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Priority Report

Cancer Research

The Neuroblastoma-Associated F1174L ALK Mutation Causes Resistance to an ALK Kinase Inhibitor in ALK-Translocated Cancers

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Abstract

The ALK kinase inhibitor crizotinib (PF-02341066) is clinically effective in patients with ALK-translocated cancers, but its efficacy will ultimately be limited by acquired drug resistance. Here we report the identification of a secondary mutation in ALK, F1174L, as one cause of crizotinib resistance in a patient with an inflammatory myofibroblastic tumor (IMT) harboring a *RANBP2-ALK* translocation who progressed while on crizotinib therapy. When present in cis with an *ALK* translocation, this mutation (also detected in neuroblastomas) causes an increase in ALK phosphorylation, cell growth, and downstream signaling. Furthermore, the F1174L mutation inhibits crizotinib-mediated downregulation of ALK signaling and blocks apoptosis in *RANBP2-ALK* Ba/F3 cells. A chemically distinct ALK inhibitor, TAE684, and the HSP90 inhibitor 17-AAG are both effective in models harboring the F1174L ALK mutation. Our findings highlight the importance of studying drug resistance mechanisms in order to develop effective clinical treatments for patients with ALK-translocated cancers. *Cancer Res; 70(24); 10038–43. ©2010 AACR.*

Introduction

Chromosomal translocations involving the anaplastic lymphoma kinase (ALK) have been detected in several human malignancies including in anaplastic large cell lymphomas (ALCL), inflammatory myofibroblastic tumors (IMT), and non–small cell lung carcinomas (NSCLC; refs. 1–3). ALK translocated fusion proteins include the entire ALK kinase domain and lead to constitutive ALK kinase activity and oncogenic transformation both *in vitro* and *in vivo* (3). Somatic mutations in the *ALK* kinase domain, including at position F1174L, have been detected in neuroblastomas and are also transforming *in vitro* and *in vivo* (4, 5). These findings have led to preclinical and clinical development of ALK kinase inhibitors including PF-2341066 (crizotinib; ref. 6). ALK kinase inhibitors lead to apoptosis *in vitro* and tumor shrinkage in mouse models of *EML4-ALK* NSCLC (7, 8). To date, significant clinical activity, including tumor shrinkage in 60% of patients, has been observed in a phase I trial of crizotinib in *EML4-ALK* NSCLC (9). Furthermore, clinical efficacy of crizotinib has been observed in an IMT patient harboring an *ALK* translocation (10). However, despite these dramatic effects, as with other kinase inhibitors, drug resistance (herein termed acquired resistance) to ALK kinase inhibitors is likely to emerge. An understanding of the acquired resistance mechanisms will be important for the development of additional ALK kinase inhibitors and/or combination therapeutic strategies.

Methods

Patients

The patients were treated in a clinical trial that was sponsored by Pfizer. Tumor biopsies were obtained under an IRB approved protocol. Both patients provided written informed consent. Total RNA was isolated using Trizol (Invitrogen) and purified using RNeasy minielute cleanup kit (Qiagen).

ALK genomic analyses

The *ALK* kinase domain was sequenced from all of the available specimens. Exon 23 of *ALK* was amplified from DNA using exon-specific primers, PCR products cloned into a TOPOTA vector (Invitrogen), transformed into bacteria, and individual clones sequenced. The PCR primers and conditions

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are available upon request. *ALK* fluorescence *in situ* hybridization (FISH) was performed using the break apart probes (Vysis LSI ALK Dual Color) as previously described (11).

Expression constructs and cell culture

The full length *RANBP2-ALK* cDNA from patient A and the *EML4-ALK* (variant 1) cDNA from the H3122 cell line were cloned into pDNR-Dual (BD Biosciences). The F1174L mutation was introduced using site-directed mutagenesis (Stratagene) with mutant-specific primers according to the manufacturer's instructions and as previously described (12). All constructs were confirmed by DNA sequencing. Retroviral infection and culture of Ba/F3 cell were performed using previously described methods (12). Polyclonal cell lines were established by puromycin selection and subsequently cultured in the absence of interleukin-3 (IL-3). Uninfected Ba/F3 cells or those expressing *EGFR* delE746_A750 or the JP1536 empty vector were used as controls.

