ROS1

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Abstract ROS1 is a receptor tyrosine kinase with *ROS1* gene fusions identified in 0.9–2.1% of non-small cell lung cancers (NSCLC), as well as a number of other malignancies. These fusions are constitutively activated, leading to significant changes in cell differentiation, proliferation, growth, and survival. The fusions can be identified by a number of methods including fluorescent in situ hybridization, immunohistochemistry, real-time PCR, and next-generation sequencing. The tyrosine kinase inhibitor crizotinib is a potent inhibitor of ROS1 and was approved by the FDA for treatment of metastatic non-small cell lung cancer (NSCLC) with *ROS1* rearrangement in March 2016. However, as with other oncogenes, patients treated with crizotinib eventually develop resistance and progressive disease. A number of different resistance mutations have been discovered, the mechanisms of which can be broken down into two major categories: mutations within the ROS1 kinase domain and bypass signaling pathways. Several additional tyrosine kinase inhibitors are under development with varying degrees of CNS penetration and efficacy against resistance mutations.

Keywords ROS1 rearrangement · ROS1 inhibitor · Lung cancer · Non-small cell lung cancer · Oncogene · Crizotinib · Ceritinib · Tyrosine kinase inhibitor (TKI) resistance · Targeted therapy · Tyrosine kinase inhibitors

Introduction

Though gene fusions in the receptor tyrosine kinase ROS1 are present in only a small percentage of non-small cell lung cancer (NSCLC), it represents a viable therapeutic target with impressive clinical benefit to ROS1 inhibitors. The first ROS1 inhibitor, crizotinib, was approved for advanced NSCLC harboring *ROS1*

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fusions in 2016. Subsequently a number of additional ROS1 inhibitors have been in development and seek to overcome resistance mutations that develop in response to crizotinib. In this chapter we provide an overview of the *ROS1* gene including its history, biology, methods of detection, and role of targeted treatment against ROS1-positive NSCLC, including the development and management of resistance mutations with newer agents targeting ROS1.

Structure and Function

Human c-ros oncogene 1 (*ROS1*) was originally discovered as a homolog of the transforming sequence of the UR2 avian sarcoma virus [1, 2] (Fig. 1). *ROS1* is located on chromosome 6q22 [4, 5]. It is a receptor tyrosine kinase that shares structural similarities to the insulin receptor tyrosine kinase, leukocyte tyrosine kinase (LTK), and anaplastic lymphoma kinase (ALK) families [6]. *ROS-1* encodes a receptor tyrosine kinase that consists of a large N-terminal extracellular domain, a hydrophobic single pass transmembrane region, and a C-terminal intracellular tyrosine kinase domain. It is relatively unique, in that its extracellular domain is composed of six repeat motifs that have high homology to the extracellular matrix and plasma protein fibronectin type III repeats, almost resembling a cell adhesion molecule. Unlike most adhesion molecules, however, the intracellular kinase domain enables ROS to directly translate adhesion engagement along intracellular signaling pathways [3].

The *ROS* receptor tyrosine kinase gene is evolutionarily conserved across multiple organisms. In *Drosophila melanogaster*, SEVENLESS (a ROS orthologue) is associated with a seven-transmembrane G-protein-coupled cell called BOSS (bride of sevenless). It is of particular importance in the developing *Drosophila* eye, where BOSS is required for differentiation of cells into photoreceptors [7, 8]. ROS expression has been examined in mouse, chicken, and rat tissue throughout various stages of development, with expression of *c-Ros* found in kidneys, small intestines, heart, lung, and male reproductive cells with restriction seen to epithelial cells [3]. In mice, testicular expression of *c-Ros* was only detected in adults with in situ hybridization of the adult testes showing expression in mature stages of development (spermatids, spermatozoa) only [9, 10]. In addition, *c-Ros* mutated male mice were noted to be infertile, though otherwise healthy. The defect was noted to be in development of the epithelia in the epididymis, especially in regionalization and terminal differentiation. No such impairment in fertility was noted in female mice. This suggests that expression may be linked to male fertility [11]. In humans, however, research has been hindered by the fact that it remains an orphan tyrosine kinase receptor, without a known ligand. Another barrier is the inability to express the full-length wild-type receptor in cellular models. There is some speculation that c-ROS1 expression may be involved in epithelial-mesenchymal interactions as well as in the cellular differentiation cascade of epithelial tissues [3].

Gene Fusion and Cancer

ROS1 gene fusions were first identified in the human glioblastoma cell line U-118 MG [12, 13]. An intra-chromosomal homozygous microdeletion of 240 kilobases on chromosome 6q21 was found to lead to fusion of the 5′ region of the *FIG* gene (fused in glioblastoma; a Golgi apparatus-associated protein) to the 3′ kinase domain of *ROS* [14–16] (Fig. 1). This fusion led to constitutive activation that was dependent on its localization to the Golgi apparatus [16]. This fusion has also been identified in a number of other malignancies, including ovarian cancer [17], cholangiocarcinoma [18], and NSCLC [19]. Additional *ROS1* gene fusions have been found in a number of other malignancies, including inflammatory myofibroblastic tumors [20, 21], gastric adenocarcinoma [22], colorectal cancer [23], angiosarcoma [24], thyroid cancer [25], atypical meningioma [26], and spitzoid melanomas [27].

