ALK Mutations Conferring Differential Resistance to Structurally Diverse ALK Inhibitors

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Abstract

Purpose—*EML4–ALK* fusions define a subset of lung cancers that can be effectively treated with anaplastic lymphoma kinase (ALK) 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 diaminopyrimidine TAE684.

Results—Here, we show that the resistance mutation, L1196M, as well as other crizotinib resistance mutations (F1174L and G1269S), are highly sensitive to the structurally unrelated ALK 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 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 show 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.

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Introduction

Lung cancers bearing epidermal growth factor receptor (*EGFR*) mutations or *EML4–ALK* fusions can be effectively treated with EGFR and anaplastic lymphoma kinase (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 echinoderm microtubule–associated protein-like 4 (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 inhibitor, PF02341066 (crizotinib), is currently undergoing evaluation in phase III clinical trials (1). Until now, only 2 resistance mutations within the *ALK* 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 diaminopyrimidine 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 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 *ALK^{F1174L}* cDNAs were kindly provided by Rogier Versteeg (Academic Medical Centre-AMC, Amsterdam, the Netherlands). Site-directed mutagenesis was carried out as described previously (20). Ba/F3 cell lines were established as described previously (20). SH-SY5Y neuroblastoma cells were cultured in Dulbecco's Modified Eagle's Medium 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 single-nucleotide polymorphism-based genotyping.

Compounds

PF02341066 (racemic mixture) was purchased from Selleck Chemicals, and TAE684 was purchased from Axon Medchem.

Immunoblotting

Immunoblotting was carried out by standard procedures (22). The following antibodies were used: phospho(p)-ERK, ERK1, andERK2 from Santa Cruz Biotechnology. Antibodies against phospho (p)-ALK Tyr1604, p-ALK Tyr1278/1282/1283 (23), phospho (p)-AKT Ser473, and total AKT were from Cell Signaling; actin from MP Biomedical, and total ALK from Cell Signaling and Bethyl Laboratories.

Mutagenesis screens

Saturation mutagenesis (24, 25) was carried out 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 interleukin 3 and presence of compound (750/1,000/1,500 nmol/L 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 were 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; 100 μg/mL) overnight and subsequently cultured in the presence of ALK inhibitor (750/1,000/1,500 nmol/L of PF02341066) to select for resistant clones. Inserts were PCR-amplified and sequenced as described earlier. For differentiation of the clonal origin of the 2 mutations found, dideoxy sequencing was carried out for each polyclonal-resistant clone separately.

For the PCR-based random mutagenesis screen, a *Not*I fragment containing the ALK^{F1174L} open reading frame was cloned into pMX-IRES-blasticidin. To generate an ALK^{F1174L} cDNA library with random mutations restricted to the kinase domain, the ALK^{F1174L} kinase domain was amplified by an error-prone PCR using the following primer pair: ALK^{Kin}-fwd GGCATCATGATTGTGTACCG and ALK^{Kin}-rev TCTTTTTGGTGGGTTTCTCTG. The PCR products were digested with the restriction enzymes *Blp*I and *Bsi*WI and cloned into the pMX–*ALK* backbone. To achieve a sufficient representation of random mutations of ALK^{F1174L}, we pooled approximately 4×10^5 bacterial clones and isolated the pooled plasmid DNA. SH-SY5Y cells stably infected with the ALK^{F1174L} mutant library were seeded at low density and incubated with TAE684 (100 nmol/L). Resistant colonies were pooled, and genomic DNA was isolated with DNAzol (Invitrogen). The ALK domain was recovered from the genomic DNA of the pooled TAE684-resistant colonies by PCR using the primers indicated earlier. 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 conducted as described previously (26) measuring cellular ATP content (CellTiter-Glo; Promega) after 96 hours of treatment.

SH-SY5Y cells were seeded at high density (4×10^3 per 384-well plate), and ALK inhibitors were added 24 hours after seeding. After 5 days, cell viability was determined with CellTiter-Blue (Promega) according to the manufacturer's recommendations. CellTiter-Blue signals were detected with the EnVision Multilabel Reader (PerkinElmer).

