Clinical Cancer Research

Crizotinib-Resistant *ROS1* Mutations Reveal a Predictive Kinase Inhibitor Sensitivity Model for *ROS1*- and *ALK*-Rearranged Lung Cancers

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Abstract

Background: The identification of molecular mechanisms conferring resistance to tyrosine kinase inhibitor (TKI) is a key step to improve therapeutic results for patients with oncogene addiction. Several alterations leading to EGFR and anaplastic lymphoma kinase (ALK) resistance to TKI therapy have been described in non–small cell lung cancer (NSCLC). Only two mutations in the ROS1 kinase domain responsible for crizotinib resistance have been described in patients thus far.

Methods: A patient suffering from a metastatic NSCLC harboring an *ezrin* (*EZR*)–*ROS1* fusion gene developed acquired resistance to the ALK/ROS1 inhibitor crizotinib. Molecular analysis (whole-exome sequencing, CGH) and functional studies were undertaken to elucidate the mechanism of resistance. Based on this case, we took advantage of the structural homology of ROS1 and ALK to build a predictive model for drug sensitivity regarding future ROS1 mutations.

Results: Sequencing revealed a dual mutation, S1986Y and S1986F, in the ROS1 kinase domain. Functional *in vitro* studies demonstrated that ROS1 harboring either the S1986Y or the S1986F mutation, while conferring resistance to crizotinib and ceritinib, was inhibited by lorlatinib (PF-06463922). The patient's clinical response confirmed the potency of lorlatinib against S1986Y/F mutations. The ROS1 S1986Y/F and ALK C1156Y mutations are homologous and displayed similar sensitivity patterns to ALK/ROS1 TKIs. We extended this analogy to build a model predicting TKI efficacy against potential ROS1 mutations.

Conclusions: Clinical evidence, *in vitro* validation, and homology-based prediction provide guidance for treatment decision making for patients with *ROS1*-rearranged NSCLC who progressed on crizotinib. *Clin Cancer Res; 22(24); 5983–91.* *©2016 AACR.*

Introduction

Alterations in tyrosine kinases or in their downstream effectors drive the activation of intracellular pathways culminating in neoplastic transformation (1, 2). As cancer cells can strictly rely on these specific alterations in order to proliferate, survive, and invade, the ability to block their function became of crucial therapeutic interest. This kind of biological dependency, termed oncogene addiction, has been documented in a broad spectrum of solid and hematologic malignancies. In non–small cell lung cancer (NSCLC), the first evidence of a targetable activating mutation was *EGFR*-mutated tumors (3). Later, *ALK* and *ROS1* gene rearrangements were documented, and fusions involving these genes lead to abnormal expression of constitutively activated kinases, thus resulting in oncogene addiction (4). Patients with *ALK*- and *ROS1*-rearranged diseases are treated with shared tyrosine kinase inhibitors (TKI). First- (crizotinib), second- (ceritinib, brigatinib), and third- [lorlatinib (PF-0646392)] generation compounds are either approved or currently under evaluation in clinical trials. As for the other TKIs, patients ultimately develop resistance to the therapy. Parallel therapeutic approaches against the two receptors can be explained by their common phylogenic origin. ALK and ROS1 are indeed evolutionary conserved and share 77% of amino acid identity in their ATP-binding sites (5, 6), specifically where TKIs exert their inhibitory activity.

If 11 different mutations in the ALK kinase domain explain a relevant part of acquired resistance to TKIs in *ALK*-rearranged tumors, only two mutations (G2032R, D2033N) have so far been reported as mechanism of crizotinib resistance in *ROS1*-rearranged NSCLC (7, 8).

Here, we describe the case of a patient suffering from an NSCLC harboring the *ezrin* (*EZR*)–*ROS1* fusion gene with acquired

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Translational Relevance

This study reveals two new ROS1 kinase domain mutations S1986Y/F detected in a patient that developed acquired resistance to crizotinib. Functional studies revealed cross-resistance to ceritinib induced by S1986Y/F mutations which can however be overcome by the third-generation TKI lorlatinib, as observed for the corresponding anaplastic lymphoma kinase (ALK) C1156Y mutation. Clinical response confirmed this result. On the basis of structural and functional homologies between ALK and ROS1 proteins, we also provide oncologists with a predictive model of sensitivity to ALK/ROS1 inhibitors for different *ROS1* mutations anticipated to appear in the clinic. With the recent approval of crizotinib for *ROS1*-rearranged non–small cell lung cancer patients, we can expect an increased number of acquired resistant mutations detected in this molecular subtype of lung cancer. Therefore, modeling theoretical sensitivity of ROS1 patients according to their mutational status can provide therapeutic guidance and generate significant clinical benefits by avoiding worthless treatments in the presence of novel resistance mutations.

crizotinib resistance. Sequencing of the tumor revealed the presence of novel S1986Y and S1986F mutations. Given the sequence homology, mentioned above, codon 1986 of ROS1 is the equivalent of codon 1156 of ALK (6). The C1156Y ALK mutation has already been reported in crizotinib-resistant patients (9, 10). This ALK mutant, also conferring ceritinib resistance in preclinical models (11), remained however sensitive to lorlatinib in both *in vitro* models (12) and in the clinical setting (10). We therefore hypothesized that the ROS1 mutations we detected would exhibit a superposable pattern of response to the different ALK/ROS1 TKIs. Experiments on engineered Ba/F3 cells confirmed our conjecture, and the patient was indeed successfully addressed to lorlatinib treatment. We therefore predicted overlapping profiles of sensitivity of *ROS1* and *ALK* corresponding mutations to the different TKIs.

