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## Supplemental information

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

**Shuyong Zhang, Dongdong Zhou, Chao Zheng, Peng Xiong, Wan Zhu, and Dexian Zheng**

## Supplementary Methods

### In vitro binding assay by ELISA

High binding 96-well plates were coated with the recombinant DR5 extra-cellular domain (ECD) from mouse, SD rat, cynomolgus monkey and human (Sino Biological Inc., Beijing, China) and blocked with 0.05% Tween-20 containing 1 % BSA in PBS (PBST). Oba01 at varying concentrations were added and incubated at 37 °C for 1.0 h, then washed with PBST and incubated with HRP-labeled donkey anti-human IgG-H&L (Abcam, ab102438) at 37 °C for 1.0 h. The excess probe was washed with PBST. Then TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution (Solarbio, Shanghai, China, PR1200) was added and incubated at room temperature for 5 to 10 min followed by adding stop solution (1.0 M $H_2SO_4$). The optical density at 450 nm was determined on SPARK 10M multiplate reader (TECAN, 1703004862, Switzerland).

### Analysis of DR5 expression in various ALL cells by flow cytometry

DR5 expression in Jurkat E6-1, Jurkat, J.gamma1, Reh, A3, MT-4, TF-1, Kasumi-1 and Daudi cells was examined by flow cytometry following immunofluorescent staining. Briefly, cells ($1\times10^6$ cells/tube) were incubated in duplicate in the presence of Oba01 (1 μg/mL) at 4°C for 30 min. Normal human IgG antibody was used as a negative control. After washing, the cells were stained with 1 μg/mL goat anti-Human IgG (H+L) cross-adsorbed secondary antibody labeled with Alexa Fluor 488 (Invitrogen) at 4°C for 1 h, and then washed twice with FCM buffer (PBS + 1% BSA). The fluorescent signals in individual samples were analyzed by flow cytometry using a FCM Calibur (ACEA, NovoCyte 2060R, USA).

### Histopathology analysis

Samples were fixed overnight in 10% NBF and transferred to Dehydration machines. Paraffin embedding and cutting of tissue sections was performed by the Embedding Machine and LEICA Semi-Automated Rotary Microtome. For HE Staining on tissue Slides were baked 60 min at 60°C and were deparaffinized with

four rinses in xylene, hydrated using an alcohol gradient. Slides were then counterstained using hematoxylin and bluing reagents. Slides were then counterstained using eosin and dehydrated using an alcohol gradient. Slides were then cleared using xylene and sealed using medium. Imaging was done on a Leica Fluorescent Microscope.

**Preclinical safety evaluation and pharmacokinetic assay**

The GLP studies were performed at JOINN Laboratories, Inc. (Suzhou, China) in compliance with the animal welfare policies and guidelines approved by the National Medical Products Administration (NMPA) of China. Sprague-Dawley (SD) rats aged 6-8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). Cynomolgus monkeys aged 3-5.5 years were purchased from Guangxi grandforest scientific primate Co. (Guangxi, China).

For acute toxicity study, SD rats and cynomolgus monkeys were randomized into four groups. SD rats were single-dosed intravenously on day 1 with vehicle, 12 mg/kg, 24 mg/kg and 48 mg/kg of Oba01, and 0.46 mg/kg MMAE (a dose equivalent to the dose administered as the Oba01 24 mg/kg), respectively (n = 5 male/5 female/group). Cynomolgus monkeys were single-dosed intravenously on days 1 with vehicle, 5 mg/kg, 10 mg/kg and 20 mg/kg of Oba01, and 0.19 mg/kg MMAE (a dose equivalent to the dose administered as the Oba01 10 mg/kg), respectively (n = 1 male/1 female/group). All animals were examined for mortality and clinical signs every day. Assessment of toxicity was based on mortality, clinical signs, food consumption, body weight, clinical and anatomic pathology. Necropsies were performed on Day 22 and tissues were routinely processed.

For multiple doses toxicity study, 40 SD rats and 8 cynomolgus monkeys (half males and half females) were randomly divided into four groups. Oba01 was intravenously administered once per 3 weeks for 6 weeks followed by a week treatment-free phase, with vehicle, 12 mg/kg and 36 mg/kg of Oba01, and 0.23 mg/kg MMAE (a dose equivalent to the dose administered as the Oba01 12 mg/kg) (n = 5

male and 5 female) in 40 SD rats and vehicle, 5 mg/kg and 15 mg/kg of Oba01, and 0.10 mg/kg MMAE (a dose equivalent to the dose administered as the Oba01 5 mg/kg) (n = 1 male and 1 female) in 8 cynomolgus monkeys. Various parameters, including clinical signs, and body weight were monitored. Two days after the last administration, all rats (half males and half females) from each group were sacrificed for gross and histopathological examination.

For pharmacokinetic (PK) analysis, six SD rats (3 male/3 female) in each group were administered single-dosed intravenously with Oba01 of 10 mg/kg. Blood samples were drawn prior to dosing on day 0, and at 5min, 1h, 4h, 8h, 24h, 48h, 72h, 120h (D6), 168h (D8), 240h (D11), 336h (D15), 408h (D18), 504h (D22), 576h (D25), 672h (D29) after dosing. Blood was collected from tail veins at specified time points, and serum was isolated and stored at -80 °C. Six cynomolgus monkeys (3 male/3 female) in each group were administered single-dosed i.v. infusion (20 min) with Oba01 of 4 mg/kg. Blood samples were drawn prior to dosing on day 0, and at 10min, 1h, 4h, 8h, 24h, 48h, 72h, 120h (D6), 168h (D8), 240h (D11), 336h (D15), 408h (D18), 504h (D22), 576h (D25), 672h (D29) after dosing. Blood was collected from vena cephalica antebrachii or vena saphena at specific time points, and serum was isolated and stored at -80 °C. Quantification of total antibody and ADC were determined by optimized ELISA. The determination of free MMAE was performed by liquid chromatography tandem mass spectrometry. The PK analysis was performed using the software WinNonlin (V6.4, Pharsight, Princeton, USA).