Following the identification of *ROS1* fusions in glioblastomas, NSCLC was the second solid tumor in which these rearrangements were identified. This landmark study characterized tyrosine kinase signaling across 41 NSCLC cell lines and over 150 NSCLC tumors using a phosphoproteomic approach. High-level ROS kinase activity was noted in one cell line and one tumor sample. When these samples were
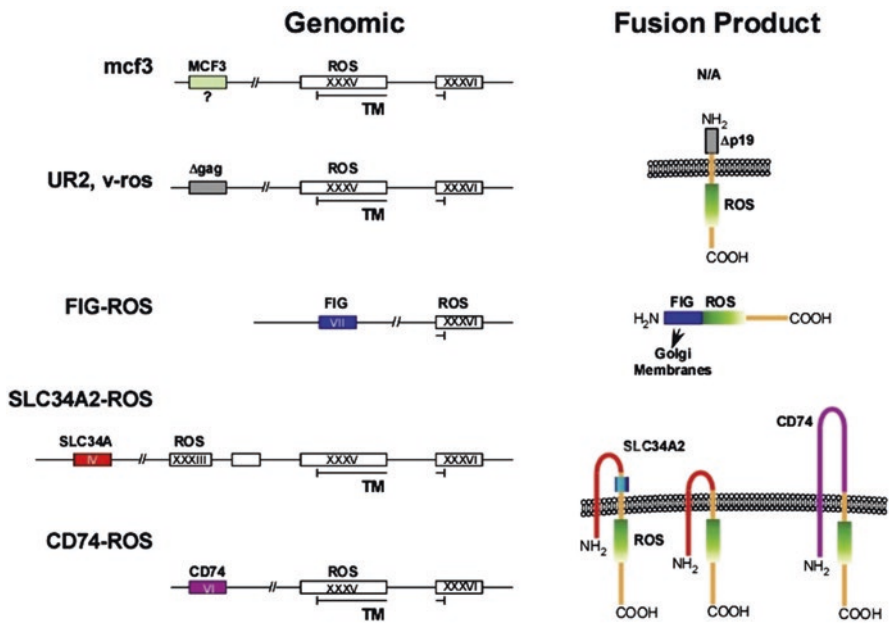

Fig. 1 ROS fusion kinases. Schematic representation of selected ROS fusions and their corresponding protein fusion product. With the exception of FIG-ROS, all of the fusion kinases are predicted to be plasma membrane bound. SLC34A2-ROS and CD740ROS fusion proteins are here illustrated to be bimembrane spanning receptors, though this has not been confirmed experimentally. (Figure from Acquaviva et al. [3])

sequenced, two novel *ROS1* fusions were identified. In the HCC78 cell line, ROS was found to be fused to the transmembrane solute carrier protein SLC34A2. This protein was found to localize to membrane fractions and display a constitutive kinase activity. siRNA against SLC34A2-ROS was found to induce apoptosis, suggesting that ROS signaling is critical for survival of these NSCLC cells. In the solid tumor, c-ROS was found to be fused to the N-terminal half a type II transmembrane protein, CD74 [28] (Fig. 1).

Since this initial publication, multiple other fusion partners have been reported in NSCLC. The *CD74-ROS1* fusion remains the most common, occurring with an estimated frequency of 32% in NSCLC. Other common fusion partners include *SLC34A2-ROS1* (17%), *TMP3-ROS1* (tropomyosin 3; 15%), *SDC4-ROS1* (syndecan 4; 11%), *EZR-ROS1* (ezrin; 6%), and *FIG-ROS1* (3%). Less common fusions, occurring at frequencies of 1% or less, include *CCDC6-ROS1* (coiled-coil domain containing 6), *LRIG3-ROS1* (leucine-rich repeats and immunoglobulin-like domains 3), *KDELR2-ROS1* (KDEL endoplasmic reticulum protein retention receptor 2 gene), *MSN-ROS1* (moesin gene), *CLTC-ROS1* (clathrin heavy chain gene), *TPD5L1-ROS1* (tumor protein D52-like gene), *TMEM106B-ROS1* (transmembrane protein 106B gene), and *LIMA1-ROS* (LIM domain and actin-binding 1 gene) [29].

Signaling Pathway

Once ROS1 becomes activated, either by its (unknown) ligand or via constitutive action from a fusion, a number of signaling pathways are triggered. The key rate-limiting step for this process is thought to be autophosphorylation of ROS1 and phosphorylation of the SH-2 domain-containing phosphatase-2 (SHP-2) [30]. Constitutive activation from a fusion then leads downstream signaling via several oncogenic pathways, including MAPK/ERK, PI3K/AKT, JAK/STAT3, and VAV3; this leads to significant changes in cell differentiation, proliferation, growth, and survival [3] (Fig. 2). Preclinical work has suggested that the activation of downstream signaling pathways depends on the fusion partner of ROS1. CD74-ROS1 but not FIG-ROS1 has been found to lead to phosphorylation of E-Syt1, which in turn led to an invasive phenotype in the CD74-ROS1 cells [31].

Epidemiology

Patients with *ROS1* fusions have been found to be more likely to be younger, female, and never-smokers than *ROS1*-negative patients—a similar profile to patients with *EGFR* activating mutations and *ALK* rearrangements [32–34]. Interestingly, however, the pattern of spread for *ROS1* rearranged disease appears to be different than that for *ALK*; *ROS* fusions are associated with lower rates of extrathoracic disease, including brain metastases, at initial metastatic diagnosis [35]. The vast majority of
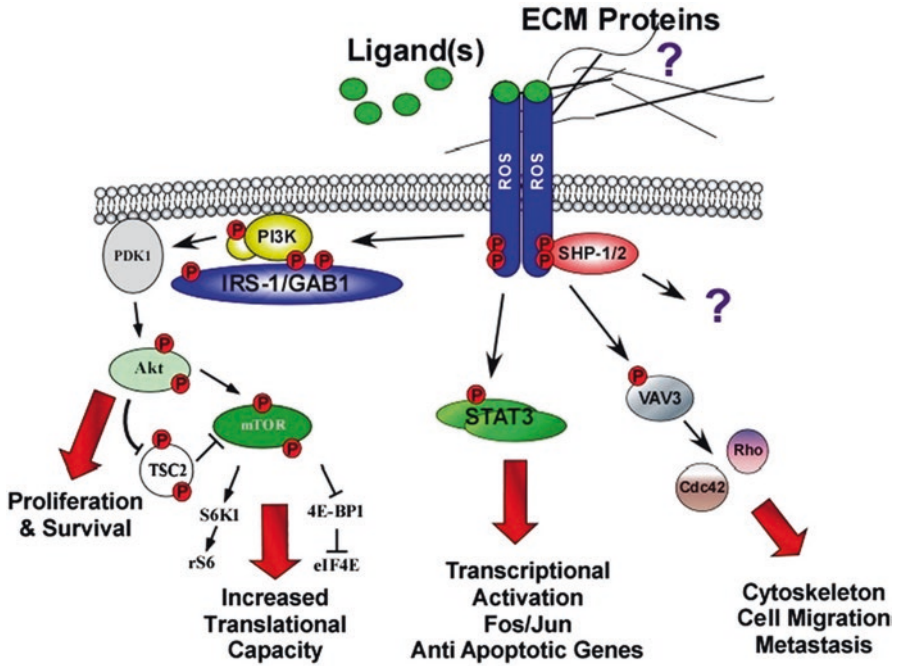
Fig. 2 ROS signaling pathways. (Figure from Acquaviva et al. [3])