Materials and Methods

Molecular analyses

Comparative genomic hybridization (CGH) analysis (Agilent technology) was performed following standard procedures (13). *ROS1* and *ALK* rearrangements were evaluated by FISH, as previously described (5, 14), and threshold for positivity attribution was defined according to current recommendations. The fusion partner of the *ROS1*-rearranged gene was identified by RNAseq analysis (Illumina technology), and *ROS1* resistance mutations were identified by whole-exome sequencing (WES; Illumina technology).

Clinical trials

The patient provided written informed consent to participate in the molecular screening studies MOSCATO (NCT01566019) and MATCH-R (NCT02517892) of serial biopsy specimens. Both trials are ongoing at Gustave Roussy Cancer Center. Lorlatinib is administered in the phase I/II clinical trial (NCT01970865), enrolling patients suffering from *ALK*- or *ROS1*-rearranged NSCLC.

Cell lines and viability assays

Ba/F3 cells were purchased from DSMZ cell bank less than 6 months ago and confirmed as mouse origin with IEF of AST, MDH, NP, PEP B, and with species PCR. Cells were confirmed Mycoplasma negative using microbiological culture, RNA hybridization, and PCR assays. Ba/F3 cells were transduced with lentiviral constructs in order to express the EZR–ROS1 fusion protein harboring different *ROS1* resistance mutations. Cell-survival assays were performed as previously described (11).

Immunoblot analyses

Native, EZR-ROS1^{WT}, EZR-ROS1^{G2032R}, EZR-ROS1^{S1986F}, and EZR-ROS1^{S1986Y} Ba/F3-expressing cells were treated with the indicated concentrations of inhibitors for 3 hours. Immunoblotting was performed as previously described (11). Phospho-ERK (T202/Y204), ERK, S6, phospho-S6 (S240/244), phospho-AKT (S473), AKT, phospho-ROS1 (Y2274), and ROS1 antibodies were obtained from Cell Signaling Technology.

ALK and ROS1 sequence alignment

ALK and ROS1 proteins share 77% of their active site amino acid sequence. Sequence alignment for ALK and ROS1 was described by Ou and colleagues (6) and obtained by Log-Expression (MUSCLE) software.

Structural characterization of ROS1$^{S1986Y/F}$ mutants

In-silico studies were performed by Life Chemicals. The initial ROS1 structure 4UXL was downloaded from the protein data bank. Mutated proteins and reconstructed gaps were generated with the Swiss-model server. Gromacs package 4.5.5 and amber99sb force field were used to perform fast simulations of the wild-type and mutant ROS1 structures. We used TIP3P water model to get the protein solved in the octahedron with a 10 Å radius. AmberTools 14 antechamber module was used to generate topology of ligands in AM1-bcc approach. A total of 25,000 steps of steepest descent energy minimization were carried before the equilibration step at constant temperature and pressure. The long-range electrostatic interactions were handled by particle-mesh Ewald algorithm, whereas constrains bond lengths by LINCS algorithm. A heat-annealing code was used for conformational mobility of the protein with periodic temperature changes for each 100 picoseconds (ps) from 100 to 320 Kelvin (K), as a modification of default parameters. The simulation time was set to 5,000 ps with integration time step of 2 femtoseconds. A free molecular dynamics (MD) simulation without any intervention in the system was then performed in a 10 ns time-lapse. Root mean square deviation (RMSD), root mean square fluctuation (RMSF), and protein–ligand interaction energies were analyzed starting from free MD with the respective Gromacs tools. Average structures were generated from last 5 ns of free MD with g-cluster tool utilizing a RMSD cut-off of 0.1 nmol/L and were then visualized with PyMol software.

Results

ROS1 S1986Y and S1986F mutations confer crizotinib resistance overcome by lorlatinib in an EZR-ROS1 NSCLC patient

In August 2010, a 63-year-old male never-smoker was diagnosed with stage IV lung adenocarcinoma with diffuse lymph node and pleural involvement. No *EGFR*, *KRAS*, *PI3K*, *BRAF*, and

HER2 mutations were found (Sanger sequencing) in the diagnostic specimen, and a search for an *ALK* translocation proved negative. First-line treatment, including cisplatin, gemcitabine, and the PARP inhibitor, iniparib (NCT01086254), started in September 2010. Therapy was administered up to the sixth cycle with good clinical and radiological responses (–44% according to RECIST 1.1 as the best response). When pulmonary disease progression was documented in April 2012, second-line treatment with pemetrexed was initiated leading to a prolonged partial response.

A new CT scan–guided biopsy of the left pleura was performed in January 2013. Histology confirmed the presence of a TTF1-positive lung adenocarcinoma, whereas CGH analysis led to the detection of *HOXA7* gene amplification and deletion of the 3' region of *ROS1*. FISH showed *ROS1* rearrangement in 40% of tumor cells (Fig. 1A), and *EZR* was identified as the *ROS1* fusion partner by RNAseq. Targeted next-generation sequencing on a panel of 50 cancer genes did not reveal any mutation. *ROS1* rearrangement was confirmed by FISH in circulating tumor cells (15).

