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REVIEW

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How receptor tyrosine kinase-like orphan receptor 1 meets its partners in chronic lymphocytic leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is the most common leukemia in western societies, recognized by clinical and molecular heterogeneity. Despite the success of targeted therapies, acquired resistance remains a challenge for relapsed and refractory CLL, as a consequence of mutations in the target or the upregulation of other survival pathways leading to the progression of the disease. Research on proteins that can trigger such pathways may define novel therapies for a successful outcome in CLL such as the receptor tyrosine kinase-like orphan receptor 1 (ROR1). ROR1 is a signaling receptor for Wnt5a, with an important role during embryogenesis. The aberrant expression on CLL cells and several types of tumors, is involved in cell proliferation, survival, migration as well as drug resistance. Antibody-based immunotherapies and small-molecule compounds emerged to target ROR1 in preclinical and clinical studies. Efforts have been made to identify new prognostic markers having predictive value to refine and increase the detection and management of CLL. ROR1 can be considered as an attractive target for CLL diagnosis, prognosis, and treatment. It can be clinically effective alone and/or in combination with current approved agents. In this review, we summarize the scientific achievements in targeting ROR1 for CLL diagnosis, prognosis, and treatment.

KEYWORDS
CLL, diagnosis, prognosis, receptor tyrosine kinase, ROR1, therapy

1 | INTRODUCTION

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a member of the ROR family, which was designated as an "orphan" receptor since its ligand was not identified for many years.[1] It is now known that ROR1 is a receptor for wingless-related integration site (WNT) family signaling molecules Wnt5a/b and Wnt16, primarly Wnt5a, involved in non-canonical WNT signaling and cell migration.[2,3] ROR1 is expressed during embryogenesis, where it is involved in skeletal and neural organogenesis,[1,4,5] diminishing during fetal development,[6] and being insignificant in healthy postnatal cells.[7] Recently, uncommon expression profiles were found in different malignancies such as Chronic Lymphocytic Leukemia (CLL), sparking interest in understanding ROR1 signaling and potential therapeutic approaches.[8]

CLL is a type of blood cancer characterized by abnormal B lymphocytes accumulation in the blood, bone marrow, lymph nodes, and other lymphoid tissues.[9] Although improved outcomes are

reported with the newly approved targeted agents for CLL treatment including the Bruton's tyrosine kinase (BTK) inhibitors ibrutinib and acalabrutinib, the BCL2 inhibitor venetoclax, and the phosphatidylinositol-3 kinase (PI3K) inhibitors idelalisib and duvelisib, acquired resistance persists on accounting of mutations in the target or the upregulation of other survival pathways such that triggered by ROR1, leading to disease progression.[10,11] Furthermore, CLL clinical and molecular heterogeneity makes it suitable for personalized medicine.[12,13] Thus, discovering new markers that predict leukemic cell survival helps in identifying patients with CLL at a high risk, characterized by a higher tumor burden, thereby facilitating appropriate therapeutic interventions. ROR1 expression in CLL and its clinical significance make it a potent target.[14–16] It can be clinically effective alone or in combination with existing treatments.[17,18]

Here, we review what is known about ROR1 including its structure, function, expression in CLL, signaling, diagnostic and prognostic roles, and in therapy resistance. We will provide updated information about anti-cancer therapies targeting ROR1 alone or in combination with other molecules.

2 | STRUCTURE AND BIOLOGY OF ROR1

In 1992, the gene encoding for ROR1 receptor was identified in SH-SY5Y, a human neuroblastoma cell line, and was initially named neurotrophic tyrosine kinase (TK) receptor-related protein 1. ROR1 gene is located on chromosome 1.[19]

ROR1 structure includes extracellular, transmembrane, and cytoplasmic sections.[1] The extracellular domain at N-terminus includes immunoglobulin-like (IG), cysteine-rich (CRD), and Kringle domains. The cytoplasmic section comprises TK, serine/threonine-rich, and proline-rich domains (PRD). The CRD binds to the ligand Wnt5a, regulating non-canonical WNT signaling.[20,21] In addition, ROR1 juxtamembrane section promotes its movement into the nucleus, where it acts as a transcription factor, influencing cytoskeleton network and cell migration.[22,23]

ROR1 importance was evident in ROR1-deficient mouse models that experienced neonatal death because of respiratory impairment indicating its role in embryogenesis.[24] Other studies demonstrated ROR1 impact on neurons' development and cell survival.[25,26]

In humans, ROR1 is normally expressed in embryo and fetus.[27] In adult stages, its expression is limited in adipose tissues, heart, lung, kidneys and some endocrine glands. It is also found minimally in the digestive tract, placenta, pancreas, skeletal muscles and immature B cells, where its role is still uncovered.[8,28,29]

3 | ROR1 ALTERATIONS IN CLL

Studies reported ROR1 overexpression in CLL compared to normal B lymphocytes.[7,30–32] In terms of cell distribution, ROR1 was found regularly expressed on leukemic cell surface,[28,33] without reported somatic mutations.[25,34]

ROR1 gene encodes a protein with a predicted molecular weight of 104 kDa.[20] It also undergoes N-linked glycosylation resulting in variants of 100, 115, and 130 kDa.[35] Notably, two major ROR1 isoforms were present in CLL cells: a 105 kDa un-glycosylated immature and a 130 kDa fully glycosylated mature isoforms. In addition, a dimerized 260 kDa form and a nuclear-restricted 64 kDa isoform exist. These isoforms are constitutively phosphorylated in CLL. Interestingly, the immature phosphorylated isoform is more expressed in non-progressive patients with CLL. However, the mature phosphorylated isoform is higher in progressive patients with CLL.[36] The 130 kDa variant, necessary for ROR1 membrane trafficking, triggers filopodia formation. ROR1 phosphorylation is necessary for the ubiquitination regulating its proteasomal degradation.[35]

Beyond CLL, ROR1 was detected in diverse hematological diseases[37–40] and solid tumors,[8,41,42] often correlated with metastases and poor outcomes.[43–45] ROR1 inhibition promoted significant apoptosis.[46–48]

4 | IMPACT OF ROR1 IN SIGNALING, DIAGNOSIS AND PROGNOSIS OF CLL

In CLL cells, ROR1 engages different signaling molecules and actives various transcription factors important for CLL cell proliferation, survival, migration, chemotaxis and drug resistance (Figure 1).