cases are adenocarcinoma, although there are rare reports of other histologies such as squamous or large cell [19, 36, 37]. The most common histologic patterns associated with *ROS1* fusion adenocarcinoma are solid growth with hepatoid cytology and acinar growth with cribriform structure. Other pathologic characteristics include mucinous features, signet ring cells, and psammomatous calcifications [38]. The *ROS1* rearrangement usually occurs without other known oncogenic drivers, although there have been rare reported cases of concurrent mutations such as EGFR, KRAS, BRAF, MET, and PIK3CA [19, 27, 39–42].

Prevalence of NSCLC *ROS1* fusion tumor ranges in the literature from 0.9% to 2.1%, though studies are always limited by their screening technique and therefore may miss cases with rare or new fusions. Worldwide prevalence is 1.9% [29].

Detection

FISH

Detection of *ROS1* rearrangements by fluorescence in situ hybridization (FISH) has been considered the gold standard. It was used in the landmark phase I study that resulted in approval of crizotinib for *ROS-1* rearranged NSCLC [42]. The

centromeric (3′) part of the fusion breakpoint is labeled with a one fluorochrome and the telomeric (5′) part with another of a different color. The criteria for ROS1 FISH identification in NSCLC is the same as that for ALK rearrangement. The first positive pattern is a classic break-apart pattern, in which there is a single fusion signal and two separated 3′ and 5′ signals. The second is an atypical pattern, with an isolated 3′ signal—usually one fusion signal and one isolated 3′ signal without the corresponding 5′ signal [43]. FISH testing may be performed either on biopsy or cytologic specimens. To be considered FISH positive, at least 15% of evaluated tumor cells must contain split or isolated 3′ signals [44, 45]. Interestingly, a limitation of FISH has been found to be an inability to detect small intrachromasomal deletions, which can lead to false-negative or false-positive results [39, 46].

IHC

Immunohistochemistry (IHC) can be used as a screening technique. It is less expensive and faster than performing FISH. The D4D6 rabbit monoclonal assay is commercially available (Cell Signaling Technology, Danvers, MA, USA). It is applied at different dilutions ranging from 1:50 to 1:1000. In a number of different studies, IHC has been found to have a sensitivity of near 100% and a specificity of between 85% and 100%; specificity varies depending on interpretive cutoffs and method used [19, 43, 45, 47–49].

Unfortunately, ROS1 IHC can be somewhat challenging to interpret. Expression can be seen in osteoclast-like giant cells adjacent to ROS-1 un-mutated tumor cells, as well as in reactive pneumocytes and alveolar macrophages [43, 49]. Staining patterns can vary depending on different intracellular localization of the ROS1 fusions [19, 43]. Results can vary depending on the performing laboratory [50]. Because of this, while it makes for an excellent screening tool, it is important to perform confirmatory testing with FISH or another testing modality.

RT-PCR

Real-time PCR (RT-PCR) utilizes specific primer sets to detect and identify known fusion variants. RT-PCR-based detection of some of the most common *ROS1* fusion genes (*SLC34A2*, *SDC*, *CD74*, *EZR*, *TPM3*, *LRIG3*, *GOPC*) at exons 32, 34, 35, and 36 has been successfully performed with a sensitivity of 100% and specificity of 85–100% with respect to FISH [41, 51]. While this is a relatively easy, rapid, and inexpensive test, it does have some drawbacks. As the list of *ROS1* fusion proteins is large and growing, RT-PCR is likely to miss rare or previously unknown variants. It can also be challenging to obtain sufficient good quality RNA from the formalin-fixed paraffin-embedded tissue samples (FFPE) [52].

In recent years, the nCounter platform (NanoString Technologies; Seattle, WA) has emerged as a clinical option. It is a multiplexed assay that can identify known fusion gene variants via the interrogation of imbalanced 5′/3′ expression levels as well as the direct detection of fusion transcript variants. It has shown a good concordance with both IHC and FISH results for *ROS1* fusion detection [53, 54].

NGS

Next-generation sequencing, or NGS, enables sensitive and specific assessments of multiple genomic regions at once, allowing for detection of both known and novel fusions [45]. Several *ROS1* fusions have been identified using NGS [55–59]. A recent study performed next-generation sequencing on 319 FFPE samples and found 100% sensitivity and specificity when compared to reference FISH assays [60]. This method allows for multiplexed detection of molecular aberrations in NSCLC in a single test instead of multiple assays; however, hybrid-based capture NGS methods may have decreased sensitivity in detecting gene fusions [45].

