Crizotinib-Resistant NPM-ALK Mutants Confer Differential Sensitivity to Unrelated Alk Inhibitors

Monica Ceccon1, Luca Mologni1, William Bisson3, Leonardo Scapozza3, and Carlo Gambacorti-Passerini1,2

Abstract

The dual ALK/MET inhibitor crizotinib was recently approved for the treatment of metastatic and late-stage ALK+ NSCLC, and is currently in clinical trial for other ALK-related diseases. As predicted after other tyrosine kinase inhibitors’ clinical experience, the first mutations that confer resistance to crizotinib have been described in patients with non–small cell lung cancer (NSCLC) and in one patient inflammatory myofibroblastic tumor (IMT). Here, we focused our attention on the anaplastic large cell lymphoma (ALCL), where the oncogenic fusion protein NPM-ALK, responsible for 70% to 80% of cases, represents an ideal crizotinib target. We selected and characterized 2 human NPM-ALK+ ALCL cell lines, KARPAS-299 and SUP-M2, able to survive and proliferate at different crizotinib concentrations. Sequencing of ALK kinase domain revealed that a single mutation became predominant at high crizotinib doses in each cell line, namely L1196Q and I1171N in Karpas-299 and SUP-M2 cells, respectively. These mutations also conferred resistance to crizotinib in Ba/F3 cells expressing human NPM-ALK. The resistant cell populations, as well as mutated Ba/F3 cells, were characterized for sensitivity to two additional ALK inhibitors: the dual ALK/EGFR inhibitor AP26113 and NVP-TAE684. While L1196Q-positive cell lines were sensitive to both inhibitors, cells carrying I1171N substitution showed cross-resistance to all ALK inhibitors tested. This study provides potentially relevant information for the management of patients with ALCL that may relapse after crizotinib treatment. Mol Cancer Res; 11(2); 122–32. ©2012 AACR.

Introduction

The interest in anaplastic lymphoma kinase (ALK) as an effective oncogenic target is rapidly growing. ALK overexpression (1, 2) or mutations (3–6) were shown to be oncogenic events in neuroblastoma, whereas several oncogenic fusion proteins involving ALK kinase domain were found in a broad range of solid and hematologic tumors. Among these, the fusion protein NPM-ALK is present in about 70% to 80% of ALK-positive anaplastic large cell lymphomas (ALCL), an aggressive non-Hodgkin T-cell Lymphoma currently treated with polychemotherapy and, sometimes, bone marrow transplantation. Moreover, fusions between TPM3-ALK or TPM4-ALK are found in approximately 50% of inflammatory myofibroblastic tumor (IMT), whereas 5% of patients with non–small cell lung cancer (NSCLC) express the EML4-ALK fusion (7). Recently, ALK was discovered to be involved at low frequencies in several diseases, such as extramedullary plasmacytoma (8), renal cell carcinoma (9), thyroid cancer (10), breast cancer, and colorectal cancer (11, 12) enlarging the population of patients that will possibly benefit from ALK inhibition. NPM-ALK has been originally recognized as the leading cause of ALCL by Morris and colleagues in 1994 (13). This fusion protein originates from the t(2;5)(p23;q35) translocation, and is composed of the first 117 amino acids of NPM1 protein, followed by a completely functional C-terminal part of ALK (residues 1,058–1,620), that comprise its catalytic domain. The NPM1 part maintains its dimerization domain and shuttling activity (14), and its function is crucial for homodimerization and consequent ALK-deregulated activation (15). After dimerization, similar to other kinases belonging to the insulin receptor family, all 3 tyrosines from the well conserved YYYYY motif in the activation loop are activated by transautophosphorylation. In particular, tyrosine 1278 plays an important role in activation and signal transduction (16). The main ALK downstream effectors are JAK3-STAT3 (17, 18) and PI3K-AKT (19) pathways, involved in cell survival and phenotypic changes, and ERK pathway (20), leading to cellular proliferation. Also, the JAK2-STAT5 pathway...
seems to be involved in NPM-ALK-mediated cellular proliferation, although the role of STAT5A and STAT5B is still controversial (21, 22).

