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Mini review

Perspectives on the development of antibody-drug conjugates targeting ROR1 for hematological and solid cancers

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Abstract

Antibody–drug conjugates (ADCs) are targeted therapeutics generated by conjugation of cytotoxic small molecules to monoclonal antibodies (mAbs) via chemical linkers. Due to their selective delivery of toxic payloads to antigen-positive cancer cells, ADCs demonstrate wider therapeutic indexes compared with conventional chemotherapy. After decades of intensive research and development, significant advances have been made in the field, leading to a total of 10 U.S. food and drug administration (FDA)-approved ADCs to treat cancer patients. Currently, ∼80 ADCs targeting different antigens are under clinical evaluation for treatment of either hematological or solid malignancies. Notably, three ADCs targeting the same oncofetal protein, receptor tyrosine kinase like orphan receptor 1 (ROR1), have attracted considerable attention when they were acquired or licensed successively in the fourth quarter of 2020 by three major pharmaceutical companies. Apparently, ROR1 has emerged as an attractive target for cancer therapy. Since all the components of ADCs, including the antibody, linker and payload, as well as the conjugation method, play critical roles in ADC's efficacy and performance, their choice and combination will determine how far they can be advanced. This review summarizes the design and development of current anti-ROR1 ADCs and highlights an emerging trend to target ROR1 for cancer therapy.

Statement of Significance: ROR1 is a promising novel target for antibody-based therapeutics and in particular, ADCs. This review highlights the three most advanced anti-ROR1 ADCs in pipelines and discusses the opportunities and challenges in developing ADCs targeting ROR1 in hematological and solid cancers.

KEYWORDS: ROR1; antibody-drug conjugates; targeted therapeutics; cancer therapy

INTRODUCTION

Chemotherapy, the dominant cancer treatment used today, uses cytotoxic small molecules to kill cancer cells but is hampered by a narrow therapeutic index due to toxicity to normal cells. With widened therapeutic windows, antibody–drug conjugates (ADCs) have emerged as one of the fastest growing drug classes for cancer therapy [1]. ADCs are composed of monoclonal antibodies (mAbs) conjugated to highly cytotoxic small molecules (payloads) through chemical linkers. This marriage of the specificity of the biological macromolecules and the cytotoxicity of the small chemical drugs mediated by stable linkers has produced tremendous clinical success for ADCs [2]. Since the first ADC, Mylotarg®, was approved for CD33 positive acute myeloid leukemia (AML) in 2000 by the US Food and Drug Administration (FDA), 11 ADCs in total have been granted market approval globally, including the first original ADC (Disitamab vedotin) developed by RemeGen Co., Ltd in China. The pace of development has increased significantly during last 2 years, with three ADCs

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approved in 2019, an additional two in 2020 (Table 1) and ~80 others undergoing clinical trials [3–6]. Notably, three ADCs targeting the same antigen, ROR1, have been acquired or licensed in the fourth quarter of 2020 [7]. First, CStone Pharmaceuticals and LegoChem Biosciences entered global licensing agreement for their ROR1 ADC 'LCB71/ABL202'. Then Merck acquired VelosBio and its ROR1 ADC 'VLS-101', and Boehringer Ingelheim acquired NBE Therapeutics and its ROR1 iADC 'NBE-002' at the cost (combined upfront and milestone payments) of 2.75 billion and 1.45 billion US dollars, respectively. These acquisitions highlight the importance of ROR1 in oncology and its emerging role as a target for ADCs.