Targeted Therapies

Crizotinib

Crizotinib (previously PF-0234106; brand name Xalkori, Pfizer, New York, USA) is a small molecule multikinase inhibitor (Table 1). It was initially developed as an inhibitor of c-MET but was further explored against a panel of over 120 diverse kinases and was found to be almost 20 times more selective for ALK and MET as compared to other evaluated kinases [61]. Following a phase I trial and initial efficacy results from a phase II trial (PROFILE 1001) that showed 50% response rates, it was approved by the FDA for use in metastatic NSCLC with *ALK* rearrangements in 2011 [62, 63]. Preclinical investigation of NSCLC cell lines, including HCC78 (*SLC34A2-ROS1*), revealed dose-dependent inhibition with crizotinib; inhibition of *ROS1* led to subsequent inhibition of its downstream targets and apoptosis of the cell line [64]. This combined with *ALK* and *ROS1*'s known homology [6] with shared high-binding affinity to crizotinib [65] and case reports of response to crizotinib in patients with *ROS1*-mutated NSCLC [33, 37] led to the incorporation of patients with *ROS-1* rearranged NSCLC into the expansion cohort of the phase I PROFILE 1001 study. This landmark study included 50 patients with *ROS1* rearranged NSCLC. Overall response rate (ORR) to crizotinib was 72% with a median duration of response (DOR) of 17.6 months, median progression-free survival (PFS) of 19.2 months, and a disease control rate (DCR) of 90% [42]. Interestingly DOR to crizotinib in *ALK*-rearranged patients is only 49.1 weeks, with a median PFS of

Table 1 Clinical trials of drugs targeting ROS1

Drug (target kinases)	Trial	Phase	Status	Enrollment: ROS1 (total)	ORR	DOR (mo)	PFS (mo)	DCR	CNS disease control	Enrollment: crizotinib resistant	Response: crizotinib resistant	Trial ID
Crizotinib (ALK, ROS1, MET)	PROFILE 1001	I	Complete	50	72%	17.6	19.2	NR	NR			NCT 585195
	Wu et al.	II	Complete	127	71.70%	19.7	15.9	NR	$N = 23$; PFS 10.2 mo			NCT 1945021
	ACSé	II	Ongoing	34	63%	NR	NR	88%	NR			NCT 2034981
	EUCROSS	II	Ongoing	34	69%	NR	NR	NR	NR			NCT 2183870
	EUROS1	Retrospective	Complete	32	80%	NR	9.1	86.70%	NR			n/a
	METROS	II	Ongoing	Not yet reported	Not yet reported							NCT 2499614
Ceritinib (ALK, ROS1)	Lim et al.	II	Complete	32	62%	21	9.3	81%	63% (5/8)	2 (6%)	0	NCT 1964157
Entrectinib (ROS1, ALK, TRK A/B/C)	ALKA-372-001	I	Complete	14 (119)	86%	NR	19	NR	100% (2/2)	6 (43%)	0	EudraCT 2012-000148-88
	STARTRK-1											NCT02097810
	STARTRK-2	I/II	Ongoing	32	75%	17.2	19.1	NR	71% (5/7)			NCT 2568267
Lorlatinib (ROS1, ALK)	Shaw et al.	I	Complete	12 (54)	50%	Not reported			60% (3/5)	7 (58%)	2/7 PR or SD	NCT 1970865
	Solomon et al.	II	Ongoing	47 (275)	36%	NR	NR	NR	56% (14/25)	NR	NR	NCT 1970865

Ropotrectinib (ROS1, ALK, TRK A/B/C)	TRIDENT	I	Ongoing	29 (65)	Not yet reported					19 (66%)	NR	NCT 3093116
DS-6051b (ROS1, TRK A/B/C)	Fujiwara et al.	I	Complete	15	58.3%[a]	NR	NR	100%	NR	4 (27%)	ORR 25% (1)	NCT 2675491
	Papadopoulos et al.	I/Ib	Ongoing	9 (35)	4/6 PR or SD	NR	NR	NR	NR	7 (78%)	2 PR, 2 SD	NCT 2279433
Brigatinib (ALK, ROS1)	Gettinger et al.	I/II	Complete	3 (137)	33% (1/3)	NR	NR	NR	NR	2 (66%)	1 SD	NCT 1449461

NR Not reported, *ORR* overall response rate, *DOR* duration of response, *PFS* progression-free survival, *DCR* disease control rate, *OS* overall survival, *CNS* central nervous system, *PR* partial response, *SD* stable disease, *n/a* not applicable

[a] 66.7% in crizotinib-naïve patients

9.7 months, suggesting that crizotinib may be a more potent inhibitor of *ROS1* than *ALK* [42, 66]. Toxicities in this study were similar to those previously described; the most common grade 3 events were hypophosphatemia (10%), neutropenia (10%), and an elevated aminotransferase activity (10%). No grade 4 or 5 events were seen [42]. Based on this study, crizotinib was approved by the FDA for treatment of metastatic NSCLC with *ROS1* rearrangement in March 2016.

There have been several other studies that yielded overall similarly promising results. The retrospective EUROS1 study identified 32 patients with advanced NSCLC who had positive ROS1 rearrangement by FISH and who had received crizotinib, 30 of whom were evaluable. ORR was 80% and DCR 86.7%, and median PFS was 9.1 months; PFS at 1 year was 44% [67]. Preliminary results from the French phase II ACSé trial prospectively looked at 34 patients with *ROS1*-rearranged NSCLC who were given crizotinib, 24 of whom were evaluable at the time of pre-liminary analysis. ORR was 63%, and DCR 88% [68]. Preliminary results from the prospective European phase II EUCROSS study similarly looked at 34 patients with *ROS1*-rearranged NSCLC (by FISH) who were given crizotinib, 29 of whom were eligible for efficacy assessment and 20 of whom had tumor tissue available for fur-ther sequencing. Of the patients who underwent additional sequencing, 19 tested positive for the ROS1 fusion. ORR was 69% in the overall trial population and 83% in those *ROS-1* positive by next-generation sequencing [69]. A phase II study in East Asian patients included 127 patients with *ROS1*-rearranged NSCLC who received crizotinib. ORR was 71.7%, with a median PFS of 15.9 months and a median duration of response of 19.7 months [70]. All studies included patients who had received varying numbers of prior therapies and who were overall fairly heavily pretreated, though crizotinib was their first tytyrosine kinase inhibitor (TKI).