Crizotinib (PF-02341066, Xalkori) was recently approved for advanced ALK+ NSCLC and is now undergoing phase II clinical trials for ALK-positive diseases different from NSCLC. However, despite encouraging results (23–25), several ALK kinase domain mutations able to confer crizotinib resistance were found in patients with NSCLC (26–29) and IMT (30). This is not surprising, as already shown by clinical experience with other tyrosine kinase inhibitors, such as imatinib in chronic myeloid leukemia (31). Second generation ALK inhibitors are currently under clinical investigation, for example, AP26113 developed by ARIAD (32), currently in phase I/II clinical trial (NCT01449461), LDK378 by NOVARTIS (33) now in phase I clinical trial (NCT01283516), and ASP3026 by ASTELLAS PHARMA (34), structurally related to NVP-TAE684, which is also in phase I trials (NCT01284192 and NCT01401504). As expected, interest in new drugs that may be able to overcome crizotinib resistance is growing fast.

In the present work, we focused our attention on ALK kinase domain mutations that may confer crizotinib resistance in ALCI, a rare non-Hodgkin lymphoma that affects both adult and pediatric patients, currently treated with standard chemotherapy or bone marrow transplantation. Two human ALCI ALK+ cell lines, Karpas-299 (K299) and SUP-M2, were grown in the presence of increasing doses of crizotinib, thus obtaining different polyclonal populations able to survive and to proliferate at well-defined drug doses. We found 2 mutations that became predominant at high crizotinib doses and, after their introduction in Ba/F3 model, we studied their biologic effects, exploring also sensitivity to 2 unrelated ALK inhibitors. Computational studies were run to provide a possible explanation for the experimental results.

Materials and Methods

Cell culture and selection of resistant cells

ALCL T-cell lymphoma cell lines Karpas-299 and SUP-M2 carrying the t(2;5) translocation and the pro-B murine Ba/F3 cell line were purchased from DSMZ, where they are routinely verified using genotypic and phenotypic testing to confirm their identity.

Cells were cultured in RPMI-1640 supplemented with 9% FBS U.S. origin (Euroclone EU0080966), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine (Invitrogen) and incubated at 37°C with 5% CO2. Ba/F3 cells’ medium was supplemented with Chinese hamster ovary cells’ supernatant (1:2,000) as a source of interleukin (IL)-3.

Crizotinib was kindly provided by Pfizer and added to the medium starting from an initial dose of 50 nmol/L. Medium was replaced with fresh RPMI-1640 supplemented with crizotinib every 48 or 72 hours, and cell number and viability were assessed by Trypan Blue count. Once the new cell line was established, medium was replaced and a higher crizotinib dose was added.

AP26113 was kindly provided by ARIAD and NVP-TAE684 was purchased from Selleck Chemicals.

Site-directed mutagenesis and Ba/F3 cell transfection

The pcDNA3.0 vector containing wild-type NPM/ALK (pcDNA3-NA) was kindly provided by Dr. S. W. Morris (St Jude Research Hospital, Memphis, TN). Site-directed mutagenesis was conducted using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) and pcDNA3-NA as a template, according to manufacturer instructions, with the following oligonucleotide sequences: CAATCCCTGCAGCCGGTT- CATTCTGCAGGACCTCATGGGAGG to insert the 4539T→A mutation and GGAAGCCCGTATCAAT- AGCAAATTCAACCACGACACGACACGACAGGCCAC and ALK-REV, CAGCTTCATCTGCTAG- GCTTG. Standard PCR was conducted using High Fidelity Taq Polymerase (Roche), according to manufacturer’s instructions. Plasmid was recovered from 30 clones per cell line using Zippy plasmid Miniprep Kit (Zymo Research). Correct insertion was verified by EcoRI (Roche) enzymatic digestion, and only clones that displayed the expected restriction map were sequenced. Sequence numbering is related to GenBank ID NM004304.0.

PCR and quantitative RT-PCR

The cells were lysed in EUROGOLD TRIFAST solution (Euroclone), and total RNA was extracted according to manufacturer’s instructions. RNA was then retrotranscribed using TaqMan Reverse Transcription reagents (Roche). Quantitative RT-PCR (qRT-PCR) was conducted using Brilliant III SYBR Green Mastermix (Agilent Technologies), according to manufacturer’s instructions. The following primers were used: NPM-FW, TGCATATTAGTGGACTGTGGACTGTGGTCATGAG. Standard PCR was conducted using High Fidelity Taq Polymerase (Roche), according to the manufacturer’s instructions, with forward TGCATATTAGTGGACTGTGGACTGTGGTCATGAG and reverse CAGAGGTCTCTGTTCGAGTC primers. Normalization was carried out using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and amplified using the following primers: GAPDH_SybrG_FWD, tgcaccaccacctgcttagc; GAPDH_SybrG_REV, GGCA- TGGACTGTGGTCATGAG.