At first, a study identified Wnt5a as the ligand of ROR1 and showed that when both are expressed in the human embryonic kidney cells, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is activated.[7] Such pathway was attenuated by anti-ROR1 antisera post-treatment. ROR1 silencing using siRNA in CLL cells results in apoptosis.[46]

Yu et al. found that stimulating CLL cells with Wnt5a induces the oligomerization of ROR1 with ROR2, an ortolog to ROR1, and the recruitment of guanine exchange factors (GEFs) such as ARHGEF1 and ARHGEF2 (which activate RhoA) and ARHGEF2 and ARHGEF6 (which activate Rac1), enhancing CLL chemotaxis and proliferation.[49] Molecular analysis revealed that the intracellular domain of ROR1, particularly the proline-rich domain (PRD) with SH3-binding sites, is necessary for the recruitment of GEFs after Wnt5a stimulation. Although ARHGEF6 contains an SH3-domain, ARHGEF1 and ARHGEF2 lack SH3-domains suggesting that other proteins (*i.e.*, adapter proteins) are required to dock with ROR1. In keeping with these observations, a study used immune precipitates after stimulating ROR1 with Wnt5a and conducted mass spectrometry, identifying the adapter protein 14-3-3ζ.[50] Upon Wnt5a binding, ROR1 interacts with 14-3-3ζ, which in turn induces the recruitment of ARHGEF2 and enhances its activity, triggering RhoA and Rac1 activation thus boosting chemotaxis and proliferation in CLL cells.

Another research found elevated Wnt5a levels in plasma of patients with CLL, causing ROR1 to activate another GEF, DOCK2, inducing the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in the mitogen-activated protein kinase (MAPK) pathway.[51]

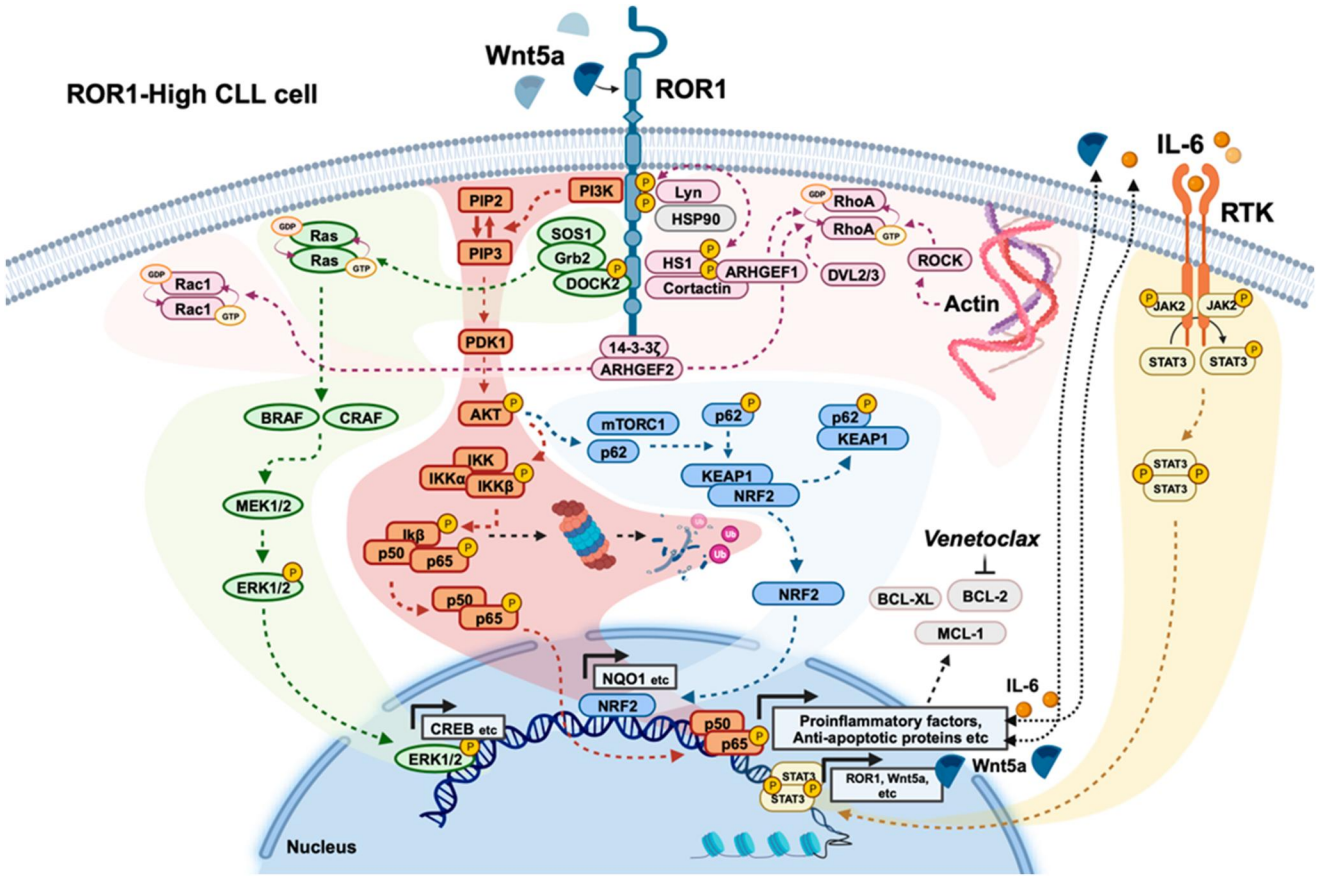

FIGURE 1 A schematic model for receptor tyrosine kinase-like orphan receptor 1 signaling in ROR1-High Chronic lymphocytic leukemia cell. Binding of Wnt5a induces the activation of different downstream pathways such as mitogen-activated protein kinase (MAPK)/ERK (green), PI3K/protein kinase B (AKT) or NFKB (orange), p62/NRF2 (blue) or janus kinase/signal transducer and activator of transcription (JAK/STAT,yellow) by recruiting different signaling proteins. Such pathways trigger cytoskeletal rearrangements (violet) associated with enhanced tumor cell migration or induce a transcriptional response (white) leading to the expression of genes which promote cell proliferation, survival, or therapy resistance. Dashed arrows and dot arrows indicate signaling pathways and factors release respectively.