Resistance to ROS1 TKI

While ROS-1 mutated tumors initially respond well to targeted therapy with crizo-tinib, most patients inevitably develop resistance. The mechanism of resistance can be broken down into two major categories: mutations within the ROS1 kinase domain or bypass signaling pathways [35].

There are two major mechanisms by which kinase domain mutations appear to confer crizotinib resistance. The first is by a gatekeeping mechanism that directly interferes with the combination of ROS1 tyrosine kinase and crizotinib, leading to resistance. The second is a solvent front mutation in the kinase domain adjacent to the crizotinib-binding site; these confer resistance via steric interference [71] (Table 2).

The first kinase domain mutation described is also the one that has been most frequently observed. A 48-year-old woman with CD74-ROS1-rearranged NSCLC was started on crizotinib with excellent response. However after 3 months of ther-apy she was found to have progressive disease. Biopsy upon progression revealed the persistence of the *ROS1* rearrangement by FISH but RT-PCR sequencing

Table 2 Crizotinib-resistant *ROS1* mutations based on preclinical data

	G2032R	L1951R	D2033N	S1986Y/F	L2026M
Mutation type	Solvent front	Solvent front	Solvent front	αC helix	Gatekeeper
Crizotinib	No[a]	No	No[a]	No	No
Ceritinib	No	No	No	No	Yes
Cabozantinib	Yes	Yes	Yes[a]	Unknown	Yes
Entrectinib	No	Unknown	Unknown	Unknown	Unclear
Lorlatinib	Unclear	Unknown	Yes	Yes[a]	Yes
Ropotrectinib	Yes[a]	Yes	Yes	Yes	Yes
DS-6051b	Unknown	Unknown	Unknown	Unknown	Unknown
Brigatinib	No	No	No	Unknown	Yes

Unclear indicates preclinical data for activity has been mixed

[a]Also supported by clinical data

revealed a c6094G→A, p.Gly2032Arg (G2032R) mutation that was not noted on her pretreatment biopsy. This mutation is analogous to the ALK G1202R mutation. Biopsy was repeated at autopsy, and all sites examined harbored this mutation, suggesting that it was an early event in the clonal evolution of resistance [72]. Crizotinib was designed to bind ROS1 at the ATP-binding site that sits within the cleft between the N and C terminal domains of the kinase [73]. Crystal structure analysis revealed that the G2032 residue sits at the solvent front of the kinase hinge (solvent-exposed region of the kinase). G2032R causes steric hindrance with the piperidine ring of crizotinib while still allowing for ATP binding and therefore oncogenic kinase activity [72]. One recent evaluation of 16 patients with *ROS1*-positive advanced NSCLC with a total of 17 repeat biopsies after progression identified G2032R mutations in 41% of the biopsy specimens [35].

Following the identification of this initial resistance mutation, a multitude of others have been identified in clinical samples. A patient with CD74-ROS1-rearranged NSCLC who progressed on crizotinib was found to acquire the solvent-front mutation D2033N. Upon crystal modeling, this mutation was noted to interfere with the favorable interaction of the ATP-binding site with the protonated piperidine region of crizotinib. It is analogous to the *ALK* D1203N mutation. This patient proceeded to respond to cabozantinib; upon crystal modeling, cabozantinib was not found to interact with this altered 2033 residue [74]. Multiple other mutations have been discovered in vitro but not yet been replicated in the clinical setting.

Additional kinase domain mutations have been reported. These include S1986Y/F, which leads to alterations in the alpha C helix of the kinase domain and therefore steric interference with drug binding [35, 75]. It is analogous to the ALK C1156Y substitution [76]. L1951R is a solvent front mutation without an analogous ALK mutation. L2026M is a gatekeeper mutation in the ATP pocket that impedes drug binding and is analogous to *ALK* L1196M [71, 77]. Interestingly when the L2026M, L1951R, and G2032R mutations were evaluated in vivo, the mutations associated with highest crizotinib resistance were those located close to the crizotinib-binding domain—G2032R and L1951 [71].

Less information is available about off-target mechanisms of crizotinib resistance. The best described is a mechanism by which cancer cells achieve resistance via activation of an alternative signaling pathway (bypass pathway). One case report describes the appearance of a *BRAF* V600E mutation in a woman with SDC-*ROS1* fusion NSCLC who had developed resistance to crizotinib; this mutation was not present on her initial biopsy. She was started on dabrafinib and trametinib but died 11 days later [78]. Similarly a new *KIT* p.D816G mutation was found after progression on crizotinib in a patient with *ROS1* fusion NSCLC [79]. Two case reports describe patients with alterations in KRAS; one described a new point mutation in *KRAS* pG12D accompanied by *KRAS* gene amplification found on progression biopsy. Patient was treated with the MEK inhibitor selumetinib as well as pemetrexed and was alive at the time of article submission [80]. The second report only noted focal *KRAS* amplification seen in a *ROS1* fusion tumor biopsy of a patient who had progressed on crizotinib, though this analysis was hampered by the lack of pretreatment sample to see if this was truly a bypass mutation [81]. Another patient with *CD74-ROS1* fusion NSCLC who had progressed on crizotinib was found on next-generation sequencing to have a novel point mutation of the *PIK3CA* gene (pL531P) that led to activation of the *m*TOR signaling pathway; patient was placed on an *m*TOR signaling pathway inhibitor but passed shortly thereafter [82]. One study that performed next-generation sequencing on 12 *ROS1* fusion patients who had progressed on crizotinib identified the same KIT D816G mutation previously characterized. It also noted a HER2 (ERBB2) mutation, though no pre-crizotinib samples were available for comparison. It also noted a β-catenin *CTNNB1 S*45F mutation that had previously been hypothesized as a potential oncogenic driver, though they were not able to evaluate pretreatment tissue in this patient to prove its presence as a bypass mutation [77, 83]. Another study created a cell line from a patient who had developed acquired resistance to crizotinib; the cell line revealed a switch in the control of growth and survival signaling pathways from ROS1 to EGFR in the resistant cell line, though this has not yet been verified in the clinic [84, 85].