Western blotting and antibodies

Cells were seeded in 12-well plates and compounds were added at different concentrations. After 4-hour treatment with the indicated drug, cells were harvested, washed once in PBS at 4°C, and resuspended in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L Na2VO4, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors]. Western blotting was performed using antibodies against NPM/ALK (C20) and phosphorylated STAT5 (Tyr694).
inhibitor cocktail (10 μmol/L benzamidine-HCl and 10 μg/mL each of aprotinin, leupeptin, and pepstatin A) followed by incubation on ice for 30 minutes. After centrifugation to remove cell debris, Laemml buffer supplemented with 10% β-mercaptoethanol was added and lysates were denatured at 97°C for 20 minutes and used for electrophoresis. Equal amounts of total proteins were loaded on 10% SDS-PAGE, transferred to nitrocellulose membrane Hybond ECL (Amersham), and incubated overnight at 4°C incubated with the chemiluminescence reagent. Secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Amersham) were incubated for 1 hour and then visualized by chemiluminescence as recommended by the manufacturer.

Anti-ACTIN antibody was purchased from Sigma; polyclonal anti-STAT3 is from Calbiochem; monoclonal anti-phospho-ALK (Y-1604), monoclonal anti-ALK (31F12) and monoclonal anti-phospho-STAT3 (Tyr 705) antibodies were from Cell Signaling Technology.

Apoptosis and MTS assay

Cells (5,000/well) were seeded in 96-well plates and exposed to serial dilutions (1:3) of the compounds, starting from 10 or 1 μmol/L concentration. Medium alone was used as a control. After 72 hours of incubation, 3H-methyl thymidine (Perkin Elmer) was added to the medium (1 μCi/well) and incubated for 8 hours at 37°C. The cells were then harvested onto glass fiber filters using a Tomtec automated cell harvester and [3H]-thymidine incorporation was measured using a filter scintillation counter (1430 MicroBeta).

Molecular modeling

The three-dimensional coordinates of wild-type (WT) human ALK catalytic domain bound to crizotinib and NVP-TAE684 and of the L1196M human ALK catalytic domain bound to crizotinib were retrieved from the Protein Data Bank (PDB codes: 2XP2, 2XB7, and 2YFX; refs. 35–37). The human L1196Q-ALK models were prepared by mutation of the desired amino acid followed by local minimization using Tripos force field (SYBYL8.0, Tripos). For this operation, the L/M1196 side chain orientation coming from WT and L1196M crystal structures were used to include in the study both possible Q1196 rotamers (here referred to as Q-R1 and Q-R2). Molecular docking with rigid protein was run with the program Surflex-Dock implemented in SYBYL8.0 (Tripos). Docking scores were calculated with the consensus-scoring program CScore implemented in SYBYL8.0 (Tripos; ref. 38).

Statistical analyses

Dose–response curves were analyzed using GraphPad Prism4 software. IC_{50} indicates the concentration of inhibitor that gives half-maximal inhibition. Densitometry values are (ALK treated/ALK untreated) divided by (ACTIN treated/ACTIN untreated) or (P-ALK treated/P-ALK untreated) divided by (ALK treated/ALK untreated). Normalized IC_{50} was calculated as the IC_{50} of the target cell line divided by the IC_{50} of the parental cell line. Therapeutic index was the ratio between IC_{50} of Ba/F3 parental cell line and IC_{50} of WT or mutant NPM-ALK–expressing Ba/F3. MTS assay results were analyzed by Student unpaired t test.

Results

Establishment of crizotinib-resistant cell lines

To establish human ALCI cell lines resistant to crizotinib, we cultured SUP-M2 and K299 in the presence of increasing doses of crizotinib. The selection started using a concentration close to the IC_{50} value (50 nmol/L for both cell lines) and gradually reached a final concentration of 1 μmol/L for K299 and 0.3 μmol/L for SUP-M2. At each step increase, after an initial drop in cell viability and absolute number (Supplementary Fig S1), a stable population able to live and proliferate in the presence of crizotinib was selected and named as CR (crizotinib resistant) followed by the crizotinib concentration [in μmol/L units] in which cells were growing. At each step, we collected and analyzed cells. To check whether resistance was due to NPM-ALK amplification or stabilization, NPM-ALK expression levels were investigated both at the transcriptional level (Table 1), using qRT-PCR, and at the protein level (Fig. 1A). While a moderate increase in NPM-ALK mRNA expression level was noted, we could not detect any significant increase of NPM-ALK protein, except for the K299CR03 cell line, in which ALK expression is quantified by densitometry as 4-fold higher than in K299 parental cell line. However, this increase in protein level was no more detectable in cell lines selected at higher crizotinib doses. Next, we

