. These data showed that EML4-ALK variant 2 has a shorter half-life compared with the other variants (Fig. 3A, Supplementary Fig. S5). Thus, binding of crizotinib to the kinase domain of EML4-ALK variant 2 accelerates protein degradation at much lower concentrations compared with variant 3a. Furthermore, our data suggested that the proportion of EML4 that is fused to ALK defines the general stability of the resulting fusion protein.

Previous studies have shown that total EML4-ALK levels decrease after treatment with HSP90 inhibitors (23–25). In light of our cycloheximide studies, we asked whether the different EML4-ALK variants display differential sensitivity to HSP90 inhibition as well. We treated Ba/F3 cells expressing EML4-ALK variants 2 and 3a with increasing concentrations of the ansamycin antibiotic derivative HSP90 inhibitor, 17-DMAG. As predicted, variant 2 was more sensitive to HSP90 inhibition compared with variant 3a, which was almost as resistant as the IL3-supplemented control (Fig. 3B). Surprisingly, Ba/F3 cells expressing EML4-ALK variant 1 were as sensitive to 17-DMAG as EML4-ALK v2–expressing cells (Fig. 3C). Thus, the intrinsic stability of the fusion variants impacts their dependency on chaperonage by HSP90 but also show that other factors (e.g., intracellular distribution, Fig. 2A) may also influence HSP90 inhibitor sensitivity. Unfortunately, the high intrinsic sensitivity of all Ba/F3 cell lines to HSP90 inhibition (evidenced by the high sensitivity of IL3-supplemented Ba/F3 cells) limits the ability to document minor differences in sensitivity (i.e., v1 vs v2). Experiments with a synthetic HSP90 inhibitor, AUY922, yielded similar results; however, the high sensitivity of IL3-supplemented controls made these experiments difficult to interpret (Supplementary Fig. S6).

To test, whether the observed EML4-ALK degradation depends on proteasome activity, we treated EML4-
ALK–expressing Ba/F3 cells with the proteasome inhibitor, bortezomib. Here, v2 and v3a showed the same sensitivity. However, this effect could not be rescued by addition of IL3 and was therefore likely to be mediated by general cellular toxicity of bortezomib in these cells (Supplementary Fig. S7). To further analyze whether proteasome inhibition could rescue ALK inhibitor–induced EML4-ALK degradation, we treated EML4-ALK–expressing NIH3T3 cells with 3 μmol/L crizotinib, 100 nmol/L bortezomib, or both compounds combined for 24 hours. However, proteasome inhibition did not reduce crizotinib-induced protein degradation, implying that the degradation process is proteasome independent (Fig. 3D).

Figure 4. ALK inhibitor sensitivity and protein stability of EML4-ALK deletion variants. A, schematic representation of EML4-ALK variants and the deletion constructs EML4-ALK del346, del702, and del223. B and C, Ba/F3 cells expressing the indicated EML4-ALK deletion variants were treated with increasing concentrations of crizotinib. Viability was determined after 72 hours by measurements of cellular ATP content and expressed as a function of compound dose relative to the viability of the DMSO-treated controls. Each data point represents the mean of 3 independent triplicate measurements, error bars indicate SEM (left). Ba/F3 cells expressing the indicated EML4-ALK deletion variants were treated with DMSO or 50 μg/mL cycloheximide for 24 hours. Whole-cell lysates were prepared and stained for total ALK levels by immunoblotting (right). CHX, cycloheximide.

Because the longest variant (variant 2) was the least stable (Fig. 3A) and showed the highest degree of sensitivity to ALK inhibition (Fig. 1A), we hypothesized that variations at the N-terminal portion of EML4-ALK have the strongest impact on protein stability and thus, kinase inhibitor sensitivity. To confirm this hypothesis, we generated 3 artificial EML4-ALK deletion variants varying in the N-terminal part and by the number of WD40 domains (Fig. 4A). We used variant 2 as a template to remove amino acids 299–346 (del346, lacking the first WD40 domain adjacent to the HELP domain) or 299–702 (del702, lacking the first 5 WD40 domains), and variant 3a was used to remove amino acids 61–223 (del223) to generate an EML4-ALK variant similar to variant 5. All
deletion constructs transformed Ba/F3 cells but showed a distinct sensitivity pattern after treatment. EML4-ALK del346–expressing Ba/F3 cells were as sensitive as variant 2–expressing cells after ALK kinase inhibition (Fig. 4B). By contrast, del702–expressing cells were much less sensitive to kinase inhibition compared with cells expressing variant 2 (Fig. 4B). Furthermore, EML4-ALK del346 was the least stable protein following cycloheximide-mediated inhibition of translation, whereas EML4-ALK del702 showed almost no degradation, similar to variant 3a (Fig. 4B). Ba/F3 cells expressing EML4-ALK del223 showed no difference in crizotinib sensitivity or protein stability compared with variant 3a (Fig. 4C). As expected, EML4-ALK del223 and v3a showed a higher sensitivity to ALK inhibition compared with BCR-ABL–expressing cells, indicating that even though these variants induced the highest degree of resistance, they still exhibit sensitivity toward ALK inhibitors (Fig. 4C).