Antibodies and Western blotting

Cell lysis, Western blotting, and immunoblotting were performed as previously described (12). Anti-phospho-ALK (DF53), anti-phospho-Akt (Ser-473), and anti-total-Akt were obtained from Cell Signaling Technology. Total ERK1/2 and phospho-ERK1/2 (pT185/pY187) antibodies were from Invitrogen. Immunoprecipitations were performed using anti-Flag-M2 agarose (Sigma-Aldrich). ALK immunohistochemistry (IHC) was performed using the mouse monoclonal antihuman CD246 (clone: ALK1, DAKO) as previously described (11).

Cell proliferation and growth assays

Crizotinib was provided by Pfizer. TAE684 was synthesized as previously described (7). Growth and inhibition of growth were assessed by MTS assay according to previously established methods (12). All experimental points were set up in 6 to 12 wells and all experiments were repeated at least 3 times.

Results and Discussion

We identified 2 patients with *ALK*-translocated cancers that developed clinical acquired resistance to crizotinib. Patient A, with IMT, achieved a partial response with crizotinib therapy lasting 8 months but subsequently developed regrowth of several tumor lesions and had these surgically removed (Supplementary Table S1; see ref. 10). Both pre- and posttreatment tumor specimens had evidence of viable tumor, expressed ALK by IHC, and contained an *ALK* translocation (Fig. 1A). There was no evidence of *ALK* amplification in the posttreatment tumor (data not shown). This patient's tumor was known to harbor the *RANBP2-ALK* translocation (10). Sequencing of the entire ALK kinase domain demonstrated that one

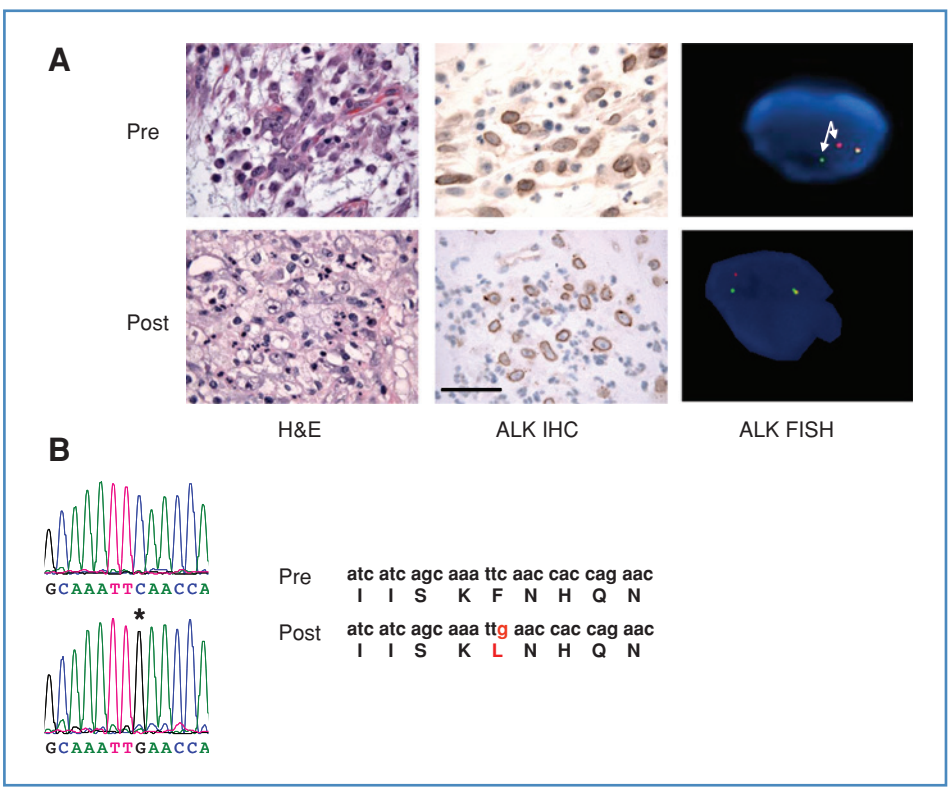

Figure 1. Tumor from crizotinib-resistant patient contains a secondary *ALK* mutation. A, comparison of pretreatment and posttreatment biopsy specimens. Both specimens contain viable tumor and both tumors express ALK by IHC that is localized to the nuclear membrane. FISH analyses demonstrate an ALK translocation (split red and green signals; arrows). Scale bar, 50 μm. B, sequence tracing from pre- and posttreatment tumor specimens. There is a C to G mutation (asterisk) in codon 3522 in exon 23 resulting in the F1174L mutation. This is not detected in the pretreatment tumor.