Receptor tyrosine kinase ROR1 is a type I single-pass transmembrane protein consisting of an extracellular region with one immunoglobulin (Ig), one frizzled (Fz) and one kringle (Kr) domain and an intracellular region harboring a pseudokinase domain [8]. A non-canonical Wnt signaling member, Wnt5a, can bind to the Fz domain and activate guanine exchange factors and the phosphoinositol-3 kinase/Jnk signaling pathway, leading to cell proliferation and enhancement of cell migration in response to chemokines [9]. As an oncofetal antigen, ROR1 is expressed physiologically in embryonic tissues and abnormally in various hematological and solid cancers, but is undetectable in most postpartum and adult tissues, making it a highly attractive target antigen for cancer therapy in general and for antibody-based therapeutics in particular [10, 11]. It is reported that high expression level of ROR1 on tumor cells correlated well with poor overall survival of patients with different cancers [9]. A phase I clinical trial (NCT02222688) has demonstrated the safety of a humanized mAb (UC-961, informally known as cirmtuzumab) targeting a unique region of ROR1 [12]. However, the naked antibody did not show significant clinical anti-tumor activity, despite its ability to block Wnt5a-induced effects on chronic lymphoblastic leukemia (CLL) cells, suggesting that inhibition of ROR1 signaling alone might be insufficient for clinical efficacy. Thus, in addition to combination therapy of ROR1 antibodies with currently available chemotherapy and immunotherapy, development of single molecule based targeted therapeutics that build upon naked antibodies, such as ADCs, are needed and may lead to more promising outcomes for cancer patients [13].

ADCs TARGETING ROR1

Although the concept of ADCs appears simplistic and straightforward in theory, the development of a safe and effective ADC is remarkably challenging. In the early era of ADC development, mouse mAbs coupled with anticancer drugs via unstable linkers had limited success because of poor stability, low potency, immunogenicity and suboptimal antigen expression profile. After decades of intensive study, significant improvements have been made in the field [14, 15], such as the use of humanized or human antibodies to reduce immunogenicity, innovative conjugation methods to generate homogeneous products, stable linkers to reduce off-target toxicity and novel release mechanisms

Table 1. Approved ADC to date

Name	Target	Format	Reactionsite	Linker	Payload	DAR	Cancer	Approval
Brentuximab vedotin(Adcetris®)	CD30	Mouse/human IgG1κ	Cys	MC-VC-PABC	MMAE	4	HL, T-NHL	2011
Ado-trastuzumab emtansine(Kadcyla®)	HER2	Humanized IgG1κ	Lys	MCC	DM1	3.5	Breast	2013
Inotuzumab ozogamicin(Besponsa®)	CD22	Humanized IgG4κ	Lys	AcBut	CAL	6	ALL	2000,2017
Gemtuzumab ozogamicin(Mylotarg®)	CD33	Humanized IgG4κ	Lys	AcBut	CAL	2.5	AML	2017
Polatuzumab vedotin-piiq(Polivy®)	CD79B	Humanized IgG1κ	Cys	MC-VC-PABC	MMAE	3.5	B-NHL	2019
Enfortumab vedotin-ejfv(Padcev®)	Nectin-4	Human IgG1κ	Cys	MC-VC-PABC	MMAE	3.8	Bladder	2019
Fam-trastuzumab deruxtecan-nxki(Enhertu®)	HER2	Humanized IgG1κ	Cys	MC-GGFG	DXd	8	Breast	2019
Sacituzumab govitecan-hziy(Trodelvy®)	TROP2	Humanized IgG1κ	Cys	CL2A	SN-38	7.5	Breast	2020
Belantamab mafodotin-blmf(Blenrep®)	BCMA	Humanized IgG1κ	Cys	MC	MMAF	4	MM	2020
Loncastuximab tesirine-lpyl(Zynlonta®)	CD19	Mouse/human IgG1κ	Cys	M-dPEG8-VA-PABC	PBD dimer	2.3	B-NHL	2021
Disitamab vedotin(爱地希®)	HER2	Humanized IgG1κ	Cys	MC-VC-PABC	MMAE	4	Gastric	2021

Abbreviations: MC-VC-PABC, maleimidocaproyl-l-valine-l-citrulline-*p*-aminobenzoyl carbamate; MCC, maleimidomethyl cyclohexane-1-carboxylate; AcBut, 4-(4′-acetylphenoxy) butanoic acid; MC-GGFG, maleimidocaproyl-glycine–glycine-l-phenylalanine-glycine; CL2A, cleavable PEG8- and triazole-containing PABC–peptide–MC linker; MC, maleimidocaproyl; M-dPEG8-VA-PABC, maleimido-dPEG8-l-valine-l-alaline-*p*-aminobenzoyl carbamate; MMAE, monomethyl auristatin E; DM1, Mertansine; CAL, calicheamicin; DXd, exatecan derivative; SN-38, active metabolite of the topoisomerase I inhibitor irinotecan; MMAF, monomethyl auristatin F; HL, Hodgkin lymphoma; HL, B-cell non-Hodgkin lymphoma; T-NHL, T cell non-Hodgkin lymphoma; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; MM, multiple myeloma; DAR, drug-to-antibody ratio.

