

# Preclinical evaluation of a novel antibody-drug conjugate targeting DR5 for lymphoblastic leukemia therapy

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**Acute lymphoblastic leukemia (ALL) is an aggressive hematological neoplasm resulting from immature lymphoid precursors. An antibody-drug conjugate (ADC), coupling a small molecule covalently with a targeting antibody, can specifically kill tumor cells. Death receptor 5 (DR5) is considered as a promising anti-tumor drug target. In this study, we describe the preclinical evaluation of a novel DR5-targeting ADC (Oba01) as a potential therapeutic against ALL. Oba01 utilizes anti-DR5 humanized monoclonal antibody (zaptuzumab) coupled via a cleavable linker to monomethyl auristatin E (MMAE). Oba01 can specifically bind to DR5 on the tumor cells and transfer into lysosome via DR5-mediated endocytosis. It then effectively releases the MMAE, which can bind to the tubulin and prevent its aggregation, thereby leading to a significant inhibition of proliferation and cell death in tumor cells. Additionally, Oba01 displays significant dose-dependent tumoricidal activity in cell-derived xenograft (CDX) and patient-derived xenograft (PDX) mouse models. More importantly, toxicity analysis of Oba01 showed a favorable safety profile, and pharmacokinetic analysis illustrated an excellent stability and tolerability in rats and cynomolgus monkeys. Taken together, our data conclusively demonstrate that Oba01 is an attractive candidate for further clinical trials in DR5-positive ALL patients.**

## INTRODUCTION

Acute lymphoblastic leukemia (ALL) is an aggressive hematological neoplasm resulting from abnormal proliferation of immature lymphoid precursors. ALL is more common in children, with a high incidence rate in the age group of 2–4 years. The current complete remission rate is more than 90%, and the disease-free survival rate is 70%–80%. In adults, 75% of ALL cases develop from B cell precursors and the remainder from T cell precursors.<sup>1–6</sup> However, the prognosis of ALL remains poor, and hence development of novel therapeutics to improve the treatment outcome is urgently needed.

Therapeutic antibodies are being actively used in clinical settings as the standard of care for the management of various solid tumors and hematological malignancies. Moreover, targeting antibody-drug conjugates (ADCs) could specifically bind to the tumor-related antigen on the cell

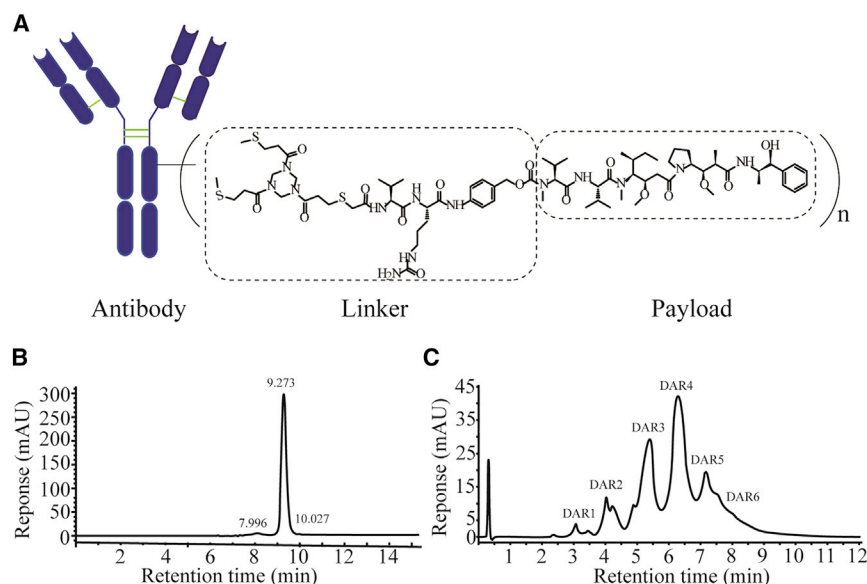
surface, and then could be endocytosed into lysosomes, where the payload of a small-molecule toxin coupled to the antibody could be effectively released by proteinase digestion and specifically kill the tumor cells.<sup>7–11</sup> Thus, ADCs have become an important strategy in development of the novel cancer therapeutics. As of now, several ADCs have been approved by the US Food and Drug Administration (FDA) or European Medicines Agency (EMA), for example, gemtuzumab ozogamicin (Mylotarg, Pfizer) for the treatment of CD33-expressing acute myelogenous leukemia,<sup>12</sup> brentuximab vedotin (Adcetris, Seattle Genetics) for the therapy of CD30-positive Hodgkin's lymphoma,<sup>13</sup> ado-trastuzumab-emtansine (Kadcyla, Genentech) for the treatment of Her2-positive breast cancer,<sup>14</sup> inotuzumab ozogamicin (Besponsa, Pfizer) for the management of CD22-expressing relapsed and refractory B cells and ALL,<sup>15</sup> polatuzumab vedotin (Polivy, Roche) for the therapy of CD79B-expressing relapsed and refractory diffuse large B cell lymphoma (R/R DLBCL),<sup>16</sup> trastuzumab deruxtecan (Enhertu, AstraZeneca/Daiichi Sankyo) for the treatment of Her2-positive breast cancer,<sup>17</sup> and enfortumab vedotin (Padcev, Seattle Genetics/Astellas) for the therapy of Nectin-4-positive bladder cancer.<sup>18</sup> Currently, more than 10 ADCs are undergoing phase III clinical trials in cancer patients.<sup>11</sup> However, there remains an unmet need to a great extent for the development of effective therapeutics against various malignant tumors, including ALL.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2/CD262), also known as death receptor 5 (DR5), is a type I membrane molecule belonging to the tumor necrosis factor receptor superfamily.<sup>19,20</sup> DR5 is highly expressed in the various cancers, such as lymphocytic leukemia, lung carcinoma, pancreatic cancer, colon cancer, breast cancer, ovarian cancer, and bladder cancer, among others, but it is minimally expressed in most of the normal cells.<sup>21–23</sup> DR5 has been considered as a promising and potential therapeutic target for the development of anticancer agents. Interestingly, various cancer therapies targeting DR5, including the recombinant soluble TRAIL (rsTRAIL) or anti-DR5 agonistic monoclonal antibodies,

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**Figure 1. Characterization of Oba01**

(A) The structure of Oba01. (B) Oba01 as characterized by size-exclusion chromatography. (C) Hydrophobic interaction chromatography depicting different drug/antibody ratio (DAR) isoforms of Oba01.

indicating that half-maximal effective concentrations ( $EC_{50}$ s) of Oba01 were 1.262, 0.4171, 0.2099, and 0.01683 nM, respectively (Figure S1). These data suggest that SD rats and cynomolgus monkeys are relevant animal species for the follow-up toxicology assessment of the Oba01 ADC before the clinical applications.