**Supplementary Tables**

**Table S1.** Source of cell lines and cell growth media

| Cell Line | Source | Origin | Cell Growth Medium |
|---|---|---|---|
| Jurkat E6-1 | Cell Bank of Chinese Academy of Shanghai Institutes for Biological Sciences (Shanghai, China) | T-cell acute lymphoblastic leukemia | RPMI 1640+10%FBS |
| Jurkat | Cell Bank of Chinese Academy of Shanghai Institutes for Biological Sciences (Shanghai, China) | T-cell acute lymphoblastic leukemia | RPMI 1640+10%FBS |
| J.gamma1 | iCell Bioscience Inc. (Shanghai, China) | T-cell acute lymphoblastic leukemia | RPMI 1640+10%FBS |
| Reh | Cell Bank of Chinese Academy of Shanghai Institutes for Biological Sciences (Shanghai, China) | non T/B acute lymphoblastic leukemia | RPMI 1640+10%FBS |
| A3 | iCell Bioscience Inc. (Shanghai, China) | Acute lymphoblastic leukemia | RPMI 1640+10%FBS |
| MT-4 | iCell Bioscience Inc. (Shanghai, China) | T- Acute lymphoblastic leukemia | RPMI 1640+10%FBS +1% β-Mercaptoethanol |
| TF-1 | iCell Bioscience Inc. (Shanghai, China) | Erythroid leukemia | RPMI 1640+10%FBS +1%GM-CSF |
| Kasumi-1 | Cell Bank of Chinese Academy of Shanghai Institutes for Biological Sciences (Shanghai, China) | Acute Myeloid Leukemia | RPMI 1640+10%FBS |
| Daudi | Cell Bank of Chinese Academy of Shanghai Institutes for Biological Sciences (Shanghai, China) | Burkitt's lymphoma | RPMI 1640+10%FBS |

**Table S2.** High molecular weight, low molecular weight and monomer designation by size exclusion chromatography showing area and percentage of each fraction occupying total area under curve at 280 nm, related to Figure 1.

| Retention Time (min) | Designation | Area280nm(mAU*s) | % Area280nm |
|---|---|---|---|
| 7.996 | HMW Aggregates | 64.01737 | 1.2496 |
| 9.273 | Monomer | 4972.25293 | 97.0538 |
| 10.027 | LMW Fragment | 86.92017 | 1.6966 |

**Table S3.** Individual DAR components as determined by Hydrophobic Interaction Chromatography showing percentage of each fraction occupying total area under curve at 280 nm, related to Figure 1.

| Retention Time (min) | Designation | Area280nm(mAU*s) | % Area280nm |
|---|---|---|---|
| 3.409 | DAR1 | 75.43899 | 1.8986 |
| 4.336 | DAR 2 | 302.80391 | 7.6206 |
| 5.42 | DAR 3 | 1012.66406 | 25.4858 |
| 6.309 | DAR 4 | 1473.97003 | 37.0954 |
| 7.149 | DAR 5 | 791.88899 | 19.9295 |
| 7.963 | DAR 6 | 316.45347 | 7.9701 |

**Table S4.** Body weight in SD rat, related to Figure 6.

| Group | Sex | Day(s) Relative to Start Date | | | |
|---|---|---|---|---|---|
| | | -1 | 7 | 14 | 21 |
| Control | Male | 220.8 ± 2.7 | 282.6 ± 2.9 | 335.2 ± 3.8 | 376.0 ± 6.4 |
| | Female | 205.8 ± 2.1 | 224.8 ± 2.7 | 242.2 ± 4.7 | 260.6 ± 7.4 |
| Oba01 12 mg/kg | Male | 221.6 ± 2.6 | 272.8 ± 5.7 | 322.4 ± 10.5 | 369.0 ± 14.4 |
| | Female | 205.8 ± 2.4 | 230.0 ± 2.1 | 240.4 ± 5.2 | 256.6 ± 5.2 |
| Oba01 36mg/kg | Male | 220.4 ± 2.1 | 245.6 ± 8.5 | 291.2 ± 11.0 | 350.0 ± 8.6 |
| | Female | 205.2 ± 3.0 | 209.4 ± 7.7 | 226.0 ± 12.3 | 252.6 ± 6.3 |
| MMAE 0.23mg/kg | Male | 222.6 ± 2.8 | 258.0 ± 3.4 | 312.0 ± 4.7 | 358.2 ± 7.0 |
| | Female | 205.2 ± 2.8 | 222.0 ± 3.5 | 241.6 ± 5.6 | 262.6 ± 8.8 |

| Group | Sex | Day(s) Relative | | | |
|---|---|---|---|---|---|
| | | 28 | 35 | 42 | 44 |
| Control | Male | 404.6 ± 6.9 | 432.8 ± 7.1 | 454.4 ± 7.6 | 437.6 ± 8.0 |
| | Female | 271.4 ± 3.0 | 275.4 ± 4.0 | 272.8 ± 3.6 | 263.2 ± 3.4 |
| Oba01 12mg/kg | Male | 389.6 ± 16.1 | 422.4 ± 18.1 | 449.2 ± 19.8 | 431.8 ± 18.0 |
| | Female | 268.2 ± 8.3 | 278.8 ± 6.8 | 273.0 ± 7.3 | 261 ± 7.7 |
| Oba01 36mg/kg | Male | 360.6 ± 9.9 | 400.4 ± 10.3 | 435.4 ± 7.2 | 413.8 ± 5.9 |
| | Female | 251.2 ± 10.1 | 247.2 ± 22.9 | 275.0 ± 5.3 | 266.0 ± 5.1 |
| MMAE 0.23mg/kg | Male | 375.6 ± 7.9 | 409.2 ± 11.4 | 435.8 ± 12.9 | 427.0 ± 14.8 |
| | Female | 268.4 ± 9.2 | 283.0 ± 11.3 | 283.4 ± 10.0 | 279.4 ± 7.8 |

