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LETTER

# DB-1310, a HER3-targeting antibody-drug conjugate, has synergistic anti-tumor activity with trastuzumab in HER2- and HER3-expressing breast cancer

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## DB-1310 and trastuzumab synergistically inhibit breast cancer (BC) cell proliferation *in vitro*

HER3 overexpression has been described in patients with HER2-positive BC[1]. We determined the levels of HER2 and HER3 expression in BC using RNA-seq data from 1,082 BC patient samples in the TCGA dataset and 67 BC cell lines in the CCLE database (**Supplementary material 1**). A positive correlation existed between HER2 and HER3 expression in the patient samples and cell lines with Pearson correlation coefficients of 0.27 ($P < 0.0001$) and 0.59 ($P < 0.0001$), respectively (**Figure S1**). This correlation suggests a potential synergistic role of HER2 and HER3 in BC tumorigenesis.

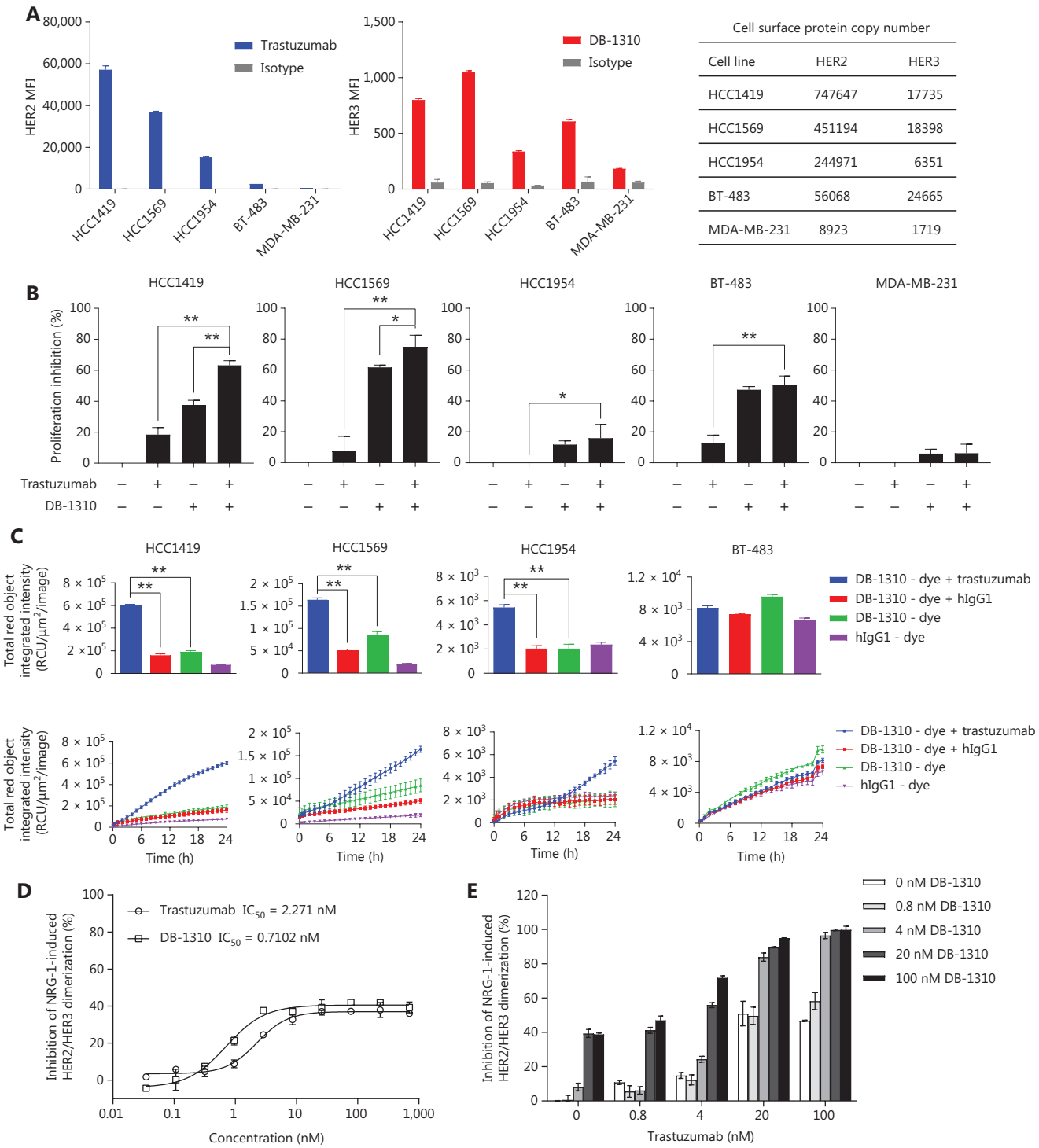
The synergistic blockage of HER2 and HER3 has demonstrated tumor suppression in BC[2]. Therefore, BC cell lines with varying HER2 and HER3 expression profiles were selected to evaluate the anti-tumor effects of DB-1310 combined with trastuzumab. DB-1310 suppressed the growth of HCC1419, HCC1569, and BT-483 cells, all of which have high HER3 expression. Trastuzumab enhanced DB-1310 anti-tumor activity in HER2 high-expression HCC1419 and HCC1569 cells, while trastuzumab alone had a minimal inhibitory effect on cell proliferation (**Figure 1A, B**). Further analysis using the Jin formula revealed that trastuzumab only acted synergistically with DB-1310 in HCC1419 and HCC1569 cell lines (**Supplementary materials 2 and 3**). Taken together, these results showed that DB-1310 combined with trastuzumab synergistically inhibit the proliferation of BC cells with high HER2 and HER3 expression.