#### Selective cytotoxicity of Oba01 in ALL cell lines

To investigate the cytotoxicity of Oba01, we first explored the binding ability of Oba01 with various ALL cell lines by using flow cytometry (FCM). It is shown that Oba01 could effectively bind to Jurkat E6-1, Jurkat, A3, J.gamma1, Reh, and MT-4 cells, but not to TF-1, Kasumi-1, and Daudi cells (Figure S2), thereby suggesting that Jurkat E6-1, Jurkat, A3, J.gamma1, Reh, and MT-4 cells can express significantly higher levels of DR5. The cytotoxicity of Oba01 was evaluated in the panel of human lymphoblastic leukemia cells by CellTiter-Glo luminescent cell viability assay. As shown in Figure 2A, Oba01 demonstrated significant cytotoxicity against Jurkat E6-1, Jurkat, J.gamma1, Reh, A3, and MT-4 cells with 50% inhibitory concentration ( $IC_{50}$ ) values of 7.927, 7.855, 3.141, 0.03766, 2.034, and 6.578 nM, respectively, but it exhibited its cytotoxic action at a concentration >100  $\mu$ M in TF-1, Kasumi-1, and Daudi cells. In contrast, MMAE was observed to be cytotoxic toward all of the ALL lines tested, and no discrimination was observed between DR5-positive and DR5-negative cells. Additionally, the non-conjugated antibody zaptuzumab also exhibited cytotoxicity toward DR5-positive ALL cell lines. However, the non-targeted ADC, anti-HER-2 ADC of hertuzumab-MC-VC-PAB-MMAE, was found to be insensitive toward both DR5-positive and DR5-negative cell lines.

have been tested in the phase I/II clinical trials for multiple tumors.<sup>24,25</sup> In a previous study, we first reported that a novel highly potent first-in-class ADC named Zapadine-1 can effectively target DR5 and displays an excellent antitumor activity against both solid tumors and hematological malignancies, but the therapeutic window of this ADC was markedly lower for effective human cancer therapy.<sup>26</sup>

In the present study, we report a comprehensive preclinical evaluation of another novel DR5-targeting ADC (designated as Oba01) for the potential use in DR5-positive ALL therapy. Oba01 utilizes the humanized DR5-specific monoclonal antibody zaptuzumab coupled via a cleavable linker to a highly toxic inhibitor of tubulin, monomethyl auristatin E (MMAE), by using ThioBridgE technology.<sup>26,27</sup> We explored its possible anti-tumor efficacy in both *in vitro* and *in vivo* models. The toxicity and pharmacokinetic (PK) analysis of Oba01 demonstrated excellent safety, stability, and tolerability in both Sprague-Dawley (SD) rats and cynomolgus monkeys, indicating that Oba01 can potentially act as an attractive therapeutic candidate for further clinical investigation in patients with DR5-positive ALL.

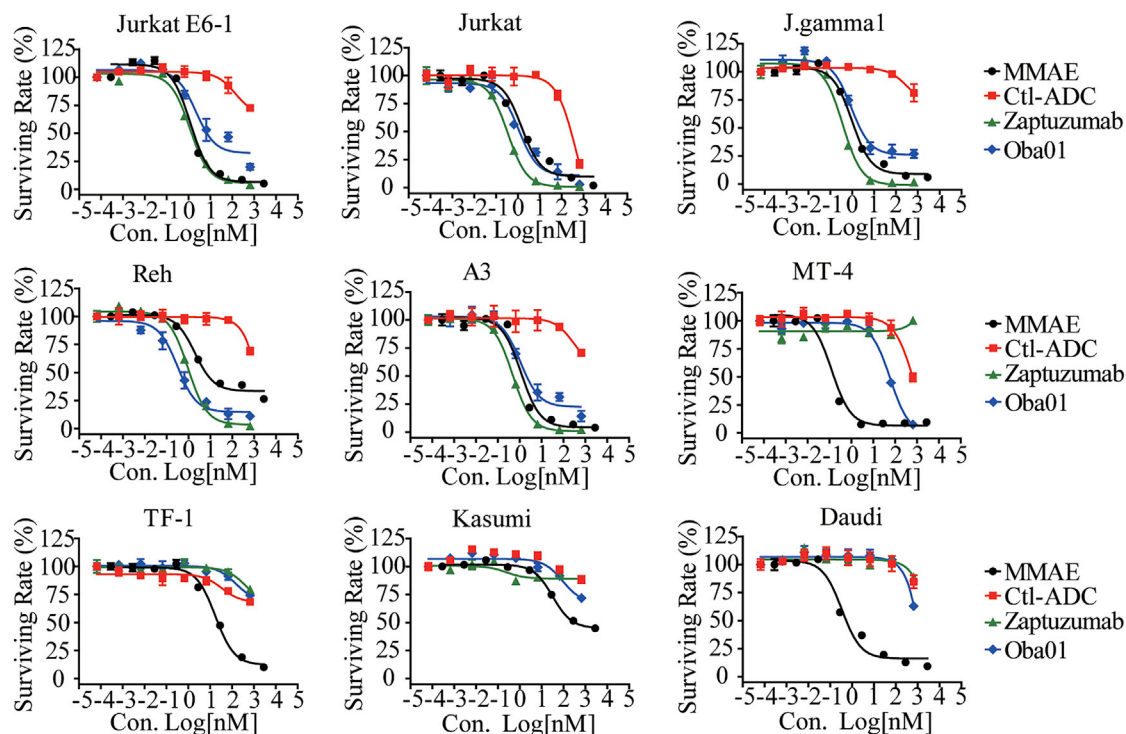
## RESULTS

### Generation of DR5-targeting ADC Oba01

Oba01 is an ADC composed of zaptuzumab, a humanized anti-DR5 antibody (immunoglobulin [Ig]G1), coupled with a cleavable valine-citrulline-dipeptide linker (PY-MAA-Val-Cit-PAB) and a highly potent microtubule-disrupting toxin, MMAE, by using ThioBridgE technology.<sup>26,27</sup> (Figure 1A). The ADC was 97.05% monodispersed as determined by size-exclusion chromatography (SEC) (Figure 1B; Table S2). The drug/antibody ratio (DAR) determined by hydrophobic interaction chromatography (HIC) was 4.0 (Figure 1C; Table S3). Additionally, the binding selectivity of Oba01 was also characterized by comparing its interactions with the extracellular domain (ECD) of DR5 from mice, SD rats, cynomolgus monkeys, and human subjects,

ADC internalization is one of the key requirements to facilitate its druggability.<sup>11</sup> As demonstrated in Figure 3A, Oba01 induced a significant time-dependent decrease in the expression of cell-surface DR5. In addition, Oba01 (green) was visible on the cells at 4°C and could be distinguished from lysosome-specific LAMP-1 that stained the lysosomes (red) in the cells (Figure 3B). After incubation at 37°C for 30 min, Oba01 was observed to be gradually internalized and visible inside of the cells, where it was found to be co-localized with LAMP-1 in the cytotoxicity of the lysosomes (Figure 3B), thereby indicating that Oba01 could bring the payload into the lysosomes through facilitating internalization.