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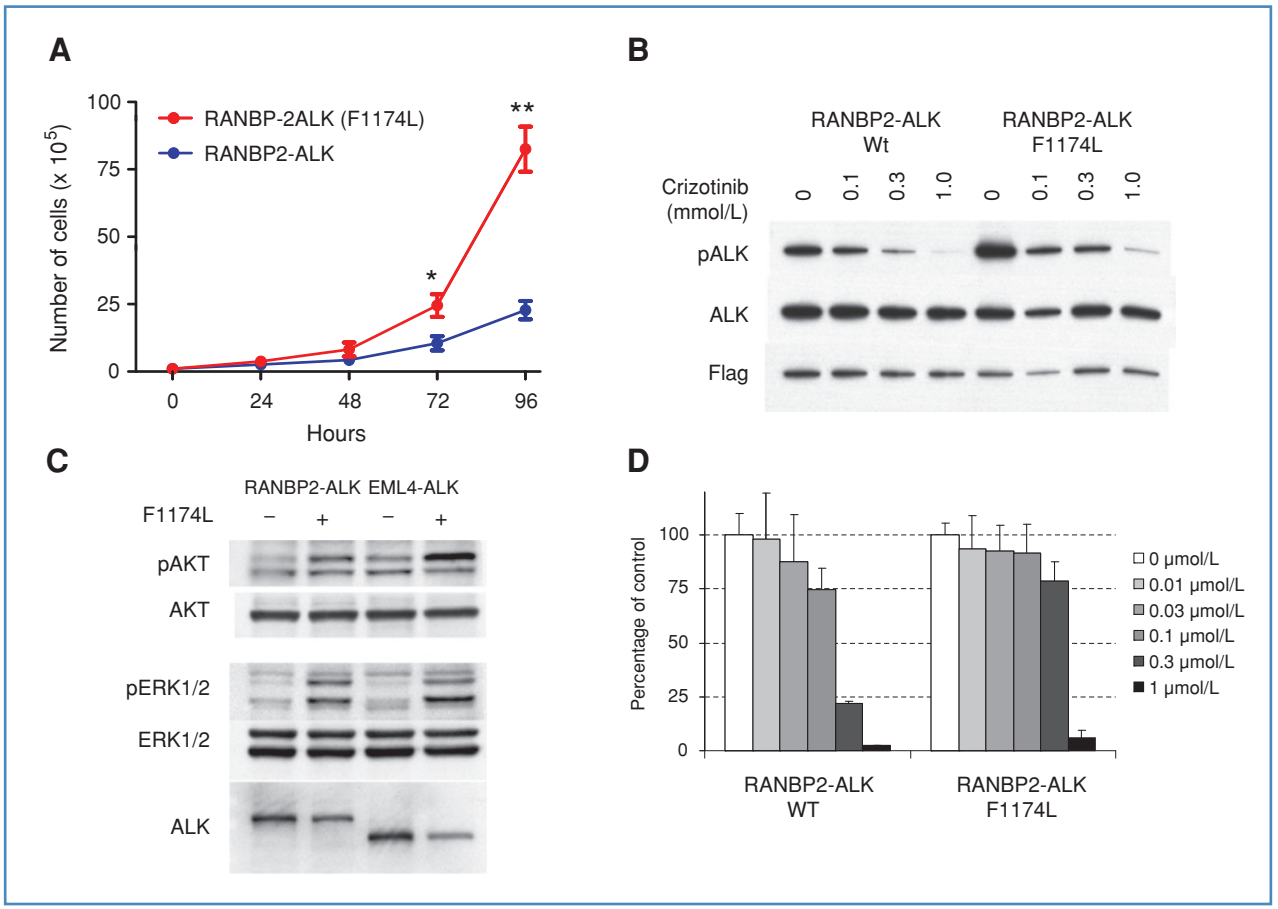