EML4–ALK-expressing cells were treated for ALK 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 carried out with 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–ALK^L1196M* and *EML4–ALK^F1174L* (13, 17, 18) in the *EML4–ALK*-positive non–small cell lung carcinoma (NSCLC) cell line H3122 (Supplementary Fig. S1A) and determined the sensitivity of the resulting mutants to the structurally unrelated ALK inhibitor TAE684. As expected, both mutations induced resistance to PF02341066, whereas *EML4–ALK^F1174L* was sensitive to higher PF02341066 concentrations. However, confirming previous reports (18), the L1196M mutant retained high sensitivity to TAE684. Furthermore, H3122^*EML4–ALK*F1174L cells were also found to be exceptionally sensitive to this structurally different compound (Supplementary Fig. S1B). 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 (asp-phe-gly) motif in *EML4–ALK*, which we predicted to induce resistance to PF02341066 but not to TAE684 due to steric hindrance (Supplementary Fig. S2). Of the resulting Ba/F3 mutants, *EML4–ALK^F1174L* (17) and *EML4–ALK^G1269S* showed an increase in ALK phosphorylation, whereas the phosphorylation levels of *EML4–ALK^L1196M* (13) were comparable with those of the wild-type kinase (Fig. 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 (Fig. 1B). Confirming and extending our results in the H3122 cells, the 3 mutants were highly sensitive to TAE684 with the 2 activating mutants *EML4–ALK^F1174L* and *EML4–ALK^G1269S* again being particularly sensitive to treatment with TAE684 compared with the original *EML4–ALK* variant (Fig. 1C). Accordingly, phosphorylation of mutated *EML4–ALK* remained unchanged after treatment with up to 2.5 μmol/L of PF02341066 but disappeared under treatment with TAE684 at concentrations as low as 30 nmol/L in all *EML4–ALK* variants (Fig. 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 Fig. S2). 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 Fig. S2). A similar mechanism of steric hindrance might be induced by L1196M, preventing the binding of PF02341066 (13) but allowing the binding of TAE684 (Fig. 2A and B). The differential resistance for *EML4–ALK^F1174L* 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 and are also able to bind the active kinase confirmation (Fig. 2A and B; ref. 27).

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 Fig. S3A). This approach yielded 2 novel resistance mutations (L1198P, 49% and D1203N, 12%) at high prevalence (Fig.2A and B, Supplementary Fig. S4). The

L1198P mutation induced a similar degree of resistance to the ALK inhibitors PF02341066 [crizotinib; concentration needed to reduce the growth of treated cells to half that of untreated cells (GI_{50}), 3,396 nmol/L] and TAE684 (GI_{50}, 624 nmol/L; Fig. 3A and B). Counting of viable cells following treatment confirmed this observation (Fig. 3C). Resistance was reflected by an increase in basal kinase activity (Supplementary Fig. S5) as well as sustained phosphorylation of ALK at concentrations up to 2.5 μmol/L of PF02341066 and 300 nmol/L of TAE684 in immunoblotting assays (Fig. 3D). The second mutation, D1203N, induced resistance to PF02341066 (GI_{50}, 3,357 nmol/L) and to TAE684 (GI_{50}, 604 nmol/L; Fig. 3A and B). This mutation led to a shift toward higher compound concentrations needed for inhibitor-mediated dephosphorylation as well, although this effect was less pronounced than in the L1198P mutation and no basal kinase activation was observed (Supplementary Fig. S6A and S6B).

As an orthogonal approach, we mutagenized Ba/F3 cells expressing *EML4–ALK v3a* with the chemical mutagen ENU (28) and selected for resistance mutations by subsequent culture in PF02341066 (Supplementary Fig. S3B). Most of the resulting resistant polyclonal cell lines expressed the mutation L1198P and were highly resistant to ALK inhibition (Supplementary Fig. S7).

We also carried out a PCR-based random mutagenesis screen on the ALK^{F1174L} kinase domain, followed by ectopic expression of mutated *ALK* in the ALK^{F1174L}-mutant neuroblastoma cell line SH-SY5Y (Supplementary Fig. S8A). The F1174L mutation increases the basal activation of ALK (Fig. 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 $EML4–ALK^{F1174L}$ (Fig. 1B). In 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 Fig. S8B). We identified approximately 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 ALK^{F1174L}, $ALK^{F1174L/L1198P}$, $ALK^{F1174L/G1123S}$, and $ALK^{F1174L/G1123D}$ in the original SH-SY5Y cell line. All 3 mutations induced a high level of resistance to TAE684 in colony formation assays (Fig. 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 (Fig. 4B, left). Immunoblotting showed that all 3 mutations prevented inhibition of extracellular signal–regulated kinase (ERK) and AKT by TAE684 (Fig. 5A). Interestingly, only the L1198P mutation, but not G1123S/D, induced crossresistance to PF02341066, as shown by colony formation assays (Fig. 4A, right), viability assays (Fig. 4B, right), and immunoblotting (Fig. 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 toward 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 (Fig. 2A and B). The L1198P mutation is located next to E1197, whose side chain forms hydrogen bonds to K1267 and R1181 (Supplementary Fig. S9). 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 toward an active conformation. This notion is compatible with our observation of increased basal *EML4–ALK* phosphorylation in this mutant (Supplementary Fig. S5) 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 analogues with different scaffolds.