To assess the possible apoptotic actions of Oba01 on Reh and TF-1 cells, FCM was used to detect annexin V/propidium iodide (PI)-positive cells.



**Figure 2. Selective cytotoxicity of Oba01 under *in vitro* conditions**

Jurkat E6-1, Jurkat, J.gamma1, Reh, A3, MT-4, TF-1, Kasumi-1, and Daudi cells were treated with increasing concentrations of Oba01, zaptuzumab, MMAE, and her-tuzumab-MC-VC-PAB-MMAE (Ctl-ADC) for 72 h. Thereafter, the cell growth and viability were determined by a CellTiter-Glo luminescent cell viability assay according to the manufacturer's instructions.

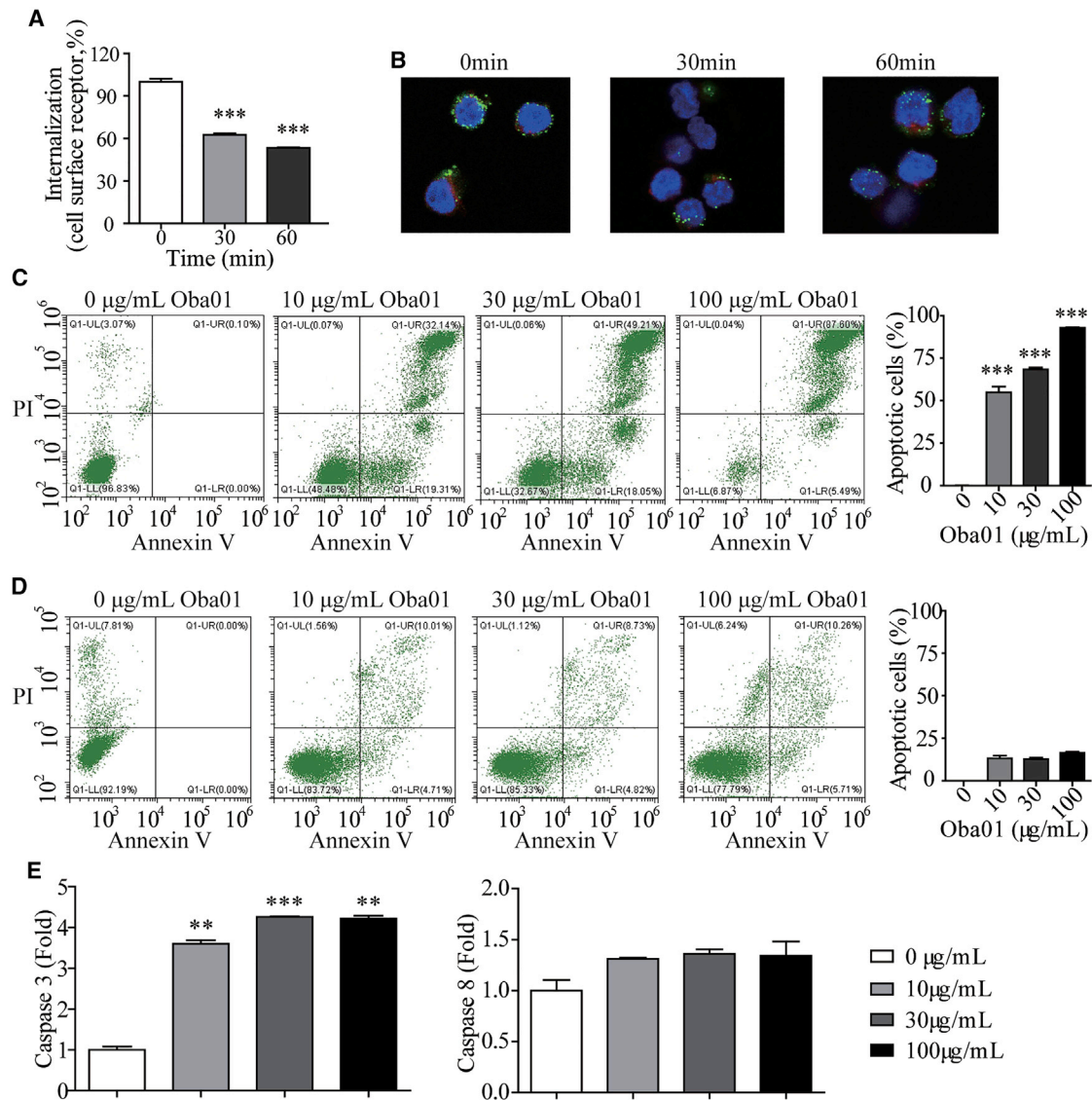
As shown in Figure 3C, there was a substantial increase of annexin V/PI-positive cells in a dose-dependent manner in DR5-positive Reh cells after Oba01 treatment for 48 h. However, Oba01 did not induce substantial apoptosis in DR5-negative TF-1 cells (Figure 3D). Caspase-3/8 activation is considered as a key biomarker of apoptosis.<sup>28,29</sup> As illustrated in Figure 3E, Oba01 caused a significant upregulation of caspase-3 activity and promoted a moderate increase in caspase-8 activity in the cellular lysate of Reh cells. Collectively, our results suggested that Oba01 could induce significant apoptosis in DR5-positive ALL cells.

#### ***In vivo* efficacy of Oba01**

Subsequently, the potential effects of Oba01 on the growth of human tumor xenografts in mice were evaluated. First, BALB/c nude mice bearing subcutaneous Reh ALL xenografts were administered Oba01 intravenously (*i.v.*) via tail vein once every three days for three times (Q3D × 3), and the tumor growth was observed for 67 days. As shown in Figure S3A, Oba01 induced a significant tumor regression at the dosages of 3 and 9 mg/kg. Complete tumor regression (CR) on day 67 was observed in one of seven animals in the 1 mg/kg oba01 dose group, four of seven animals in the 3 mg/kg dose group, and six of seven animals in the 9 mg/kg dose group. Thereafter, the tumor-bearing mice were administered Oba01 intravenously via tail vein once every week for four times (Q1W × 4) and the tumor size was observed for 35 days. As shown in Figure S3C, Oba01 displayed an impressive and significant

anti-tumor activity in a dose-dependent manner. The CR was noted in six of eight animals in the 3 mg/kg group on day 35, and in eight of eight animals in the 9 mg/kg group on day 35. Meanwhile, the tumor-bearing mice were intravenously administered Oba01 via tail vein once every two weeks for three times (Q2W × 3) and the tumor sizes were monitored for 43 days. As shown in Figure S3E, Oba01 induced substantial tumor regression at doses of 4 and 8 mg/kg, and the CR was seen in four of six animals in the 8 mg/kg group on day 43. Moreover, no notable body weight changes were observed in all animals during the experimental process (Figures S3B, S3D, and S3F).