**Table S5.** Body weight in cynomolgus monkeys, related to Figure 6.

| Group | Sex | Day(s) Relative | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | -2 | 7 | 11 | 14 | 21 | 28 | 32 | 35 | 42 | 44 |
| Control | Male | 4.06 | 4.12 | - | 4.10 | 4.08 | 4.20 | - | 4.14 | 4.06 | 4.08 |
| | Female | 2.54 | 2.46 | - | 2.50 | 2.52 | 2.54 | - | 2.58 | 2.56 | 2.56 |
| Oba01 5mg/kg | Male | 2.48 | 2.48 | - | 2.50 | 2.50 | 2.58 | - | 2.58 | 2.50 | 2.54 |
| | Female | 2.54 | 2.60 | - | 2.56 | 2.52 | 2.70 | - | 2.64 | 2.62 | 2.62 |
| Oba01 15mg/kg | Male | 2.84 | 2.88 | 2.72 | - | - | - | - | - | - | - |
| | Female | 2.80 | 2.74 | - | 2.70 | 2.68 | 2.68 | 2.54 | - | - | - |
| MMAE 0.10mg/kg | Male | 3.94 | 3.92 | - | 3.76 | 3.84 | 3.94 | - | 3.98 | 4.02 | 4.00 |
| | Female | 2.46 | 2.40 | - | 2.36 | 2.42 | 2.46 | - | 2.54 | 2.36 | 2.32 |

**Table S6.** Primary blood-chemistry parameters in SD rats

| Group | Sex | ALT (U/L) | AST (U/L) | ALP (U/L) | GGT (U/L) | CK (U/L) | LDH (U/L) | CHO (mM) | TG (mM) |
|---|---|---|---|---|---|---|---|---|---|
| Ctl | male | 73±8 | 190±47 | 167±31 | 0.4±0.5 | 426±167 | 817±362 | 1.99±0.24 | 0.54±0.12 |
| | female | 53±12 | 134±20 | 64±14 | 1±0 | 461±254 | 938±565 | 2.37±0.60 | 0.61±0.11 |
| 12 mg/kg | male | 78±5 | 170±17 | 182±41 | 0.8±0.4 | 328±70 | 616±166 | 2.14±0.24 | 0.51±0.12 |
| Oba01 | female | 60±4 | 147±16 | 68±14 | 0.8±0.4 | 430±163 | 943±380 | 2.43±0.69 | 0.47±0.15 |
| 36 mg/kg | male | 103±11 | 293±57 | 257±67 | 3.4±2.2 | 812±426 | 1352±721 | 2.98±0.63 | 0.64±0.21 |
| Oba01 | female | 211±145 | 564±377 | 114±35 | 4.5±5.5 | 942±318 | 1922±746 | 3.51±0.54 | 0.74±0.13 |
| 0.23 mg/kg | male | 118±34 | 327±73 | 243±45 | 1.8±1.6 | 503±182 | 867±553 | 3.12±0.40 | 1.45±0.51 |
| MMAE | female | 96±15 | 273±17 | 75±18 | 1.2±0.4 | 492±77 | 713±170 | 2.67±0.44 | 1.85±0.55 |

**Table S7.** Primary blood-chemistry parameters in male cynomolgus monkeys

| Group | Day | ALT (U/L) | AST (U/L) | ALP (U/L) | GGT (U/L) | CK (U/L) | LDH (U/L) | CHO (mM) | TG (mM) |
|---|---|---|---|---|---|---|---|---|---|
| 0 mg/kg (Oba01) | -1 | 163 | 190 | 623 | 63 | 7560 | 1160 | 3.29 | 0.59 |
| | 8 | 140 | 48 | 676 | 53 | 286 | 542 | 3.25 | 0.93 |
| | 29 | 82 | 50 | 703 | 60 | 323 | 427 | 3.69 | 0.98 |
| | 43 | 62 | 46 | 635 | 60 | 286 | 363 | 3.98 | 0.60 |
| | 44 | 123 | 144 | 573 | 57 | 7145 | 1180 | 3.38 | 0.52 |
| 5 mg/kg (Oba01) | -1 | 71 | 47 | 435 | 59 | 362 | 539 | 3.81 | 0.45 |
| | 8 | 71 | 46 | 419 | 53 | 174 | 565 | 3.80 | 0.48 |
| | 29 | 81 | 45 | 463 | 59 | 227 | 508 | 4.11 | 0.50 |
| | 43 | 64 | 37 | 450 | 61 | 242 | 446 | 4.42 | 0.64 |
| | 44 | 63 | 57 | 382 | 54 | 422 | 516 | 3.53 | 0.45 |
| 15 mg/kg (Oba01) | -1 | 55 | 80 | 381 | 56 | 761 | 479 | 3.33 | 0.47 |
| | 8 | 62 | 152 | 398 | 61 | 1410 | 1602 | 5.17 | 0.34 |
| | 11 | 170 | 728 | 238 | 60 | 62000 | 6580 | 2.22 | 0.79 |
| 0.10 mg/kg (MMAE) | -1 | 86 | 88 | 739 | 96 | 396 | 494 | 2.51 | 0.37 |
| | 8 | 89 | 28 | 703 | 72 | 165 | 406 | 2.50 | 0.31 |
| | 29 | 49 | 23 | 759 | 71 | 154 | 374 | 2.47 | 0.35 |
| | 43 | 39 | 28 | 689 | 75 | 215 | 316 | 2.59 | 0.46 |
| | 44 | 79 | 130 | 641 | 73 | 3415 | 977 | 2.31 | 0.44 |