## Trastuzumab enhances DB-1310–HER3 complex internalization and blocks HER2–HER3 dimerization

ADC cytotoxicity is mediated through internalization and payload release[3]. Therefore, whether increased DB-1310 internalization enhances tumor growth inhibition with combination treatment was determined. The uptake of DB-1310, a critical step for ADC efficacy, was significantly increased in the presence of trastuzumab in HCC1419, HCC1569, and HCC1954 cells, all of which highly express HER2 (**Figure 1C**). This enhancement was not due to changes in HER3 expression. Indeed, trastuzumab treatment did not change both surface and total HER3 levels.

NRG-1, an HER3 ligand, promotes HER2/HER3 heterodimerization in tumor growth and contributes to HER2-targeted therapy resistance[4,5]. The ability of DB-1310 and trastuzumab to inhibit this process was determined. DB-1310 and trastuzumab alone partially blocked NRG-1-induced HER2/HER3 dimerization with a maximum inhibition rate of approximately 40% (**Figure 1D**) and combination treatment completely inhibited dimerization (**Figure 1E**). This finding revealed that DB-1310 plus trastuzumab treatment efficiently disrupts HER2/HER3 dimerization and contributes to synergistic anti-tumor effects *in vitro*.

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Cell surface protein copy number

| Cell line | HER2 | HER3 |
|---|---|---|
| HCC1419 | 747647 | 17735 |
| HCC1569 | 451194 | 18398 |
| HCC1954 | 244971 | 6351 |
| BT-483 | 56068 | 24665 |
| MDA-MB-231 | 8923 | 1719 |

**Figure 1** Synergistic anti-proliferation of DB-1310 plus trastuzumab in *in vitro* models with high HER2 and HER3 expression. (A) The cell surface HER2 and HER3 on BC cell lines was measured by FACS. The fluorescence signal was detected and analyzed using FACSC. The translation into the HER2 and HER3 copy number was calibrated with BD Quantibrite™ PE beads. (B) BC cells were treated with PBS control, 100 nM DB-1310, 100 nM trastuzumab, or a combination of the two for 7 d. Cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay. Proliferation inhibition was compared with the single-agent group. (C) DB-1310 (100 nM) or human IgG1 isotype control was conjugated with IncuCyte® Human Fabfluor-pH Red Antibody Labeling Reagent followed by the indicated cell treatment. DB-1310-HER3 complex

internalization was analyzed using the IncuCyte® Live-Cell Analysis System and quantified using the IncuCyte software. The top panel represented the internalization results 24 h after treatment with the labeled complex. PathHunter cells with HER2 and HER3 expression were treated with DB-1310 and trastuzumab alone (D) or in combination (E) at the indicated concentrations in the presence of 0.3 μM NRG-1. Inhibition of ligand-induced HER2/HER3 dimerization was analyzed using the PathHunter® Kinase Dimerization Assay Kit. The results are representative of three different experiments and expressed as the mean ± SEM. *$P$ < 0.05, **$P$ < 0.01. HER2, human epidermal growth factor receptor 2; HER3, human epidermal growth factor receptor 3; BC, breast cancer; FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity; $IC_{50}$, half-maximal inhibitory concentration; hIgG1, human immunoglobulin G1; SEM, standard error of the mean; PBS, phosphate-buffered saline.