Upon phosphorylation, ERK1/2 moves from cytoplasm into the nucleus to induce transcription factors enhancing CLL-cell proliferation. Interestingly, ROR1-expressing CLL cells have higher levels of DOCK2 and activated ERK1/2 with respect to ROR1-lacking CLL cells. A complex of DOCK2, a cytoplasmic protein, Growth Factor Receptor Bound Protein 2 (Grb2) and son of sevenless homolog 1 (SOS1) was then identified at position 808 of ROR1 in CLL cells upon Wnt5a stimulation. This complex induces the activation of Ras, which activates MAPK pathway that in turn activates ERK1/2, thus enhancing CLL-cell proliferation, potentially contributing to the adverse prognosis for high ROR1-expressing CLL cells.[52]

Subsequent studies discovered that Wnt5a prompts ROR1 to bind with both cortactin, and its homolog, hematopoietic lineage cell-specific protein 1 (HS1), that are then subject to tyrosine phosphorylation at Y421 and Y378 respectively, recruit ARHGEF1, and activate RhoA, enhancing chemokine-directed migration in CLL cells.[53,54] Cortactin and HS1, are involved in regulating actin cytoskeletal assembly and remodeling. Both are upregulated and linked with poor prognosis in CLL.[55–57] Interestingly, when both cytoskeletal proteins were silenced, the impact on blocking the Wnt5a-enhanced chemokine-directed migration was significantly greater compared to silencing either protein alone, indicating that both are important in such pathway and that shortage of one cannot be completely compensated by the other.

Daneshmanesh et al. demonstrated that anti-ROR1 mouse mAb targeting the CRD domain induced dephosphorylation of ROR1 and downstream signaling molecules including p85 isoform of PI3K, protein kinase B (AKT), mechanistic target of rapamycin complex 1 (mTOR) and CREB resulting in apoptosis in primary CLL cells compared to untreated CLL cells.[58] The PI3K pathway, known for its constitutive activation in CLL cells leading to AKT activation and downstream phosphorylation, was involved.[59]

Of note, the PI3K/AKT/mTOR and the previous mentioned ERK1/2 pathways, involved with ROR1, are interested by an interaction with the HSF1/HSP70 axis in CLL cells. In this context, the role of ROR1 within this partnership certainly deserves to be further investigated.[60,61]

Khan et al. identified DVL1, 2 and 3 in leukemic B cells but not in normal peripheral blood mononuclear cells.[62] Treatment of CLL cells with the anti-CRD ROR1 mouse mAbs induced ROR1, DVL 2 and 3

dephosphorylation at both serine- and tyrosine-residues. DVLs may induce the activation of Rho-associated protein kinase (ROCK), molecule involved in cell adhesion and migration.[63]

Furthermore, a study found that the levels of ROR1 and pSTAT3 were more elevated in CLL cells within the leukemia microenvironment where the expression of Wnt5a by Nurse like cells (NLCs) suggests higher Wnt5a concentrations in lymphoid organs than the circulation.[64] In fact, NLC expresses Wnt5a to induce ROR1-dependent phosphorylation of p65 and the expression of NF-κB targeted genes, thus the production of proinflammatory cytokines, predominantly IL6 and autocrine STAT3 activation. Activated STAT3 binds to ROR1 promoter, increasing ROR1 expression in leukemic B cells expressing ROR1 and potentially amplifying Wnt5a/ROR1 signaling through a positive feedback loop. This signaling induces other cytokines/chemokines (IL-8, CCL3, CCL4) promoting disease progression.[65] The ROR1 inhibitor cirmtuzumab blocks the ability of Wnt5a to activate NF-κB, inhibiting the expression of proinflammatory factors, which help recruit non neoplastic accessory cells to the leukemia microenvironment, as well as inhibiting the activation of STAT3.[66] Treated patients showed lower plasma IL6 levels, indicating that ROR1 signaling significantly leads to NF-kB activation in CLL cells expressing ROR1 in vivo. Given IL6's crucial role in CLL cell proliferation and disease aggressiveness,[67] targeting pathways associated with increased IL6 could have potential beneficial clinical effects.

CLL is a disease recognized by dependence on B-cell receptor (BCR) signaling and high ROR1 expression, where both represent therapeutic targets. Thus, understanding their interplay is of direct therapeutic relevance. Recently, a study unveiled that Lyn kinase interacts with the Tyrosine kinase domain of ROR1, phosphorylating tyrosine-residues (Y645 and Y646) within this domain impacting surface ROR1 during chemotaxis of CLL.[68–71] Dasatinib, a Lyn inhibitor, can attenuate this process. Lyn induced-ROR1 phosphorylation results in its binding to E3 ubiquitin ligase c-CBL, upregulated in CLL cells,[72] that recognizes phosphotyrosine of proteins and triggers their degradation.[73,74] Accordingly, pivotal phosphatases expressed in B lymphocytes are inactivated in CLL cells.[75] Lyn effect on ROR1-induced migration also includes action toward two cytoskeletal proteins HS1 and cortactin, which are Lyn substrates[55–57], that then undergo tyrosine phosphorylation, the recruitment of ARHGEF1, and activation of RhoA eventually leading to CLL cell migration.[53,54] Importantly, Lyn activity and ROR1 surface dynamics are negatively correlated explaining how low-Lyn activity BCR-inhibited cells can survive through ROR1 dependence.