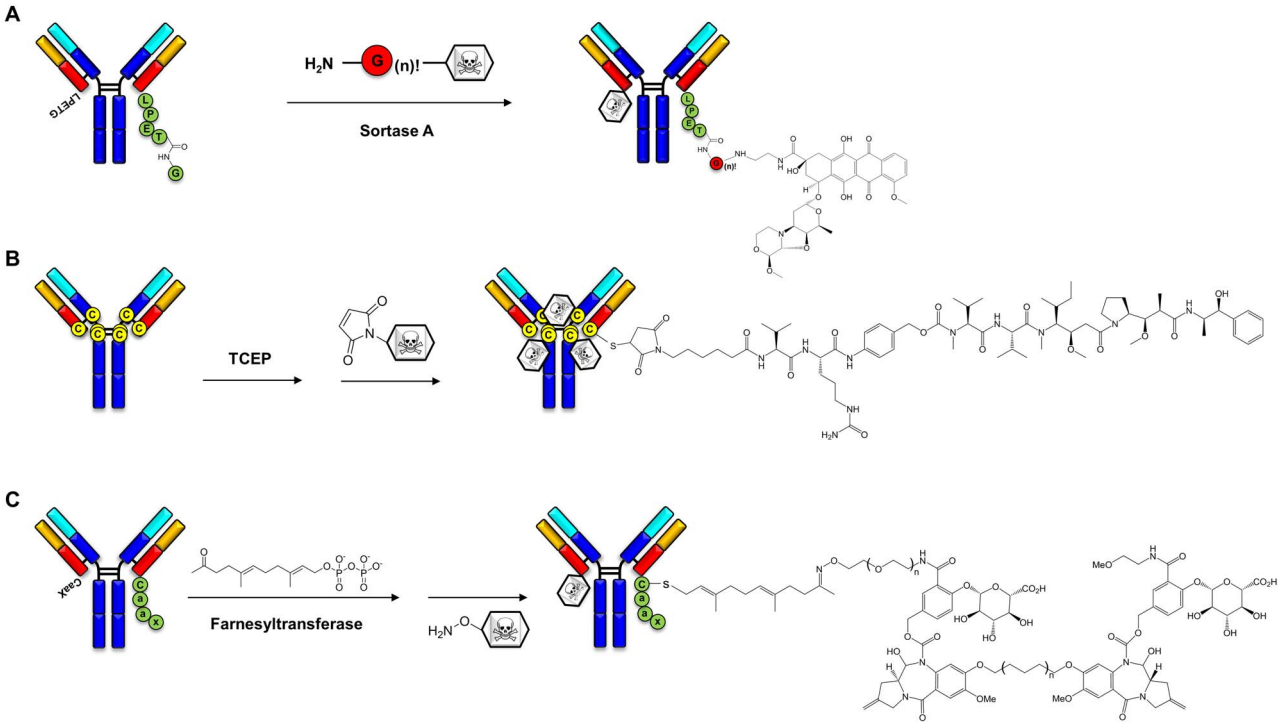

Figure 1. Potential conjugation strategies of the three anti-ROR1 ADCs. (**A**) NBE-002, a homologous ADC generated by Sortase A-mediated SMAC-technology™ to attach $Gly_{(n)}$-EDA-PNU-159682 to a humanized antibody. (**B**) VelosBio101, a humanized antibody coupled with MMAE via the 'MC-VC-PABC' linker. (**C**) LCB71/ABL202, a homologous ADC produced by Farnesyltransferase-mediated ConjuALL™ to introduce geranyl ketone pyrophosphate (GKPP) to a human antibody and subsequent oxime ligation reaction to load the dPBD prodrug.

for selective delivery, leading to the production of safer and more effective next-generation ADCs, including those targeting ROR1.