Figure 2. Impact of F1174L on growth and signaling in Ba/F3 cells harboring *RANBP2-ALK*. A, IL-3–independent Ba/F3 cells expressing *RANBP2-ALK* F1174L proliferate faster compared to cells expressing *RANBP2-ALK*. *, $P < 0.05$; **, $P < 0.001$. B, Ba/F3 cells with indicated genotypes were treated with increasing concentrations of crizotinib for 6 hours. Cell extracts were immunoprecipitated with an anti-FLAG antibody followed immunoblotting to detect the indicated proteins. C, presence of F1174L mutation in the background of an ALK translocation leads to enhanced AKT and ERK 1/2 signaling. Cell extracts were immunoblotted to detect the indicated proteins. D, Ba/F3 cells were treated with crizotinib at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. There is a significant effect of the F1174L mutation at 300 nmol/L ($P < 0.001$).

of the clinically progressing tumor lesions contained a F1174L mutation (Fig. 1B). This was not detected by direct sequencing, or by cloning and sequencing of individual clones, in the 2 other progressing lesions, or by direct sequencing in two other tumors that were clinically stable at the time of surgery (Supplementary Table S1). Furthermore, it was not detected in the pretreatment tumor specimen even by cloning and sequencing of individual clones (Supplementary Table S1). Patient B, with *EML4-ALK* NSCLC, achieved a partial response with crizotinib treatment but developed acquired resistance following 5 months of therapy. At the time of progression, a liver biopsy of a growing lesion was performed (Supplementary Table S1). *EML4-ALK* variant 1 was identified by RT-PCR, but no secondary mutations in *ALK* were detected in the acquired resistance tumor specimen.

We next evaluated the biologic impact of the F1174L mutation. Both *RANPB2-ALK* and *RANBP-ALK F1174L* led to IL-3 independent growth of Ba/F3 cells (Fig. 2A) but the growth was faster in the presence of the F1174L mutation. This increased growth rate was mirrored by a greater baseline ALK phosphorylation of RANBP2-ALK F1174L compared with RANBP2-ALK (Fig. 2B) and by increased downstream AKT and ERK 1/2 phosphorylation (Fig. 2C). The *RANB2-ALK* F1174L cells were significantly more resistant to crizotinib (Fig. 2D) and the F1174L mutation diminished crizotinib-mediated inhibition of ALK signaling and blocked apoptosis (Supplementary Fig. S1A and B). We also introduced the F1174L mutation into the background of *EML4-ALK* found in NSCLC (3). Similar to *RANPB2-ALK*, the *EML4-ALK* F1174L Ba/F3 cells grew faster (Fig. 3A), had a greater baseline ALK phosphorylation (Fig. 3B), and were more resistant to crizotinib growth inhibition than *EML4-ALK* Ba/F3 cells (Fig. 3C). Consistent with these findings on growth, greater concentrations of crizotinib were required to inhibit ALK phosphorylation in the *EML4-ALK* F1174L cells compared with those with *EML4-ALK* alone (Fig. 3B). Collectively, our studies demonstrate that