Finally, phosphorylated ROR1 was identified as an interactor for another Heat Shock Protein, that of 90 kDa (HSP90),[76,77] leading to an αC-β4 loop formation, stabilizing ROR1. The inhibition of HSP90 leads to a ubiquitin-proteasome-dependent ROR1 degradation subsequently depleting ROR1 signaling in CLL. All the above-mentioned pathways were shown to be inhibited by cirmtuzumab. The detailed list of ROR1 interactors is summarized in Table 1.

In the diagnostic context, ROR1 showed selective expression on CLL cells with insignificant expression on normal B cells. This differential expression allows for accurate detection of CLL using flow cytometry.[7,29,33,80] Studies on patients with CLL undergoing various treatments demonstrated the consistent expression of ROR1 regarding the stage of the treatments allowing the use of ROR1 as a diagnostic marker independently from the disease stage,[80] but alone not enough for CLL diagnosis since it is expressed in other malignancies, including acute lymphoblastic leukemia,[37,81] mantle cell lymphoma (MCL),[38,39] and diffuse large B cells lymphoma (DLBCL).[40] However, other consensus biomarkers combination is required for CLL differential diagnosis such as CD19, CD5, CD20, CD23, Kappa, and Lambda whereas "Recommended" markers potentially useful for differential diagnosis in borderline cases could be: CD43, CD79b, CD81, CD200, CD10, and ROR1.[82] Yet less than 10% expression indicates the disease absence.[83] 95% of patients with CLL express ROR1 and the remaining 5% lack detectable ROR1.[14] Quantitative immune fluorescence studies reported surface ROR1 molecules range from 1000 to 11,000 per cell.[33,84] Importantly, after all treatment combinations, ROR1 was consistently present on residual CLL cells, aiding in detecting measurable residual disease (MRD) as alongside markers such as CD5 and CD19.[85–87] ROR1 evaluation can substitute the evaluation of the light chain, a marker used to detect CLL cells, not MRD. Furthermore, using recursive partitioning, a study defined a threshold for distinguishing CLL cells with elevated-level versus low-level ROR1 surface expression. This threshold corresponds to 6.2×10^3 immune fluorescent molecules per cell, established in 797 patients with CLL, that distinguishes ROR1-High and ROR1-Low subgroups with differing median treatment-free survival (TFS), validated in 771 patients.[14] Currently, ROR1 is included in the diagnostic panel recommended by the European Research Initiative on CLL and European Society for Clinical Cell Analysis.[82]

In a prognostic context, monoclonal antibodies (mAbs) against ROR1 indicated a significantly higher expression of ROR1 in CLL cells from patients with progressive disease, associated with shorter overall survival (OS) as compared with non-progressive disease exhibiting low ROR1 expression. Particularly, the rate of ROR1 expression was also worthily higher in patients with unmutated immunoglobulin heavy chain variable region (U-IGHV) versus altered IGHV (M-IGHV) genes.[15] Notably, non-progressive patients in the study were untreated, whereas most progressive patients had received prior therapy. However, as disease shifted from a non-progressive to a progressive, cells expressing ROR1 increased. Although treatment could explain this difference, a study reported that the expression and the mean fluorescence intensity of ROR1 were maintained by CLL cells regardless the type of treatment received in GIMEMA trials: ibrutinib + rituximab (CLL1114), venetoclax + rituximab (CLL1518), fludarabine + cyclophosphamide + ofatumumab (CLL0911); ibrutinib + ofatumumab (CLL1215). Particularly, this expression was detected always on residual CLL cells.[86] Transgenic mice expressing both ROR1 and TCL1 developed lymphocytosis and splenomegaly resembling human CLL. In such cells, ROR1 and TCL1, a coactivator of

TABLE 1 List of ROR1 interactors.

Interactor	ROR1 (domain)	Pathway/Main effectors	Effects	Refs
ROR2	Intracellular domain	ARHGEF1/2/6 (activation of RhoA and Rac1)	Proliferation and migration	49
14-3-3ζ	Serine-857 residue within the RSPS857SAS motif in the C-terminal serine/threonine domain	ARHGEF2 (activation of RhoA and Rac1)	Proliferation and migration	50
DOCK2	Proline-808 residue within the P-X-X-P-motifs of the PRDs of ROR1	MAPK pathway and activation of ERK1/2 (induction of transcription factors: CREB, Jun, HIF-1, Ets-1, Elk, etc.)	Proliferation and survival	51,52
HS1 and cortactin	Proline-841 residue within the P-X-X-P-motifs of the PRD of ROR1	ARHGEF1 (activation of RhoA)	Planar cell polarity, filopodia formation and chemokine-directed migration	53,54
PI3k		PI3K/AKT/mTOR pathway and activation of NRF2 (expression of ROS detoxifying enzymes: NQO1)	Survival, detoxification program, anti-oxidant response, anti-apoptotic response, resistance to therapy	17,58
		PI3K/AKT pathway and activation of NF-kB (induction of proinflammatory factors: IL6, IL8, CCL2, CCL3, CCL4, and CXCL1 etc., and anti-apoptotic proteins: MCL1, BCLXL)	Activation of JAK2/STAT3 pathway and autocrine activation of ROR1 pathway, survival, anti-apoptotic response, and resistance to therapy	64,78,79
Lyn	TKD of ROR1	Induction of ROR1 phosphorylation at tyrosine- residues, namely Y645 and Y646	Control of the dynamics of surface ROR1 during chemotaxis	68
HSP90	ELHHPNIV binding motif	Formation of an αC-β4 loop	ROR1 stabilization and protection from proteasomal degradation	76

Abbreviations: CREB, cyclic AMP receptor binding protein (CREB); Elk, E-26-oncogene; ERK1/2, extracellular signal-regulated kinase 1/2; Ets-1, E-twenty-six; HIF-1, Hypoxia-inducible factor-1; MAPK, Mitogen-activated protein kinase; NQO1, NAD(P)H quinone oxidoreductase 1; PRD, Proline rich domain; ROR1, Receptor tyrosine kinase-like orphan receptor 1; ROR2, Receptor tyrosine kinase-like orphan receptor 2; ROS, reactive oxygen species; TKD, Tyrosine kinase domain.

