Genomic Alterations of Anaplastic Lymphoma Kinase May Sensitize Tumors to Anaplastic Lymphoma Kinase Inhibitors

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Abstract

Selective kinase inhibitors have had a substantial impact on the field of medical oncology. Whereas these agents can elicit dramatic clinical responses in some settings, their activity is generally limited to a subset of treated patients whose tumor cells harbor a specific genetic lesion. We have established an automated platform for examining the sensitivity to various molecularly targeted inhibitors across a large panel of human tumor-derived cell lines to identify additional genotype-correlated responses that may be clinically relevant. Among the inhibitors tested in a panel of 602 cell lines derived from a variety of human cancers, we found that a selective inhibitor of the anaplastic lymphoma kinase (ALK) potentely suppressed growth of a small subset of tumor cells. This subset included lines derived from anaplastic large cell lymphomas, non–small-cell lung cancers, and neuroblastomas. ALK is a receptor tyrosine kinase that was first identified as part of a protein fusion derived from a chromosomal translocation detected in the majority of anaplastic large cell lymphoma patients, and has recently been implicated as an oncogene in a small fraction of non–small-cell lung cancers and neuroblastomas. Significantly, sensitivity in these cell lines was well correlated with specific ALK genomic rearrangements, including chromosomal translocations and gene amplification. Moreover, in such cell lines, ALK kinase inhibition can lead to potent suppression of downstream survival signaling and an apoptotic response. These findings suggest that a subset of lung cancers, lymphomas, and neuroblastomas that harbor genomic ALK alterations may be clinically responsive to pharmacologic ALK inhibition. [Cancer Res 2008;68(9):3389–95]

Introduction

Tyrosine kinases are now widely recognized as attractive targets for therapy. The clinical success of several selective kinase inhibitors including imatinib, erlotinib, sunitinib, and lapatinib has shown that this strategy may be broadly applicable to a variety of hematologic and epithelial malignancies. However, it is also becoming clear that such treatments are largely beneficial to a subset of patients whose tumor cells harbor activating mutations of genes encoding the target kinase (1). Thus, imatinib, which inhibits the ABL, KIT, and platelet-derived growth factor (PDGF) receptor kinases, is effective in chronic myelogenous leukemias, which harbor the BCR-ABL oncogenic kinase fusion, and in gastrointestinal tumors that harbor mutationally activated KIT or PDGF receptors (2). Similarly, most non–small-cell lung cancer patients that respond to the epidermal growth factor receptor (EGFR) kinase inhibitor erlotinib harbor activating EGFR mutations (3). Ongoing cancer genome analyses continue to reveal novel genetic lesions that give rise to activated kinases in a variety of cancers, and many of these may represent attractive targets for therapy.

We have recently reported the development of an automated high-throughput platform for profiling a very large panel of human tumor-derived cell lines to identify subsets that exhibit exquisite sensitivity to a variety of molecularly targeted inhibitors with potential anticancer activity (4). Those findings showed the power of this strategy to reveal genotype-correlated sensitivities that may be useful in guiding clinical testing of novel therapeutic compounds. Here, we describe the profiling of 602 cancer cell lines for sensitivity to a selective inhibitor of the anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase first identified as part of an NPM-ALK fusion protein expressed in a subset of patients with anaplastic large cell lymphoma (5). Our studies revealed that a small subset of cell lines harboring ALK gene alterations are highly sensitive to ALK inhibition (5, 6). These include cells derived from non–small-cell lung cancers and anaplastic large cell lymphomas, where ALK translocations have previously been reported, as well as from neuroblastomas, where ALK gene amplification has been described (7, 8). Our findings indicate that selective ALK kinase inhibitors may be useful in the clinical management of a subset of patients with diverse tumor types that harbor ALK gene alterations.

Materials and Methods

Human cancer cell lines and cell viability assays. Human cancer cell lines were obtained from commercial vendors and were maintained and tested for viability using an automated platform, as previously described (4).

Protein detection. Immunodetection of proteins following SDS-PAGE was done using standard protocols. Equal lane loading was assessed using a β-tubulin antibody (Sigma). The Akt, ALK, extracellular signal–regulated kinase (Erk)-1/2, phospho-Erk1/2 (T202/Y204), phospho-ALK (Y1604), signal transducers and activators of transcription 3 (STAT3), and phospho-STAT3 (S727) antibodies were from Cell Signaling Technology. The phospho-Akt (S473) antibody was from BioSource International. The poly(ADP-ribose)
polymerase antibody was from BD Biosciences. All antibodies were used at a 1:1,000 dilution, except for the β-tubulin antibody, which was used at 1:10,000 dilution.