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

The 2 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. Although 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 (i) likely to sterically impede ATP binding and/or (ii) alter the dynamics of the glycine-rich loop and thus perturb interactions with inhibitors that require a particular conformation of the loop for binding (Fig. 2A and 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 on the basis of 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 and one of the 2 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 diaminopyrimidine-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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 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.
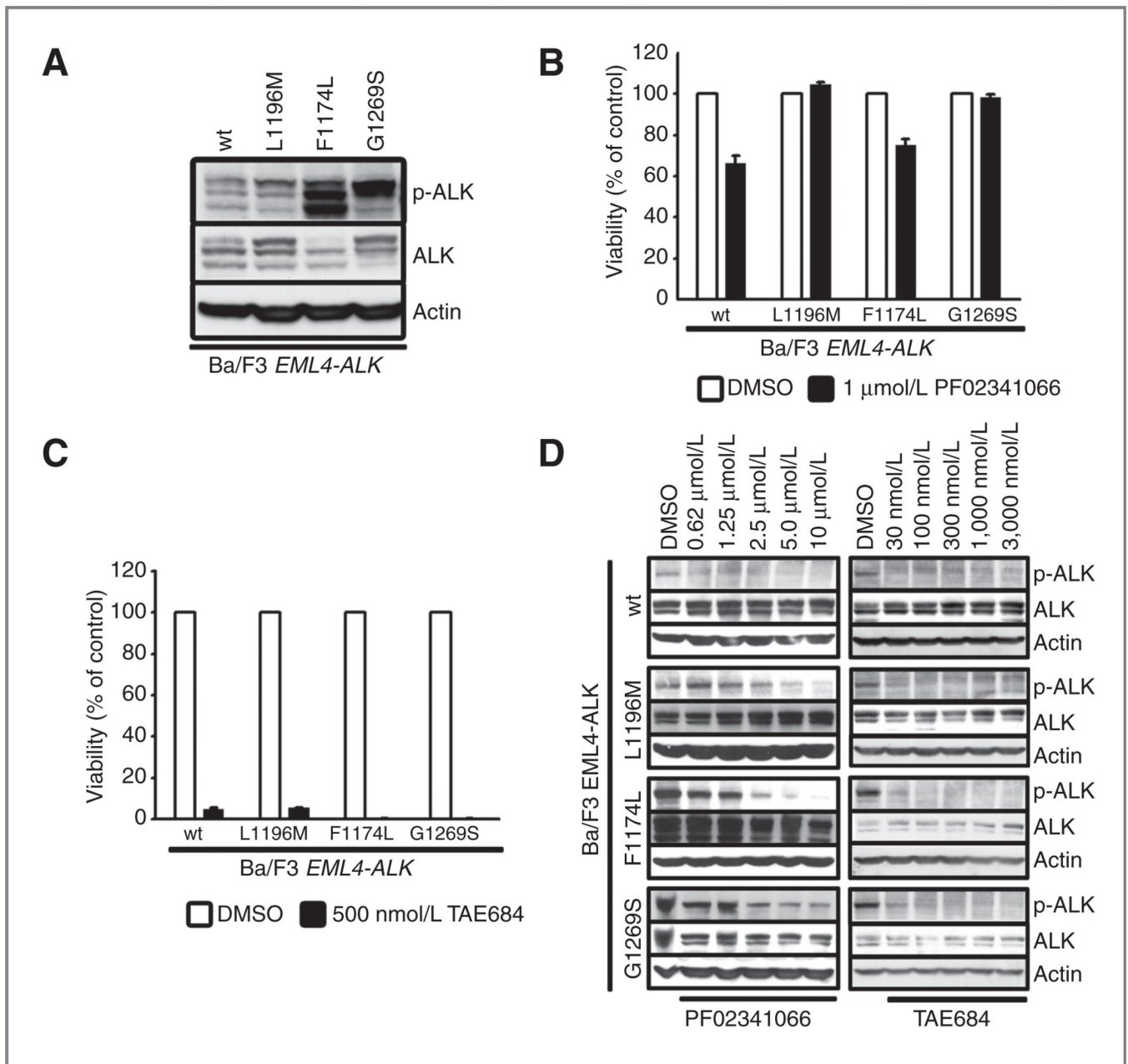
Figure 1.
PF02341066 resistance mutations show differential kinase activity and sensitivity to structurally diverse ALK inhibitors. A, cellular extracts of Ba/F3 cells expressing the indicated *EML4–ALK* cDNAs were prepared and analyzed for p-ALK, ALK, and actin protein levels by immunoblotting. B, *EML4–ALK*-expressing Ba/F3 cells were treated with PF02341066 with the indicated concentrations. After 48 hours of treatment, trypan blue–negative (viable) and trypan blue–positive (dead) cells were counted in triplicate. C, *EML4–ALK*-expressing Ba/F3 cells were treated with TAE684 with the indicated concentrations. After 48 hours of treatment, trypan