Treatment Efficacy and Resistance Mechanisms Using the Second-Generation ALK Inhibitor AP26113 in Human NPM-ALK-Positive Anaplastic Large Cell Lymphoma

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Abstract

ALK is a tyrosine kinase receptor involved in a broad range of solid and hematologic tumors. Among 70% to 80% of ALK+ anaplastic large cell lymphomas (ALCL) are caused by the aberrant oncogenic fusion protein NPM-ALK. Crizotinib was the first clinically relevant ALK inhibitor, now approved for the treatment of late-stage and metastatic cases of lung cancer. However, patients frequently develop drug resistance to Crizotinib, mainly due to the appearance of point mutations located in the ALK kinase domain. Fortunately, other inhibitors are available and in clinical trial, suggesting the potential for second-line therapies to overcome Crizotinib resistance. This study focuses on the ongoing phase I/II trial small-molecule tyrosine kinase inhibitor (TKI) AP26113, by Ariad Pharmaceuticals, which targets both ALK and EGF. Two NPM-ALK+ human cell lines, KARPAS-299 and SUP-M2, were grown in the presence of increasing concentrations of AP26113, and eight lines were selected that demonstrated resistance. All lines show IC50 values higher (130 to 1,000-fold) than the parental line. Mechanistically, KARPAS-299 populations resistant to AP26113 show NPM-ALK overexpression, whereas SUP-M2-resistant cells harbor several point mutations spanning the entire ALK kinase domain. In particular, amino acid substitutions: L1196M, S1206C, the double F1174V+L1198F and L1122V+L1196M mutations were identified. The knowledge of the possible appearance of new clinically relevant mechanisms of drug resistance is a useful tool for the management of new TKI-resistant cases.

Implications: This work defines reliable ALCL model systems of AP26113 resistance and provides a valuable tool in the management of all cases of relapse upon NPM-ALK–targeted therapy. Mol Cancer Res; 13(4); 775-83. ©2014 AACR.
to the positive selection of mutant clones have already been determined in several Crizotinib-treated NSCLC and ALCL patients (20, 23–26). To effectively overcome Crizotinib resistance, the development of second-generation ALK inhibitors is exponen-
tially growing (27). Currently, several ALK inhibitors are already in clinical trial: the phase II/III ceritinib (LDK-378, Novartis), the phase I/II alecinib (CH5424802, Roche), and the phase I ASP3026 (Astellas). In this work, we decided to focus our attention on the dual ALK/EGFR inhibitor AP26113 because, after Crizotinib, it was the first inhibitor that entered in clinical trials. Preliminary data showed that this inhibitor is 10-fold more potent than Crizotinib and active against the gatekeeper mutant (28). Unfortunately, the chemical structure remains undis-
closed. In this article, we selected 8 new human NPM-ALK+ cell lines, 4 derived from KARPAS-299 and 4 from SUP-M2, able to live and proliferate at high AP26113 doses (K299AR300A, K299AR300B, K299AR300C, K299AR300D, SUP-M2AR500A, SUP-M2AR500B, SUP-M2AR500C, and SUP-M2AR500D). For KARPAS-299–derived cell lines, we observed oncogene over-
expression as the main resistance mechanism, whereas in SUP-
M2–derived cell lines, we identified several point mutations located within the NPM-ALK kinase domain, which could explain drug resistance. We also found an L1196M mutation in two of four SUP-M2–derived cell lines, but it had no role in conferring resistance at high drug doses. To find a way to overcome AP26113 resistance, we explored the cross-resistance of our KARPAS-299–derived cell lines and mutated Ba/F3 NPM-
ALK against other clinically relevant ALK inhibitors nowadays available, namely Crizotinib, ceritinib, alecinib, and ASP3026. KARPAS-299–derived cell lines are highly resistant to all inhibi-
tors tested, whereas all mutants studied were targetable with at least a compound, except S1206C. Collectively, our data predict in a human cell-based model the appearance of different mechanisms of resistance to AP26113, and we explored different ways to overcome resistance using a set of clinically relevant ALK inhibitors. This kind of knowledge is a powerful tool to manage clinical cases of Crizotinib and AP26113 relapse.