**Kinase inhibitors.** TAE684 and BMS-536924 were synthesized as previously described (9, 10). PF-2341066 was synthesized at Pfizer Pharmaceuticals. WZ-5-126 is a recently developed inhibitor with selective ALK inhibitory activity,5 and the in vitro profile of inhibitory activity against a panel of kinases was done by Ambit Biosciences (Supplementary Table S1).

**Cell cycle analysis.** Cells were pulsed with 10 μmol/L bromodeoxyuridine (BrdUrd) for 1 to 2 h before collection, centrifuged to remove supernatant, and fixed in ice-cold 70% ethanol. The cells were washed with PBS/0.5% bovine serum albumin (BSA) and incubated in denaturing solution (2 mol/L HCl) for 20 min at room temperature. After a further wash with PBS/0.5% BSA, the cells were resuspended in 0.1 mol/L sodium borate for 2 min at room temperature. After an additional wash, the cells were suspended in anti-BrdUrd monoclonal antibody for 20 min (1:500; Becton Dickinson) per manufacturer's instructions. Cells were washed in PBS/0.5% BSA and the pellet was resuspended in FITC-conjugated antitumour IgG (1:50; Vector Laboratories) for 20 min. After an additional wash in PBS/0.5% BSA, the cells were stained with 10 μg/mL propidium iodide (Sigma) and treated with RNase A (Sigma) before two-dimensional fluorescence-activated cell sorting analysis using CellQuest software (Becton Dickinson).

**RNAi studies.** Two shRNA species targeting sequences downstream of the common ALK breakpoint were expressed from the pLKO1 lentiviral vector (target sequences: 
\[GAGCTGGTCATTACGAGGATA-3\] and 
\[GTGATAAATACAAGGCCCAGA-3\]¶). Cells were infected with the viruses (1:500; Vector Laboratories) for 20 min. For an additional wash in PBS/0.5% BSA, the cells were stained with 10 μg/mL propidium iodide (Sigma) and treated with RNase A (Sigma) before two-dimensional fluorescence-activated cell sorting analysis using CellQuest software (Becton Dickinson).

**Fluorescence in situ hybridization.** Two-color fluorescence in situ hybridization (FISH) was done on 3:1 methanol/acetic acid-fixed cell lines or on formalin-fixed paraffin-embedded tumor tissue using the LSI ALK Dual Color, Break Apart Rearrangement Probe (Abbott-Vysis) following the manufacturer's protocols. Images were captured with an Olympus BX61 fluorescent microscope equipped with a charge-coupled device camera, and analysis was done with Cytovision software (Applied Imaging).

**PCR detection of ALK fusion products.** RNA was extracted from cell lines using RNA STAT-60 according to the manufacturer's instructions (Tel-Test, Inc.) and reverse transcription was carried out with the AffinityScript Multi-Temperature cDNA Synthesis Kit (Stratagene). PCR was then done using the AmpliTaq Gold PCR Master Mix (Applied Biosystems). Primer sequences are listed in Supplementary Fig. S1.

**DNA sequencing.** Genomic DNA was isolated from cell lines using the Gentra purification system according to the manufacturer’s protocol. The entire ALK coding sequence (exons 1–29) was amplified from genomic DNA by PCR with primers (Supplementary Fig. S2). PCR products were purified and subjected to bidirectional sequencing using BigDye v1.1 (Applied Biosystems) in combination with an ABI3100 sequencer (Applied Biosystems). Electropherograms were analyzed using Sequence Navigator software (Applied Biosystems).

**Data analysis.** The sensitivity of each cell line to various concentrations of kinase inhibitors was calculated as the fraction of viable cells relative to untreated cells. Data were subjected to nonlinear regression analysis using GraphPad Prism Software version 3.0 (GraphPad Software, Inc.) to obtain IC₅₀ values.