AKT, were found to complex, leading to AKT activation, increased proliferation and resistance to apoptosis.

The recursive partitioning study reported a considerably shorter TFS and OS in ROR1-High cases in U-IGHV/M-IGHV independent manner.[14] ROR1 ligand, Wnt5a, may represent a diagnostic and prognostic marker. Patients with CLL have elevated amounts of Wnt5a in their plasma but absent in normal subjects.[14] In addition, within the CLL microenvironment, accessory cells, such as NLCs, also express elevated levels of Wnt5a stimulating CLL cells.[64] Wnt5a might act in an autocrine way to circumvent such microenvironmental regulation.[88]

5 | ROR1 AS TARGET FOR THERAPY IN CLL

Several therapeutic strategies that potentially target ROR1 molecule, especially for CLL therapy, have been developed using different approaches including specific mAbs, mAbs-based strategies and small molecule inhibitors. They have been evaluated in preclinical studies and clinical trials, either alone and/or in combination with recently approved agents (*e.g.*, ibrutinib, idelalisib, venetoclax) as summarized in Tables 2 and 3, and Figure 2.

Blocking the ligand binding site by mAbs calls the immune system to remove malignant cells.[99] Various mAbs against ROR1 were designed, as detailed in Table 2.[15,89,90,100] Cirmtuzumab (formerly UC-961) is a humanized immunoglobulin G1 mAb, currently called zilovertamab, with strong affinity and selectivity for the ROR1 functional epitope on its extracellular section.[101] This antibody demonstrated the capacity to internalize and inhibit Wnt5a-induced survival impacts on leukemic cells. In vivo experiments demonstrated its stability, long serum half-life, and its capability to guide conjugated drugs to lysosomal compartments. Phase 1 trials in patients with CLL showed its long half-life, without dose-limiting toxicity, and was successful in inhibiting ROR1 signaling.[84]

Based on mAbs targeting ROR1, drug conjugated antibody (ADC), bispecific T cell engager (BiTE), and chimeric antigen receptor (CAR) T cells were created.

Zilovertamab Vedotin (formerly VLS-101) is an ADC functioning as a cell division inhibitor, hindering tubulin synthesis.[102,103] It effectively targets malignant cells expressing ROR1 in patient-derived xenograft models that were obtained from Richter syndrome patients.[91] This ADC recognizes ROR1 leading to rapid internalization of monomethyl auristatin E; causing cell-cycle arrest and apoptotic tumor death.[103,104] NBE-002, ADC, displayed efficient anti-tumor activity in patient-derived xenografts of solid tumors.[105]

BT-1, ADC, used to deliver a toxin that selectively promoted apoptosis of ROR1-positive MCL cells.[90]

TABLE 2 ROR1-targeted modalities in preclinical studies.

Drug	Description	Disease	Refs
Anti-ROR1 mAbs	Inhibitors	CLL	15
	3B8 against Ig-domain of ROR1, **1C11 and 1D8** against CRD of ROR1, **4C10 and 4A7** against KD of ROR1, **R11, R12, Y31** chimeric rabbit/human Fab and IgG1, **2A2 and 2D11** against N-terminal epitopes of ROR1	CLL, MCL	89
		CLL	90
Zilovertamab Vedotin (formerly VLS–101)	ADC: cirmtuzumab-linker-MMAE	RS	91
BT-1 immunotoxin	ADC: anti-ROR1 antibody (2A2-IgG) + truncated *Pseudomonas* exotoxin A (PE38)	CLL, MCL	90
2A2-miR-29b-ILP	ADC: anti-ROR1 antibody immunoliposome (2A2-ILP) + miR-29b	CLL	92
2A2-OSU-2S-ILP	ADC: anti-ROR1 (2A2-ILP) + OSU-2S	CLL	93
		MCL	38
KAN0173631 KAN0438063 KAN0438175 T	Small molecule inhibitors	CLL	94
KAN0439834	Small molecule inhibitor	CLL	95
KAN0441571 C	Small molecule inhibitor	DLBCL	40
ARI-1	Small molecule inhibitor	NSCLC	96
Strictinin	Small molecule inhibitor	TNBC	97

Abbreviations: CCL, Chronic lymphocytic leukemia; CRD, Cysteine-rich domain; DLBCL, Diffuse large B-cell lymphoma; Ig, immunoglobulin; KD, Kringle domain; mAb, monoclonal antibody; MCL, Mantle cell lymphoma; MMAE, Monomethyl auristatin E; NSCLC, Non-small-cell lung cancer; ROR1, Receptor tyrosine kinase-like orphan receptor; RS, Richter Syndrome; TNBC, Triple negative Breast cancer.

2A2-miR-29b-ILP, ADC, used to deliver regulatory RNA, was generated by encapsulating miR-29b associated with enhanced survival, in an anti-ROR1 antibody immunoliposome.[92] These nanoparticles mediated potent internalization and uptake of miR-29b selectively to ROR1-expressing CLL cells and exerted anti-leukemic activity both in vitro and in vivo.

2A2-OSU-2S-ILP, used to deliver OSU-2S sphingosine analog with an anti-tumor activity, was generated by coupling a mAb against ROR1 to nanoparticles containing OSU-2S. This ADC induced apoptosis in ROR1-positive malignant cells in preclinical studies.[38,93]

Another strategy of targeting ROR1 includes BiTEs, genetically engineered recombinant antibodies that can simultaneously bind two different epitopes or antigens. NVG-111 is a bispecific antibody that can simultaneously bind both ROR1 and CD3 molecules used to promote the activation of cytotoxic T cells through the T cell CD3 complex and selectively target the activated T cells to ROR1-positive malignant cells, with promising results in CLL and solid tumors in preclinical models.[106–108]

CAR-T cells targeting ROR1 proved effective outcomes in patients with ROR1^{pos} hematological and solid malignancies.[28,98,109]

The application of small-molecule TK inhibitors (TKIs), ATP-competitive inhibitors, is one of the most effective approaches for cancer treatment targeting the catalytic domains in TKs.[110]

Small-molecule TKIs (*e.g.*, KAN0173631, KAN0438063 and KAN0438175T) inhibit ROR1 cytoplasmic TK domain, inducing CLL cell apoptosis and ROR1 dephosphorylation.[94] KAN0439834 promotes CLL cell apoptosis without affecting healthy B and T cells, targets Wnt5a-induced ROR1 phosphorylation, and reduces CD45^{+}/CD19^{+}/ROR1^{+} cells in the spleen when administered to NOD-SCID mice xenotransplanted with human CLL cells.[95] KAN0441571C is a second-generation small molecule inhibiting ROR1 with enhanced pharmacological characteristics, which inhibits diffuse large B-cell lymphoma in a zebrafish.[40] It synergizes with venetoclax for DLBCL elimination in vitro.