blue–negative (viable) and trypan blue–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 6 hours of ALK inhibitor treatment. wt, wild-type.
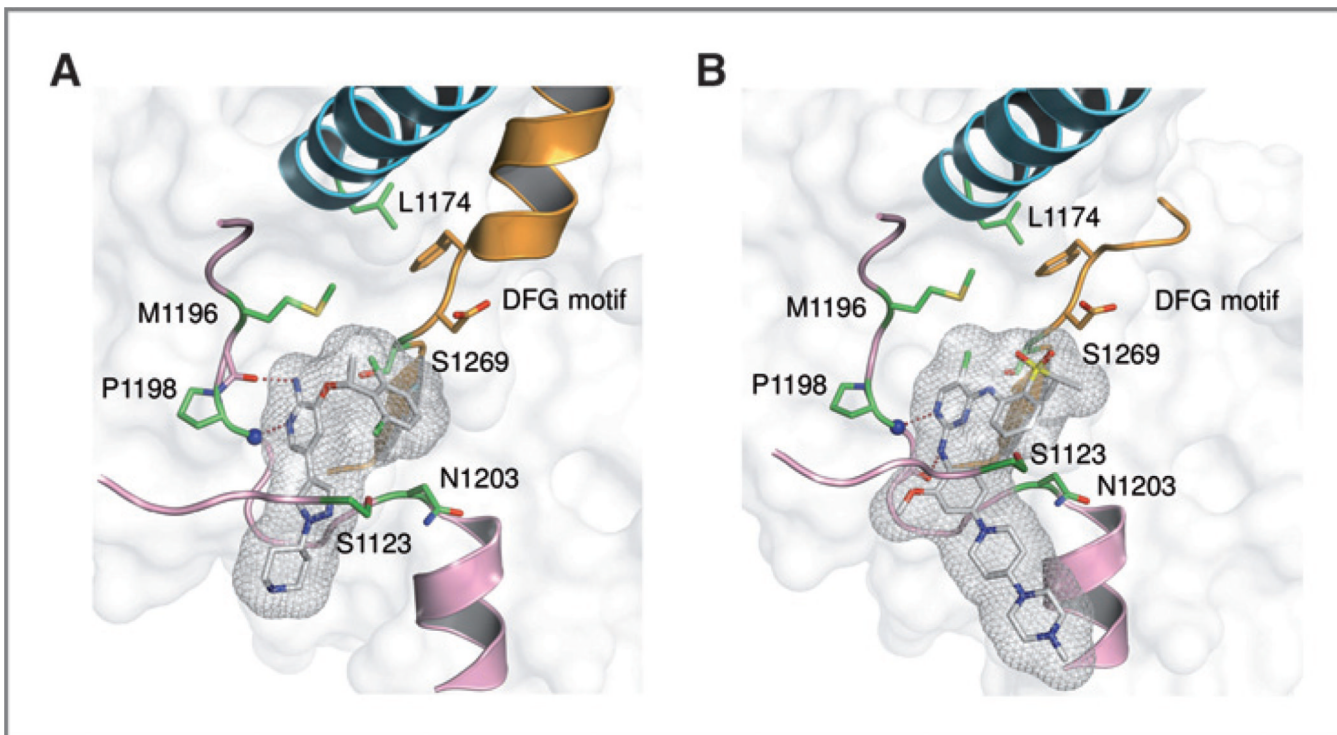
Figure 2.
ALK resistance mutations that confer resistance PF02341066 and/or TAE684. A, crystal structures showing PF02341066 bound to ALK. Amino acids that confer resistance to ALK inhibitors if mutated (G1123S, F1174L, L1196M, L1198P, D1203N, and G1269S) are indicated. B, crystal structures showing TAE684 bound to ALK. Amino acids that confer resistance to ALK inhibitors, if mutated (G1123S, F1174L, L1196M, L1198P, D1203N, and G1269S), are indicated.
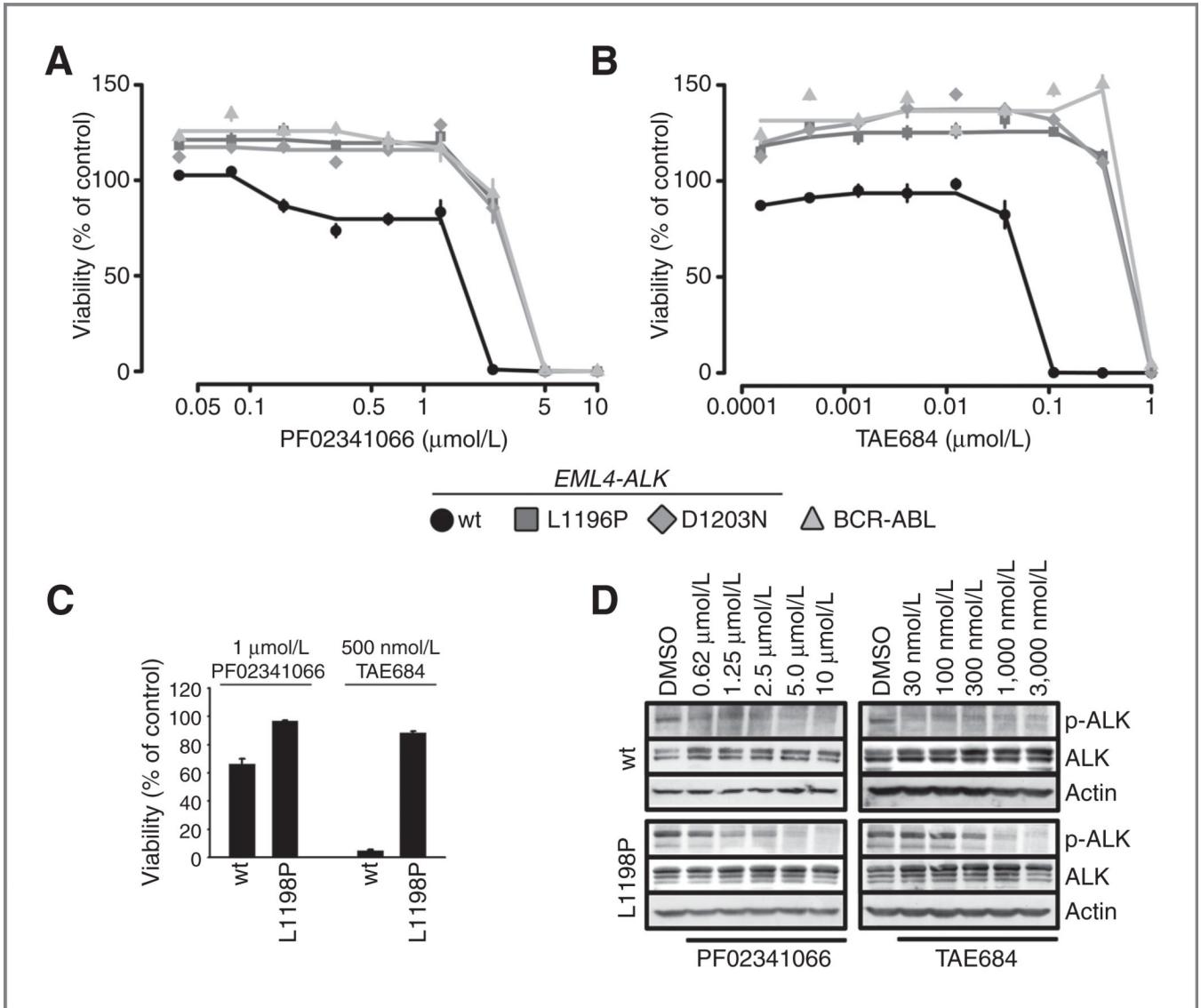
Figure 3.