NBE-002

Currently, most of the approved ADCs are heterogeneous mixtures of drugs produced by chemical conjugation of payloads to the mAbs through lysine or cysteine residues. In contrast, the ROR1-targeting next-generation ADC, NBE-002 is generated by enzymatic conjugation of a humanized antibody (huXBR1-402) with the anthracycline PNU-159682 through sortase A-mediated site specific transpeptidation reaction at pre-defined drug-to-antibody ratio (DAR), resulting in a purer and more homogenous product with more stable biophysical and pharmacokinetic profiles (Fig. 1A; [16–18]). HuXBR1-402 was derived from a chimeric rabbit/human mAb (named XBR1-402) selected by phage display from naïve rabbit antibody libraries. XBR1-402 binds to epitopes in the Ig/Fz domains of human ROR1 with single-digit nanomolar affinity (Kd = 5.8 nM; [19]). This lead clone outperformed more than a dozen other rabbit mAbs selected from naïve or immune rabbit antibody libraries and murine mAb 2A2 and humanized mAb UC-961 [20–22], when loaded with PNU-159682 by sortase-enzyme mediated antibody conjugation technology (SMAC-technology™) in their ability to kill ROR1 positive cancer cells. XBR1-402 was humanized without loss of potency when constructed as an ADC while maintaining comparable affinity [23].

The SMAC-technology™ utilizes Sortase A, a transpeptidase enzyme derived from *Staphylococcus aureus* that can catalyze the ligation of oligo-glycine-containing molecules to proteins with a sequence motif (LPXTG; [24]). When the motif sequence LPETG was introduced to the C-termini of heavy and light chains of immunoglobulin proteins, a variety of cytotoxic payloads (e.g. MMAE and maytansine) with different linkers containing a penta-glycine tag was site-specifically conjugated with an average DAR of 3.2, ranging between 3.05 and 3.53, suggesting a conjugation efficiency per attachment site of roughly 80% under non-optimized reaction conditions [25, 26]. In the case of NBE-002, a highly potent PNU-159682 derivative comprising an ethylene-diamino (EDA) linker connecting a glycine stretch to the carbonyl group at C13 of the anthracycline structure (Gly_n-EDA-PNU) is the payload of preference to attach to huXBR1-402 to generate NBE-002. PNU-159682 is a liver metabolite of nemorubicin that can inhibit DNA topoisomerase II with three orders of magnitude higher potency compared with the parental molecule [27, 28]. Interestingly, in comparison with other payloads, conjugation efficiencies of anthracycline-based linker-payloads (including Gly_5-EDA-PNU) by SMAC-technology™ were significantly higher, with DAR ranging between 3.7 and 3.9, which could be efficiently increased to 4 by a simple StrepTactin affinity chromatography step to remove under-conjugated ADC species still containing a Strep-tag fused downstream of the LPETG motif sequence. Notably, the non-cleavable ADCs (targeting HER2 or CD30) with Gly_5-EDA-PNU have shown similar potency to cleavable

ones with the linker-payload, Gly_3-vc-PAB-PNU, but have reduced off-target toxicity compared with constructs using a cleavable linker [29]. It is possible that ADCs with non-cleavable linker-payloads such as Gly_5-EDA-PNU have less bystander killing efficacy against tumor cells with lower or no target expression. However, ADCs with PNU-159682, including NBE-002, have been demonstrated to induce sustained and long-lasting adaptive anti-tumor immunity in immunocompetent mouse models, in addition to direct anti-tumor activity observed for the conjugates. It was found that NBE-002 was able to increase the filtration of T cells into tumors and turn 'cold' tumors into 'hot' tumors, making them responsive to checkpoint inhibitors against PD-1 and CTLA-4 [30, 31]. Therefore, NBE-Therapeutics' ADCs are termed immune-stimulatory ADCs, or iADCs. To test the potency of NBE-002, this iADC has been evaluated in different preclinical cancer models, including acute lymphoblastic leukemia (ALL), CLL, AML, and triple negative breast cancer (TNBC), lung adenocarcinoma, ovarian carcinoma and a variety of sarcomas. In these studies, NBE-002 was found to display significant anti-tumor activity in each indication [16–18, 23, 30, 32]. NBE-002 is currently in phase 1/2 clinical trial (NCT04441099) to evaluate the safety and immunogenicity in patients with advanced solid tumors (carcinoma and sarcoma), especially triple-negative breast cancer. The initial results from this study are expected for the third or fourth quarter of 2021.