**Table S8.** Primary blood-chemistry parameters in female cynomolgus monkeys

| Group | Day | ALT (U/L) | AST (U/L) | ALP (U/L) | GGT (U/L) | CK (U/L) | LDH (U/L) | CHO (mM) | TG (mM) |
|---|---|---|---|---|---|---|---|---|---|
| 0 mg/kg (Oba01) | -1 | 35 | 62 | 372 | 78 | 236 | 387 | 2.68 | 0.57 |
| | 8 | 26 | 38 | 337 | 73 | 171 | 351 | 2.70 | 0.42 |
| | 29 | 28 | 38 | 345 | 85 | 209 | 331 | 2.80 | 0.39 |
| | 43 | 32 | 46 | 389 | 91 | 223 | 348 | 3.01 | 0.59 |
| | 44 | 35 | 44 | 367 | 93 | 284 | 428 | 3.07 | 0.36 |
| 5 mg/kg (Oba01) | -1 | 101 | 184 | 246 | 60 | 690 | 632 | 3.11 | 0.52 |
| | 8 | 112 | 73 | 299 | 50 | 352 | 473 | 3.87 | 0.57 |
| | 29 | 61 | 48 | 293 | 62 | 218 | 434 | 4.09 | 0.56 |
| | 43 | 39 | 36 | 283 | 56 | 235 | 327 | 3.72 | 0.45 |
| | 44 | 49 | 69 | 244 | 52 | 793 | 492 | 3.21 | 0.36 |
| 15 mg/kg (Oba01) | -1 | 201 | 335 | 403 | 61 | 1725 | 1320 | 1.92 | 0.58 |
| | 8 | 221 | 358 | 454 | 63 | 4020 | 2340 | 2.59 | 0.56 |
| | 29 | 48 | 102 | 553 | 61 | 245 | 969 | 1.89 | 0.38 |
| 0.10 mg/kg (MMAE) | -1 | 59 | 38 | 304 | 32 | 142 | 241 | 3.65 | 0.53 |
| | 8 | 70 | 31 | 223 | 26 | 149 | 252 | 3.16 | 0.53 |
| | 29 | 38 | 18 | 259 | 28 | 90 | 170 | 3.20 | 0.53 |
| | 43 | 40 | 19 | 221 | 29 | 89 | 171 | 3.63 | 0.56 |
| | 44 | 60 | 48 | 222 | 29 | 1148 | 395 | 3.51 | 0.69 |

**Table S9.** PK parameters in SD rats related to Figure 6.

| Analyte | | $t_{1/2}$ h | $T_{max}$ h | $C_{max}$ μg/mL | $AUC_{last}$ h·mg/mL | $AUC_{inf}$ h·mg/mL | $MRT_{last}$ h |
|---|---|---|---|---|---|---|---|
| Total ADC | male | 238 | 0.0833 | 209 | 9.63 | 10.0 | 108 |
| | female | 140 | 1.00 | 177 | 9.62 | 9.82 | 117 |
| Total Ab | male | 209 | 0.0833 | 222 | 12.0 | 12.8 | 135 |
| | female | 148 | 1.00 | 185 | 12.3 | 12.9 | 152 |
| MMAE | male | 66.6 | 24 | 0.237 | 19.6 | 28.6 | 52.4 |
| | female | NA | 48 | 0.233 | 20.4 | NA | 49.2 |

**Table S10.** PK parameters in cynomolgus monkey related to Figure 6.

| Analyte | | $t_{1/2}$ h | $T_{max}$ h | $C_{max}$ μg/mL | $AUC_{last}$ h·mg/mL | $AUC_{inf}$ h·mg/mL | $MRT_{last}$ h |
|---|---|---|---|---|---|---|---|
| Total ADC | Mean | 136 | 101 | 96.7 | 6.07 | 6.17 | 0.444 |
| | SD | 38.0 | 3.82 | 13.7 | 0.785 | 0.847 | 0.272 |
| Total Ab | Mean | 167 | 105 | 123 | 7.35 | 7.63 | 0.333 |
| | SD | 49.8 | 4.34 | 17.2 | 1.10 | 1.34 | 0.00 |
| MMAE | Mean | 115.4 | 0.13 | 68.29 | 14.75 | 25.80 | 40.00 |
| | SD | 42.68 | 0.03 | 11.33 | 2.86 | 3.54 | 12.39 |

**Supplementary data**

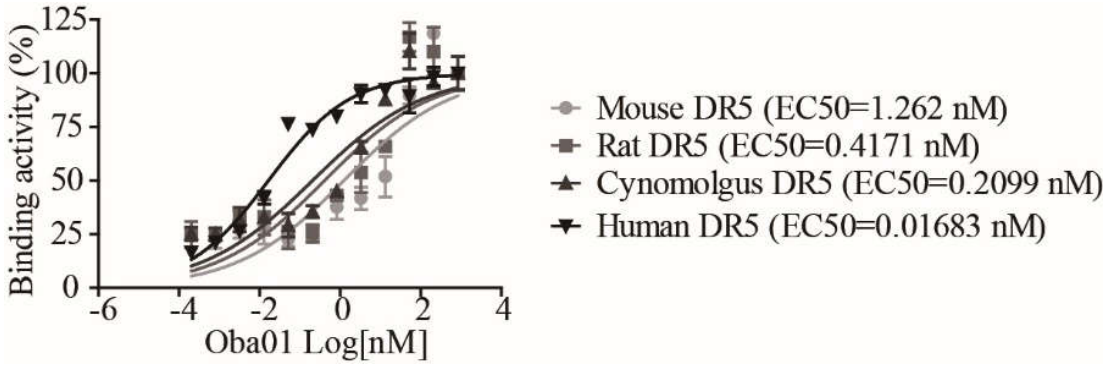


**Figure S1.** Binding activity of Oba01 to different species DR5. The binding activity of Oba01 in the recombinant mouse, SD rat, cynomolgus monkey and human DR5 antigen as estimated by ELISA.