After a total of 17 pemetrexed cycles from April 2012 to September 2013, manifest bilateral lung progression was detected, and third-line therapy with off-label crizotinib, 250 mg twice daily, was initiated. Previous coughing symptoms disappeared a few days after treatment initiation, and after 40 days of treatment, CT scans depicted a profound thoracic response, with clear shrinkage of parenchymal lesions dimensions. Crizotinib exerted a clinical activity maintained over time, with the best radiological response achieved in March 2014, after 6 months of therapy (–75% according to RECIST 1.1).

Disease control and excellent clinical tolerability persisted over 22 months of TKI therapy, and disease progression was first detected in July 2015; crizotinib administration was maintained beyond tumor progression with still a major clinical benefit. In order to explore the molecular events explaining crizotinib secondary resistance, a new CT scan–guided biopsy was performed on a left lung cancer nodule. WES analysis uncovered the presence of molecular alterations in exon 37 of the *ROS1* gene, corresponding to the ROS1 tyrosine kinase domain. Mutations consisted of serine-to-tyrosine and to-phenylalanine substitutions in codon 1986 (S1986Y/F) of *ROS1* (Fig. 1B). The specimen analyzed contained 70% of cancer cells, and tyrosine and phenylalanine substitutions were found respectively in 18% and 17% of WES reads. No other relevant molecular alterations were identified (Supplementary Table S1).

The patient was therefore enrolled in the phase I/II clinical trial evaluating lorlatinib activity (NCT01970865), given the strong *in vitro* evidence of the activity of this compound against the analogous C1156Y ALK mutation.(12) As depicted in Fig. 1B, although not strictly conserved, native 1986 ROS1 and 1156 ALK codons respectively harbor serine (S) and cysteine (C) amino acid residues, whose structures are markedly similar. A single missense mutation generates the substitution of serine to tyrosine (Y)/phenylalanine (F) for ROS1 and from cysteine to tyrosine for ALK. Tyrosine and phenylalanine have comparable structures with a benzoic group, indicating that they act in a superposable manner modifying ROS1 S1986Y/F and ALK C1156Y conformations. Lorlatinib administration, 100 mg daily, started at the beginning of December 2015 with a suboptimal tolerance profile, characterized by grade I hypercholesterolemia and peripheral neuropathy. The first evaluation CT scan performed 40 days after treatment initiation showed an impressive disease response, confirmed at the second radiologic evaluation at the third month of lorlatinib treatment (–89% according to RECIST 1.1; Fig. 1C). Disease remains controlled without any signs of progression after 6 months of therapy (June 2016).

Functional characterization of novel ROS1 mutations

In order to investigate the effect of *ROS1* mutations on ROS1 sensitivity to TKIs, we generated Ba/F3 cells expressing EZR-ROS1^{WT}, EZR-ROS1^{G2032R}, EZR-ROS1^{S1986F}, and EZR-ROS1^{S1986Y}. The cells were treated with increasing concentrations of crizotinib, ceritinib, and lorlatinib (Fig. 2A). The results of viability assays confirmed the superiority of lorlatinib ($\cong$7–10-fold lower IC_{50}) against ROS1^{WT}-expressing cells compared with crizotinib and ceritinib. The latters showed similar IC_{50} values in inhibiting ROS1^{WT}, with contrast to the pronounced biological superiority of ceritinib compared with crizotinib against the native ALK form (11). As suggested by structure analogies with ALK, S1986F/Y substitutions in the ROS1 kinase domain led to crizotinib and ceritinib resistance. However, lorlatinib maintained its strong inhibitory activity against S1986F and S1986Y mutations (Fig. 2A). The first and most reported ROS1 G2032R mutation (7, 16, 17) engendered resistance to the three compounds (Fig. 2A).

Immunoblot analyses of ROS1 and downstream pathway activation corroborated the cell survival assays. Lower doses of lorlatinib, compared with crizotinib and ceritinib, were required to decrease ROS1 phosphorylation, whereas only a high concentration of lorlatinib appeared to have a mild effect on ROS1^{G2032R} phosphorylation (Fig. 2B). Crizotinib and ceritinib had only a mild effect on ROS1 phosphorylation in cells harboring S1986F/Y mutations, which led to persistent S6 phosphorylation and cell survival. Only lorlatinib was able to completely switch off ROS1, AKT, and ERK signaling leading to inhibition of S6 phosphorylation in the presence of S1986Y or S1986F mutations (Fig. 2B).

Taken together, these *in vitro* results revealed crizotinib and ceritinib resistance conferred by ROS1 S1986F/Y mutations, against which lorlatinib maintained its inhibitory potency.

Structural basis for S1986Y/F mutations impact on ROS1 structure and TKI binding

We performed molecular studies to acquire information about the conformational changes induced by the two uncovered mutations on ROS1 structure. In the absence of ligands, we observed that mutant structures underwent some motions with comparison to the wild-type ROS1 protein (Fig. 3). Importantly, we noticed that these mutations do not directly occur within the enzyme active site, but do actually impact on the alphaC helix of the kinase domain, causing its movement with a following change in the tip of the close glycine-rich region, involving the residues from 1950 to 1960 (Fig. 3). As suggested by their similar structures, S1986Y and S1986F substitutions generated perfectly comparable conformations in ROS1 kinase domain.