**Materials and Methods**

**Cell lines, inhibitors, and selection of AP26113-resistant cell lines**

The human NPM-ALK+ KARPAS-299 and SUP-M2 cell lines bearing the t(2;5) translocation and the pro-B murine cell line Ba/F3 were purchased from DSMZ, where they are routinely veri-

**Site-directed mutagenesis**

pcDNA3.0 bearing human WT NPM-ALK (pcDNA3.0 NA) was kindly provided by Dr. S.W. Morris (St Jude Research Hospital, Memphis, TN). Site-directed mutagenesis on pcDNA3-NA was performed using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer’s instructions. Primers used for mutagenesis were as follows: NPM-ALK L1122V FW: 5’-ATCACCGCTACCGGATGCGGCGCGCC-3’; NPM-
ALK S1206C FW: 5’-GAGACACTCAAGTCTCTCCGAGAGACCCGCC-3’; NPM-
ALK L1196M FW: 5’-GTTCATCGTGCTGAGTGTAGGCTAGGGGAG-3’. PCR products were sent to MWG Operon for sequencing. Quantitative real-time PCR (qRT-PCR) was performed as previously described (29). Housekeeping genes used for normalization were murine HPRT FW: 5’-TCAGTCAGCGCAGGCGAGGCAATAAAA-3’; REV: 5’-GGGGCGTCATCAGCTCAAGCAG-3’. Deep sequencing was performed as previously described (20).

**PCR, quantitative RT-PCR, and sequencing**

An NPM-ALK fragment encompassing the breakpoint and comprising the whole kinase domain was amplified by PCR using high-fidelity Taq polymerase (Roche), according to instructions. Primers used were FW: 5’-TGATATATAAACTTTCGAGACCC-3’; REV: 5’-CTGCAAAACAGGGAGCCGTAC-3’. PCR products were sent to Eurofins MWG Operon for sequencing. Quantitative real-time PCR (qRT-PCR) was performed as previously described (29). Housekeeping genes used for normalization were murine HPRT FW: 5’-TCAGTCAGCGCAGGCGAGGCAATAAAA-3’; REV: 5’-GGGGCGTCATCAGCTCAAGCAG-3’. Deep sequencing was performed as previously described (20).

**Exome sequencing**

Exome libraries were generated from 1 µg of genomic DNA extracted with a PureLink Genomic DNA kit (Life Technology). Genomic DNA was fragmented to a size of 200 to 500 bp and processed according to the standard protocol for the Illumina TruSeq DNA Sample Preparation Kit. Genomic libraries were then enriched with the Illumina TruSeq Exome Enrichment Kit. Libraries were sequenced on an Illumina Genome Analyzer IIX with 76-bp paired-end reads using Illumina TruSeq SBS kit v5.

**Bioinformatics**

Image processing and base calling were performed using the Illumina Real Time Analysis Software RTA v1.9.35 or newer. Qseq files were deindexed and converted to the Sanger FastQ file format using in-house scripts. FastQ sequences were aligned to the human genome database (NCBI Build 36/hg18) using the Burrows-Wheeler NGS short-reads aligner tool. The alignment files (SAM format) were processed with SAMtools (30): they
were initially filtered by proper pair, then converted into the binary BAM alignment format, sorted, and indexed. Removal of duplicates was performed using the SAMtools rmdup command. Unique BAM files were then converted into the mpileup format. mPileup data generated from paired cancer and control samples were cross-matched using a dedicated in-house software tool. Copy number and allelic imbalance/loss of heterozygosity analyses from whole-exome data were performed using CEQer (31).