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5 N.S. Gray, unpublished data.
and phosphorylation was completely abolished following treatment with TAE684. Thus, the ALK kinase seems to have become activated by virtue of genomic rearrangement in these cells. Autophosphorylation of ALK leads to the activation of multiple signaling pathways that contribute to cell survival and transformation (15). Significantly, treatment of each of these lines with TAE684 resulted in a dramatic inhibition of Akt and Erk1/2 phosphorylation (Fig. 1D), suggesting that ALK activation in these cells is coupled to the engagement of downstream survival effectors.

ALK shares a high degree of homology with the insulin-like growth factor receptor (IGF-IR), which has also been implicated...
in tumorigenesis (16), and significant expression of IGF-IR was detected in both of the TAE684-sensitive non–small-cell lung cancer cell lines (Fig. 1C). However, treatment of both lines with an IGF-IR inhibitor, BMS-536924 (IC$_{50}$ 100 nmol/L), had no effect on cell viability (Supplementary Fig. S5). Moreover, these cells were similarly sensitive to another selective ALK inhibitor, WZ-5126 (IC$_{50}$ 3.4 nmol/L; Supplementary Table S1), suggesting that the observed effects of TAE684 in these cells are mediated through ALK inhibition (Supplementary Fig. S5). Cell cycle analysis of the NCI-H3122 cell line following treatment with TAE684 revealed a dramatic increase in the sub-G$_1$ apoptotic fraction of cells as early as 24 hours after treatment, suggesting a cytotoxic response to ALK inhibition (Supplementary Fig. S6A). Poly(ADP-ribose) polymerase cleavage was also evident in this cell line following treatment with TAE684 (Fig. 1E). Notably, the TAE684 response in the NCI-H2228 cell line seems to be cytostatic rather than apoptotic (data not shown). Thus, ALK kinase inhibition in tumor cells harboring ALK genomic lesions may lead to either a cytostatic or cytotoxic outcome, potentially depending on additional genetic features.

TAE684 sensitivity in neuroblastoma cells correlates with ALK gene amplification and rearrangement. The cell line profiling data also revealed a preponderance of neuroblastoma-derived cell lines among the most TAE684-sensitive lines (Fig. 1A).
ALK expression has previously been reported in a large fraction of neuroblastomas (11), and rare cases of ALK gene amplification have also been described (7, 8). Therefore, we examined the 17 neuroblastoma cell lines that were screened with the ALK inhibitor using an ALK FISH probe to detect gene rearrangements. Two of the most TAE684-sensitive cell lines showed either ALK gene rearrangement (KELLY) or substantial amplification of intact ALK (NB-1; Fig. 2A). Although FISH analysis of the KELLY line revealed a clear chromosomal split within the ALK gene, the molecular nature of the gene rearrangement remains unknown. Curiously, phosphorylated ALK was difficult to detect in the KELLY cell line, suggesting that very low levels of protein may be driving downstream signaling in these cells. However, KELLY cells, as well as H3122 non–small-cell lung cancer cells, were effectively killed following infection with either of the two different lentiviruses that encode ALK-specific shRNAs, confirming the requirement for ALK in these cells (Fig. 2C). Thus, in these cell lines with genomic ALK alterations, ALK signaling seems to be coupled to key downstream survival effectors. Moreover, as early as 6 hours after treatment with TAE684, there was evidence of poly(ADP-ribose) polymerase cleavage in the NB-1 cell line (Fig. 2C), indicating that, as in non–small-cell lung cancer cells harboring ALK translocations, neuroblastoma cells with activated ALK also undergo an apoptotic response to kinase inactivation by TAE684. Previous studies that made use of ALK-specific siRNAs to reduce ALK protein expression showed a similar requirement for ALK in a neuroblastoma cell line exhibiting ALK gene amplification (7).

To assess the potential clinical significance of these cell line findings in primary neuroblastomas, we used FISH to detect ALK gene abnormalities in 10 pediatric neuroblastoma samples. Among the 10 cases analyzed, we identified 1 case with marked...
amplification of ALK, similar to that seen in the NB-1 cell line (Fig. 2D). Although this represents a small sample size, a previous report identified ALK gene amplification in 8 of 85 primary neuroblastoma specimens, suggesting an ~10% frequency of this genotype in human neuroblastomas (7).