We further tested the efficacy of a single intravenously administered dose in Reh xenografts. As shown in Figures 4A, 4B, and S4A, Oba01 elicited tumor regression followed by a marked delay in tumor progression. The CR was seen in nine of nine animals in the 8 mg/kg group on day 12, and this group remained free of palpable tumors until day 21, when the experiment was ended. The CR was also observed in two of nine animals in the 2 mg/kg group and one of nine animals in the 4 mg/kg group on day 21. However, the free form of MMAE (0.08 mg/kg, an equal mole of MMAE in the dosage of Oba01 of 4 mg/kg) exhibited no significant inhibitory effect on the tumor growth. The non-conjugated antibody zaptuzumab (8 mg/kg) also showed a significant suppression of the tumor growth in the mouse xenograft model, but it was comparable to Oba01 of 2 mg/kg, thereby



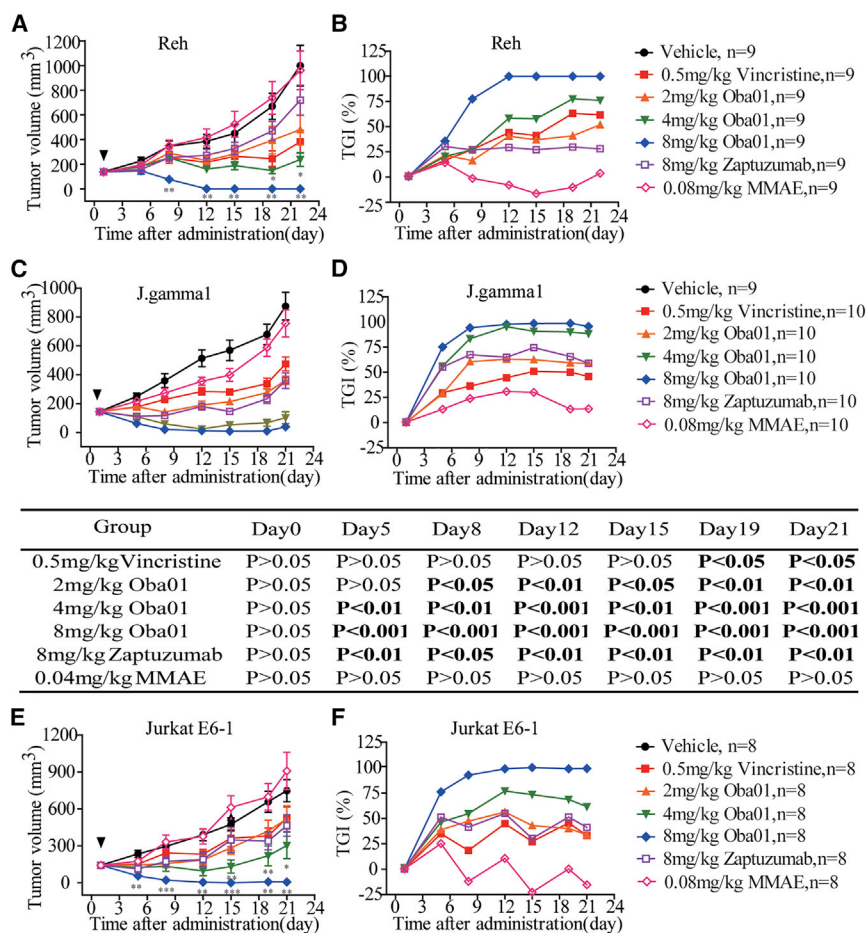
**Figure 3. Oba01 induces endocytosis, apoptosis, and caspase-3/8 activation in lymphoblastic leukemia cells**

(A) DR5 expression was detected on the Reh cell surface by flow cytometric analysis. (B) Internalization and lysosomal localization of Oba01 in Reh cells by a laser scanning confocal microscope. (C and D) Reh (C) and TF-1 (D) cells were incubated with Oba01 for 2 days, and then cell death was detected by using an annexin V-FITC/PI apoptosis kit by flow cytometry. The percentages of annexin V/PI-positive cells are shown in the bar charts. (E) Reh cells were exposed to 10  $\mu\text{M}$  Oba01 for 2 days. Then, caspase-3 and caspase-8 activation levels were analyzed by using a caspase-3 or caspase-8 activity assay kit. The p values were found to be two-tailed. \*\* $p < 0.01$ , \*\*\* $p \leq 0.001$ , versus vehicle control.

indicating that the Oba01 could significantly and dose-dependently suppress the tumor growth in Reh mouse xenografts.

Next, anti-tumor efficacy of Oba01 was validated in two other subcutaneous xenograft models of ALL J. gamma1 and Jurkat E6-1 cells. BALB/c nude mice bearing the xenografts were given by a single intravenous injection of various dosages of Oba01, and the tumor sizes were observed for 21 days. As shown in Figures 4C, 4D, and S4B, Oba01 displayed an impressive and substantial anti-tumor activity in a dose-

dependent manner. In the J.gamma1 model, 8 mg/kg Oba01 resulted in CR in 5 out of 10 animals. A significant tumoricidal activity was also observed in the 4 mg/kg group with a complete response in 50% of the mice bearing J.gamma1 xenografts (5 of 10). Moreover, in the Jurkat E6-1 model, 8 mg/kg Oba01 resulted in CR in 7 out of 8 animals. Additionally, tumoricidal activity was also observed in the 4 mg/kg group with a complete response in 37.5% of the mice bearing Jurkat E6-1 xenografts (three of eight), and 2 mg/kg Oba01 resulted in CR in one out of eight animals (Figures 4E, 4F, and S4C). However, in



**Figure 4. Antitumor activity of Oba01 in different sets of tumor models**

(A–F) Transplanted tumor volumes were assessed in J.gamma1 (A and B), Reh (C and D), and Jurkat E6-1 (E and F) cells. BALB/c nude mice bearing xenografts (tumor size approximately averaged 150–200 mm<sup>3</sup>) were intravenously (i.v.) injected with saline, 2.0, 4.0, and 8.0 mg/kg Oba01, or 0.5 mg/kg vincristine, 8.0 mg/kg zaptuzumab, or 0.08 mg/kg MMAE (the dose of MMAE equal to 4 mg/kg Oba01) once every 3 weeks. The dosing frequency is shown by arrowheads in the figures. The p values were found to be two-tailed. \*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001, versus vehicle control.

the ratio of human CD45<sup>+</sup> cells to the live cells in the mouse peripheral blood as the mouse tumor burden index was detected weekly. The data demonstrated that the ratios of CD45<sup>+</sup> cells to the live mouse cells were significantly and rapidly decreased to almost zero after administration of Oba01 but not those of the vehicle control (Figure 5B). Importantly, there was no visible influence on the body weights in the animals treated with Oba01 (Figure 5C). These results further indicated that Oba01 could significantly suppress tumor growth in the ALL PDX model without exhibiting any side effects.