Western blotting and antibodies

Cells were seeded in complete medium in 12-well plate, and compounds were added at different concentrations. After 4 hours, cells were harvested, washed once in PBS at 4°C, and resuspended in Laemmli buffer 1× supplemented with 10% β-mercaptoethanol (100 μL/10⁵ cells) and denatured at 97°C for 20 minutes before electrophoresis. Equal volumes were loaded on 10% SDS-PAGE, transferred to nitrocellulose membrane Hybond ECL (Amersham), and incubated overnight at 4°C with primary antibody (1:1,000 dilution in BSA 2.5%). Secondary horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies (Amersham) were incubated for 1 to 2 hours and then visualized by chemiluminescence as recommended by the manufacturer. Monoclonal anti-phospho-ALK (Y-1604), monoclonal anti-ALK (31F12), and monoclonal anti–phospho-STAT3 (Tyr 705) antibodies were from Cell Signaling Technology. Anti-ACTIN antibody was purchased from Sigma; polyclonal anti-STAT3 is from Calbiochem.

Proliferation assay

Five thousand cells per well were seeded in 96-well plates in the presence of serial dilutions (1:2 or 1:3) of each compound, starting from a concentration of 10 μmol/L or 1 μmol/L, based on drug potency and specific cell line sensitivity. Incubation with radioactive-labeled thymidine and radioactivity detection was performed as previously described (29).

Software and statistical analysis

Dose–response curves were analyzed using GraphPad Prism 5 software. IC₅₀ indicates the concentration of inhibitor that gives half-maximal inhibition. Densitometry values are calculated as follows: total ALK level for each well is normalized on its own loading control (ALK/ACTIN). Values are the average of at least three independent wells. P-ALK value is normalized both on the untreated sample and on the total ALK level: (P-ALK treated/ P-ALK untreated) divided by (ALK treated/ALK untreated). Relative resistance (RR) index was calculated as the ratio between mutant and WT IC₅₀ values (32). qPCR data were analyzed using the ΔΔCT method, normalized on the proper housekeeping gene. ALK kinase domain was drawn using PDB viewer software (PDB code:3LCS).

Results

Establishment and characterization of human AP26113-resistant cell lines

To obtain a reliable resistance model, we grew two different human NPM-ALK–positive cell lines, KARPAS-299 and SUP-M2, in the presence of increasing AP26113 doses (29). We divided each cell line into four different subpopulations, referred to as resistant populations, referred to as KARPAS-299 and SUP-M2 cells. In our experience, for a highly drug-resistant population, an IC₅₀ value of at least 10-fold compared with parental cells was expected. In fact, all AP26113-resistant cell lines showed a RR index higher than 100 (Table 1). Among all cell lines established, we decided to focus our attention on the highest AP26113 dose–resistant populations, referred to as KARPAS-299 and SUP-M2. These values were used to establish a resistance model of ALK kinase domain was drawn using PDB viewer software (PDB code:3LCS).

Identification of resistance mechanism

Western blot analysis revealed an increase in NPM-ALK expression, quantified by densitometry at 9.4 ± 2.1, 6.0 ± 1.3, 7.1 ± 1.8, and 6.5 ± 0.5-fold compared with parental cells for K299AR300A, K299AR300B, K299AR300C, and K299AR300D cells, respectively (Supplementary Fig. S2). Notably, in the first three cell lines (A, B, and C), the basal phospho-ALK signal was higher than in parental cells (Fig. 1A). This hyperactivation could be simply due to the increased NPM-ALK expression rather than to an intrinsic NPM-ALK hyperactivation, as highlighted in Supplementary Fig. S2. To further confirm that in AP26113-resistant cell lines, NPM-ALK targeting was ineffective, the phosphorylation status in Tyr705 of the NPM-ALK downstream effector STAT3 was also assessed. In all AP26113-resistant KARPAS-derived cell lines, STAT3 P-Tyr705 was present at higher drug doses than the one observed for parental cells and correlated with the persistent NPM-ALK phosphorylation (Fig. 1A). qRT-PCR confirmed that oncogene overexpression was present also at transcriptional level, because a 3.7-, 16-, 25-, and 5.1-fold increase of NPM-ALK transcript was detected in K299AR300A, B, C, and D, respectively, compared with parental (Fig. 2A; Table 2). Values are obtained upon normalization on the proper housekeeping gene. Of note, protein and mRNA levels correlated. Moreover, a FISH experiment

