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ARTICLE

HER3 upregulation reduces DS-8201 sensitivity in HER2-positive tumor cells by ATR/CHK1/FoxO1 signaling cascade

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The anti-HER2 antibody–drug conjugate (ADC) DS-8201 presents new hope for patients with advanced HER2-positive tumors. Its clinical application, however, is hindered by serious adverse reactions and reduced efficacy following long-term treatment. In this study, we investigated the factors influencing the sensitivity of DS-8201 and developed effective combination regimens to optimize its therapeutic efficacy. We showed that HER3 upregulation diminished the sensitivity of HER2-positive tumor cells to DS-8201. We found that DS-8201 treatment activated DNA damage repair responses in BT-474 cells, in which the ATR kinase pathway induced the expression of the HER3 transcription factor FoxO1, leading to increased HER3 levels. This process was triggered by the payload component of DS-8201, the topoisomerase I inhibitor DXd, rather than the antibody. Based on this finding, we showed that combining DS-8201 with either a HER3-targeting antibody (SIBP-03) or an ATR inhibitor (BAY1895344) resulted in significant synergistic antitumor efficacy without substantial toxicity in vitro or in vivo. Overall, this study revealed that the ATR/FoxO1/HER3 pathway plays a critical role in modulating the efficacy of DS-8201, suggesting that combining DS-8201 with ATR or HER3 inhibition represents a promising therapeutic strategy for HER2-positive cancers.

Keywords: HER2-positive tumors; DS-8201; DNA damage response; FoxO1; HER3 upregulation; ATR inhibitor

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INTRODUCTION

The receptor tyrosine kinase ERBB-2, also known as HER2, is a member of the EGFR family, which includes EGFR, HER3, and HER4 [1]. The homo- or heterodimerization of HER2 results in the autophosphorylation of tyrosine residues, activating multiple signaling pathways that promote cell proliferation and tumorigenesis [2]. HER2 amplification and overexpression serve as oncogenic drivers in various malignancies, such as breast, lung, and gastric cancers [3].

HER2 is a critical therapeutic target in HER2-positive tumor cells [4]. In recent years, several antibody–drug conjugates (ADCs), such as T-DM1 and DS-8201, have been developed and have demonstrated favorable clinical outcomes [4, 5]. DS-8201 is an innovative HER2-targeting ADC composed of a humanized anti-HER2 antibody, an enzymatically cleavable peptide-based linker, and a potent topoisomerase I inhibitor (DXd) as the cytotoxic payload. Compared with earlier HER2 ADCs, T-DM1 and DS-8201 offer several significant advantages, including a favorable bystander effect, a short systemic half-life to minimize systemic exposure, and complete retention of the antibody-dependent cellular cytotoxicity (ADCC) of trastuzumab [6, 7]. DS-8201 has shown high efficacy in HER2-targeted breast cancer patients. Furthermore, it expands the benefit of HER2-targeted therapy from HER2-positive tumors to those with low HER2 expression [8].

However, although DS-8201 demonstrated superior efficacy, nearly half of the patients did not respond favorably in multiple trials. For example, in one clinical trial (NCT03505710), only 55% of patients with HER2-mutated non-small cell lung cancer (NSCLC) achieved an objective response to DS-8201 [9]. Additionally, the toxicity of DS-8201 in clinical applications must be considered. Treatment with DS-8201 has been associated with adverse reactions such as nausea, vomiting, decreased lymphocyte and neutrophil counts, and anemia [6]. Moreover, nearly 15% of patients develop interstitial lung disease (ILD), with a fatality rate as high as 2.7% [6]. These adverse events often necessitate dose reductions or even the discontinuation of treatment, significantly impacting the clinical application of DS-8201 [10, 11].

Given the clinical limitations of DS-8201, in-depth mechanistic studies are essential for the development of novel therapeutic strategies. However, few combination regimens involving DS-8201 have been successfully implemented. There is an urgent need to design effective treatment regimens that enhance clinical efficacy and reduce the toxicity of DS-8201 in patients.

HER3, a preferred dimerization partner of HER2, binds to HER2 to form the most oncogenic heterodimer in the EGFR family [12]. It exhibits high catalytic activity for the PI3K/AKT pathway and activates a series of intracellular signal transduction cascades [13]. Research has demonstrated that knocking down HER3 in HER2-positive breast cancer animal models significantly inhibits or even eliminates breast tumors [14, 15], highlighting the significant role of HER3 in HER2-positive tumors. As the predominant dimerization partner of HER2, HER3 itself has virtually no intracellular tyrosine

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kinase activity and is instead activated through transphosphorylation by its dimerization partner [2]. Clinical trials have shown that HER3 antibodies exhibit limited efficacy when used as monotherapy; however, they possess a favorable safety profile and are frequently administered in combination with other HER receptor-targeted therapies [16].