Surprisingly, the most TAE684-sensitive neuroblastoma cell line identified in our panel, SH-SY5Y, showed no evidence of either ALK gene rearrangement by FISH or ALK coding sequence mutation by DNA sequencing (data not shown). However, TAE684 treatment of these cells effectively suppressed Akt and Erk1/2 phosphorylation (Fig. 2C). Significantly, a separate analysis of tumor cell sensitivity to the IGF-IR inhibitor BMS-536924 in 256 cell lines from a variety of tissue types revealed that, as with TAE684, the majority of cell lines were drug resistant, but SH-SY5Y was notably among the most sensitive cell lines (Fig. 3A). As mentioned above, the ALK kinase domain exhibits a high degree of sequence homology with the IGF-IR kinase, and TAE684 inhibits phosphorylation of IGF-IR in in vitro kinase assays at concentrations of 10 to 20 nmol/L (9). In addition to expressing ALK, a large fraction of the neuroblastoma cell lines also express IGF-IR (Fig. 3B). Although KELLY and SH-SY5Y both express significant levels of IGF-IR, a comparison of their sensitivities to TAE684, WZ-5-126, and BMS-536924 showed that in KELLY cells the predominant target of TAE684 is ALK, whereas in the SH-SY5Y cell line it appears to be IGF-IR (data not shown). Indeed, treatment of SH-SY5Y cells with the IGF-IR inhibitor BMS-536924 resulted in a dramatic suppression of Akt phosphorylation (Fig. 3C). Previous studies have also implicated IGF-IR as a potential therapeutic target in neuroblastoma cells, including SH-SY5Y cells (17). We also noted that two of the neuroblastoma lines without obvious ALK gene alterations (SK-N-FI and SK-N-SH) exhibited TAE684 sensitivity but did not respond to BMS-536924, raising the possibility that these cells harbor more subtle ALK lesions or that another target of TAE684 confers sensitivity in those lines. Taken altogether, these findings suggest that a subset of neuroblastomas with ALK gene amplification or rearrangement may be clinically responsive to selective ALK kinase inhibitors. Moreover, our findings raise the possibility that a dual inhibitor of ALK and IGF-IR, such as TAE684, may be clinically active in a subset of neuroblastomas that includes those with either ALK or IGF-IR dependency.

Anaplastic large cell lymphoma–derived cells with ALK translocations are sensitive to ALK kinase inhibition. Anaplastic large cell lymphoma is the tumor type where ALK translocations have been most frequently detected (5). Our cell line profiling screen with TAE684 included two anaplastic large cell lymphoma–derived cell lines (SU-DHL-1 and Karpas-299), and both have previously been shown to express a fusion protein resulting from the NPM-ALK translocation. Significantly, these lines were among the most TAE684-sensitive cell lines detected in our screen (Fig. 1A), and we confirmed the presence of the NPM-ALK fusion.
translocation in these cells by both PCR and FISH analysis (Supplementary Fig. S7). Furthermore, TAE684 potently suppressed cell viability and ALK phosphorylation, as well as the phosphorylation of downstream survival effectors, in both lines (Fig. 4A and B).

Because TAE684 is currently not being tested as a clinical agent, we also examined the activity of PF-2341066, a dual MET/ALK kinase inhibitor currently undergoing phase I clinical testing (18). In the two anaplastic large cell lymphoma lines tested, as well as the neuroblastoma line NB-L, PF-2341066 was able to inhibit proliferation and ALK-mediated signaling in these cell lines at clinically achievable doses, although the inhibitory effects were not as substantial as those seen with TAE684 (Fig. 4A and B). Moreover, potent suppression of Akt and Erk signaling was also seen in PF-2341066–treated NB-L neuroblastoma cells. Similar trends in sensitivity to both TAE684 and PF-2341066 were also evident in the non–small-cell lung cancer cell line NCI-H3122 and the neuroblastoma line KELLY (Supplementary Fig. S8). Together, our cell line findings suggest that ALK gene rearrangements associated with specific chromosomal translocations or gene amplification are well correlated with sensitivity to selective ALK kinase inhibition, and that clinical testing of PF-2341066 in anaplastic large cell lymphoma, non–small-cell lung cancer, and neuroblastoma may be warranted.

Concluding remarks. Our collective observations from cell line profiling analysis with the selective ALK kinase inhibitor TAE684 have revealed that a subset of human cancer-derived cell lines harboring ALK gene rearrangements and/or amplifications are exquisitely sensitive to ALK kinase inhibition (Supplementary Fig. S9). Moreover, in these cell lines, ALK activation seems to be warranted.

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