We also performed conformational studies involving protein–inhibitor complexes (Supplementary Fig. S1). With the intrinsic limits of the complexity concerning the mutation site, as already reported for the corresponding ALK C1156Y substitution (11), S1986Y/F mutations prevent crizotinib access to the kinase domain, whereas lorlatinib can fix and maintain its ability to

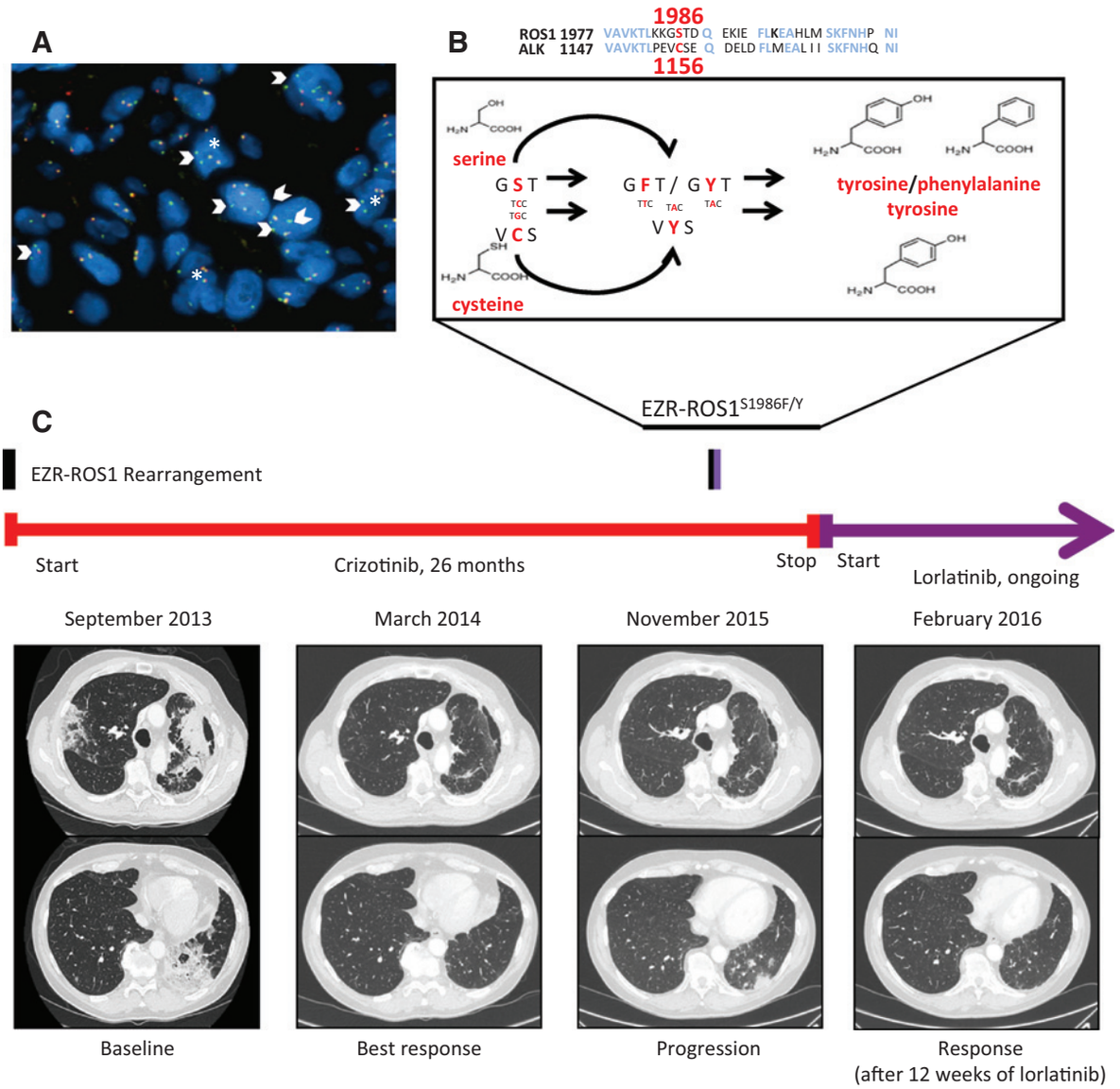


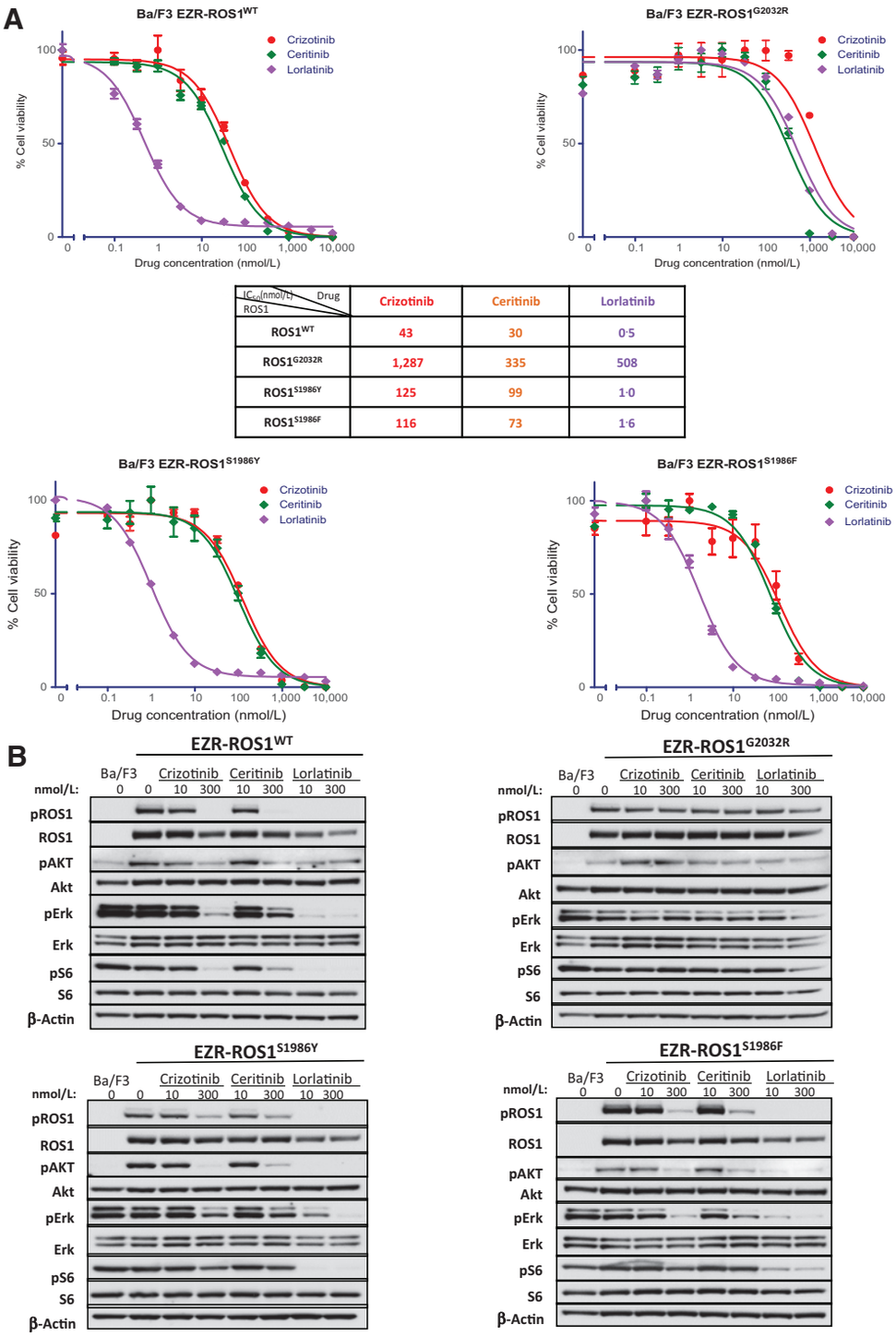
Figure 1.

Novel ROS1 crizotinib-resistant mutations clinically overcome by lorlatinib. **A,** FISH analysis showed *ROS1* split signal in 40% of cells, with a copy-number gain of both the native (red and green probes leading to yellow signal, asterisks) and the rearranged (green signal, arrowheads) *ROS1* genes. **B,** Analogy between ROS1 and ALK amino acidic sequences. The serine (S) at codon 1986 of ROS1 kinase domain corresponds to the 1156 ALK cysteine (C). S1986Y and S1986F ROS1 substitutions emerge from TCC to TTC and TAC missense mutations, respectively. Although residues of 1986 ROS1 and 1156 ALK codon do not match, both serine and cysteine are structurally close nucleophilic amino acids. The same conclusions can be drawn from the similarity between tyrosine (Y) and phenylalanine (F) aromatic conformations. Structural proximity between amino acids involved in respective *ROS1* and *ALK* substitutions suggests similar effects on protein structure and upon steric interferences. **C,** Radiological evolution of lung lesions under ALK-TKIs treatment. After having undergone two systemic treatment lines, in September 2013, patient started crizotinib treatment, which led to deep and prolonged response upon diffuse lung metastatic sites. Disease progression was documented in July 2015 and confirmed 4 months later. Patient was successfully addressed to lorlatinib as a second ALK/ROS1 TKI.

induce a conformational shift in both wild-type and mutated ROS1 forms. In the case of crizotinib binding, the positioning of the glycine-rich loop tip of the alphaC helix is inside-out, whereas it does present oppositely in the case of lorlatinib interaction, potentially explaining the differential capacity in inhibiting ROS1 mutants (Supplementary Fig. S1).

Prediction of ROS1 mutations identification and inhibitory profiles

All mutations *loci* reported so far in crizotinib-resistant *ROS1*-rearranged NSCLC [G2032R (7), D2033N (8), and the present S1986Y/F, chronologically] have already been reported in the clinic at the corresponding ALK kinase domain codons (G1202R,



IC50(nmol/L) ROS1 \ Drug	Crizotinib	Ceritinib	Lorlatinib
ROS1WT	43	30	0·5
ROS1G2032R	1,287	335	508
ROS1S1986Y	125	99	1·0
ROS1S1986F	116	73	1·6

Figure 2.