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Normalized ALK expression</th>
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<td>K299</td>
<td></td>
</tr>
<tr>
<td>K299CR01</td>
<td>1.7</td>
</tr>
<tr>
<td>K299CR03</td>
<td>2.68</td>
</tr>
<tr>
<td>K299CR06</td>
<td>4.19</td>
</tr>
<tr>
<td>K299CR1</td>
<td>4.58</td>
</tr>
<tr>
<td>SUP-M2</td>
<td>1</td>
</tr>
<tr>
<td>SUPM2CR02</td>
<td>3.76</td>
</tr>
<tr>
<td>SUPM2CR03</td>
<td>1.52</td>
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</table>
evaluated cell proliferation rates in the presence of crizotinib for each cell line (Fig. 1B). As expected, IC\textsubscript{50} values increased in proportion to the amount of crizotinib in which cell lines were selected, except for K299CR1 (Table 2). We also checked for NPM-ALK activity in the presence of increasing doses of crizotinib, by analyzing its phosphorylation status. We found that in all resistant cell lines, NPM-ALK phosphorylation was detectable at drug concentrations higher than in the parental cell line, and the increase was proportional to the resistance index (Supplementary Fig. S2A). To assess if ALK intrinsic activity in our resistant cell lines is higher than the one observed in parental cells, we measured ALK phosphorylation status of untreated cells by densitometry and then we normalized this value on ALK expression levels (Supplementary Fig. S2B). In two cell lines, K299CR1 and SUPM2CR03, we observed a slight increase in ALK phosphorylation signal (2.17- and 1.84-fold, respectively). To investigate the presence of point mutations and to assess their frequency within the resistant populations, we sequenced the NPM-ALK kinase domain. Interestingly, we found that in both cell lines, a single NPM-ALK kinase domain mutation became predominant at high doses of crizotinib. In K299 cells, the 4539T\textrarr;A transversion, leading to L1196Q substitution, reached a frequency of 86\% at 0.6 \mu mol/L concentration (K299CR06), whereas in SUPM2CR03 cell line, the double 4464-65TC\textrarr;AT mutation, responsible for the I1171N substitution, was present in 100\% of the analyzed clones (Table 3 and Supplementary Fig. S3).

### Human cell lines carrying L1196Q and I1171N mutations show different sensitivity to other ALK inhibitors

To further investigate the role of mutations found at higher crizotinib doses, we focused our attention on those cell lines expressing the relevant mutation at higher frequency, that is, K299CR06 and SUPM2CR03. We confirmed both cell lines to be resistant to crizotinib and then we challenged them with 2 additional ALK inhibitors: the dual ALK/EGFR inhibitor AP26113 and the diaminopyrimidine NVP-TAE684. We evaluated cellular proliferation in the presence of increasing drug concentrations (Fig. 2A). IC\textsubscript{50} values obtained are summarized in Table 4. In K299CR06 cells, bearing at high frequency the L1196Q substitution, the IC\textsubscript{50} values observed with AP26113 and NVP-TAE684 were 4- and 13-fold higher than in parental cells, respectively, but the absolute values were still low nanomolar (<20 \mu mol/L). In SUPM2CR03 cells, bearing the I1171N substitution, the IC\textsubscript{50} values for AP26113 and NVP-TAE684 were 112 nmol/L and 52 nmol/L, respectively, that is 8- and 10.4-fold higher than in the parental cell line (Table 4). We tested NPM-ALK autophosphorylation by Western blot analysis in the presence of increasing doses of all inhibitors, confirming cell proliferation data (Fig. 2B). We also assessed

| Table 2. IC\textsubscript{50} values obtained by \textsuperscript{3}H-thymidine incorporation test for each crizotinib-resistant human cell line are summarized |
|---------------------|-----------------|-----------------|
| Cell line          | IC\textsubscript{50} (\mu mol/L) | Normalized      |
| K299               | 0.023           | 1.0             |
| K299CR01           | 0.27            | 11.7            |
| K299CR03           | 0.45            | 19.6            |
| K299CR06           | 0.948           | 41.2            |
| K299CR1            | 0.726           | 31.6            |
| SUP-M2             | 0.056           | 1.0             |
| SUPM2CR01          | 0.26            | 4.6             |
| SUPM2CR02          | 0.25            | 4.5             |
| SUPM2CR03          | 0.388           | 6.9             |