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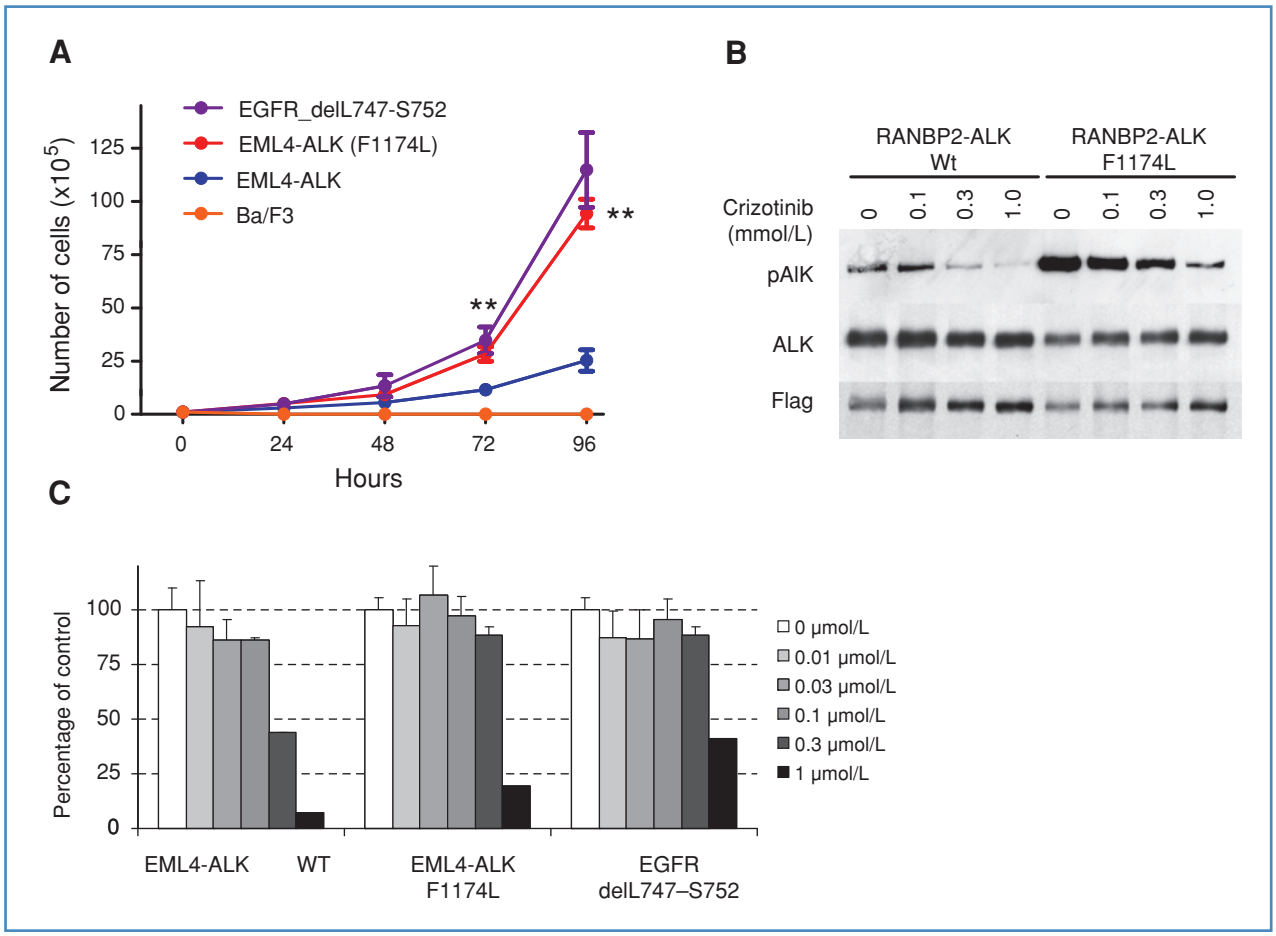

Figure 3. Impact of the F1174L mutation on growth and signaling in Ba/F3 cells expressing *EML4-ALK*. A, IL-3–independent Ba/F3 cells expressing *EML4-ALK* F1174L proliferate faster than cells expressing *EML4-ALK*. **, $P < 0.001$. B, *EML4-ALK* and *EML4-ALK* F1174L Ba/F3 cells were treated with increasing concentrations of PF-02341066 for 6 hours. Cell extracts were immunoprecipitated with an anti-FLAG antibody followed immunoblotting to detect the indicated proteins. C, Ba/F3 cells were treated with crizotinib at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. There is a significant effect of the F1174L mutation at 300 nmol/L ($P < 0.001$).

the F1174L mutation imparts both biologic and drug resistance properties to cancers harboring *ALK* translocations. Furthermore, patients with neuroblastoma harboring the F1174L *ALK* mutation may have a transient or no clinical benefit from crizotinib treatment using the current dosing schedules (13).