| Table 1. IC₅₀ values obtained for AP26113-resistant KARPAS-derived cell lines and SUP-M2-derived cell lines |
|----------------|----------------|----------------|
| Cell line     | IC₅₀ (μmol/L) | RR Index |
| K299          | 0.001         | 1         |
| K299AR300A    | 0.1799        | 179.9     |
| K299AR300B    | 0.205         | 201.5     |
| K299AR300C    | 0.1224        | 132.4     |
| K299AR300D    | 0.1804        | 180.4     |
| SUPM2         | 0.004         | 1         |
| SUP-M2AR500A  | 0.9984        | 249.6     |
| SUP-M2AR500B  | 0.9068        | 226.7     |
| SUP-M2AR500C  | 0.4491        | 112.275   |
| SUP-M2AR500D  | 0.4071        | 101.775   |

value, calculated with a preliminary proliferation assay as 13 nmol/L for KARPAS-299 and 19.4 nmol/L for SUP-M2. Each cell line was challenged with five or six sequential drug doses (Supplementary Fig. S1). An independent cell line was established on drug potency and specificity of drug resistance. Incubation with radioactive-labeled thymidine and radioactivity detection was performed as previously described (29).

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revealed that the increase in NPM-ALK overexpression is due to gene amplification (Fig. 2B). Because the IC_{50} value observed for all resistant cell lines is extremely high, we explored the presence of low-frequency point mutations in ALK kinase domain as an additional resistance mechanism by deep sequencing. The results excluded this hypothesis (data not shown). NPM-ALK overexpression in K299AR300D was less evident than in the other cell lines. Moreover, the band corresponding to P-Tyr1604 ALK disappears at 300 nmol/L, suggesting low molecular resistance to AP26113, despite the fact that STAT-3 phosphorylation remains also at high drug doses and the cells RR index was 180. Whole-exome sequencing and copy-number analysis of this cell line highlighted that the increase in ALK expression was due to NPM-ALK amplification. In addition, some huge copy-number alterations were present. Also missense mutations were detected, but none of them involved proteins clearly related to tumorigenesis or drug resistance (Supplementary Fig. S3).

Also in SUP-M2–derived resistant cells, we could observe a great increase in IC_{50} value compared with SUP-M2 parental cell line, and, as expected, this corresponded to a persistent P-Tyr1604

Figure 1.
Human cell lines characterization. Parental or resistant KARPAS-299 (A) or SUP-M2 (B) cell lines were incubated for 4 hours in the presence of increasing AP26113 concentrations: 0, 100, 300, and 1000 nmol/L. P-ALK (Tyr1604), ALK, P-STAT3 (Tyr705), STAT3, and βACTIN expression levels were assessed by Western blot.

Figure 2.
Mechanism of AP26113 resistance in KARPAS and SUP-M2 cell lines. A, NPM-ALK expression at transcriptional level in KARPAS and SUP-M2 cells grown respectively at AP26113 concentration of 300 and 500 nmol/L were investigated by quantitative real-time PCR. B, gene amplification is shown by FISH analysis. C, chromatograms related to all mutations found in AP26113-resistant SUP-M2–derived cell lines are shown and compared with the parental SUP-M2 cells.
mements, a Ba/F3 cell line expressing the single NPM-ALK D1203N able to survive in the absence of IL3. Moreover, the double L1196M+D1203N mutant is highly AP26113 resistant, so it is possible that both mutations are required for an advantageous selection and drug resistance. We detected also the presence of the P1139S mutation in one clone of 15, but it is not able to confer high AP26113 resistance (IC50 = 0.015 μmol/L; RR index = 2.14).

Interestingly, F1174V and L1198F mutations were not able to induce resistance to AP26113 singularly, but their cooperation was necessary.

Cross-resistance to other ALK inhibitors

to explore a possible way to overcome AP26113 resistance, we tested the sensitivity of KARPAS-derived cell lines and mutated NPM-ALK—expressing Ba/F3 cells to other clinically relevant ALK inhibitors: Crizotinib, ceritinib, alecitinib, and ASP3026. As expected, all KARPAS-299—derived cell lines are highly resistant to all inhibitors, according to the proliferation assay (Tables 8 and 9). These data are consistent with the observation that in K299AR300A, B, and C cell lines, a general mechanism of drug resistance, oncogene amplification, has been selected.