Functional characterization of ALK/ROS1 TKIs inhibitory potential across different *ROS1* mutations. **A,** Cell viability assays upon Ba/F3 cells harboring EZR-ROS1^{WT}, EZR-ROS1^{G2032R}, EZR-ROS1^{S1986Y}, and EZR-ROS1^{S1986F} constructs, respectively. Ba/F3 cells were treated with the indicated doses of crizotinib, ceritinib, or lorlatinib for 48 hours. After the incubation, the cell survival was assayed by Cell-Titer-Glo. As depicted by survival curves and IC_{50} table, lorlatinib displayed superior inhibitory activity against EZR-ROS1^{WT}, EZR-ROS1^{S1986Y}, and EZR-ROS1^{S1986F} compared with crizotinib and ceritinib. The central table resumes median values obtained from crizotinib, ceritinib, and lorlatinib treatment of the four mutants. Data are representative of three independent biological replicates. **B,** Immunoblot analyses show differential activity of crizotinib, ceritinib, and lorlatinib upon intracellular signaling inhibition in EZR-ROS1^{WT}, EZR-ROS1^{G2032R}, EZR-ROS1^{S1986Y}, and EZR-ROS1^{S1986F} Ba/F3 cells. Cells were treated with the indicated concentrations of TKI for 3 hours. Lysates were probed with antibodies directed against the indicated proteins. Inhibition of ROS1 and intracellular intermediates of MAPK and PI3K-AKT-mTOR pathways was achieved at lower doses for lorlatinib (10 nmol/L) compared with crizotinib and ceritinib in ROS1^{WT} and ROS1$^{S1986Y/F}$ cells. Experiments were repeated three times.

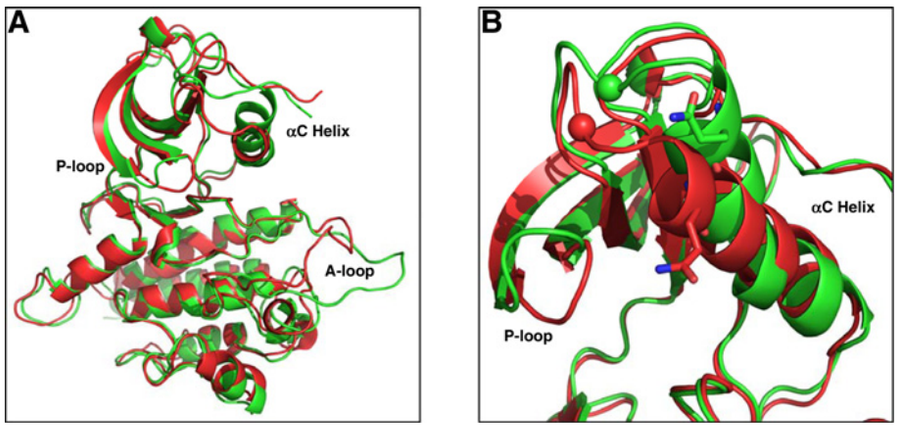


Figure 3.

Visual representation of wild-type and S1986Y/F mutants ROS1 structures. **A,** Alignment of the complete structures of the wild-type (green) and mutated (red) ROS1 forms. No difference was noticed between tyrosine or phenylalanine mutations at 1986 codon. The conformational changes generated by the mutations mainly involve three structural elements of ROS1 N-lobe: the alphaC helix, the P-loop (harboring the glycine-rich region within codons 1950–1960), and the A-loop. **B,** Detailed representation of the alphaC helix displacement due to the different state of the glycine-rich region of P-loop in the mutated protein. 1986 codons are marked with spheres. The images were created by PyMol software (www.pymol.org).

D1203N, and C1156Y, respectively; Fig. 4). In addition to a perfect match in involved amino acids, functional consequences, in terms of structure and TKI sensitivity, overlapped between ROS1 S1986Y/F and ALK C1156Y, as depicted above (Figs. 1B and 2). Therefore, ROS1 and ALK kinase domains do not only share phylogenic origin and structure homology, but also mutational hotspots and TKI sensitivity. We can therefore predict that any of the ALK-resistant mutations could be identified in *ROS1*-rearranged tumors and confer specific TKI resistance. Those mutations would be the 1981Tins, L1982F, S1986Y/F (reported here), M2001T, F2004C/V, L2026M, G2032R (7), D2033N (8), and G2101A (Fig. 4).

With regard to ALK analogy, the 1981Tins, L1982F, S1986Y/F, F2004C/V, and D2033N ROS1 mutations (homologous to the 1151Tins, L1152R, C1156Y, F1174C/V, and D1203N ALK mutations, respectively) would be ceritinib resistant and lorlatinib sensitive (Fig. 4; refs. 11, 12).The M2001T, the gatekeeper L2026M, and the G2101A ROS1 mutations (corresponding to I1171T, L1196M, and G1269A ALK mutations, respectively) would still be prone to ceritinib inhibition (Fig. 4), due to smaller dimensions allowing access to the ATP-binding pocket (11). Remarkably, recent evidence confirmed our predictions concerning ceritinib lack of activity against D2033N and L1982F mutations (8, 16), as we functionally validated here for S1986Y/F mutants and ceritinib efficacy against the L2026M gatekeeper mutation (18).

Of note, not all described ALK secondary mutations can be acquired in ROS1 kinase domain. An arginine (R) residue cannot substitute the leucine (L) of L1982 ROS1 codon as a consequence of a single nucleotide base mutation as observed for ALK L1152R mutation. However, Katayama and colleagues have reported a L1982F mutation conferring crizotinib and ceritinib resistance in a mutagenesis screening (16). ROS1 cannot either acquire the homologous of L1198F (resistant to ceritinib and lorlatinib, crizotinib re-sensitizing; ref. 10) and S1206Y ALK mutation, as a the result of a single-nucleotide modification. Similarly, M2001 ROS1 codon can give rise to M2001T but not to M2001S or M2001N as reported for ALK I1171 codon (Supplementary Table S2; refs. 19, 20).