Crizotinib is administered continuously daily (250 mg BID), reaching a median through plasma concentration of 57 nmol/L of free drug and is clinically effective at this dosing in *ALK* rearranged IMT and NSCLC (9, 10, 13). Our preclinical studies suggest that higher doses of crizotinib could be used to overcome the F1174L-mediated resistance mechanism (Fig. 2D). This could potentially be achieved using intermittent administration of higher doses of crizotinib, to achieve a higher C_{max}, sufficient to inhibit ALK phosphorylation in the presence of F1174L. Similarly, some imatinib resistance mutations (including F359V, M244V, Q252H, and E355G), many of which effect the conformational change in ABL, cause a relative drug resistance that can be overcome by higher drug doses *in vitro* and in some cases clinically by using higher doses of imatinib (14, 15). In order to develop additional therapeutic strategies, we evaluated a structurally unrelated ALK kinase inhibitor, TAE684, and the heat shock protein (HSP) 90 inhibitor 17-AAG in the crizotinib resistant models (7, 16). Although the F1174L mutation slightly increased the IC_{50} for TAE684 against RANBP-ALK Ba/F3 cells (59 nmol/L with F1174L; 22 nmol/L without), the IC_{50} was still substantially below the concentrations of crizotinib (IC_{50} 200 nmol/L) required to inhibit growth and ALK phosphorylation in the parental RANBP-ALK Ba/F3 cells (Figs. 2B and D and 4A). Similar findings were observed for EML4-ALK Ba/F3 cells (Fig. 4A and data not shown). The effects of TAE684 on growth were also mirrored at the level of ALK phosphorylation (Fig. 4B). Recent clinical studies have identified antitumor activity of the HSP90 inhibitor IPI-504 in *ALK*-translocated NSCLC (17). We thus evaluated the effects of the HSP90

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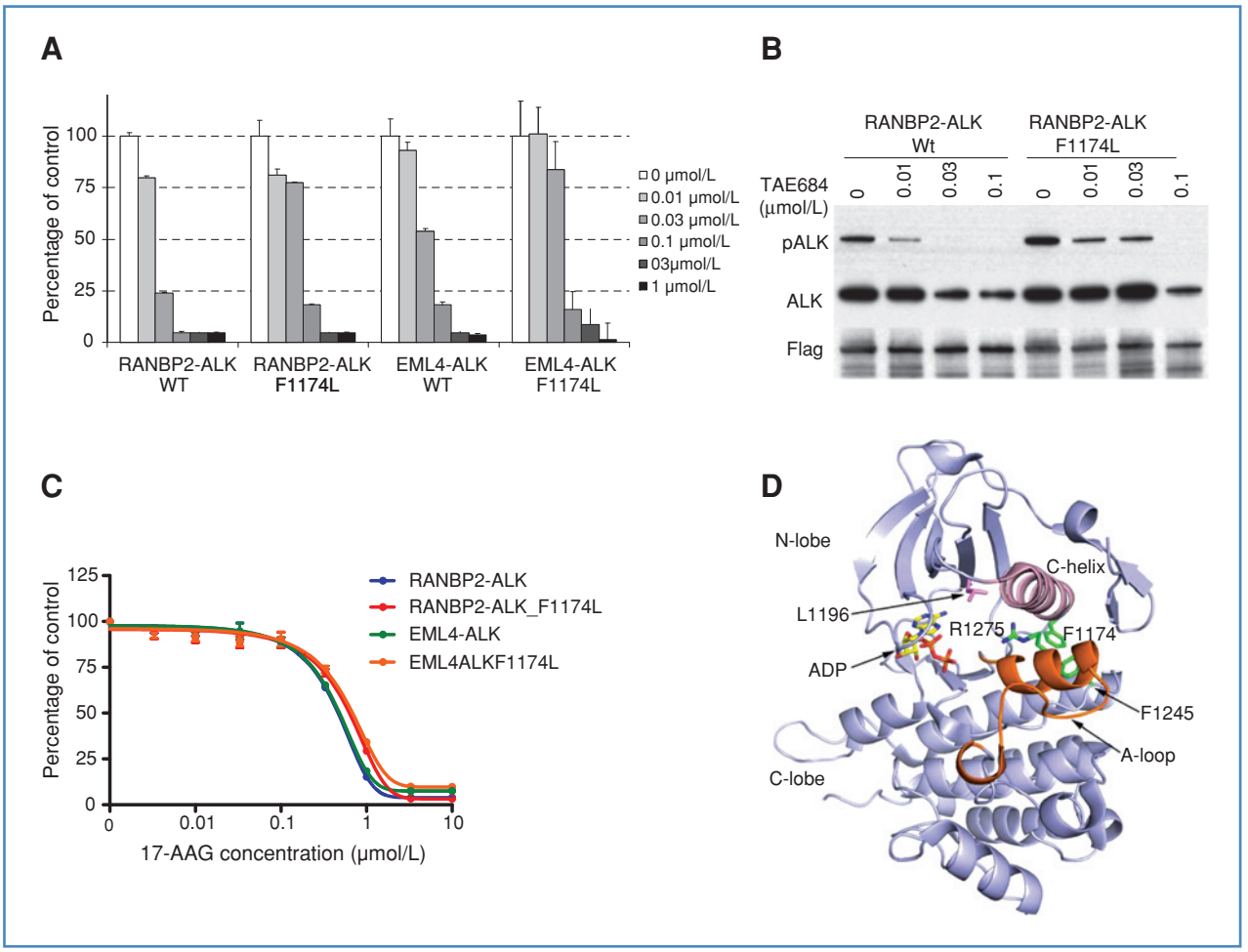