We also challenged Ba/F3 NPM-ALK WT and mutated cell lines with all the ALK inhibitors (Tables 4 and 5). Cells carrying the NPM-ALK L1122V mutation are moderately resistant to Crizotinib and resistant to alecitinib, ceritinib, and ASP3026 (RR index = 2.6, 5.3, 9.1, and 4.9, respectively); our data about the gatekeeper mutant L1196M suggest a moderate resistance to AP26113, Crizotinib, and alecitinib (RR = 2.1, 3.4, and 2.9), confirming our previous data (29), resistance against ASP3026 and sensitivity to ceritinib. Combination of L1122V with the L1196M substitution increases the resistance values of all drugs, especially of AP26113, thus giving an impressive advantage upon treatment with all compounds that directly inhibit the target. Ba/F3 NPM-ALK bearing the S1206C substitution are resistant to ceritinib, and ceritinib (RR = 4.3, 5.7, and 4.1) and highly resistant to ASP3026. The double F1174V+L1198F mutant is resistant to all drugs except crizotinib, with an RR index of 12.2 and 10 for AP26113 and ceritinib and 9.0 and 5.8 for alecitinib and ASP3026, respectively. Interestingly, the single F1174V mutation is completely sensitive to all drugs, AP26113 included, but

Table 2. Values obtained for NPM-ALK expression by QRT-PCR, in terms of absolute number and fold change compared with parental cells.

Table 3. Mutations reported by direct sequencing.

Table 4. IC50 values obtained by proliferation assay for each Ba/F3 NPM-ALK WT or mutagenized cell line are summarized.
resistant to alectinib and ASP3026, whereas the single L1198F is per se resistant to all drugs except Crizotinib. Together, these two mutations cooperate in conferring higher resistance to AP26113. Moreover, we explored the cross-resistance of other two mutations found at lower frequencies in SUP-M2AR500B cell line, namely P1139S and D1203N. Although P1139S mutant is sensitive to all drugs except ceritinib (RR index = 3.5), we could not establish an IL3-independent Ba/F3 cell line carrying the single D1203N substitution. On the other hand, the double L1196M + D1203N mutant is highly resistant to AP26113 (RR index = 33.2), confirming that the latter may be the driver mutation for this compound. Cell proliferation data are confirmed by Western blot analysis (Supplementary Fig. S5).

In conclusion, according to these data, we can foresee that for each mutation, alone or in combination, except S1206C, there is a clinically available ALK inhibitor able to overcome acquired resistance to AP26113.

**Discussion**

In the last 4 years, the management of ALK-related diseases has successfully changed, thanks to the availability of a new ALK inhibitor, Crizotinib. However, as expected, the problem arising from drug resistance soon became reality, in fact several patients relapsed after Crizotinib treatment, mainly because of the positive selection of point mutations. So, other different ALK inhibitors became necessary to clarify this issue (Supplementary Fig. S3). For all NPM-ALK overexpression was less evident in KARPAS-derived cell lines, both at protein and at transcriptional levels, mechanism. NPM-ALK overexpression was less evident in KARPAS-derived cell lines, both at protein and at transcriptional levels, and is clearly due to NPM-ALK amplification. The presence of copy-number alterations spread throughout all the genome may explain the drug resistance. However, further studies will be necessary to clarify this issue (Supplementary Fig. S3).

### Table 5.

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<th>Clone</th>
<th>Mutations</th>
<th>Substitutions</th>
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<tbody>
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<td>#1</td>
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<td>4544 C&gt;T</td>
</tr>
<tr>
<td>#2</td>
<td>4472 T&gt;G</td>
<td>4544 C&gt;T</td>
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<td>4544 C&gt;T</td>
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<tr>
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<td>4472 T&gt;G</td>
<td>4544 C&gt;T</td>
</tr>
<tr>
<td>#5</td>
<td>4472 T&gt;G</td>
<td>4544 C&gt;T</td>
</tr>
</tbody>
</table>