Discussion

Growing attention is currently being devoted to the identification of resistance mechanisms to targeted therapies, to better guide treatment strategies and impact favorably on patient outcomes. We report two novel mutations in the ROS1 kinase domain capable of conferring crizotinib resistance but allowing lorlatinib efficacy. Using Ba/F3 cells expressing native or mutated *EZR-ROS1*, we functionally demonstrated that S1986F/Y substitutions confer crizotinib and ceritinib resistance. Lorlatinib, more potent against WT ROS1 compared with crizotinib and ceritinib, maintained strong growth inhibition of $ROS1^{S1986F/Y}$ cells (Fig. 2A). Immunoblot analyses confirmed that lorlatinib was the only ALK/ROS1 TKI tested capable of completely switching off ROS1 and downstream signaling phosphorylation in engineered Ba/F3 cells (Fig. 2B). Early clinical data supported lorlatinib strong efficacy by reporting remarkable tumor response rates, including partial and complete intracranial remissions (21).

We moreover confirmed that G2032R, the first documented and most reported ROS1 secondary mutation, confers resistance to crizotinib and ceritinib. Lorlatinib activity against this mutation ($IC_{50} \cong 500$ nmol/L) suggested that the third-generation compound would not be able to reverse resistance in the clinic. As other *in vitro* data (18) suggested a potential lorlatinib efficacy on this mutation, clinical activity remains to be sorted out. Recent evidence suggests potential activity of board-spectrum TKIs such as foretinib and cabozantinib against G2032R ROS1 mutation (16–18, 22). Similarly, cabozantinib has recently been shown to overcome D2033N, the other ROS1-resistant mutation, both in *in vitro* models and in a patient (8). The therapeutic index of these mutitargeted kinase inhibitors against the G2032R ROS1 mutation has not yet been clinically investigated.

Crystallographic features and docking studies for ROS1 kinase domain have already been described (7, 22). Regarding the

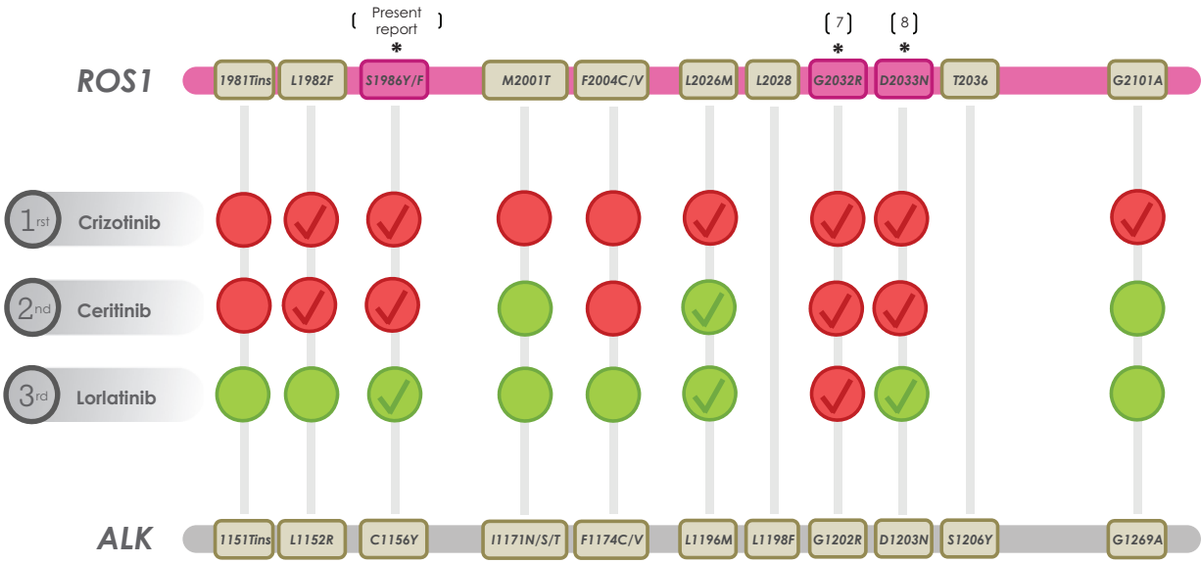


Figure 4.

Modeling theoretical sensitivity of ROS1 patients according to their mutational status. The present figure depicts the validated (√) and predicted activity patterns of crizotinib, ceritinib, and lorlatinib against *ROS1* mutations homologous to reported *ALK*-resistant mutations. Magenta boxes contain ROS1 mutations already reported in NSCLC patients. Red and green circles indicate resistance or sensitivity of the mutated protein, respectively. ROS1 mutations homologous to L1198F and S1206Y ALK ones cannot develop as a result of a single-nucleotide substitution (Supplementary Table S2).

current S1986F/Y mutations, Shaw and colleagues recently described structural alteration of the analogous C1156Y ALK mutation (10). Generating co-crystal structures of the native or mutated kinase domains coupled with crizotinib or lorlatinib, the authors observed that substitution at codon C1156 would probably not directly affect drug binding, due to its relatively remote distance from the inhibitor binding site. The structural studies we performed revealed a similar situation concerning S1986Y/F ROS1 mutations, supposed to trigger a conformational shift in the alphaC helix and the glycine-rich region close to the active site of the enzyme (Fig. 3). The latter event appears to impact on crizotinib binding while allowing the access and the inhibitory activity of lorlatinib into the kinase domain (Supplementary Fig. S1).