Less is known about the potential for phenotypic changes leading to resistance. In EGFR and ALK fusion cancers, histologic transformation from adenocarcinoma to small cell cancer has been observed as a mechanism for TKI resistance, but this has yet to be demonstrated in ROS1 fusion NSCLC [86]. One preclinical study that took tumor tissue from NSCLC patients who had progressed on crizotinib noted evidence of epithelial to mesenchymal transition by way of upregulated vimentin and downregulated E-cadherin. A similar finding was noted in HCC78CR1-2 cell clones, though they also harbored a L2155S mutation that had previously been found to confer crizotinib resistance in cell lines [85].

Ceritinib

Ceritinib (previously LDK378l, brand name Zykadia; Novartis Pharmaceuticals) is an oral small molecule tyrosine kinase inhibitor of ALK [87] (Table 1). Preclinical studies suggested that it would inhibit ROS1 as well [88, 89]. A Korean phase II

study evaluated 32 patients with advanced NSCLC who tested positive for *ROS1* rearrangement by FISH. All but two of them were crizotinib-naïve. They received ceritinib 750 mg daily. ORR was 62%, with 1 complete response (CR) and 19 partial responses (PR). DOR was 21 months, with a DCR of 81%. Median PFS was 9.3 months overall and 19.3 months in crizotinib-naïve patients. Median overall survival (OS) was 24 months. Eight patients entered the trial with metastases to the brain; intracranial disease control was obtained in five (63%) of them, with an intracranial ORR of 25%. Of note, at the beginning of the trial two patients who had previously received treatment with crizotinib were enrolled. Neither were available for objective response—one passed due to suspected leptomeningeal disease, and one withdrew from the trial 2 weeks after their first dose due to grade 3 weakness and anorexia. However, neither of them showed signs of clinical improvement after initiation of ceritinib, and the protocol was subsequently amended to only enroll crizotinib-naïve patients who had previously been treated with at least one chemotherapeutic agent [89].

Adverse events in this study were primarily grade 1-2, the most common of which were diarrhea (78%), nausea (59%), and anorexia (56%)—all of which occurred at higher frequencies than with crizotinib [42, 89]. A recent randomized phase I study of 137 patients with metastatic *ALK*-mutated NSCLC found that ceritinib 450 mg taken with a low-fat meal resulted in fewer GI toxicities as compared with the standard 750 mg taken fasting and was associated with comparable plasma levels when assessed pharmacokinetically [90]. Ceritinib is not FDA approved for management of *ROS1*-rearranged advanced NSCLC, but it is noted as an option for front-line therapy per NCCN guidelines.

Cabozantinib

Cabozantinib (PF-06463922; brand name Cabometyx; Exelixis, Alameda, CA) is an oral multikinase inhibitor with CNS penetration (Table 1). It is FDA approved for use in medullary thyroid cancer and as a second-line agent in advanced renal cell carcinoma. In vitro studies found it to exhibit excellent activity against both the wild-type ROS1 fusion and the G2032R and G2026M mutations at concentrations less than 30 nmol/L—a dose much lower than what is clinically achievable [71, 91]. It has been found to inhibit *CD74-ROS1*-transformed Ba/F3 cells with more potency than entrectinib, brigatinib, lorlatinib [92], or foretinib [71].

One case report described a 50-year-old woman with metastatic NSCLC who progressed after platinum-based therapy and was found to have a *ROS1* fusion. She was treated with crizotinib and progressed, at which point she was found to have the *ROS1* D2033N mutation within her ROS1 kinase domain. She was started on crizotinib 60 mg orally daily and achieved PR by 4 weeks and near CR by 12 weeks (92% reduction in disease burden). At the time of paper publication, she remained on therapy (near 8 months duration). In vitro analysis of CD74-ROS1 cells with D2033N mutation found significantly more suppression with cabozantinib than

ceritinib, brigatinib, and lorlatinib, though they remained highly sensitive to foretinib [74]. A recent abstract evaluated HCC78R cell lines with SLC34A2-ROS1 and ABC-20 cell lines harboring CD74-ROS1 (resistant to crizotinib). NGS evaluation found both an upregulation of HB-EGF and activation of the EGFR signaling pathway as well as an upregulation of AXL. The combination of cabozantinib and gefitinib was found to inhibit the growth of HCC28R tumors in an in vivo NOG mice model [93].

Unfortunately, cabozantinib is associated with a number of toxicities. The landmark METEOR trial which evaluated its use in renal cell carcinoma noted that 71% of patients experienced grade 3 or 4 events, the most common of which were hypertension (15%), diarrhea (13%), fatigue (11%), and palmar-plantar erythrodysesthesia syndrome (8%). Sixty-two percent of patients required dose reductions [94].

Entrectinib

Entrectinib (RXDX-101, Ignyta Pharmaceuticals, San Diego, CA, USA) is a small molecule that inhibits the tyrosine kinases TRKA/B/C, ROS1, and ALK (Table 1). It has a preclinical median inhibitory concentration (IC50) of 7 nm against ROS1, higher than crizotinib [95, 96]. Entrectinib was specifically designed to cross the blood-brain barrier [95]. Two recent phase I studies (ALKA-372-001 and STARTRK-1) evaluated entrectenib in patients with advanced solid tumors. Fourteen patients with *ROS1*-rearranged solid tumors (all NSCLC except one melanoma) were evaluated. These patients were all crizotinib-naïve. ORR was 86%, with an intracranial ORR of 100% (in the two ROS1 fusion patients evaluated). Median PFS was 19 months. Interestingly, six patients with ROS-1-rearranged disease who had previously received crizotinib were not observed to have any response to entrectinib [97]. Preliminary phase II data was recently reported, in which 32 patients with ROS1 fusion proven NSCLC (by NGS) who were naïve to prior TKI therapy were given 600 mg by mouth of entrectinib daily in 4 weeks cycles. ORR was 75% with three complete responses, intracranial ORR 71%, median PFS 19.1 months. The most common treatment-related adverse events were fatigue/asthenia (34%), dysguisia (34%), and dizziness (24%) [98]. There has been no preclinical activity demonstrated against *ROS1* resistance mutations G2032 or L2026; this combined with the lack of response in crizotinib-pretreated patients as noted above suggests that entrectinib's role in treating crizotinib-resistant disease may be limited unless progression is only in the CNS [92].