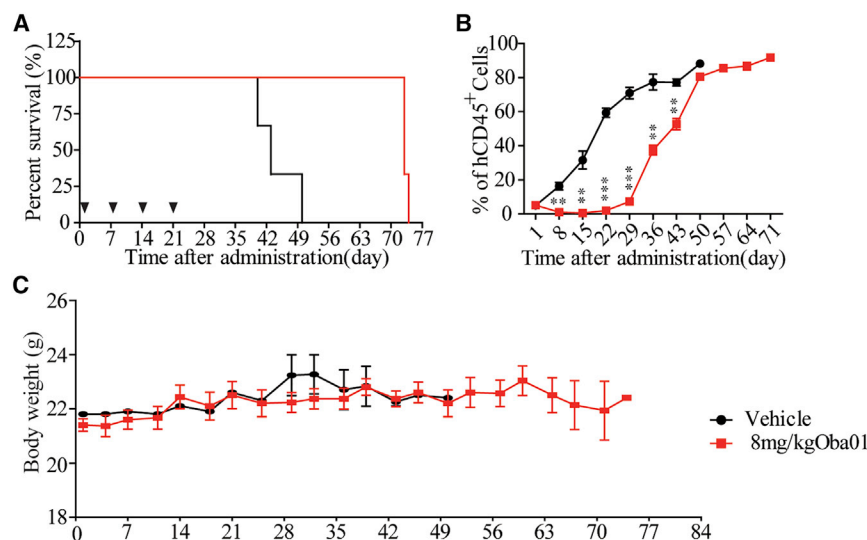
**Safety evaluation in rats and cynomolgus monkeys**

As mentioned above, all of the mice that were administered Oba01 in the mouse xenograft models appeared healthy and no notable body weight changes were observed. To further investigate the toxicity of Oba01 *in vivo*, in an acute toxicity study, a single dose of Oba01 (12, 24, and 48 mg/kg) and MMAE (0.46 mg/kg, an equal mole of MMAE in the dosage of Oba01 of 24 mg/kg) was intravenously injected into SD rats. As shown in Figure 6A, the maximal toxic dose (MTD) in SD rat was greater than 24 mg/kg, and the lethal dose was 48 mg/kg. However, the toxicity of Oba01 in SD rats was lower than that of the dose equivalent of MMAE alone. In addition, the acute toxicity study of Oba01 in cynomolgus monkeys was also conducted. A single dose of Oba01 (5, 10, and 20 mg/kg) and MMAE (0.19 mg/kg, an equal mole of MMAE in the dosage of Oba01 of 10 mg/kg) was intravenously administered into cynomolgus monkeys, respectively. As shown in Figure 6D, the MTD in cynomolgus monkeys was greater than 10 mg/kg, and the lethal dose was 20 mg/kg. Additionally, the toxicity of Oba01 in cynomolgus monkeys was lower than that of the dose equivalent of MMAE alone.

the two models, the free-form MMAE (0.08 mg/kg, an equal mole of MMAE in the dosage of 4 mg/kg Oba01) exhibited no substantial inhibitory effects, and the non-conjugated antibody zaptuzumab (8 mg/kg) also significantly suppressed the tumor growth. All of the treatment modalities did not lead to a notable change in the body weights of the mice (Figure S5). In addition, histopathological images of the mouse organ dissections clearly showed that Oba01 did not exhibit any notable adverse effects on the heart, liver, spleen, lung, and kidney (Figures S6–S8). Taken together, these data clearly demonstrate that Oba01 could significantly and dose-dependently exert an excellent and potent therapeutic efficacy in the different tumor xenograft mouse models of ALL.

To further confirm the efficacy of Oba01 in ALL mouse models, a T lymphocyte leukemia of the PDX model was established in non-obese diabetic (NOD)-severe combined immunodeficiency (SCID) mice by using HuKemia AL7174 patient lymphocyte leukemic cells. Thereafter, Oba01 was injected through the tail vein of mice (0 and 8 mg/kg, Q1W × 4). The results of this experiment demonstrated that the mice injected with 8.0 mg/kg of Oba01 survived for more than 71–74 days, but the mice that were administered with vehicle survived for 40–43 days after administration (Figure 5A). Moreover,

To further check the toxicity of Oba01 *in vivo*, a multiple-dosage toxicity study was performed in SD rats and cynomolgus monkeys. As shown in Figure 6B and Table S4, the MTD was more than 12 mg/kg, and the lethal dose was 36 mg/kg in SD rats, and there



**Figure 5. *In vivo* anti-tumor activity of Oba01 in acute lymphocyte leukemia PDX model AL7174**

(A) Survival rate of AL7174 mice. (B) Tumor burden of AL7174 mice. (C) Body weight of AL7174 mice. The dosing frequency is shown by arrowheads in the figures. The p values were found to be two-tailed. \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , versus vehicle control.

were no Oba01-related effects on body weight. In the 36 mg/kg Oba01 group, the animals showed some toxic reactions, such as wasting, arching back, sparse coat, swelling, pallor, scab, tissue defect, and fluffy coat, and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), cholesterol (CHO), creatine kinase (CK), and lactate dehydrogenase (LDH) were elevated (Table S6). Additionally, in the 0.23 mg/kg MMAE group, the body weight and food intake decreased. The toxicity of Oba01 of 12 mg/kg in SD rats was lower than that of the dose equivalent of MMAE alone. In addition, the MTD was more than 5 mg/kg and the lethal dose was 15 mg/kg in cynomolgus monkeys as shown in Figure 6E and Table S5, and there were no obvious abnormal changes in body weight in each dose group. In the 15 mg/kg Oba01 group, ALT, AST, CHO, CK, and LDH were elevated (Tables S7 and S8). Also, the toxicity of Oba01 of 5 mg/kg in cynomolgus monkeys was lower than that of the dose equivalent of MMAE alone. Therefore, a multiple-dose toxicity study of Oba01 in rats and cynomolgus monkeys showed excellent safety and tolerability *in vivo*.

#### Pharmacokinetics in rats and cynomolgus monkeys

The quantitation of total antibody, Oba01, and free-form MMAE was carried out in SD rat serum following a single-dose administration of 10 mg/kg (Figure 6C; Table S9) and in the serum of cynomolgus monkeys after a single-dose administration of 4 mg/kg (Figure 6F; Table S10). The pharmacokinetic parameters of Oba01 in SD rats and cynomolgus monkeys were basically the same as the total antibody, and area under the concentration-time curve (AUC) from time zero to the last quantifiable concentration ( $AUC_{last}$ ), AUC from time zero to infinity ( $AUC_{inf}$ ) were slightly lower than the total antibody, and half-time ( $t_{1/2}$ ), mean residence time (MRT) elimination parameters of Oba01 were slightly different from the total antibody, and the elimination of Oba01 was slightly faster as compared to the total antibody. The content of MMAE in Oba01 was 1.9%, and the system exposure of MMAE in SD rats and cynomolgus monkeys was about 1:100,000