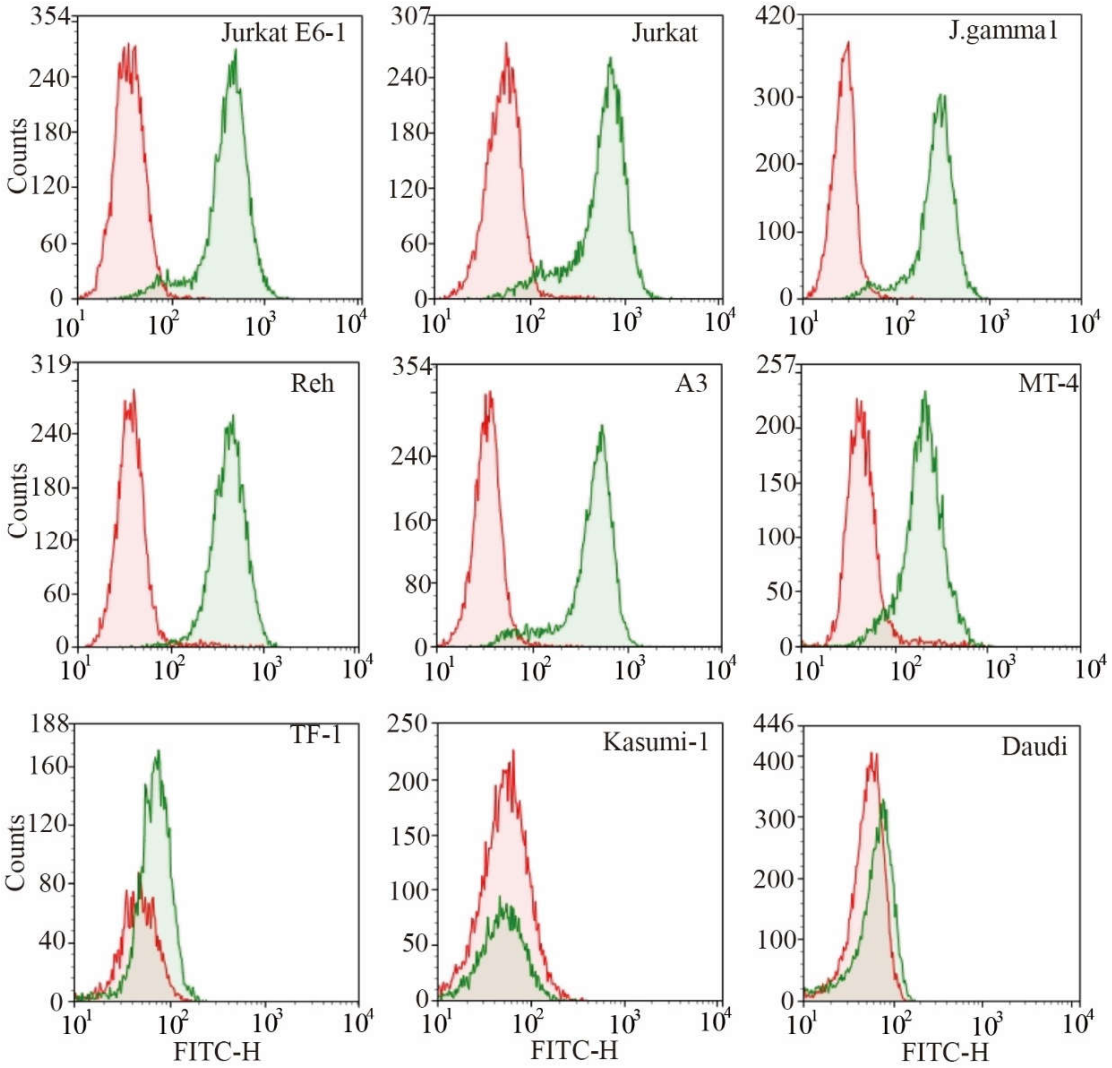


**Figure S2.** The binding specificity of Oba01 in the Jurkat E6-1, Jurkat, J.gamma1, Reh, A3, MT-4, TF-1, Kasumi-1 and Daudi cells. Anti-human IgG antibody was used as control and the cell-associated fluorescence was determined by FACS.