Lorlatinib

Lorlatinib (PF-06463922, Pfizer Oncology, Groton, CT, USA) is an oral TKI that targets both ALK and ROS1 with high affinity and good CNS penetration [91] (Table 1). Phase I data has been published looking at lorlatinib in NSCLC with *ALK*

or *ROS1* rearrangement; patients were allowed to have both CNS disease and prior TKI therapy. In this study, 12 patients had *ROS1* rearrangements, 7 of whom were pretreated with crizotinib. ORR was 50% [99]. Preliminary data has been presented from the phase II component of this study; the ROS1 cohort contained 47 patients, each of whom was treated with lorlatinib 100 mg daily. Regardless of prior treatment, ORR was 36%, with intracranial ORR 56%. The most common treatment-related adverse events and grade 3/4 adverse events were hypercholesterolemia (81%/16%) and hypertriglyceridemia (60%/16%) [100].

Lorlatinib is intriguing because of its activity against several crizotinib-resistant mutations. Dong et al. published a case report of a 57-year-old gentleman with a history of stage IIIB lung adenocarcinoma who initially went into remission following platinum-based chemotherapy but then relapsed and was found to have an EZR-ROS1 mutation. He initially responded well to crizotinib, with PFS of 6 months. After disease progression, he was started on lorlatinib 100 mg daily with favorable response after 3 months; he remained on drug at time of article publication [101]. Mutation type was not assessed in that publication, but another case report described an excellent response to lorlatinib in a patient who had the crizotinib- and ceritinib-resistant mutations S1986Y/F [75]. Additional cell-based assays have described sensitivity in the setting of D2033N [74] and L2026M [91] mutations. It is less clear what the role of lorlatinib is in the setting of the G2032 mutation; in preclinical studies this mutation has been found to significantly reduce lorlatinib's potency though activity still remained overall robust. *ROS1*-rearranged BA/F3 cells with the G2032 mutation have been found to have an IC50 of 508 nM as compared to 0.5 nM in wild-type *ROS1* [91].

Ropotrectinib

Ropotrectinib (TPX-0005; TP Therapeutics, San Diego, CA, USA) is a next-generation ROS1 inhibitor, a novel three-dimensional macrocyle with a much smaller size (MW <370) than current ROS1 inhibitors (Table 1). It was specifically designed to overcome resistance mutations. Preclinical studies have shown activity against gatekeeper and solvent mutations, including G2032R, D2033N, L2026M, S1986F/Y, L1951R, and kinases involved in bypass signaling such as focal adhesion kinase, SRC proto-oncogene, and non-receptor tyrosine kinase [102, 103]. Preliminary results have been reported from the phase I TRIDENT study. It included patients with *ALK*, *ROS1*, or *NTRK1-3* fusion-positive advanced solid tumors. Patients could be either TKI pretreated or naïve, and brain metastases were allowed. At the time of report of preliminary results, 29 ROS1 patients were enrolled. Confirmed PR have been observed in both TKI-naïve and pre-treated ROS1/NTRK+ patients at all dose levels, including one crizotinib refractory ROS1 G2032R+ patient with untreated CNS metastases. Median duration of clinical PR was 6.7 months with 88% (7 out of 8) responses ongoing. Toxicities have been tolerable, with the majority of adverse events remaining at grade 1–2; most common include dysgusia (38%), dizziness (35%), paresthesia (24%), and nausea (12%) [104].

DS-6051b

DS-6051b (Daiichi Sankyo, Japan) is an oral small molecule tyrosine kinase inhibitor that has demonstrated preclinical activity against *ROS1* and *NTRK1-3* rearrangements [105] (Table 1). A phase I trial evaluated 15 Japanese patients with NSCLC harboring *ROS1* fusions. ORR was 58.3% in the 12 patients with target lesions and 66.7% in the 9 patients who were crizotinib-naïve; DCR was 100%. Common toxicities included transaminitis (80%), diarrhea (53.3%), and nausea (46.7%). Maximal tolerated dose and recommended phase II dose was 600 mg by mouth daily [106]. Preliminary data was recently presented for a phase I trial of DS-6051b in advanced solid tumors conducted in the United States. 35 patients were enrolled, with 31 tumors evaluable. Nine patients had ROS1 fusions, including seven patients who had NSCLC and who had previously received crizotinib. Of the six evaluable NSCLC *ROS1*-rearranged patients who had previously received crizotinib, two patients had PR, and two had stable disease (SD). DS-6051b was noted to be tolerable up to 800 mg by mouth daily, with the primary adverse events being gastrointestinal (89%) [107].