Another molecule, ARI-1, binds the extracellular CRD of ROR1, blocks Wnt5a binding, halting non-small cell lung cancer cell growth in vitro as well as in vivo.[96]

Strictinin is an extract of *Myrothamnus flabellifolius* that binds the intracellular domain of ROR1. This compound prevented AKT phosphorylation and survival of breast cancer cells.[97]

DB03208, a naturally occurring organic compound interacts unspecifically with ROR1 and blocks its function.[111]

6 | ROR1 ROLE IN DRUG RESISTANCE MECHANISM AND COMBINATION THERAPY

Agents including the BTK inhibitor ibrutinib, the BCL2 inhibitor venetoclax, and the PI3K inhibitors idelalisib and duvelalisib, have been confirmed in the treatment of CLL. However, the success of these targeted agents is limited to the acquired resistance in relapsed CLL, caused by mutations in the target or by the upregulation of other survival pathways.[10]

TABLE 3 ROR1-targeted drugs in clinical trials.

Title	Condition or disease	Results	Reference
UC-961 (cirmtuzumab) in relapsed or refractory chronic lymphocytic leukemia NCT02222688, phase 1 Drug: cirmtuzumab (mAb)	R/R CLL. $n = 26$ patients received cirmtuzumab (up to 20 mg/kg) without dose-limiting toxicity, 4 biweekly infusions of cirmtuzumab prolonged time to next treatment (TTNT)	Median time to next treatment (TTNT) (following CLL progression) of 259 days (8.6 months), considering that pa- tients had progressive disease requiring treatment upon study enrollment.	(84)
Study of cirmtuzumab and paclitaxel for metastatic or locally advanced, unresectable breast cancer NCT02776917, phase 1 Drug: cirmtuzumab + paclitaxel	Breast neoplasms. Actual enrollment $n = 22$ participants	Ongoing	
A study of cirmtuzumab and ibrutinib in patients with B-cell lymphoid malignancies NCT03088878, phase 1, 2 Drug: cirmtuzumab + ibrutinib	B-cell CLL; SLL; MCL. Estimated enrollment = 160 participants	Ongoing	
Cirmtuzumab consolidation for treatment of patients with detectable CLL on venetoclax (venetoclax) NCT04501939, phase 2 Drug: cirmtuzumab Drug: venetoclax	CLL. Estimated enrollment = 16 participants	Ongoing	
A study of zilovertamab vedotin (MK-2140) (VLS-101) in participants with hematologic malignancies (MK-2140-001) NCT03833180, phase 1 Drug: zilovertamab vedotin	CLL, MCL, FL, MZL, DLBCL, richter Transformation,BL, LPL; TCL; NHL, ALL, AML, MW. Estimated enrollment = 330 participants	Ongoing	
A study to evaluate zilovertamab vedotin (MK-2140) for relapsed or refractory diffuse large B-cell lymphoma (DLBCL) (MK-2140-004) NCT05144841, phase 2 Biological: MK-2140 (zilovertamab vedotin)	R/R DLBCL estimated enrollment = 100 participants	Ongoing	
A study of zilovertamab vedotin (MK-2140) in combination with standard of care in participants with relapsed or refractory diffuse large B-cell lymphoma (rrDLBCL) (MK-2140-003) NCT05139017, phase 2, 3 Biological: zilovertamab vedotin; rituximab Drug: Gemcitabine; oxaliplatin; bendamustine	R/R DLBCL estimated enrollment = 420 participants	Ongoing	
A study of zilovertamab vedotin (MK-2140) (VLS-101) in participants with solid tumors (MK-2140-002) NCT04504916, phase 2 Drug: zilovertamab vedotin (MK-2140) (VLS-101)	TNBC, NSCLC, ER-positive BC, PR-positive BC, ER-negative BC, PR-negative BC, HER2-negative BC, ER-positive BC, PtR-OC, gastric cancer, pancreatic cancer. Estimated enrollment = 210 participants	Ongoing	
NBE-002 in patients with advanced solid tumors NCT04441099, phase 1, 2 Drug: NBE-002	Advanced solid tumor, advanced cancer TNBC. Estimated enrollment = 100 participants	Ongoing	
First in human study of NVG-111 in chronic lymphocytic leukemia and mantle cell lymphoma NCT04763083, phase 1, 2	CLL, SLL, MCL. Estimated enrollment = 90 participants	Ongoing	

(Continues)

TABLE 3 (Continued)

Title	Condition or disease	Results	Reference
Drug: NVG-111; NVG-111 (RP2D)			
Genetically modified T-cell therapy in treating patients with advanced ROR1+ malignancies NCT02706392, phase 1 Biological: ROR1 CAR-specific autologous T-lymphocytes	Hematopoietic and lymphoid cell neoplasm, malignant solid neoplasm, metastatic NSCLC, metastatic TNBC, recurrent ALL, recurrent MCL, R-CLL, stage III - IIIA - IIIB - NSCLC AJCC v7, stage IV NSCLC AJCC v6 and v7, stage IV BC AJCC v6 and v7, unresectable NSCLC	Terminated due to slow accruals	(98)

Abbreviations: AJCC, American Joint Committee on Cancer; ALL, Acute lymphoblastic leukemia; AML, Acute myeloid leukemia; BL, Burkitt Lymphoma; CLL, Chronic lymphocytic leukemia; FL, Follicular lymphoma; LPL/WM, Lymphoplasmacytic lymphoma or Waldenström's macroglobulinemia; MCL, Mantle cell lymphoma; MZL, Marginal zone lymphoma; NHLs, Non-Hodgkin's Lymphomas; NSCLC, Non-small-cell lung cancer; PR-BC, ER-BC, progesterone (PR) or estrogen (ER) receptor-positive/receptor-negative breast cancer; PtR-OC, Platinum-resistant Ovarian Cancer; ROR1, Receptor tyrosine kinase-like orphan receptor; R/R DLBCL, Relapse/refractory Diffuse large B-cell lymphoma; RT, Richter Transformation; SLL, Small lymphocytic lymphoma; TCL, T cell lymphoma; TNBC/HER2 Negative-BC, Triple-negative Breast Cancer or HER2 negative Breast cancer.