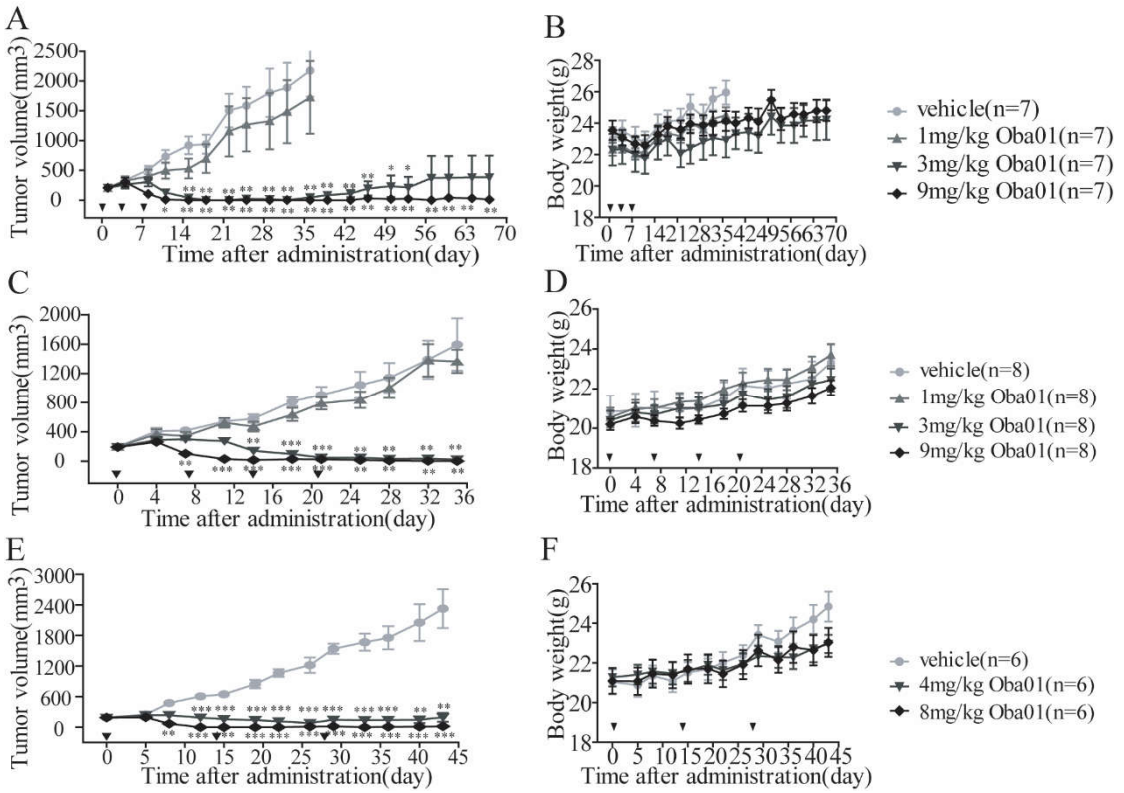


**Figure S3. In vivo antitumor efficacy of Oba01 in subcutaneously implanted Reh xenograft model.** The transplanted tumor volume and mouse body weights were assessed after administration. BALB/c nude mice bearing xenografts of human Reh lymphocyte leukemia were intravenously injected saline and Oba01 once every three days for 3 different times (Q3D×3) at 1, 3 and 9 mg/kg (A), once every week for 4 times (Q1W×4) at 1, 3 and 9 mg/kg (B), and once every two weeks for 3 times (Q2W×3) at 4 and 8 mg/kg (C), respectively. The dosing frequency has been shown by triangular 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.

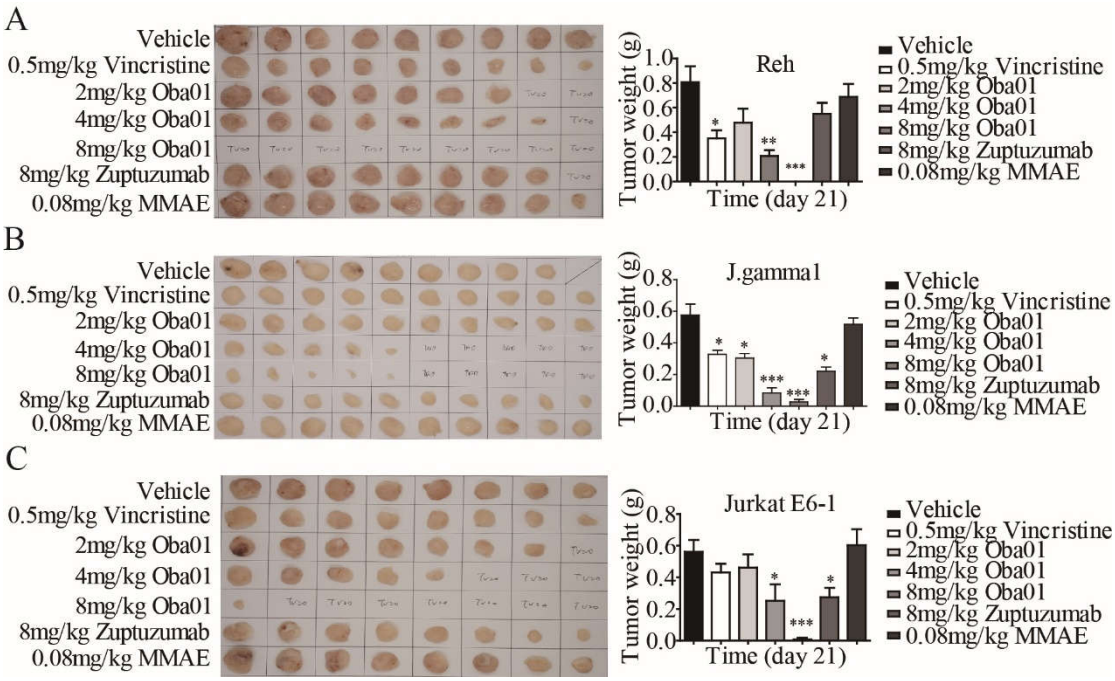


**Figure S4.** Efficacy of Oba01 in the mouse xenografts of human acute lymphocyte leukemia cells J.gamma1 (A), Reh (B) and Jurkat E6-1 (C), related to Figure 4 as indicated above. The representative transplanted tumor images and tumor weights were assessed at the end of the experiment. The *p* values were found to be two-tailed, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. versus vehicle control.