In this study, we aimed to identify factors influencing the sensitivity of DS-8201 and develop effective combination regimens to optimize its therapeutic efficacy. We discovered that DS-8201 induced HER3 upregulation, which contributed to reduced sensitivity in HER2-positive cell lines. This HER3 upregulation was mediated by the ATR-mediated DNA damage repair pathway, which is activated by the payload component of DS-8201, DXd. Furthermore, our findings demonstrate that combining HER3-targeting antibodies or ATR inhibitors significantly enhances the efficacy of DS-8201 both in vitro and in vivo. These findings provide preliminary evidence supporting the potential clinical application of DS-8201 combination therapy strategies in HER2-positive cancer.

MATERIALS AND METHODS

Antibodies and reagents

DS-8201 (Enhertu) was purchased from Daiichi Sankyo (Tokyo, Japan). SIBP-03 was provided by the Shanghai Institute of Biological Products (Shanghai, China). Cetuximab (Erbitux) was obtained from Merck KGaA (Darmstadt, Germany). Trastuzumab (Herceptin) and T-DM1 (Kadcyla) were purchased from Roche (Basel, Switzerland). BAY1895344 was purchased from Selleck Co. (Shanghai, China). DXd, exatecan, and SN-38 were purchased from MCE (Shanghai, China). DM1 was purchased from Meilunbio, Inc. (Dalian, China). Actinomycin D (Act D) was purchased from Sigma (#A1410-5MG, St. Louis, MO, USA).

Antibodies against HER3 (#12708S), phospho-HER3 (Tyr1289, #4791S), FoxO1 (#2880S), phospho-ATM (Ser1981, #13050S), phospho-CHK2 (Thr68, #2197S), ATR (#13934S), phospho-ATR (Thr1989, #30632S), phospho-CHK1 (Ser317, #12302S), normal rabbit IgG (#2729), PARP (#9532S), and phospho-histone H2A.X (Ser139, γH2A.X, #2577S) were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody against phospho-DNA-PK was obtained from Abcam (Ser2056, ab18192; Cambridge, UK). The antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Protein Tech (#60004-1-Ig, Rosemont, IL, USA). The antibody against poly(ADP-ribose) (PAR) was obtained from Trevigen (#4336-BPC-100, Gaithersburg, MD, USA). The antibody against histone H3 was obtained from ABclonal (#A17562, Wuhan, China). Goat anti-rabbit IgG (H&L chain-specific peroxidase conjugate, #401315), goat anti-mouse IgG (H&L chain-specific peroxidase conjugate, #401215), and enhanced chemiluminescence reagents (#WBKLS0500) were purchased from Merck Millipore (Billerica, MA, USA).

Cell lines and cell culture

BT-474, SK-BR-3, MDA-MB-453, KPL-4, Calu-3, and HEK293T cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cell lines were authenticated by short tandem repeat analysis and cultured according to instructions provided by the ATCC.

Flow cytometry analysis

Antibodies were labeled with Dylight 488 NHS Ester from Thermo Fisher (#46403, Waltham, MA, USA) and then incubated with the cells on ice for 1 h with protection from light. After centrifugation, the supernatant was discarded, the cells were resuspended in phosphate-buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, and 1.8 mmol/L KH_2PO_4, pH 7.4), and fluorescence was measured using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell proliferation assay

Effects on cell proliferation were determined using sulforhodamine B (SRB) assays, as previously described [17]. The combination index (CI), which characterizes the interaction between two medications as synergistic (CI < 1), additive (CI = 1), or antagonistic (CI > 1), was calculated by applying the median effect principle using CalcuSyn software.

Western blot analysis

After collection, the cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, and 1 mM dithiothreitol). The blots were cut prior to hybridization with antibodies. Western blotting was carried out as previously described [18].

Lentiviral overexpression

The HER3-pLenti plasmid was constructed by cloning the ORF nucleotide sequence of ERBB3 (NM_001982) human tagged ORF clone (#RC209954, Origene, USA) into the pLenti-C-Myc-DDK-IRES-Puro lentiviral gene expression vector (#PS100069, Origene, USA). The HER3-pLenti plasmid was subsequently transfected into HEK293T cells using Lipofectamine® 2000 Reagent (#11668-019, Thermo Fisher Scientific, Waltham, MA, USA). Lentiviral supernatants were collected at 24, 48, and 72 h posttransfection. The target cells were then transfected with lentiviral supernatants at 37 °C after being filtered through 0.45-μm filters. Twenty-four hours later, the viral supernatants were removed, and the cells were cultured in medium supplemented with puromycin (#MB2005, Meilunbio Inc., Dalian, China) for 7 days to generate stable cell lines. After several passages, colonies were harvested, and the HER3 expression level was verified by Western blot analysis.