NOTE: Each value derives from 2 independent experiments.
the inhibition of STAT3, an ALK downstream target, after each drug administration at different doses. In NPM-ALK–positive cells, STAT3 phosphorylation is ALK-dependent and recapitulates ALK activation. Moreover, ALK phosphorylation status was further analyzed in K299CR06 cells in the presence of lower doses of AP26113 and NVP-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dose (µmol/L)</th>
<th>Mutation</th>
<th>Substitution</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K299</td>
<td>0.1 µmol/L</td>
<td>4539T→A</td>
<td>L1196Q</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4485A→G</td>
<td>N1178S</td>
<td>3</td>
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<td></td>
<td></td>
<td>4596C→T</td>
<td>P1215L</td>
<td>3</td>
</tr>
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<td></td>
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<td>4936G→A</td>
<td>M1328I</td>
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<td>0.3 µmol/L</td>
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<td>L1196Q</td>
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<tr>
<td>SUP-M2</td>
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<td>I1171N</td>
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<tr>
<td></td>
<td>0.3 µmol/L</td>
<td>4464–65TC→AT</td>
<td>I1171N</td>
<td>100</td>
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Figure 2. Human cell lines carrying dominant NPM-ALK mutations were characterized. A, dose–response curves obtained by 3H-thymidine incorporation assay after 72-hour treatment with crizotinib, AP26113, and NVP-TAE684. IC50 values related are summarized in Table 4. B, ALK and STAT3 phosphorylation status was assessed by Western blot analysis after 4-hour incubation with different concentrations of crizotinib, AP26113, or NVP-TAE684. C, apoptosis was quantified after 72-hour treatment with crizotinib, AP26113, and NVP-TAE684 using Annexin V–PI staining and analyzed by flow cytometry. D, after 72 hours of incubation with crizotinib, AP26113, and NVP-TAE684, cell viability was analyzed by MTS assay. K299CR06 viability was significantly reduced after AP26113 (P < 0.0001) and NVP-TAE684 (P = 0.0008) exposure but not in response to crizotinib, whereas SUPM2CR03 were not affected by any kind of treatment. Values obtained derive from at least 2 independent experiments.
TAE684, and we observed a complete loss of NPM-ALK phosphorylation at 10 nmol/L AP26113 and NVP-TAE684, in agreement with values obtained by thymidine incorporation rate (Supplementary Fig. S4). We then studied induction of apoptosis in the presence of 300 nmol/L crizotinib, 20 nmol/L AP26113, or 20 nmol/L NVP-TAE684, using ANNEXIN V–PI staining and extended the analysis to cell viability at the same drug doses using MTS assay. We found that in K299CR06 cells treated with crizotinib apoptosis was comparable with the untreated control, whereas significant apoptosis was induced in the K299 parental line (P < 0.05). In contrast, K299CR06 cells treated with AP26113 or NVP-TAE684 were as sensitive as the parental cells. SUPM2CR03 survived in the presence of all drugs (Fig 2C). Interestingly, no apoptosis was noted in SUPM2CR03 cells even at higher concentrations of AP26113 and NVP-TAE684 (100 nmol/L; Supplementary Fig. S5). MTS assay confirmed Annexin data, (Fig. 2D), and these results are consistent with the resistance profiles previously found.

Together, these data indicate that the L1196Q mutation is highly resistant to crizotinib but sensitive to AP26113 and

Table 4. IC₅₀ values, expressed in μmol/L, obtained for K299CR06 and SUPM2CR03 cell lines and the same values normalized on parental cell line, are summarized

<table>
<thead>
<tr>
<th></th>
<th>Crizotinib</th>
<th>AP26113</th>
<th>NVP-TAE684</th>
</tr>
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<tbody>
<tr>
<td>IC₅₀</td>
<td>Norm</td>
<td>IC₅₀</td>
<td>Norm</td>
</tr>
<tr>
<td>IC₅₀</td>
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<td>K299CR06</td>
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<td>SUP-M2</td>
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<td>SUPM2CR03</td>
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NOTE: All values shown derive from at least 2 independent experiments.