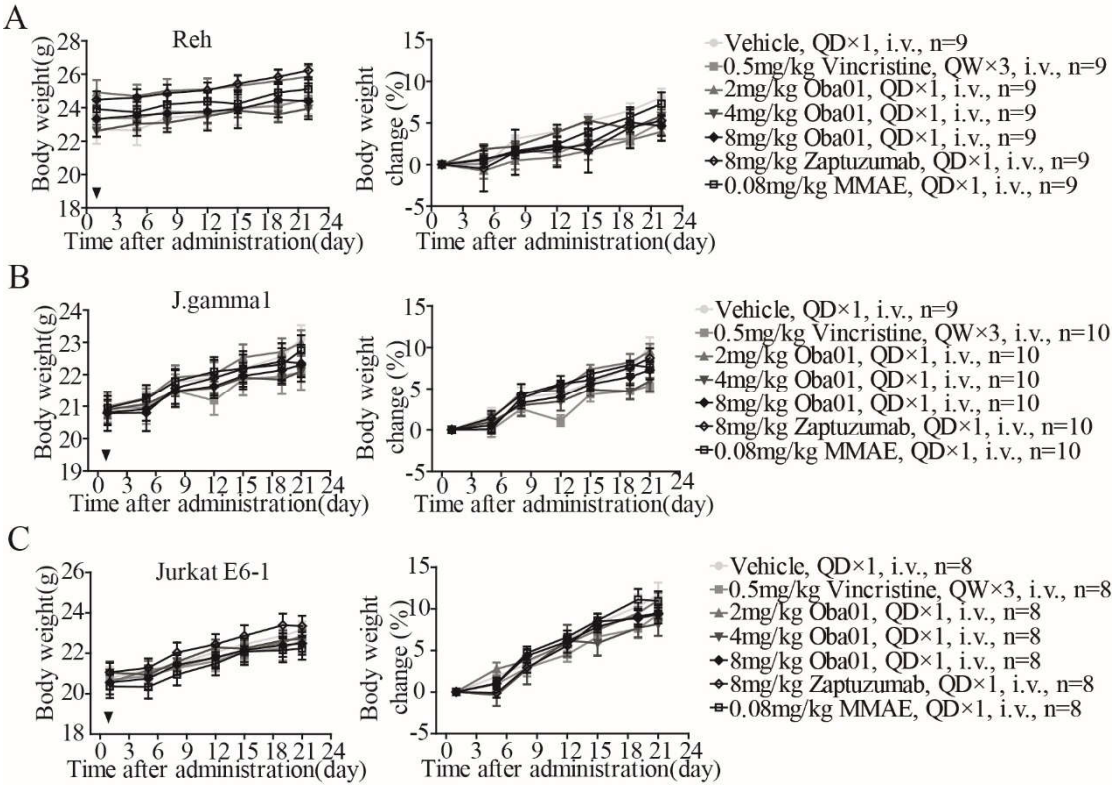


**Figure S5.** Efficacy of Oba01 in mouse xenografts of human acute lymphocyte leukemia cells Reh (A), J.gamma1 (B) and Jurkat E6-1 (C), related to Figure 4 as indicated above. Representative transplanted tumor mouse body weights and the changes of the body weights were assessed twice every week. The dosing frequency has been shown by triangular arrowheads in the figures.

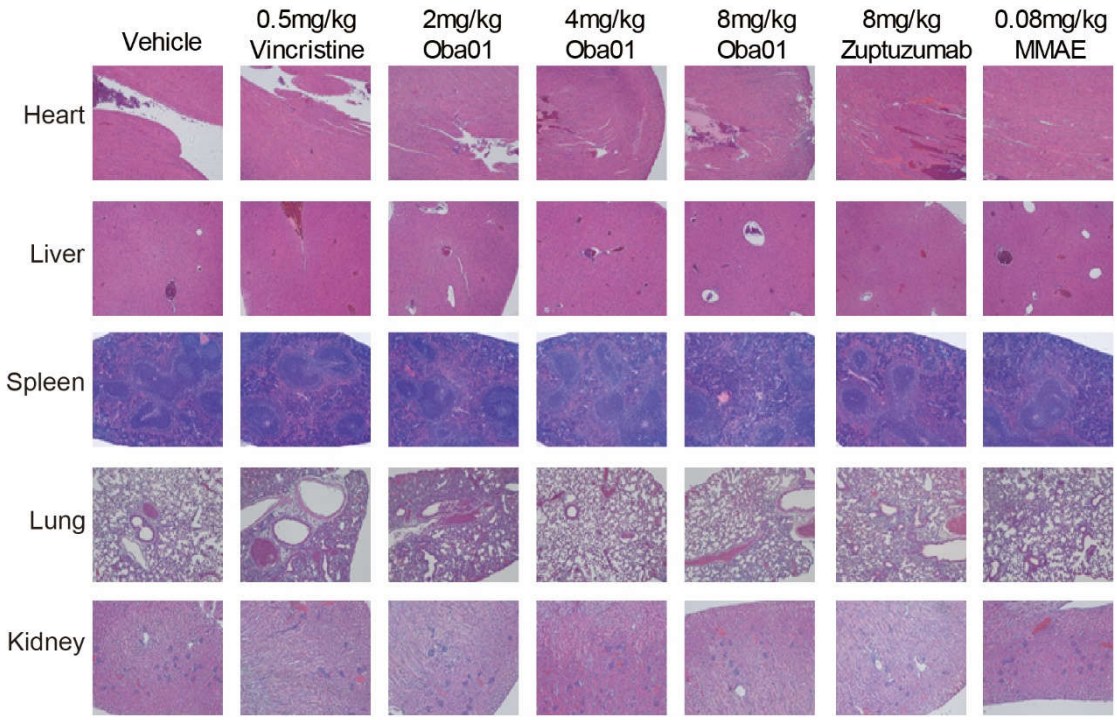


**Figure S6**. Oba01 had no effects on heart, liver, spleen, lung and kidney in human Reh mouse CDX models. H&E staining assay for the evaluation of pathological changes in these organs of human Reh mouse CDX models, related to Figure 4. Images captured at 40× magnification. Scale bars = 50 μm.