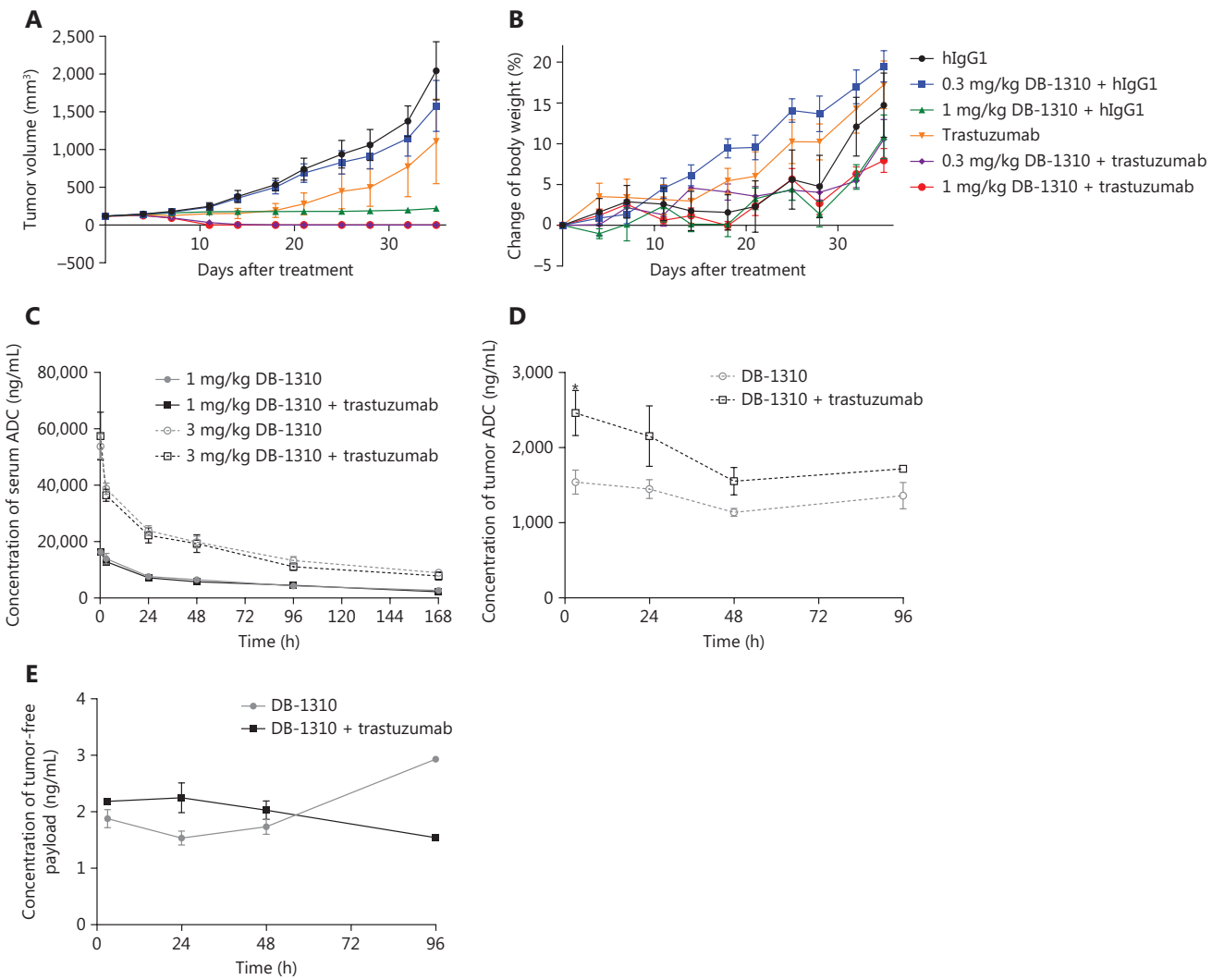



**Figure 2** DB-1310 combined with trastuzumab showed synergistic tumor inhibition *in vivo*. NOD/SCID mice inoculated with HCC1569 cells were intravenously administered with vehicle control or DB-1310 and/or trastuzumab at the indicated doses on day 0 ($n$ = 5 mice/group). Tumor size (A) and body weight (B) during treatment were monitored twice weekly. Tumor volume was measured as the (length × width$^2$)/2. Blood and tumor tissue samples were collected at indicated time points for single-dose pharmacokinetic profiles. The concentrations of ADC (DB-1310) in the serum (C) and tumor tissues (D) were quantified by ELISA. (E) The intratumoral concentration of the free payload was assessed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The results represent two different experiments and are expressed as the mean ± SEM. ADC, antibody-drug conjugate; SEM, standard error of the mean.