**NOTE:** For each clone, the mutations and consequent aminoacidic substitutions are reported.
Table 7. Clonal sequencing of SUPM2AR500B

<table>
<thead>
<tr>
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<th>Mutations</th>
<th>SUP-M2AR500B</th>
<th>Substitutions</th>
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The AP26113 resistant clone #15 is sensitive to AP26113 (RR index = 1.8). Thus, we can speculate that, in this clone, other unknown mechanisms may cooperate in its positive selection. F1174 is located at the end of the αC helix (Supplementary Fig. S6) and lies in a hydrophobic cluster composed by F1098, F1271, and F1245. Mutations involving F1174 were recognized as activating in neuroblastoma and phenylalanine substitution with a leucine was found in an IMT patient that relapsed after Crizotinib treatment (24). Clones carrying cysteine, valine, or isoleucine in residue 1174 instead of phenylalanine were selected at AP26113 100 nmol/L and 200 nmol/L in a previous Ba/F3 screen (34). In our screening, Ba/F3 cells bearing the single mutation are completely sensitive to all drugs studied, including AP26113. L1198F alone in our Ba/F3 cells was sufficient to confer resistance to AP26113, alecinib, ceritinib, and ASP3026; however, it was completely sensitive to Crizotinib. A methionine substitution in this position was predicted to confer resistance to AP26113 at 100 nmol/L (34), whereas a proline was predicted to confer crizotinib resistance in an in vitro screening (36). This residue corresponds to the Abl F317, a site that, if mutated, induces resistance to several TKIs. Notably, in our model, L1198F and F1174V cooperate in conferring resistance against AP26113. Finally, S1206 is located into the αD helix. A tyrosine substitution was found in an NSCLC patient that relapsed after Crizotinib treatment, whereas substitutions with an arginine, an isoleucine, or a glycine were predicted in vitro as resistant to AP26113. Notably, the S1206C was the only mutation detected at AP26113 500 nmol/L, indicating that this residue has a key role in conferring high AP26113 resistance (34). All data obtained by 3H thymidine incorporation test were validated by Western blot (Figs. 1 and 3), evaluating both NPM-ALK and downstream target STAT3 phosphorylation in Tyrosine 705. The pattern of STAT3 phosphorylation recapitulates the one found for NPM-ALK; moreover, in some cases it appears even stronger, likely because NPM-ALK-driven signaling is amplified while transduced. Targeting the molecular chaperon HSP90 has been proposed as an alternative way to hit NPM-ALK and overcome TKI’s resistance, because NPM-ALK is a well-known HSP90 client. For this reason, we tested all our NPM-ALK-overexpressing KARPAS-derived cell lines and Ba/F3 cells bearing all single and double mutations against the HSP90 inhibitor 17-AAG (Supplementary Table S1). AP26113-resistant KARPAS cells seemed to be more sensitive to 17-AAG than to other TKIs, whereas all mutations except the S1206C were sensitive to the inhibitor, and this could be due to the vast heterogeneity of HSP90 clients, because other molecules impaired by HSP90 inhibition may cooperate in cell survival and proliferation. Overall, our cross-resistance experiments revealed that, except for S1206C, all point mutations detected may be targeted simply switching to another inhibitor, already available in clinic. In the light of these data, we could speculate that most of the efforts should be directed in finding a new inhibitor, able to target mutations involving the S1206 residue. This knowledge, together with all data nowadays available on Crizotinib resistance, is a useful tool to manage cases of
Disclosure of Potential Conflicts of Interest

Professor C. Gambacorti-Passerini is principal investigator of the trial A8081013 sponsored by Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Concept and design: M. Ceccon, L. Mologni, C. Gambacorti-Passerini

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

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ceccon, L. Mologni, R. Piazza, C. Gambacorti-Passerini

Writing, review, and/or revision of the manuscript: M. Ceccon, L. Mologni, C. Gambacorti-Passerini

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

Study supervision: M. Ceccon

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References


Correction: Treatment Efficacy and Resistance Mechanisms Using the Second-Generation ALK Inhibitor AP26113 in Human NPM-ALK–Positive Anaplastic Large Cell Lymphoma

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