Figure 3. Characterization of Ba/F3 cell lines stably transfected with WT or mutated human NPM-ALK. A, cell lines were cultured for 72 hours in the presence of crizotinib, AP26113, and NVP-TAE684 at different concentrations; ³H-thymidine incorporation was then measured. Curves derive from at least 2 independent experiments. Corresponding IC₅₀ values are summarized in Table 5. B, ALK phosphorylation status in the presence of increasing doses of crizotinib, AP26113, and NVP-TAE684. Activation of the main ALK downstream effector STAT3 is also shown. ACTIN was used as a loading control.
NVP-TAE684, whereas the I1171N mutant is resistant to these drugs.

Effects of crizotinib, AP26113, and NVP-TAE684 in Ba/F3 cells carrying NPM-ALKL1196Q and NPM-ALKI1171N mutants

To evaluate the actual biologic role of L1196Q and I1171N mutations out of the context where they were identified, we studied their effects in the Ba/F3 cellular model transfected with human NPM-ALK. We introduced both mutations in Ba/F3 NPM-ALK cell lines (Supplementary Fig. S6) and we selected 2 new cell lines, named Ba/F3 NA L1196Q and Ba/F3 NA I1171N. Both new cell lines were able to grow in the absence of IL-3 in the medium and showed comparable NPM-ALK expression levels (data not shown). We evaluated their proliferation when exposed to crizotinib, AP26113, and NVP-TAE684 (Fig. 3A). All IC50 values and relative therapeutic indexes are summarized in Table 5. Both cell lines are resistant to crizotinib, but show different sensitivity to the 2 other compounds. While Ba/F3 NA L1196Q are sensitive to both AP26113 and NVP-TAE684, with an IC50 of 9 nmol/L, Ba/F3 NA I1171N are resistant to both drugs, with an IC50 of 329 and 759 nmol/L, respectively. We also checked the phosphorylation status of ALK and its downstream effector STAT3 after treatment with several drug doses (Fig 3B). Resistance to crizotinib was confirmed for both cell lines, whereas sensitivity to AP26113 and NVP-TAE684 differed: in Ba/F3 NA L1196Q, phosphorylated (p)-ALK and pSTAT3 signals disappear after 4 hours at 0.1 μmol/L AP26113 and NVP-TAE684, whereas in Ba/F3 NA I1171N cells, the band is still present at 0.3 μmol/L of both drugs. This finding is consistent with IC50 values previously shown. As a comparison, the well-known mutant L1196M was analyzed and showed a similar pattern to L1196Q (Fig 3B). These data are in line with results from the original human crizotinib-resistant cell lines, K299CR06 and SUPM2CR03.

Computational studies

The results obtained with crizotinib (PF-02341066) and NVP-TAE684 on WT-, L1196Q-, and I1171N-ALK in Ba/F3 cells were investigated computationally. As AP26113 structure is not available, we could not extend our analysis to this drug.

The inhibitory trend of crizotinib and NVP-TAE684 in L1196Q-ALK cells is similar to the one observed with the L1196M mutant. The consistent difference in activity between the 2 inhibitors was then studied through molecular docking into the L1196Q-mutant model.

The protocols were validated by docking the ligand back to its binding pocket in the crystal structure. Crizotinib established hydrogen bonds with backbone atoms from 2 hinge region residues, M1199-NH and E1197-CO, with low all-atoms root-mean-square deviation (RMSD) compared with the crystallographic orientation and with a score of 7.16 (Table 6). Crizotinib was then docked into the L1196Q-ALK model using both Q-R1 and Q-R2 rotamers (see Material and Methods section). Crizotinib did not dock into the Q-R1 model, whereas it docked into Q-R2 model in BM2 mode, with a score of 6.73 (Table 6). Thus, in the case of crizotinib, the possibility of docking into the ATP site is clearly affected by the type of Q-rotamer used. In cells, the inhibitor is less active towards the L1196Q mutant compared with WT. This might be caused by the flexibility and the size of the gatekeeper

Table 5. IC50 values, expressed in μmol/L, obtained for each Ba/F3 cell line and relative therapeutic index (TI) calculated as IC50 parental line/IC50 mutant, are summarized

<table>
<thead>
<tr>
<th></th>
<th>CRIZOTINIB</th>
<th>AP26113</th>
<th>NVP-TAE684</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50</td>
<td>Norm</td>
<td>T.I. IC50</td>
</tr>
<tr>
<td>Ba/F3</td>
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NOTE: IC50 values obtained derive from at least 2 independent experiments.