## DB-1310 binds HER3 with a unique epitope and partially blocks NRG1 binding to HER3

The binding epitope is crucial for HER3-targeting antibody to block ligand binding, heterodimerization, and HER3 uptake[6]. DB-1310 bound to a unique epitope on HER3 that is distinct from the epitopes recognized by patritumab and elgemtumab (**Figure S2A**). The cryo-EM structure of the DB-1310 Fab fragment binding to the HER3 extracellular domain (HER3-ECD) with a maximal resolution of 3.29 Å revealed that DB-1310 extensively interacts with domain I of the HER3-ECD. Moreover, the DB-1310 Fab fragment binding to the HER3-ECD was shown to be spatially separated from the ligand-binding site located between domains I and III, and the HER2/HER3 dimerization interface of domain II (**Figure S2B**). The observed interface included hydrogen bonds and ionic interactions between the HER3 residue (S128, R132, D153, C156, R181, and S182) and the heavy chain (S50, Y53, Y70, Y73, D118, D120, and Y121) or the HER3 residue (S95, T96, and R132) and the light chain (Y51, G110, and D111; **Figure S2C-E**). DB-1310 demonstrated partial blocking of NRG-1 to HER3 despite the non-overlapping binding site with the ligand, NRG-1 (**Figure S2F, G**). These data indicated that DB-1310 binds a unique epitope of HER3-ECD domain I with a potential allosteric effect on receptor function.

## DB-1310 combined with trastuzumab exerts synergistic tumor suppression *in vivo*

The tumor suppressive activity of DB-1310 plus trastuzumab was further investigated *in vivo* using the HCC1569 xenograft model, which highly expresses HER2 and HER3 (**Figure 1A**) and is resistant to trastuzumab. Tumor-bearing mice were treated with DB-1310 (0.3 or 1 mg/kg biweekly), trastuzumab (20 mg/kg weekly), or both for 3–5 weeks. Significant tumor volume inhibition was noted with DB-1310 in combination with trastuzumab treatment compared to the control and single-agent groups (**Figure 2A**), which suggested superior synergy on inhibition of proliferation *in vitro*. Because this combination was not approved for clinical use and the safety profile was uncertain, the toxicity of the combination was also assessed by monitoring body weights in the HCC1569 mice model. No obvious weight loss was recorded in the two combination groups (**Figure 2B**). Pharmacokinetic studies revealed a dose-dependent increase in the circulating DB-1310 concentration with a similar pharmacokinetic profile between DB-1310 monotherapy and DB-1310 and trastuzumab combination therapy (**Figure 2C**). Importantly, DB-1310 enrichment in tumor tissues was significantly enhanced when DB-1310 was combined with trastuzumab treatment (**Figure 2D**) and increasing free payload accumulation (**Figure 2E**). These results confirmed that combination therapy caused BC tumor regression by enhancing DB-1310 tumor enrichment and payload release (**Supplementary material 4**).

## Conclusions

In conclusion, the current study showed synergistic anti-tumor activity of DB-1310 combined with trastuzumab against BC with HER2 and HER3 expression through enhanced DB-1310 internalization, disruption of HER2/HER3 dimerization, and improved DB-1310 enrichment in the tumor tissues. The preclinical efficacy provides a strong rationale for further clinical evaluation of this combination therapy in BC treatment and has the potential to overcome trastuzumab resistance.

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## Ethics statement

All animal procedures were performed in accordance with the applicable Chinese legislation and approved by the Institutional Animal Care and Use Committee (IACUC) of Crownbio Co., Ltd. (Approval No. AN-2204-05-1364).

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## Conflict of interest statement

No potential conflicts of interest are disclosed.

## Author contributions

Conceived and designed the analysis: Xi Li, Yang Qiu and Haiqing Hua.
Performed the research: Xi Li and Liwen Liang.
Supervised the study and fund support: Zhongyuan Zhu.
Wrote the manuscript: Xi Li.
All authors read and approved the final manuscript.

## Data availability statement

The data generated in this study are available upon reasonable request from the corresponding author.

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