Gene knockout using CRISPR/Cas9

ATR and *FOXO1* knockout BT-474 cells were generated by CRISPR/Cas9-mediated genome editing. The guide RNAs (gRNAs) used for *ATR* and *FOXO1* knockout were designed on the Synthego website (https://design.synthego.com/#/). The sequences of the sgRNAs are listed in Supplementary Table S1.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from the cells using TRIzol reagent (#9109, TAKARA Bio, Inc., Kyoto, Japan). The total RNA was subsequently reverse transcribed into complementary deoxyribonucleic acid (cDNA) on the T100 Thermal Cycler PCR system (Bio-Rad Laboratories, Hercules, California, USA) using HiScript® III ALL-in-one RT SuperMix, which is suitable for quantitative real-time polymerase chain reaction (#R333-01, Vazyme Biotech Co., Ltd., Nanjing, China). Quantitative real-time PCR was then performed on the StepOne-Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with 2 × ChamQ Universal SYBR qPCR Master Mix (#Q711-02, Vazyme Biotech Co., Ltd., Nanjing, China). GAPDH was used as the internal reference gene. The primer pairs used for qPCR are listed in Supplementary Table S2.

Chromatin immunoprecipitation (ChIP) and ChIP–qPCR assays

BT-474 cells were formaldehyde-crosslinked (1%), and the reaction was quenched with glycine. Chromatin was isolated by lysing the cells with buffer I (0.5 M EDTA, 0.5 M EGTA, 1 M HEPES, and 10% Triton X-100) and buffer II (0.5 M EDTA, 0.5 M EGTA, 1 M HEPES, and 5 M NaCl). The chromatin was subsequently sonicated to shear it into fragments ranging from approximately 200 to 500 bp. The samples were immunoprecipitated using IgG, anti-histone H3 antibody, or anti-FoxO1 antibody and subsequently captured using Protein G Sepharose 4 Fast Flow (#17-0618-01, GE, USA). The levels of immunoprecipitated DNA fragments were evaluated by qPCR. Qualitative analysis of the ChIP–qPCR results was performed

by 3% agarose gel electrophoresis. The primers used are listed in Supplementary Table S3.

In vivo study

Female BALB/cA nude mice (5–6 weeks old) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). Human tumor xenografts were generated by subcutaneously implanting BT-474 cells into the right flank of each animal. The mice were randomly assigned to treatment groups once the tumor volume, calculated as (length × width^2)/2, reached ~100 mm^3. Animals in the treatment groups received the indicated medications alone or in combination, whereas animals in the control group received the vehicle. Tumor growth inhibition (TGI) was calculated as TGI (%) = $(V_t - V_{t0})/(V_c - V_{c0}) \times 100$. In cases where tumor regression occurred, TGI (%) = $100 - (V_t - V_{t0})/V_{t0} \times 100$. V_t and V_{t0} are the tumor volumes in the treatment group at each treatment day and the initial day, respectively, and V_c and V_{c0} are the tumor volumes in the vehicle control group at each treatment day and the initial day, respectively. At the end of the study, tumor tissues were collected, and HER3, phospho-HER3, ATR, phospho-ATR, and γH2A.X protein levels were measured by Western blot analysis.

The animal experiments were performed in accordance with the Institutional Animal Care and Use Committee Guidelines of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. All procedures were approved by the local ethics review committee of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA) and are presented as the means ± SDs (in vitro) or means ± SEMs (in vivo). Student's *t* test was used to determine the significance of differences between two groups. A P value < 0.05 was considered statistically significant.

RESULTS

Inhibiting HER3 sensitizes DS-8201

To explore the antiproliferative characteristics of the HER2-targeted ADC DS-8201 across different cell types, we first systematically compared the expression levels of HER2, as well as those of EGFR and HER3, in several tumor cell lines (Fig. 1a). We then evaluated the antiproliferative effects of DS-8201 in these cell lines (Fig. 1b). Unexpectedly, despite BT-474 cells exhibiting the highest HER2 expression, they demonstrated relatively low sensitivity to DS-8201 treatment, with a maximum proliferation inhibition rate of only approximately 50% (Fig. 1b). Consistent with DS-8201, BT-474 cells exhibited the lowest sensitivity to DXd among these cell lines (Fig. S1). The maximum inhibition rate of DS-8201 on other HER2-positive tumor cells reached 85%–95% (Fig. 1b). In contrast, another HER2-targeted ADC, T-DM1, almost completely suppressed the proliferation of BT-474 cells at a concentration of 10 μg/mL (Fig. 1c). These findings suggest that high HER2 expression levels alone are insufficient to predict sensitivity to DS-8201. Therefore, it is essential to investigate rational treatment strategies to increase the effectiveness of DS-8201.