Table 6. Docking scores of crizotinib and NVP-TAE684 on human WT and L1196Q ALK

<table>
<thead>
<tr>
<th></th>
<th>WT-ALK (PDB 2XP2)</th>
<th>WT-ALK (PDB 2XB7)</th>
<th>L1196Q-ALK (Q-R1)</th>
<th>L1196Q-ALK (Q-R2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crizotinib</td>
<td>7.16</td>
<td>-</td>
<td>ND</td>
<td>6.73</td>
</tr>
<tr>
<td>NVP-TAE684</td>
<td>-</td>
<td>5.67</td>
<td>4.47</td>
<td>5.63</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not defined.
glutamine side chain not favoring energetically the binding of the inhibitor. (Fig. 4B).

The same procedure was used with NVP-TAE684 starting with the validation of the protocol. The inhibitor docked into WT-ALK model establishing 2 hydrogen bonds with only one hinge region amino acid, M1199-NH and –CO, with low all-atoms RMSD compared with the crystallographic orientation and with a score of 5.67 (Table 6). Then, NVP-TAE684 was docked in L1196Q-ALK model using again Q-R1 and Q-R2 rotamers. In contrast to crizotinib, NVP-TAE684 docked into both models in a BM1 mode with a score of 4.47 (Q-R1) and 5.63 (Q-R2; Table 6). In the latter case, the ligand establishes a second hydrogen bond between its sulphonic group and the side chain of Q1196. (Fig. 4C). In cells, the inhibitor is active against both WT and mutant forms. This can be explained by thermodynamically favorable binding poses into the ATP site independently of the orientation of the gatekeeper glutamine side chain.

Residue I1171 is distant from the ATP binding site and thus, mutations were not studied with molecular docking because they were unable to affect WT docking outputs. In addition, for both inhibitors the activity in cells against this mutant is similar. Hence, we tried to find out a common cause, not related to the structure of the ligand, explaining the observed decrease in the biologic response. The hydrophobic residue I1171, together with C1182, F1271 (DGF motif), H1247 (HRD motif), and the polar D1311 (F-helix) define the hydrophobic R-spine (regulatory spine) connecting the 2 lobes of the kinase (Fig. 4D; refs. 39, 40). All structural motifs important in the catalytic cycle are connected to the F-helix, which in this case, is represented by the aspartic acid D1311. (39, 40). The R-spine, together with the salt bridge E1167-K1150, stabilizes the active conformation of the ALK (35, 36). According to our model, mutations that alter this hydrophobic assembly, like I1171N, decrease the stability of the inhibitor-bound DFG in conformation. In addition, the mutation of I1171 to Asn allows the formation of a hydrogen bond between the side chain of N1171 and the backbone carbonyl CO of V1180, as previously reported in literature (36). The novel interaction maintains the R-spine compact, balancing the disruptive effect of the polar Asn residue on the hydrophobic assembly. This will ultimately stabilize the activated conformation while destabilizing inhibitors binding. The described
destabilizing event takes place independently of the structure and binding mode of the inhibitor. This explains both crizotinib (BM2 mode) and NVP-TAE684 (BM1 mode) decreased activity in I1171N-ALK cells.