VelosBio101

Similar to Adcetris®, Polivy® and Padcev®, VelosBio101 (VLS-101) is an ADC that exploits a proteolytically cleavable linker, mc-vc-PAB, used to connect the mAb to an inhibitor of tubulin polymerization, monomethyl auristatin E (MMAE; [13]). VelosBio101 utilizes the ROR1-targeting antibody UC-961 (Fig. 1B), a humanized antibody binding to an intradomain epitope of ROR1 with high affinity (Kd = 2 nM) and has direct cytotoxic activity against various ROR1 positive tumor cells through blocking the binding of Wnt5a to ROR1 [22]. Particularly, UC-961 has been evaluated in a clinical study (NCT02222688) demonstrating the safety and potency to suppress ROR1 signaling and stemness signatures in patients with CLL. However, the naked antibody alone did not show significant clinical anti-tumor activity in these patients [12].

Due to the distinctive epitope recognized by UC-961, this antibody triggers obvious internalization of ROR1 upon binding, prompting its construction as an ADC to deliver cytotoxic payloads specifically to ROR1 positive tumor cells. After conjugation via formation of thioether bonds following reduction of two of the four interchain disulfides between the light chain and the heavy chain, an average of four MMAE molecules can be coupled to each UC-961 molecule, with a distribution ranging from 0 to 8 [13]. Using Richter syndrome (RS) cells as target cells, VLS-101 was shown to potently induce cell cycle arrest and apoptosis in *ex vivo* models and to block *in vivo* tumor growth in both subcutaneous and systemic RS-PDX (Patient Derived Xenografts) models. In a phase 1 study in hematological malignancies (NCT03833180), VLS-101 was well tolerated at 2.5 mg/kg every 3 weeks and seemingly effective in patients with advanced mantle cell lymphoma (MCL) or diffuse large B-cell lymphoma (DLBCL) as reported at the American Society of Hematology annual meeting in December 2020. PK studies revealed a mean ADC half-life of ~2.5 d, and no neutralizing anti-drug antibodies (ADA) were detected [33]. A Phase 2 study (NCT04504916) in patients with solid tumors, including breast and lung cancer, started in October 2020 and is estimated to be completed in September 2022.

LCB71/ABL202

LCB71/ABL202 is another homogeneous anti-ROR1 ADC co-developed by two South Korean biopharmaceutical companies, LegoChem Biosciences and ABL Bio. Although it has not yet advanced to the clinic, preclinical studies have attracted significant attention in the field and have led to the first global commercial deal for anti-ROR1 ADC development. China-based CStone Pharmaceuticals acquired the exclusive rights for the development and commercialization of LCB71/ABL202 outside South Korea. LCB71/ABL202 is based on a human antibody against ROR1 and uses another enzyme-mediated site-specific conjugation technology known as ConjuALL™ developed by LegoChem Biosciences (Fig. 1C; [3]). This conjugation approach utilizes the enzyme farnesyltransferase, which natively catalyzes the prenylation of the cysteine residue present within a CaaX motif (where 'a' stands for any aliphatic amino acid, and 'X' for any amino acid that determines specificity for a particular prenyltransferase; [34]). The ConjuALL™ technology exploits the substrate promiscuity of farnesyltransferase enzyme to introduce a geranyl ketone pyrophosphate (GKPP) group to install a ketone handle for subsequent chemoselective ligations to alkoxyamine derivatives. Previous results indicate that the catalytic efficiency of GKPP incorporation was >95% [35]. As oximes are highly resistant to hydrolytic cleavage in an aqueous solvent at physiological pH, the attached ketone handle introduced in the first step is harnessed for chemoselective ligation to alkoxyamine derivatives in the second step to conjugate a β-glucuronide-linked pyrrolobenzodiazepine dimer (dPBD), in which the DNA reactive imine at the N10-position of dPBD is modified with a β-glucuronidase-cleavable trigger and a self-immolative *o*-nitrophenol carbamate group to provide a hydrophilic masking of the toxic moiety as prodrug [36, 37]. Glucuronide linkages are known to be stable in circulation and can be cleaved by lysosomal β-glucuronidase that is highly expressed in tumor cells and the tumor microenvironment, allowing for selective cleavage and release of free PBD in cells and ultimately causing DNA damage and cell death [38]. The resulting homogeneous and stable LCB71/ABL202 with a defined DAR (DAR = 2) is expected to advance into clinical trials soon.