Brigatinib

Brigatinib (AP26113, brand name Alunbrig; ARIAD Pharmaceuticals, Cambridge, MA, USA) is an inhibitor of both *ALK* and *ROS1* fusion NSCLC (Table 1). In preclinical studies, it was found to inhibit viability of *CD4-ROS1*-expressing Ba/F3 cells with an IC50 of 7.5 nM, as compared to a IC50 of 9.8 nM in *EMLA4-ALK* cells [108, 109]. It was FDA approved for use in metastatic crizotinib-resistant *ALK* fusion NSCLC in April 2017. A single armed phase I/II trial evaluated patients with advanced malignancies including *ALK*-rearranged NSCLC refractory to currently available therapies. Three patients in this study had *ROS1*-rearranged NSCLC. Two of these patients had previously received crizotinib; one had progressive disease (PD), and one had SD. The single crizotinib-naïve ROS1-rearranged NSCLC patient experienced a partial response and was continuing to receive brigatinib at the time of data cutoff (21.6 mo of therapy) [110]. In a phase II trial of 222 patients with advanced ALK fusion NSCLC that had progressed on crizotinib, common treatment-related adverse events were noted to be nausea (33/44%), diarrhea (19/38%), headache (28/27%), and cough (13/34%) (brigatinib 90 mg daily/180 mg daily). A subset of patients were noted to have early onset pulmonary events (all grades 6%; grade $\geq$ 3 3%) [111]. Preclinical work examining *CD74-ROS1* transformed BA/F3 cells in vivo has revealed that brigatinib exhibits activity against L2026M [92], but does not fare as well against D2033N [74], G2032R [71, 92, 109], or L1951R [71].

Foretenib

Foretinib (GSK1363089; GlaxoSmithKline) is an oral multikinase inhibitor that targets MET, VEGFR-2, RON, KIT, and AXL kinases. Preclinical data suggested that it was a potent inhibitor of *ROS1* fusions. It also demonstrated effective inhibition against the G2032 mutation at clinically feasible concentrations [112]. However it has been found to be less potent and effective than cabozantinib, and further development of the drug was discontinued [113].

Chemotherapy and Immunotherapy

Pemetrexed (formerly LY231514, brand name Alimta, Eli Lilly, Indianapolis, Indiana, USA) is a folate-based antimetabolite that exerts its activity via inhibition of enzymes critical in purine and pyrimidine synthase. These include thymidine synthase (TS), dihydrofolate reductase, and glycinamine ribonucleotide formyl-transferase [114]. Multiple studies have found that patients with *ALK* fusions have improved outcomes as compared to their wild-type colleagues [115]. The same appears to be true for patients with *ROS1* gene rearrangements. One retrospective study of 25 patients who had received pemetrexed (with or without bevacizumab) for 12 months or longer as therapy for their advanced stage non-squamous NSCLC included 5 patients with a *ROS1* gene rearrangement. Median OS was 42.2 months with median PFS of 22.1 months; patients with an oncogenic driver mutation (including but not limited to ROS1) had a statistically significant improvement in their PFS ($p = 0.006$) and OS ($p = 0.001$) compared to wild type [115]. Another retrospective study looked at four patients with metastatic NSCLC and FISH-detected ROS1 rearrangement who received pemetrexed. PFS ranged from 18 to more than 47 months [116]. A different retrospective study evaluated 253 patients with advanced NSCLC who were screened for driver mutations using RT-PCR. 19 patients (7.5%) had *ROS1* fusions. These patients were noted to have a better ORR (57.9%, $p = 0.026$), DCR (89.5%, $p = 0.033$), and PFS (7.5 mo; $p = 0.003$) as compared to patients with other driver mutations. Interestingly, while low levels of TS have historically been considered a favorable marker for pemetrexed efficacy in NSCLC, in this population this effect was not seen [117].

Although, PD(L)1 blockade has revolutionized the treatment of advanced NSCLC both as single agent and in combination with chemotherapy, no clear data currently exists suggesting the efficacy of immunotherapy specifically in patients with *ROS1* gene rearrangements. A phase I/II study evaluating the safety and tolerability of nivolumab plus crizotinib in the treatment of patients with metastatic NSCLC and ALK fusions was stopped early due to the degree of toxicity observed [118]. A recent phase II trial evaluated the use of pembrolizumab in NSCLC patients with both EGFR mutated and PD-L1-positive disease; study was similarly terminated early due to lack of efficacy even in patients with PD-L1 expression ≥50% [119]. Extrapolating

from EGFR-mutant NSCLC which, like ROS1, is also associated with patients who have not smoked, a recent meta-analysis evaluating three trials found that the use of single agent PD(L)1 inhibitors failed to improve overall survival in the *EGFR* mutant NSCLC, though survival was improved in wild-type lung cancers [120]. Another meta-analysis similarly revealed that in *EGFR*-mutated patients with metastatic NSCLC, PD-1/PD-L1 therapy is inferior to EGFR TKI in terms of progression-free survival [121]. Malignancies associated with tobacco smoking are frequently associated with a higher tumor mutational load and smoking-associated signatures that may underlie their improved response to immune checkpoint blockade [122]. As patients with driver oncogene mutations such as ROS1 are much less likely to have a history of tobacco smoking and low tumor mutational burden compared to patients with smoking-associated lung cancers, it is possible that this may explain inferior outcomes to single agent immunotherapy in these oncogene-driven tumors.

Interestingly, the recent IMpower150 trial that evaluated the addition of atezolizumab to the combination of carboplatin, paclitaxel, and bevacizumab (BCP) in patients with metastatic non-squamous NSCLC found a significantly improved PFS and OS as compared to the non-atezolizumab arm. This included patients who had received TKIs, irrespective of *ALK* or *EGFR* mutational status [123]. In the subgroup analysis, addition of atezolizumab in patients with *EGFR* exon19 deletion or L858R mutation led to improved PFS (HR 0.41; 95% CI 0.22–0.78) vs BCP alone [124]. Hopefully, one would expect comparable results in ROS1 NSCLC.

Conclusions

With the data currently available, first-line therapy for patients with advanced *ROS1*-rearranged NSCLC should be crizotinib. While resistance mutations such as G2032R can pose a treatment challenge, there are a number of next-generation TKIs that may assist in the management of these patients. Although immunotherapy likely does not appear to provide benefit as a monotherapy, immune therapy combinations warrant further study. Additional studies will need to be performed to fully define the role of next-generation TKIs, combination-targeted therapies against ROS1, and bypass tract mechanisms or resistance in *ROS1*-rearranged NSCLC that become resistant to crizotinib.

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