Novel ALK resistance mutations confer resistance PF02341066 and TAE684. A, polyclonal Ba/F3 cultures resistant to PF02341066 were treated with increasing concentrations of PF02341066. Viability was determined after 96 hours by measurements of cellular ATP content and expressed as a function of compound dose relative to dimethyl sulfoxide (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^{wt}* or *EML4–ALK^{L1198P}* were treated with 1 μmol/L of PF02341066 or 500 nmol/L of TAE684. Cells were stained with trypan blue, and the fraction of viable cells was counted after 48 hours of treatment. D, whole-cell lysates of Ba/F3 stably expressing *EML4–ALK^{wt}* or *EML4–ALK^{L1198P}* were treated with different concentrations of ALK inhibitors. Levels of ALK phosphorylation were monitored by immunoblotting. Actin was used as loading control. wt, wild-type.
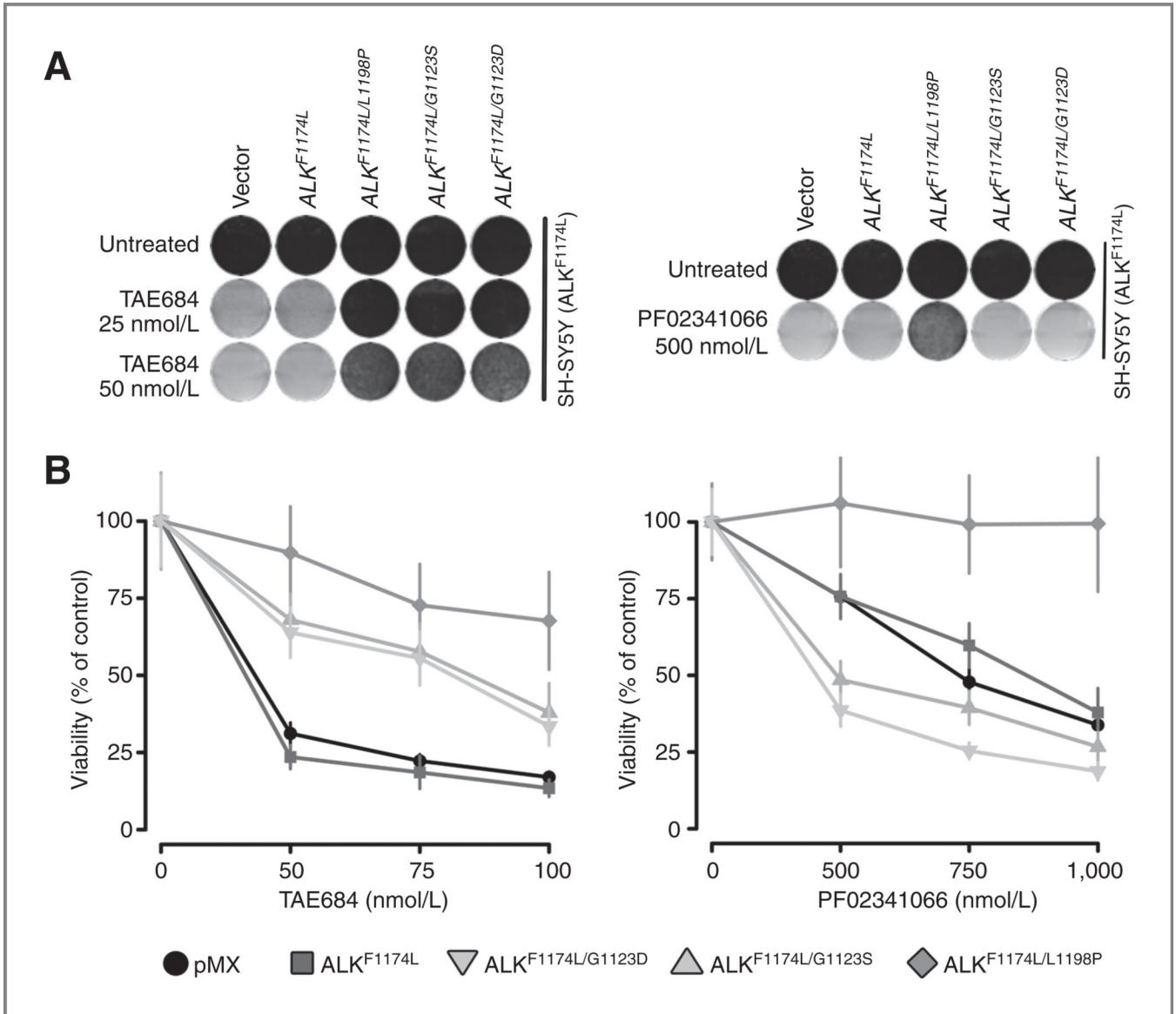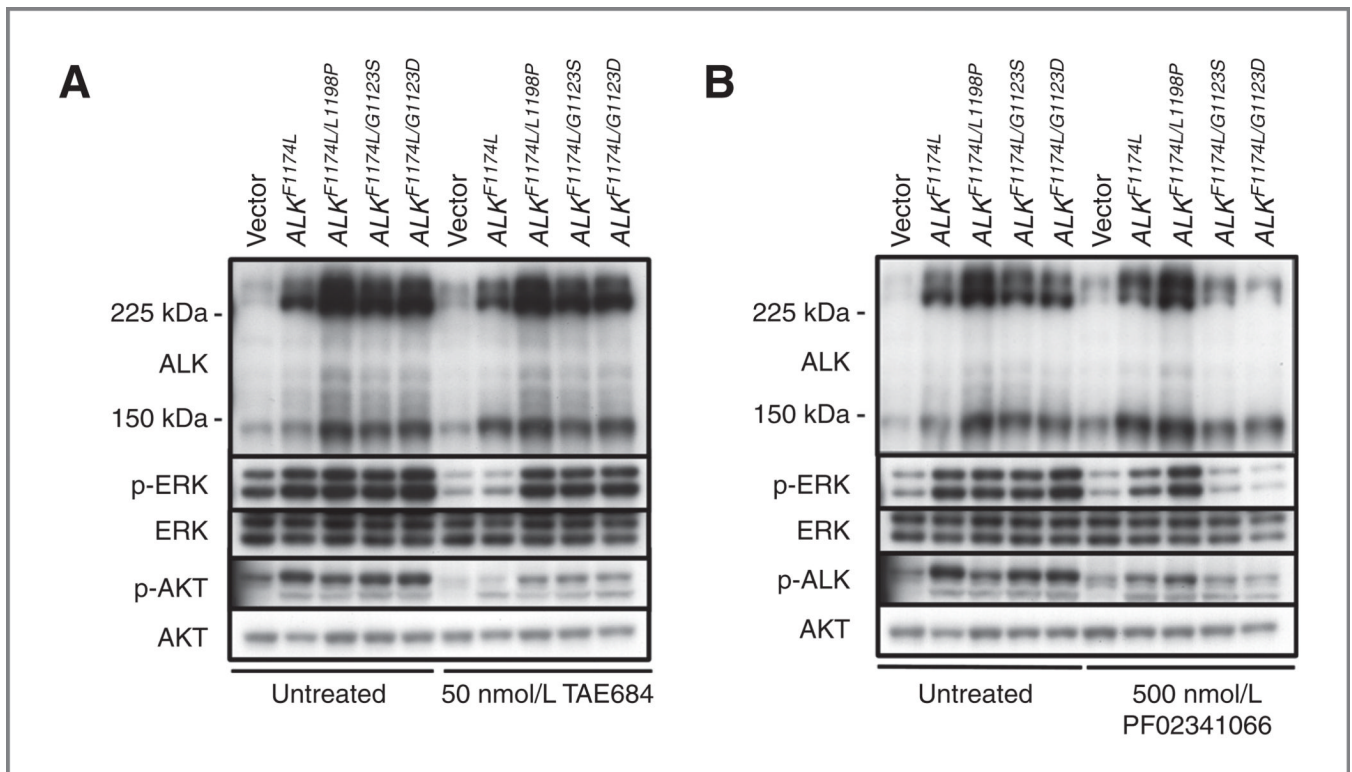
Figure 4.
G1123S/D and L1198P mutations induce distinct resistance to ALK inhibitors in *ALK^F1174L*-expressing SH-SY5Y cells. A, SH-SY5Y cells stably expressing the indicated resistance mutations were treated with the indicated doses of TAE684 (left) or PF02341066 (right). After 2 weeks of treatment, 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 resorufin conversion after 5 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, p-ERK, ERK, p-AKT,
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, p-ERK, ERK, p-AKT, and AKT by immunoblotting.