To test, whether other fusion partners and absolute protein size of the resulting fusion dictate sensitivity to ALK inhibition, we tested the sensitivity of KIF5b-ALK and NPM1-ALK–expressing Ba/F3 cells to crizotinib (Fig. 5A). Ba/F3 cells expressing KIF5b-ALK were highly sensitive to ALK inhibition, whereas NPM1-ALK–expressing cells showed an intermediate sensitivity compared with variant 2 and variant 3a of EML4-ALK (Fig. 5B). Thus, the actual fusion partner and the domain composition—rather than the size of the fusion protein—dictate sensitivity to ALK inhibition. Cycloheximide treatment revealed a similar protein stability of the KIF5b-ALK and NPM1-ALK fusion proteins, despite the differences in ALK inhibitor sensitivity. Compared with EML4-ALK v2, both ALK fusion proteins were much more stable, indicating only a correlation between ALK inhibitor sensitivity and protein stability in EML4-ALK–expressing cells (Fig. 5C).

Because sensitivity to ALK kinase inhibition did not correlate with sensitivity to HSP90 inhibition, suggesting different mechanisms (Fig. 1A, Fig. 3B and C), we asked whether a combination of ALK and HSP90 inhibitors might induce additive cytotoxic effects. To this end, we treated Ba/F3 cells expressing EML4-ALK v1, EML4-ALK v2, and v3a as well as KIF5b-ALK and BCR-ABL with increasing concentrations of crizotinib, 17-DMAG, or both compounds combined, each at the same concentrations. Interestingly, in all ALK fusion gene–expressing cells, the combination of both compounds induced synergistic cytotoxicity (Fig. 6A and B, Supplementary Table S1; ref. 26). However, we note that the degree of synergy varied depending on the respective ALK fusion variant, with only minimal synergistic cytotoxicity observed in cells expressing EML4-ALK v1. As expected, in BCR-ABL–expressing cells, only treatment with 17-DMAG–induced cytotoxicity, which could not be enhanced further by combination treatment (Fig. 6A and B).

Collectively, our data showed that variations at the N-terminal part of ALK fusion genes strongly impact protein stability, HSP90 and kinase inhibitor sensitivity.
Discussion

Here, we report differences in the sensitivity of different EML4-ALK variants to 2 structurally diverse ALK inhibitors crizotinib (aminopyridine) and TAE684 (diaminopyrimidine). These differences do not seem to be mediated by differential compound activity on the kinase. By contrast, we find that various parts of EML4 that are fused to ALK in the different variants influence overall fusion protein stability, inhibitor-induced protein degradation, and drug sensitivity. These observations are also supported by analyses of artificial variants of various lengths as well as of the fusion genes KIF5b-ALK and NPM1-ALK that recapitulate our findings. However, a correlation between protein stability and ALK inhibitor sensitivity could only be shown for EML4-ALK.

We speculate that protein-folding properties of the somatically acquired (i.e., not evolutionary developed) fusions might lead to an open conformation, leaving several hydrophobic residues exposed. These exposed residues vary depending on the respective fusion variant and are likely to recruit HSP90 and other chaperones, which are essential for a stabilization of these proteins. This stabilization can effectively be disturbed by ALK or HSP90 inhibitors, initiating a proteasome-independent degradation of the fusion proteins. As a consequence, combined treatment of an ALK and HSP90 inhibitor induced synergistic cytotoxicity in all ALK fusion gene-expressing cells. The more similar the single drug GI50 values were, the higher the synergistic effect in the combination treatment with equal drug concentrations.

It remains to be seen whether our in vitro observations translate into the clinic. The previously published patient cohort that was treated with crizotinib included only one specified patient whose tumor expressed EML4-ALK variant 2 (2). This patient showed an intermediate response of 57%. Thus, analyses of larger cohorts will be required to afford a sufficiently powered analysis of amount and/or duration of response as a factor of individual fusion variants. Further complicating such an analysis is the fact...
that ALK fusions are currently diagnosed by FISH. Importantly, such FISH assays only allow detecting the general presence of an ALK rearrangement but cannot identify which particular ALK fusion variant is present. In light of our findings here, it may be necessary to establish more specific diagnostic tests that can specifically identify the ALK fusion present. We hope that future studies will monitor patient outcomes according to specific ALK variant status, which would allow us to address this question.

In summary, we found that different EML4-ALK variants as well as other ALK fusion genes exhibit differential sensitivity to ALK inhibition. In addition, the sensitivity to HSP90 inhibition varied substantially depending on the respective ALK fusion and could be further enhanced by combined ALK/HSP90 inhibitor treatment. Such combinations might therefore be promising to test in the clinic. Furthermore, dosing of ALK and HSP90 inhibitors might need to be adapted to specific genetic variants of EML4-ALK to enhance tumor control.

Disclosure of Potential Conflicts of Interest
C.M. Lovly is a consultant and an advisory board member of Abbott. W. Pao is a recipient of commercial research grant from AstraZeneca, Enzon, Symphogen, and Xcovery. He is also a consultant and an advisory board member of MolecularMD, AstraZeneca, Bristol-Myers Squibb, Symphony Evolution, Clovis Oncology. R.K. Thomas has received commercial research grant from AstraZeneca, Merck, EOS. He is also a consultant and an advisory board member of Sanofi-Aventis, Merck, Bayer, Lilly, and Roche. Boehleringer Ingelheim, Johnson & Johnson, AstraZeneca, Atlas-Biologics. No potential conflicts of interest were disclosed by the other authors.

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