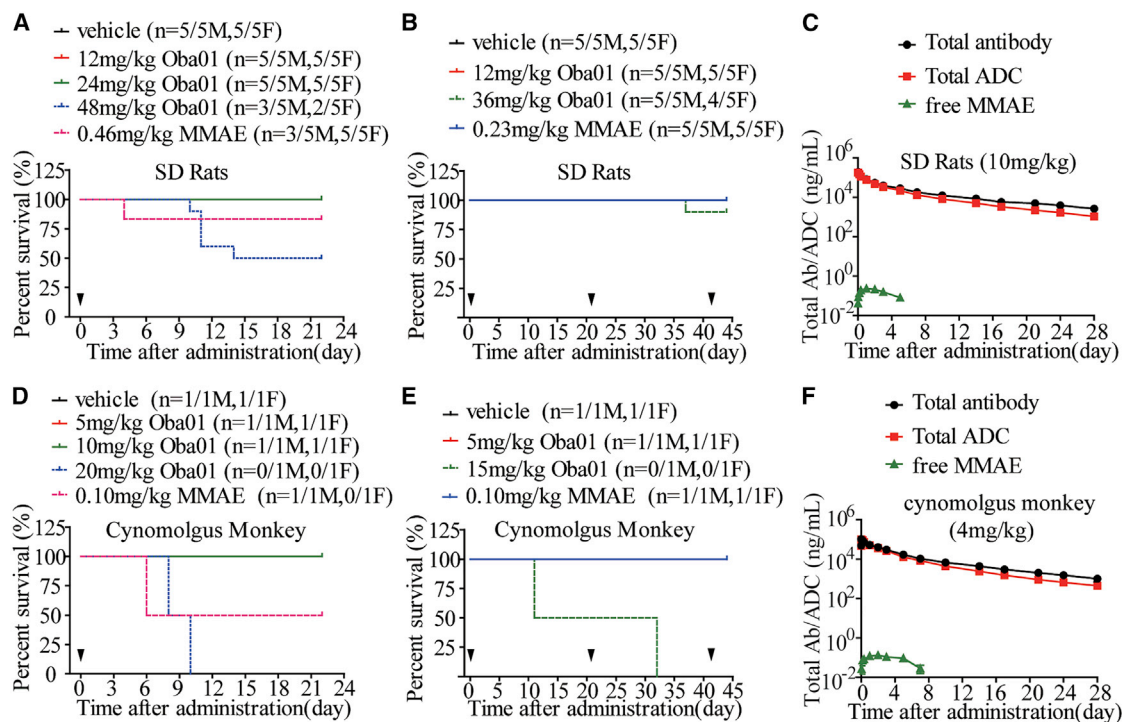
of Oba01, thereby indicating that Oba01 was substantially stable *in vivo* whereas the free form of MMAE was rapidly eliminated. Overall, the pharmacokinetic data clearly demonstrated that Oba01 was well tolerated in the rats and the monkeys with a favorable safety profile.

#### DISCUSSION

DR5 has been found to act as a potentially attractive therapeutic target for the development of anticancer agents, as it is exclusively expressed on the cell surface in various carcinomas, including lymphocytic leukemia, lung carcinoma, pancreatic cancer, colon cancer, breast cancer, ovarian cancer, and bladder cancer, among others, but with minimal expression in most normal cells.<sup>6,21,23</sup> DR5 can mediate apoptosis or autophagic cell death through initiating an intrinsic or extrinsic signaling pathway in tumor cells.<sup>23,30</sup> Anti-cancer therapies targeting DR5, including the use of recombinant soluble TRAIL (rsTRAIL) and anti-DR5 agonistic monoclonal antibodies, have been in clinical trials (phase I/II) for the treatment of multiple tumors.<sup>24,25</sup> We have also previously reported that the humanized anti-DR5 monoclonal antibody, zaptuzumab, has significant efficacy against lung carcinoma under *in vitro* and *in vivo* settings.

The concept of ADC has been well explored, and MMAE-based ADCs (such as brentuximab vedotin, polatuzumab vedotin, and enfortumab vedotin) are especially found to play an important role as anti-cancer therapeutics in the ADC arena.<sup>10</sup> Thus, exploiting molecular targets such as a DR5-targeting ADC can serve as a useful pharmacological strategy for developing therapeutics to improve antitumor activity in various malignancies. We have recently reported that Zapadine-1 ADC using MMAD as payload could display an excellent antitumor activity against both solid tumor and hematological malignancies, but the therapeutic window was found to be relatively lower for its application in human cancer;<sup>26</sup> however, at present, no ADC using MMAD has been reported to enter the clinical development stage. This suggests that MMAE-based Oba01 ADC targeting DR5 may offer different clinical opportunities once tested in cancer patients.

In this study, we evaluated the functional characterization and potential antitumor activities of Oba01 on ALL cell lines *in vitro* and *in vivo*. We showed using FCM assays with Oba01 that DR5 was highly expressed on the cell surface of most lymphoblastic leukemia cell lines. It has been reported that immunohistochemistry (IHC) analysis of anti-DR5 (AD5-10) demonstrated that DR5 was highly expressed



**Figure 6. Preclinical safety and pharmacokinetics studies of Oba01 *in vivo***

(A and D) An acute toxicity study was performed after single dosing intravenously, and survival rates in SD rats (A) and cynomolgus monkeys (D) were analyzed. (B and E) A long toxicity study was performed after multiple dosing intravenously, and survival rates in SD rats (B) and cynomolgus monkeys (E) were analyzed. (C) Quantitation of total antibody, Oba01, and free MMAE was carried out in the serum from SD rats treated with a single intravenous administration of 10 mg/kg Oba01. (F) Quantitation of total antibody, Oba01, and free MMAE was carried out in the serum from cynomolgus monkeys treated with a single intravenous administration of 4 mg/kg Oba01. The dosing frequency is shown by arrowheads in the figures.

on the tissue arrays of most blood malignancies.<sup>22</sup> We observed that Oba01 selectively and significantly inhibited the proliferation of DR5-positive human lymphocyte leukemia cells, but not that of DR5-negative tumor cells. In contrast to Oba01, MMAE was found to be cytotoxic against all of the cell lines tested and did not discriminate between DR5-positive and DR5-negative cells. The results from the apoptosis analysis in Reh and TF-1 cells demonstrated that Oba01 could induce significant apoptosis in DR5-positive lymphocyte leukemia cells. Furthermore, it has been reported that the delivery of toxin payloads by cytotoxic antibodies is limited via efficient internalization of the antigen-ADC complex.<sup>31</sup> We noted that Oba01 could effectively bind to DR5 on the tumor cells and enter into the lysosomes via DR5-mediated endocytosis, where it releases the small molecule toxin MMAE from Oba01 by the degradation of proteases, which can then bind to the tubulin proteins to prevent tubulin aggregation, thus leading to a substantial inhibition of tumor cell proliferation and eventually resulting in tumor cell death. The findings further indicated that Oba01 displayed a high selectivity and specificity toward DR5-positive tumor cells.