In their report, Shaw and colleagues reported two mutations within the same ALK allele, C1156Y/L1198F (10). In our case, the two mutations at the exact same position can either be on two distinct translocated ROS1 alleles within the same cell or in two individual cells. In addition to being technically challenging to investigate, it did not seem crucial here to examine whether the two mutations were in the same cells. Indeed, the two mutants displayed identical sensitivity to the different TKIs tested, and cell sensitivity would be the same with one or two mutants of the ROS1 protein.

Along with the functional characterization of the novel S1986F/Y ROS1 mutations, the present study focuses on the transposability of ALK-validated therapeutic approaches to *ROS1* secondary mutations. Based on ALK and ROS1 similarity in kinase domain sequences, common pharmacologic susceptibility, and homologous hotspots *loci* involved in molecular events, we developed a model predicting sensitivity of 9 ROS1 mutants to different generations of ALK/ROS1 TKIs (Fig. 4). Six of these putative ROS1 secondary mutations have been studied for some TKI in preclinical models, and the data the authors obtained matched our prediction based on ALK knowledge (Fig. 4; refs. 7, 8, 16–18). We cannot however exclude that other ROS1 mutations could occur, and functional studies will be required to directly assess TKI sensitivity. If 11 different mutations conferring resistance to TKIs have already been reported in *ALK*-rearranged NSCLC thus far (9–11, 19, 23, 24), three secondary mutations have been documented in *ROS1*-rearranged tumors [G2032R (7), D2033N (8), and the present S1986Y/F]. This discrepancy is ostensibly due to the number of patients with *ALK*-rearranged disease, about 4 to 5 times higher compared with *ROS1*-rearranged NSCLC (21), and the fact that therapeutic targeting against ALK began earlier.

Besides ROS1 kinase domains mutations, crizotinib resistance has been reported to emerge due to EGFR (25), RAS (26), or KIT signaling activation (27). Remarkably, the three events have also been reported in *ALK*-rearranged tumors and suggest the potential of dual target inhibition (19, 23). Pharmacokinetic issues can account for a significant part of resistance to crizotinib and other inhibitors in *ALK*-rearranged NSCLC, and the equivalent scenario can be depicted for tumors harboring *ROS1* fusion gene. The significant number of patients developing brain metastases during crizotinib treatment (28) reflects the scarce penetration of the compound across the blood–brain barrier (29). The novel ALK/ROS1 inhibitors have been designed to obtain a better performance over brain metastases and are indeed showing remarkable results, with a special mention accorded to lorlatinib (12, 21). Recently, *ALK*-rearranged cells have been proven to adapt to both crizotinib and ceritinib treatment by the overexpression of the p-glycoprotein, whereas lorlatinib and the second-generation ALK inhibitor alectinib are not suitable substrates for this efflux pump (30).

Anyhow, as further exhaustion of novel generation compounds does eventually lead to disease progression, treatment

combinations with downstream signaling cascade inhibitors, cytotoxic chemotherapies, or antiangiogenic antibodies represent potential strategies to approach the issue of acquired resistance (31).

As previously suggested (18), our experiments indicate that crizotinib and ceritinib display similar potency on WT ROS1. This is in contrast with the superior efficacy of ceritinib compared with crizotinib on WT ALK both in preclinical and clinical studies (11, 28, 32). Therefore, the ability of ceritinib to overcome crizotinib resistance in ROS1-rearranged tumors without secondary mutations, as it is the case for ALK-positive patients, would need to be directly addressed in the clinic.

Besides defining the pharmacologic susceptibility of ROS1 S1986F/Y mutations, the present report highlights the unavoidable contribution of molecular analyses of specimens obtained at disease progression under TKI therapy. The availability of tumor material thus far allows us to address treatment decisions and to undertake functional validations. The current study enabled us to confirm that the ROS1 S1986Y/F mutations confer resistance to both crizotinib and ceritinib and allowed more efficient patient treatment by avoiding a ceritinib therapy, which would very likely not have afforded a clinical benefit. It is actually challenging to drive specific indications for the treatment of *ROS1*-rearranged NSCLC patients, given the relative small incidence (1%–2% of all NSCLC), potentially designating ROS1 tumors as a "rare molecularly subtype of common cancers" (33). Given the absolute incidence of NSCLC, the number of patients harboring *ROS1* rearrangement worldwide is still relevant. Validated data concerning ALK inhibition can be advantageously transposed to guide treatment decisions in *ROS1*-rearranged tumors. The current widespread access to deep molecular analyses would ostensibly reveal, for every patient, the specific *ROS1* mutation responsible for crizotinib resistance, when it is actually due to kinase domain modifications. We therefore provide a predictive model which finds in the clinical setting its best field of both validation and exploitation. However, performing repeated biopsies in patients is not always feasible. A noninvasive method to detect molecular alterations conferring ALK and ROS1 resistance to TKIs, such as that finely developed for EGFR NSCLC with cell-free circulating tumor DNA (34), would be of inestimable interest.

Disclosure of Potential Conflicts of Interest

D. Planchard is a consultant/advisory board member for Pfizer. B. Besse reports receiving commercial research grants from Novartis and Pfizer. J. Remon is a consultant/advisory board member for osepharma. J-C. Soria is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

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