Figure 4. Therapeutic strategies against cancers harboring the F1174L crizotinib resistance mutation. A, Ba/F3 cells were treated with TAE684 at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. B, *RANBP2-ALK* and *RANBP2-ALK* F1174L Ba/F3 cells were treated with increasing concentrations of TAE684 for 6 hours. Cell extracts were immunoprecipitated with an anti-FLAG antibody followed by immunoblotting to detect the indicated proteins. C, IL-3–independent Ba/F3 cells harboring ALK translocations with or without the F1174L mutation are equally sensitive to the HSP90 inhibitor 17-AAG. Viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. D, ribbon diagram depicting the crystal structure of ALK kinase in the inactive conformation in complex with ADP. The side chains of F1174 and selected other neuroblastoma mutations are shown in green, and the gatekeeper residue L1196 is shown in magenta. Note that F1174 is not in contact with the ATP-binding cleft. Interestingly, these neuroblastoma mutations cluster along a helix formed in the activation loop (A-loop, colored orange) in this inactive structure. The mutations may destabilize this helix to promote the active conformation. They may also affect the position of the C-helix (pink), which is known to play a key regulatory role in some kinases. Figure is drawn from PDB ID 3LCT (18).

inhibitor 17-AAG in models harboring the F1174L mutation (Fig. 4C). Ba/F3 cells with and without F1174L were equally sensitive to 17-AAG *in vitro* (Fig. 4C). As many ALK inhibitors are in preclinical development and several HSP90 inhibitors currently undergoing clinical development, our findings provide direct therapeutic strategies for patients that develop crizotinib resistance.

The structural basis for the crizotinib resistance of the F1174L mutation is not entirely clear. Examination of the recently published crystal structure of ALK in an inactive conformation reveals that the F1174L mutation is not in direct contact with the ATP-binding pocket, where both crizotinib and TAE684 are expected to bind (Fig. 4D; see refs. 18, 19). Thus, the F1174L mutation is unlikely to confer resistance via direct steric interactions. Crizotinib is known to bind an *inactive* conformation of MET (20) and a recently released crystal structure in complex with ALK indicates that it binds a distinct inactive conformation in ALK (PDB ID 2XP2). The activating F1174L mutation must promote the active conformation of the kinase and therefore may disfavor binding of crizotinib analogous to some imatinib resistance mutations in ABL (14). A more definitive understanding of the mechanism of resistance, and the differential effect of the mutation on crizotinib versus TAE684, will require detailed binding and structural studies of these inhibitors with the F1174L mutant.

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This study highlights the need to study drug resistance mechanisms from cancer patients treated with kinase inhibitors. The molecular, cellular, and structural understanding of drug resistance mechanisms will continue to reveal therapeutic insights for the development of future anticancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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