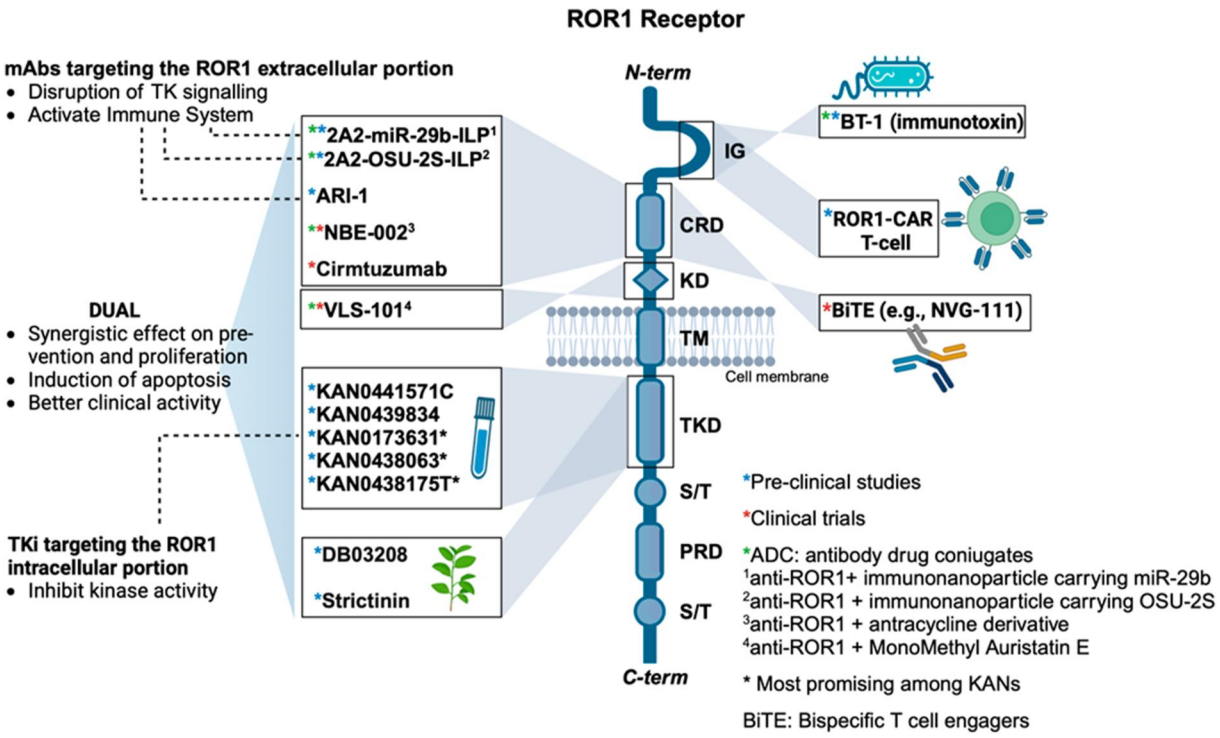

FIGURE 2 Therapeutic modalities targeting ROR1. The most advanced anti-ROR1 monoclonal antibody (mAb) is cirmtuzumab. Other antibody-based targeting strategies include anti-ROR1 antibody drug conjugates (ADC) that can deliver toxin (BT-1), regulatory RNA (2A2-miR-29b-ILP) or cytotoxic drugs (2A2-OSU-2S-ILP, NBE-002); bispecific T cell engagers (BiTE) such as NVG-111; and chimeric antigen receptor-engineered (CAR) T cells. ROR1 can also be targeted with small molecules inhibitors including KANs, ARI-1, Strictinin and DB03208. ROR1, receptor tyrosine kinase-like orphan receptor 1.

In venetoclax resistance, recent studies highlighted Wnt5a as a factor in the CLL microenvironment contributing to resistance to venetoclax.[64,112,113] In ROR1-high CLL cells, Wnt5a promotes the expression of NF-κB target genes and thus a significantly greater amount of p62 activating mTORC1 signaling and NRF2 to promote the expression of reactive oxygen species (ROS) detoxifying enzymes thus protecting tumor cells from drug-induced oxidative stress.[114–116] The expression of NFR2 target genes, such as NAD(P)H quinone oxidoreductase 1 (NAD(P)H quinone oxidoreductase 1), contributes to a drug-resistant phenotype, whose ablation enhanced the sensitivity of CLL cells to be eliminated by drugs that induce ROS production, such venetoclax.[117] Several evidences support that ROR1 recruits PI3K to induce the transformation of phosphatidylinositol[4,5]-bisphosphate (PIP2) to phosphatidylinositol[3–5]-trisphosphate (PIP3), leading to the activation of 3-phosphoinositide-dependent protein kinase 1, thus to the phosphorylation of AKT and the activation of NF-κB thereby the accumulation of p62 (in a IKKβ-dependent manner). NF-κB

activation also activates mTORC1. p62 binds and increases the activation of mTORC1. Phosphorylated p62 attaches to the Kelch-like ECH Associated protein 1 (KEAP1), thus prohibiting KEAP1 from sequestering NRF2.[63]

A study found that the loss/down-regulation of miR-15/16 upregulates BCL2 and ROR1.[118] High ROR1 levels coincide with high BCL2 expression. Cirmtuzumab potentiates venetoclax cytotoxicity for high-level ROR1 CLL cells suggesting synergistic ROR1 and BCL2 targeting potential.[78] Wnt5a neutralization counters this signaling, indicating its role in NLCs-CLL cell interaction. These findings reveal resistance mechanisms and suggest Wnt5a as a potential therapeutic target.