**Discussion**

In the present work, we selected human ALK+ lymphoma cell lines resistant to different concentrations of crizotinib, a dual ALK and MET inhibitor developed by Pfizer. Crizotinib has recently been approved for the treatment of advanced lung cancer and is in clinical trial for other ALK-related diseases. We preferred human cell lines over the broadly used Ba/F3 cells for our screening, because they represent a more relevant biologic model of the disease. Analysis of resistant cell lines and following cloning and direct sequencing of NPM-ALK kinase domain showed an interesting scenario, where in both cell lines, a single mutant clone becomes predominant at higher crizotinib doses. After sequencing, we found L1196Q in 85.7% of screened K299CR06 clones and I1171N in 100% of SUPM2CR03 clones. The frequency of the L1196Q clone diminished in the K299CR1 population (58%), maybe because other mechanisms of resistance involving different effectors were randomly selected while subculturing. In fact, detailed analysis of K299CR1 population revealed that 33% of clones carry WT NPM-ALK kinase domain, compared with only 14% of K299CR06 clones (data not shown). However, we can exclude gene amplification and mRNA or protein stabilization as causes of resistance (Table 1 and Fig. 1A). We can also exclude that observed resistance was related to enhanced ALK intrinsic activity, despite we observed a slight increase in basal ALK phosphorylation status (Supplementary Fig. S2B). We could not select a SUP-M2 cell line at crizotinib concentrations higher than 0.3 μmol/L. We hypothesize that the selected I1171N substitution was sufficient to confer resistance at this dose, but not at higher doses. This hypothesis is consistent with the work of Zhang and colleagues who, in a different cellular model (Ba/F3 EML4-ALK), could observe I1171N appearance, but this substitution disappeared at doses more than 720 nmol/L (41). Whether such mutation will have a clinical significance, remains to be verified. However, if we refer to imatinib experience in CML context, some mutations with a low in vitro resistance profile are indeed clinically relevant (42, 43), so we cannot exclude that, despite I1171N mutation was found at a relatively low crizotinib dose, it may have a significant role in the clinic. Of note, the reported plasma concentration of crizotinib at the recommended dose (250 mg twice daily) is 0.57 μmol/L (44). From a patient’s viewpoint, I1171N-mutant cells may respond to a crizotinib dose increase. Leucine 1196 is the so-called “gatekeeper” residue (45); it is a part of the N-lobe binding to ATP, and it is located in the vicinity of the inhibitor (Fig. 4A). In ALK, mutation of L1196 to methionine (L1196M) leads to clinical forms of cancer drug resistance (26). As reported in literature, crizotinib is less active against the L1196M mutant. In contrast, NVP-TAE684 maintains the same activity as on the WT kinase (41). In our screening, a different mutation of the gatekeeper was selected by crizotinib, namely a L1196Q substitution. The same mutant has been recognized as an important feature of tyrosine kinases. Molecular studies led to the hypothesis that the high resistance observed toward all inhibitors in our NPM-ALK I1171N cell lines may be due to an alteration of the kinetics of ALK structural plasticity, independently from the structure of the inhibitor and its binding mode. Mutation of hydrophobic Ile to a polar Asn residue is likely to be destabilizing for the R-spine and for the conformation that is bound by the inhibitors. However, the additional hydrogen bond with V1180 will possibly compensate and contribute to maintain an activated state of the kinase. Alternatively, as proposed by Bossi and colleagues (36), the mutation may have no effect on inhibitor binding, but simply increase catalytic efficiency: according to this model, the N1171-V1180 hydrogen bond stabilizes the R-spine and, adds to the E1167-K1150 salt bridge, and shifts the thermodynamic equilibrium toward a fully active conformation. In neuroblastoma, I1171N is an activating mutation and thus, the plasticity of the ALK-KD plays a major role in favoring a particular conformation of the mutant. In any case, I1171N is predicted to modify the structure of the kinase-inhibitor complex. More detailed computational studies need to be conducted to increase our understanding of I1171N and other resistant ALK-mutant forms distant from the ATP site.

AP26113 is a dual ALK and EGFR inhibitor developed by ARIAD now in phase I/II clinical trial (NCT01449461). Preclinical data presented at AACR 2010 by Zhang and colleagues (32) and Rivera and colleagues (47) were promising, showing a potent, selective, and orally active compound. AP26113 may soon become a second-line therapy for ALK-related diseases. While cells carrying L1196Q substitution are slightly less sensitive to AP26113 compared...
with the parental cell lines, the IC_{50} values are still low nanomolar (Tables 4 and 5) so we consider these cell lines as sensitive to the drug, as was also shown by cell death assays in Fig. 2C and 2D. In contrast, cells carrying the I1171N mutation are resistant to AP26113. Moreover, this mutation showed persistent ALK phosphorylation in Western blotting and protected from cell death. Unfortunately, AP26113 structure is not available. So, we could not conduct computational studies to explain the molecular mechanisms underlying our biologic results. However, these data provide important information to guide the oncologist towards the most effective therapeutic option for those patients carrying I1171N mutation.

In conclusion, we selected 2 mutations in the ALK kinase domain of NPM-ALK that confer resistance to crizotinib, using human ALK+ lymphoma cell lines as a model. While L1196Q was sensitive to crizotinib-unrelated compounds AP26113 and NVP-TAE684, I1171N was resistant to both drugs, encouraging or excluding all related compounds as possible therapeutic options in case of relapse resistant to both drugs, encouraging or excluding all related compounds as possible therapeutic options in case of relapse.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Ceccon

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ceccon, L. Mologni, W. Busson, L. Scapozza

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Scapozza

Study supervision: L. Mologni, C. Gambacorti-Passerini

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