Furthermore, the antitumor activity of Oba01 was evaluated in human acute lymphocyte leukemia xenograft mouse models. It was observed that Oba01 effectively eliminated the tumors and prevented

recurrence, especially in the 4.0 and 8.0 mg/kg groups, and it even cleaned up tumors in Reh, J.gam1, and Jurkat E6-1 CDX models, thereby displaying a significant dose-dependent efficacy and potent anti-tumor activity in tumor-bearing mice. In contrast, free-form MMAE (0.08 mg/kg, an equivalent MMAE content to Oba01 of 4 mg/kg) displayed no significant inhibitory effect on tumor growth. However, zaptuzumab antibody alone (8 mg/kg) as a single agent suppressed tumor growth in this model but had a limited therapeutic effect comparable to the Oba01 at a dose of 2 mg/kg, thus indicating that Oba01 could significantly suppress the tumor growth in a dose-dependent manner. We also showed that Oba01 could significantly inhibit tumor growth in an ALL PDX model.

There have been several therapeutic anti-DR5 antibodies in clinical trials (phase I/II); however, Camidge et al.<sup>32</sup> reported that drozitumab monotherapy failed to show objective response in the patients with advanced solid tumors. It was also reported that the combination of conatumumab with carboplatin and paclitaxel for the treatment of non-small cell lung cancer (NSCLC), with panitumumab for metastatic colorectal cancer, or with doxorubicin for unresectable soft tissue sarcoma were found to be not effective despite being well tolerated.<sup>33</sup> The main reason for this inefficacy may be that the complex mechanism of DR5-mediated signaling pathways might produce an

anti-apoptotic effect downstream of DR5 signaling. Thus, it is important that the DR5 targeting ADC cytotoxicity primarily depends on DR5 expressed on the cell membrane, rather than the intracellular DR5-mediated signaling cascades. Additionally, the anti-DR5 antibody can specifically and effectively target tumors, and this tumor-targeting capability exerts an important role for the distribution of toxin payload to kill the tumor cells. Therefore, Oba01 ADC not only retains high tumor specificity, comparable DR5 affinity, and excellent therapeutic efficacy, but it also avoids the complex intracellular signal transduction network, which might produce a sustained anti-apoptotic effect produced by using naked anti-DR5 antibody alone.

Moreover, compared to Zapadacine-1 ADC, Oba01 demonstrated a better safety profile in the acute toxicity study in both rats and cynomolgus monkeys. Preliminary safety evaluation demonstrated that the MTD of Oba01 in SD rats was found to be more than 24 mg/kg, and the lethal dose was 48 mg/kg. The MTD of Oba01 in cynomolgus monkeys was more than 10 mg/kg, and the lethal dose was 20 mg/kg. Additionally, in single-dose toxicity of MMAE in SD rats and cynomolgus monkeys, the toxicity of Oba01 was found to be significantly lower than that of the dose-equivalent MMAE alone. The therapeutic dosages in the mouse CDX model were as low as 2–8 mg/kg, which was approximately equal to less than 0.2–0.8 mg/kg in humans, thus indicating that the therapeutic window of Oba01 was excellent for human cancer therapy. Therefore, in contrast to Zapadacine-1, Oba01 could display significantly less toxicity and better safety in clinical studies.

Meanwhile, in the analysis of pharmacokinetic profiles of Oba01, total antibody and free MMAE were measured by ELISA and liquid chromatography-tandem mass spectrometry (LC-MS/MS), respectively. In SD rats and cynomolgus monkeys, the pharmacokinetic parameters of Oba01 were basically similar to those of the total antibody.  $AUC_{last}$  and  $AUC_{inf}$  were slightly lower than the total antibody, and  $t_{1/2}$  MRT elimination parameters of Oba01 were slightly different from the total antibody. Finally, the conjugated MMAE concentrations decreased more rapidly than did the total antibody due to rapid antibody elimination and cytotoxic drug deconjugation.<sup>34–36</sup> Additionally, the level of free MMAE released from the ADC may be associated with significant loss of efficacy or even increased toxicity. Our data demonstrated that very low levels of free MMAE were detected for Oba01 in the systemic circulation, and the elimination of Oba01 *in vivo* was slightly faster than the total antibody. This reflected the potential stability of the linker and relatively limited deconjugation of MMAE from the conjugated zaptuzumab, which prevented induction of severe systemic toxicity. Therefore, we demonstrated that Oba01 could effectively release MMAE from Oba01 in the target tumor cells, and it was found to have favorable safety and pharmacokinetic profiles with excellent stability in both rats and cynomolgus monkeys.

Taken together, these preclinical findings demonstrate that Oba01 possesses a significant potential and dose-dependent tumoricidal ac-

tivity in CDX and PDX mouse models, and a favorable safety profile, stability, and tolerability in SD rats and cynomolgus monkeys. Future studies are being planned to further demonstrate that Oba01 is an attractive and promising drug candidate for exploration in clinical settings in patients with DR5-positive lymphoblastic leukemia.

## MATERIALS AND METHODS

### Cell lines and animals

The human cell lines used in this study along with cell growth media are shown in Table S1. The identities of cell lines were validated by short tandem repeat (STR) DNA fingerprinting. The cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (North China Pharmaceutical, Shijiazhuang, China) in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO<sub>2</sub> at 37°C.

BALB/c nude mice (5–6 weeks old) were purchased from GemPharmatech (Jiangsu, China). All animals were housed and maintained in a specific pathogen-free (SPF) grade of animal care facility. Five- to 6-week-old mice at initiation of the experiment were maintained with standard laboratory chow and water *ad libitum*. All animal experiments were approved and performed in full compliance with guidelines approved by the Animal Care Committee of the Center for Experimental Animals, Obio Technology (Shanghai, China).

### Antibodies and Oba01 ADC

The fully humanized DR5-specific monoclonal antibody (zaptuzumab)<sup>27,30</sup> and its ADC (Oba01) were generated by Yantai Mabplex International Bio-Pharmaceutical (Shandong, China).<sup>37</sup> Oba01 was produced by enabling zaptuzumab coupled with a highly toxic inhibitor of tubulin, MMAE, via a proteinase cleavable linker by ThioBridge technology and formulated in 5 mM histidine, 175 mM trehalose, and 0.035% (w/v) Tween 20 (pH 6.0). The solution was filtered (0.22 μm) and stored at –80°C.<sup>37</sup>

Characterization of Oba01 was performed by size-exclusion chromatography (1200 series high-performance liquid chromatography [HPLC], Agilent Technologies, Wilmington, DE, USA) and hydrophobic interaction chromatography (HIC) as described by Hamblett et al.<sup>37,38</sup> The identification for peaks corresponding to ADCs with 2, 4, and 6 mol of toxin per mole of antibody was accomplished. The drug/Ab ratio was determined by peak area integration.