Furthermore, a study[119] analyzed CLL cells with high-level ROR1 expression before and over a year after venetoclax therapy, observing increased ROR1 levels post-treatment despite ongoing therapy, linked to rising MRD. ROR1-driven Wnt5a signaling upregulates genes including BCL2L1 encoding BCL-XL, contributing to enhanced venetoclax resistance. A case of venetoclax resistance without BCL2 alterations but having high BCL-XL levels was reported.[112] In primary CLL cells, Wnt5a potentiated their resistance to venetoclax, which was reversed by zilovertamab. Ongoing phase II trial evaluates the combination of cirmtuzumab with venetoclax in CLL cells of patients who received venetoclax for more than 1 year.

In ibrutinib resistance, studies reported that although ibrutinib may inhibit leukemic cells to enter the protective CLL microenvironment, Wnt5a remained at elevated amounts in the plasma of resistant patients[49,84]; such patients displayed activated Rac1, which declined when cells are cultured without serum, unless supplemented with exogenous Wnt5a.[18] High-dose ibrutinib, blocking BTK and BCR pathways, couldn't counter Wnt5a-induced Rac1 activation, but such effect was suppressible with cirmtuzumab. In vivo experiment using mouse model engrafted with histocompatible ROR1-positive leukemia, or human CLL xenografts, treated with cimrtuzumab and ibrutinib approved more effective clearing of leukemia cells than those treated with either agent.[18]

Ibrutinib potential is limited to impairing ERK1/2 and DOCK2 phosphorylation induced by BCR ligation, lacking influence over ROR1-dependent phosphorylation of such proteins caused by elevated plasma-levels of Wnt5a and thereby enhancing their growth via a ROR1/DOCK2-dependent route other than of BTK.[51,64]

Liu et al. reported that treating leukemic B cells with HSP90 inhibitor led to ROR1 degradation and enhancement of ibrutinib activity in vivo and in vitro.[76] Indeed, ibrutinib inhibits BTK, BLK, LCK or Lyn whose degradation is induced by HSP90 inhibitor, but not ROR1.[76]

Additionally, ibrutinib, insulates BCR signaling but doesn't affect Wnt5a-triggered phosphorylation of HS1 and cortactin, amplifying the CLL cell migration.[53,54]

These studies show that blocking ROR1 survival signaling, that appears unaffected by ibrutinib, supports the logic for clinical assessment of cirmtuzumab in patients with CLL. A phase 1/2 trial combined ibrutinib and cirmtuzumab, showing good tolerability and efficacy.[120]

T cells from at least 6-month ibrutinib-treated patients with CLL, co-cultured with autologous CLL cells and anti-ROR1 BiTE showed better cytotoxicity compared to non-ibrutinib-treated patients with CLL T cells.[121] Acalabrutinib showed similar effects in CLL models.[122]

7 | CONCLUSIONS AND PERSPECTIVES

In recent years, significant progress has been achieved in treating CLL with the rise of targeted agents such as BCL2 inhibitors, BTK inhibitors, and PI3K inhibitors. Although these agents have revolutionized CLL therapy and improved patients' outcomes, the issue of acquired resistance remains a challenge for recurrent and relapsed CLL. This can arise from mutations of the target or the overexpression of alternative cell survival pathways. One of these pathways is triggered by ROR1, a Wnt5a receptor. Its distinct expression profile on CLL cells and minimal presence on normal B cells makes it an appealing therapeutic targeting CLL, resulting in a heightened concern in depicting ROR1 signaling and targeted therapies against ROR1. ROR1 is involved in different signaling pathways important in CLL cell proliferation, survival, migration and drug resistance by calling different signaling proteins and activating various transcription factors. Approximately 95% of patients with CLL express ROR1, and anti-ROR1 mAb can distinguish CLL cells from normal B lymphocytes. ROR1 is consistently expressed in patients with CLL undergoing various treatment combinations, particularly on residual leukemic cells, making it a definite and reliable marker to diagnose MRD together with other markers. Due to the high expression of ROR1 in other malignancies, this marker alone does not seem to be enough for CLL diagnosis. But less than 10% of ROR1 expression could indicate disease remission. For that, mAb against ROR1 is proposed as a complement to be added in the diagnostic panel to increase the specificity and sensitivity of CLL detection. ROR1 has clinical significance in CLL; high level of ROR1 is strongly correlated with accelerated disease progression and adverse outcome. Particularly, the frequency of ROR1 expression is also significantly higher in patients with U-IGVH versus M-IGHV. Additionally, its ligand Wnt5a may represent a diagnostic and prognostic marker for CLL. Interestingly, patients with CLL have elevated plasma Wnt5a levels, not detected in healthy adults. Moreover, Wnt5a expression is significantly higher in U-IGHV patients, those with poor prognosis with respect to the mutated counterparts. ROR1 selective expression on CLL cells offers targeted therapy potential, minimizing side effects. Effective approaches include ROR1-targeting mAbs, ADC, BiTE, CAR T cells and small-molecule inhibitors. Interestingly, Lyn role in BCR-ROR1 interplay explains BCR inhibition leading to ROR1-dependent survival, contributing to drug resistance. This has clinical and therapeutic significance. Thus, combining BCR and ROR1 targeting holds promise in CLL and other cancers. Finally, ROR1-directed therapies, alone or with approved agents, show potential in CLL cell elimination and trials continue to test these approaches in CLL and ROR1-related diseases. Interestingly, ibrutinib enhances

ROR1 BiTE cytotoxicity by restoring T cell function. These findings pose a challenge to optimize drug combinations and sequencing in treatment strategy.

AUTHOR CONTRIBUTIONS

All authors contributed to drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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