PERSPECTIVE

Unlike most of the antigens targeted by the approved ADCs that are either expressed solely by solid or hematological

tumor cells, ROR1 is widely expressed in a variety of both solid and hematological tumor cells, making it extremely attractive as target for more indications and potentially larger markets for drug development. In addition to naked antibodies and ADCs, small molecule inhibitors (e.g. KAN0439834 and KAN0441571C by Kancera, DB03208, strictinin, ARI-1), bispecific antibodies (e.g. NVG-111 by Novalgen) and CAR-Ts (e.g. Cirtuzumab-based CAR-T by Oncternal Therapeutics and JCAR-024 by Juno Therapeutics) have been developed to target ROR1 [9, 39, 40]. Notably, the phase 1 CAR-Ts based on a rabbit mAb R12 for patients with ROR1+ TNBC and NSCLC (NCT02706392) did not cause obvious toxicity to normal tissues after robust expansion in the peripheral blood, indicating the safety of ROR1 as target despite its low expression in the parathyroid and pancreatic islets [41]. This is very encouraging for development of therapeutics, including ADCs, to target this antigen. However, different from other antigens such as HER2, with hundreds of thousands of copies on tumor cells, the expression level of ROR1 in most tumor cells are usually less than ~5000 copies per cell [42], leading to the requirements to use a more potent antibody to trigger robust internalization, a more toxic payload, novel conjugation methods for greater drug loading and delivery (higher DAR), a linker that can release the payloads more efficiently, or a combination of approaches to generate more potent ADCs. For NBE-002 and LCB71/ABL202 particularly, both have highly potent payloads to overcome the low expression level of ROR1 on tumor cells. To minimize the risk of off-target toxicity caused by immature release of the drugs during circulation, both are using very stable linkers to ensure the release of drugs only in tumor cells. Note that although VLS-101 has less potent payload compared with the other two, VLS-101 showed very strong anti-tumor effects in blood cancer, probably due to a higher DAR and strong internalization and potential inhibition of Wnt5a signaling mediated by the antibody UC-961.

Due to unique site-specific conjugation strategy, both NBE-002 and LCB71/ABL202 are homogeneous ADCs with defined DARs, which is favored for manufacturing. In addition, the stable linkers in both ADCs also confer better pharmacokinetic profiles. Although the product of VLS-101 is not homogeneous and might have stability problems similar to Adcetris®, Polivy® and Padcev®, a phase 1 study has already demonstrated the safety and clinical efficacy in patients with MCL and DLBCL, and is the most advanced anti-ROR1 ADC that is now in phase 2 clinical trials. Regardless, all 3 anti-ROR1 ADCs look very promising, and the 3 recent major commercial deals will likely foster rapid development of multiple therapeutic modalities targeting ROR1, in addition to ADCs. Since the choice of mAb, the conjugation site, the linker and the payload all play critical roles in ADC design, novel components and combinations in the development of anti-ROR1 ADCs are expected in the future.

DATA AVAILABILITY STATEMENT

All data included in this review are available upon request by contact with the corresponding author.

CONFLICT OF INTEREST STATEMENT

HP is named inventor on a patent [23] claiming rabbit and humanized rabbit antibodies including XBR1-402 and huXBR1-402.

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