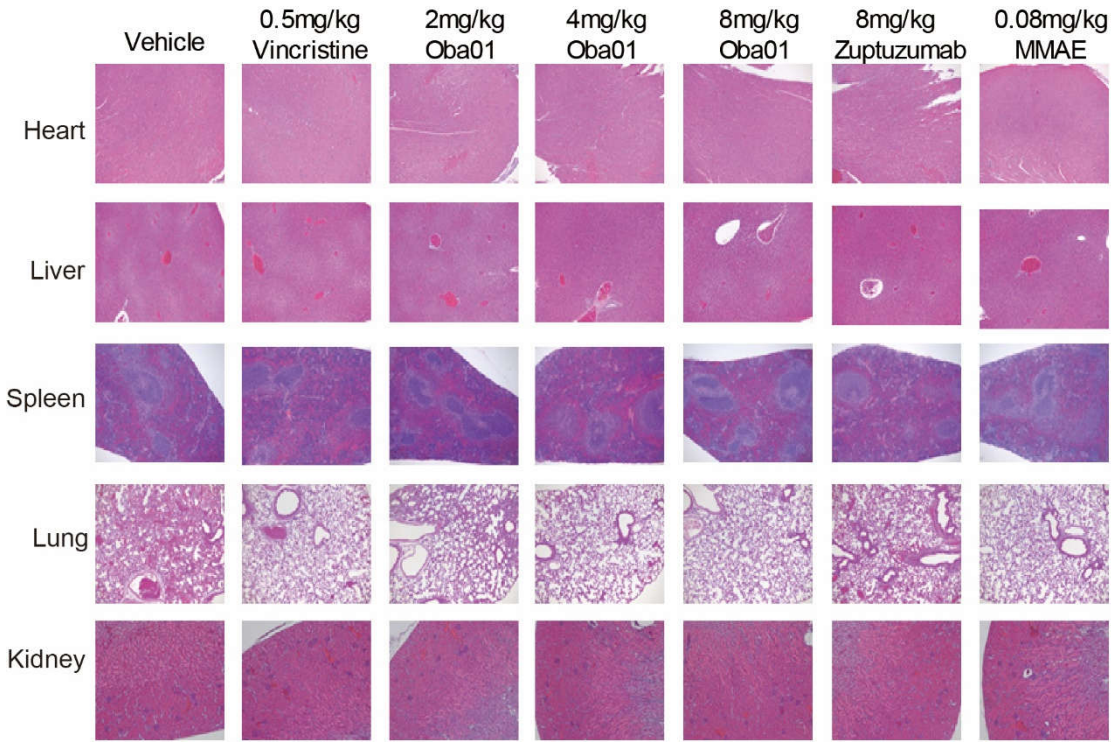


**Figure S7.** Oba01 had no effects on heart, liver, spleen, lung and kidney in human J.gammal mouse CDX models. H&E staining assay for the evaluation of pathological changes in these organs of human J.gammal mouse CDX models, related to Figure 4. Images captured at 40× magnification. Scale bars = 50 μm.

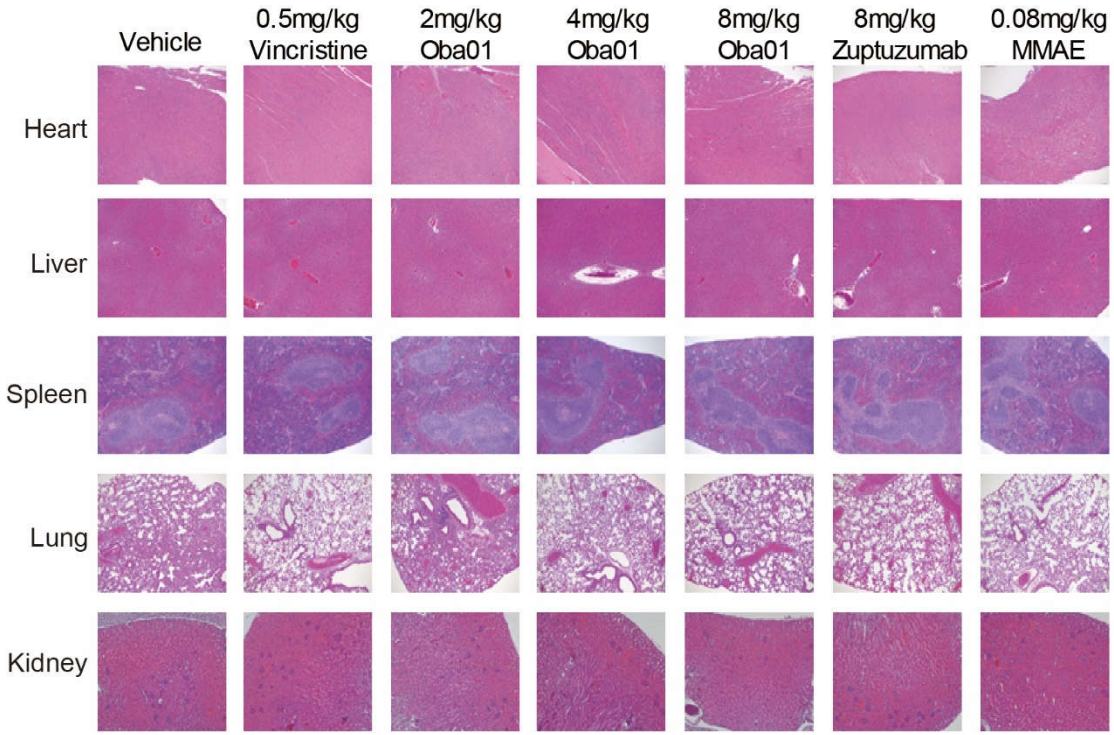


**Figure S8.** Oba01 had no effects on heart, liver, spleen, lung and kidney in human Jurkat E6-1 mouse CDX models. H&E staining assay for the evaluation of pathological changes in these organs of human Jurkat E6-1 mouse CDX models, related to Figure 4. Images captured at 40× magnification. Scale bars = 50 μm.