### *In vitro* cytotoxicity assay

To determine *in vitro* anti-tumor activity, various lymphoblastic leukemia cell lines were treated with a 10-fold dilution series of ADC in triplicate in RPMI 1640 media supplemented with 10% heat-inactivated bovine serum (Gibco) for 3 days. Cell viability was measured with a CellTiter-Glo luminescent cell viability assay kit (Promega, G7572) according to the manufacturer's instructions, and absorbance was determined using a SPARK 10M multiplate reader (Tecan, Switzerland). Untreated cells served as controls. The cell survival rate (%) was calculated using the following formula:  $(A_{sample}/A_{control}) \times 100\%$ . The IC<sub>50</sub>



was calculated using a non-linear regression analysis using GraphPad Prism 5.0.

### Internalization assay

Reh lymphocyte leukemia cells were harvested and resuspended at a density of  $1 \times 10^6$ /mL in RPMI 1640 medium. 2.0  $\mu$ g/mL Oba01 was thereafter incubated with the cells at 4°C for 1 h, and the cells were washed with RPMI 1640 medium. Then, the cells were incubated at 37°C for the indicated time intervals. Thereafter, the cells were washed three times in ice-cold RPMI 1640 medium and fixed with 4% paraformaldehyde for 10 min. The cells were then incubated with 1  $\mu$ g/mL goat anti-human IgG (H+L) cross-adsorbed secondary antibody labeled with Alexa Fluor 488 (Invitrogen) at 4°C for 1 h, and thereafter washed twice with FCM buffer (PBS + 1% BSA) and resuspended in the ice-cold FCM buffer. The cell membrane staining was analyzed with a FCM Calibur (ACEA, NovoCyte 2060R, USA).

To examine Oba01 internalization and trafficking to the lysosome, approximately  $1 \times 10^6$  Reh cells in the logarithmic growth phase were added on the Lab-Tek chambered cover glass (Thermo Fisher Scientific, Waltham, MA, USA). The cells were then incubated the next day with 2.0  $\mu$ g/mL Oba01 at 4°C for 1 h, and thereafter the cells were washed twice with ice-cold RPMI 1640 medium and incubated at 37°C for the indicated time intervals followed by washing twice with ice-cold RPMI 1640 medium. The cells were then fixed with 4% paraformaldehyde and permeabilized with 0.05% Tween 20 in PBS for 10 min at 4°C. The cells on the cover glass were washed twice with ice-cold RPMI 1640 medium and incubated with 1  $\mu$ g/mL LAMP1 (D2D11) XP rabbit monoclonal antibody (mAb) at 4°C for 1 h. The cells were washed twice with ice-cold RPMI 1640 medium and then incubated with 1  $\mu$ g/mL goat anti-human IgG (H+L) cross-adsorbed secondary antibody labeled with Alexa Fluor 488 (green) (Invitrogen) and goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody labeled with Alexa Fluor 568 (red) at 4°C for 1 h. The nuclei were then counterstained with Hoechst 33342, washed twice with PBS, and covered with the coverslip. Images were captured by using a laser scanning confocal microscope (SP8, Leica, Germany).

### Cell apoptosis

To evaluate apoptosis of Reh lymphocyte leukemia cells treated with Oba01, the cells were seeded at a density of  $2 \times 10^5$  cells/well and exposed to Oba01 with various concentrations (0, 10, 30, and 100  $\mu$ g/mL) for 48 h. The cells treated with medium alone were used as controls. Cell death was detected by using an annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis kit (Beyotime Biotechnology, Shanghai, China) and measured by a FCM Calibur (Beckman, CytoFLEX LX, USA). Caspase-3 or caspase-8 activity was then determined by using caspase-3/8 activity assay kits (Beyotime Biotechnology, Shanghai, China) and measured by a microplate reader (SpectraMax M5, Molecular Devices, USA).

### *In vivo* therapeutic efficacy analysis in ALL CDX models

$1 \times 10^7$  Reh, J.gam1, or Jurkat E6-1 cells suspended in 200  $\mu$ L of PBS were injected subcutaneously into the right flank of BALB/c

nude mice. When the tumor volume reached 100–200 mm<sup>3</sup>, the mice were randomized into four or seven groups and injected intravenously with Oba01 at various doses at Q3D  $\times$  3, Q1W  $\times$  4, Q2W  $\times$  2, and Q3W  $\times$  1, respectively. Physiological saline was administered as a vehicle control. The tumor sizes were measured twice a week with calipers, and tumor volumes were determined according to the formula: tumor volume (mm<sup>3</sup>) = (length  $\times$  width<sup>2</sup>)  $\times$  0.5. T/C was calculated as (treated tumor volume/control tumor volume)  $\times$  100%. The inhibition rate of the tumor growth (TGI) was calculated as (1 – treated tumor volume/control tumor volume)  $\times$  100%.

### *In vivo* anti-tumor activity in ALL PDX model

To establish the T lymphocyte leukemia PDX model, HuKemia AL7174 cells (Crown Bioscience, Beijing, China) were thawed out from liquid nitrogen in a 37°C water bath rapidly and transferred into a 50-mL Falcon tube containing 40 mL of pre-warmed RPMI 1640 medium. The cells were pelleted by centrifugation at 1,000 rpm for 7 min, washed twice with ice-cold PBS, and then re-suspended into ice-cold PBS. The cells at a density of  $2 \times 10^6$  were intravenously injected into NOD-SCID mice for tumor development, denoted as day 0. After inoculation, eye blood was collected weekly from the animals, and the human CD45<sup>+</sup> cells in the mouse peripheral blood mononuclear cells (PBMCs) were stained and determined by fluorescence-activated cell sorting (FACS) to monitor the tumor burden. When the average of the tumor burden reached about 5%, the mice were randomly divided into two different groups (n = 3). The day of grouping was denoted as day 1. The mice were intravenously injected with vehicle and 8 mg/kg Oba01 at days 1, 7, 14, and 21, respectively. Physiological saline was administered as the vehicle control. The tumor burden of each group was determined and analyzed accordingly. In addition, animals were monitored daily for vital signs, including weight, mobility, activity, appetite, appearance and grooming, hydration, color of limbs, and color of mucosa, among others.

### Preclinical safety and pharmacokinetic studies

GLP (good laboratory practice) studies were conducted by JOINN Laboratories (Suzhou, China) in compliance with the animal welfare policies and guidelines approved by the National Medical Products Administration (NMPA) of China, including the toxicity study and pharmacokinetic study. The detailed methods are provided in the [Supplemental materials and methods](#).

### Statistical analysis

Results of all experiments are presented as mean values  $\pm$  standard deviation (SD) by GraphPad Prism 5 software. IC<sub>50</sub> values were determined by nonlinear regression analysis of concentration response curves using SPSS 16.0. The statistical significance between two groups was determined using a two-way ANOVA followed by a Student's t test. For all tests, p values less than 0.05 were considered statistically significant.

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omto.2021.04.013>.

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## AUTHOR CONTRIBUTIONS

S.Z. designed the study, planned the experiments, analyzed and interpreted the data, and wrote and edited the manuscript. W.Z., C.Z., P.X., and D.Z. made efforts on acquisition of data, development of methodology, interpretation of data, and carrying out all of the experiments. D.Z. conceived the project and reviewed and edited the manuscript. All authors contributed to and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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