Given that HER2 can form not only homodimers but also heterodimers with EGFR or HER3, which activate tumor cell survival signaling pathways [19–21], we hypothesized that EGFR or HER3 might influence therapeutic sensitivity to DS-8201. To test this hypothesis, we evaluated the inhibitory effects of DS-8201 on BT-474 cell proliferation in combination with cetuximab (an anti-EGFR antibody) or SIBP-03 (an anti-HER3 antibody). Our findings demonstrated that while cetuximab did not enhance the antiproliferative effects of DS-8201 (Fig. 1d), SIBP-03 significantly increased the sensitivity of BT-474 cells to DS-8201 treatment (Fig. 1e). These findings indicate that the efficacy of DS-8201 might be modulated by HER3. Interestingly, SIBP-03 did not affect the sensitivity of cells to another anti-HER2 ADC, T-DM1, which shares the same antibody, trastuzumab, but carries a distinct payload, DM1, suggesting that the enhancement of efficacy induced by HER3 inhibition is specific to DS-8201 (Fig. 1f).

We subsequently aimed to validate whether this sensitization effect could be generalized to other HER2-expressing tumor cells. As expected, the inhibition of HER3 also affected the sensitivity of SK-BR-3 and MDA-MB-453 cells to DS-8201 (Fig. 1g). Overall, these data suggest that HER3 influences the sensitivity of cells to DS-8201 and that inhibiting HER3 can augment the antiproliferative activity of DS-8201 against HER2-expressing tumor cells.

HER3 is upregulated upon DS-8201 treatment

To elucidate the mechanism by which HER3 inhibition sensitizes cells to DS-8201, we first examined the changes in HER3 expression in BT-474 cells following DS-8201 treatment over different time intervals. DS-8201 treatment for a short time induced slight downregulation of HER3 and phospho-HER3 (2–6 h) (Fig. 2a). Then, HER3 recovered to baseline levels (6–17 h) and was gradually upregulated (up to 48 h). In contrast, T-DM1 treatment resulted in sustained inhibition of HER3 and phospho-HER3 (Fig. 2a), which was consistent with the effects observed with trastuzumab treatment (Fig. 2a). Conversely, DXd induced a persistent increase in HER3 and phospho-HER3 (Fig. 2a). We further evaluated changes in extracellular HER3 levels by FACS analysis and observed consistent results (Fig. 2b). These findings suggest that DS-8201 has a dual mechanism of action, combining the effects of both the antibody trastuzumab and the payload DXd.

As DS-8201 induced HER3 expression, we next explored whether the HER3-targeted antibody SIBP-03 could reverse this effect. SIBP-03 (10 μg/mL) significantly reversed the upregulation of HER3 induced by DS-8201 or DXd and demonstrated synergistic effects with trastuzumab (Fig. 2c). These results indicate that the HER3-targeted antibody SIBP-03 effectively counteracts the upregulation of HER3 signaling triggered by DS-8201, providing a mechanistic explanation for why HER3 inhibition sensitizes cells to DS-8201.

To further explore the significance of HER3 upregulation in DS-8201-treated cells, we stably transfected human *HER3* into the BT-474, SK-BR-3, and KPL-4 cell lines (Fig. 2d). We observed that exogenous overexpression of HER3 resulted in a 10%–20% reduction in the inhibitory effects of DS-8201 on the proliferation of BT-474 cells and even an approximately 100-fold increase in the IC_{50} of DS-8201 in SK-BR-3 and KPL-4 cells (Fig. 2d). These findings further confirm that HER3 plays a critical role in modulating sensitivity to DS-8201. The combination of HER3 inhibitors with DS-8201 represents a promising therapeutic strategy that could enhance the efficacy of DS-8201, optimize treatment regimens, and potentially improve overall clinical outcomes.

DS-8201-induced HER3 expression is mediated by FoxO1

To investigate the mechanism underlying HER3 induction by DS-8201, we examined whether this effect was mediated by transcriptional activation. We analyzed the HER3 mRNA levels in BT-474 cells after treatment with DS-8201. DS-8201 treatment induced the upregulation of HER3 mRNA, which peaked at 8 h posttreatment (increased nearly threefold) (Fig. 3a), with a time lag of approximately 6 h between transcription and translation. Furthermore, actinomycin D (Act D), a transcription inhibitor [22, 23], completely reversed the upregulation and activation of HER3 by DXd (Fig. 3b). Interestingly, treatment with Act D abolished the upregulation of HER3 induced by DS-8201 (Fig. 3b), leaving only the inhibitory effects observed with trastuzumab treatment (Fig. 2c). These results suggest that DS-8201 elevates HER3 levels by enhancing HER3 transcription and that inhibiting the transcription process can block HER3 induction, shifting the effects of DS-8201 from induction to inhibition.

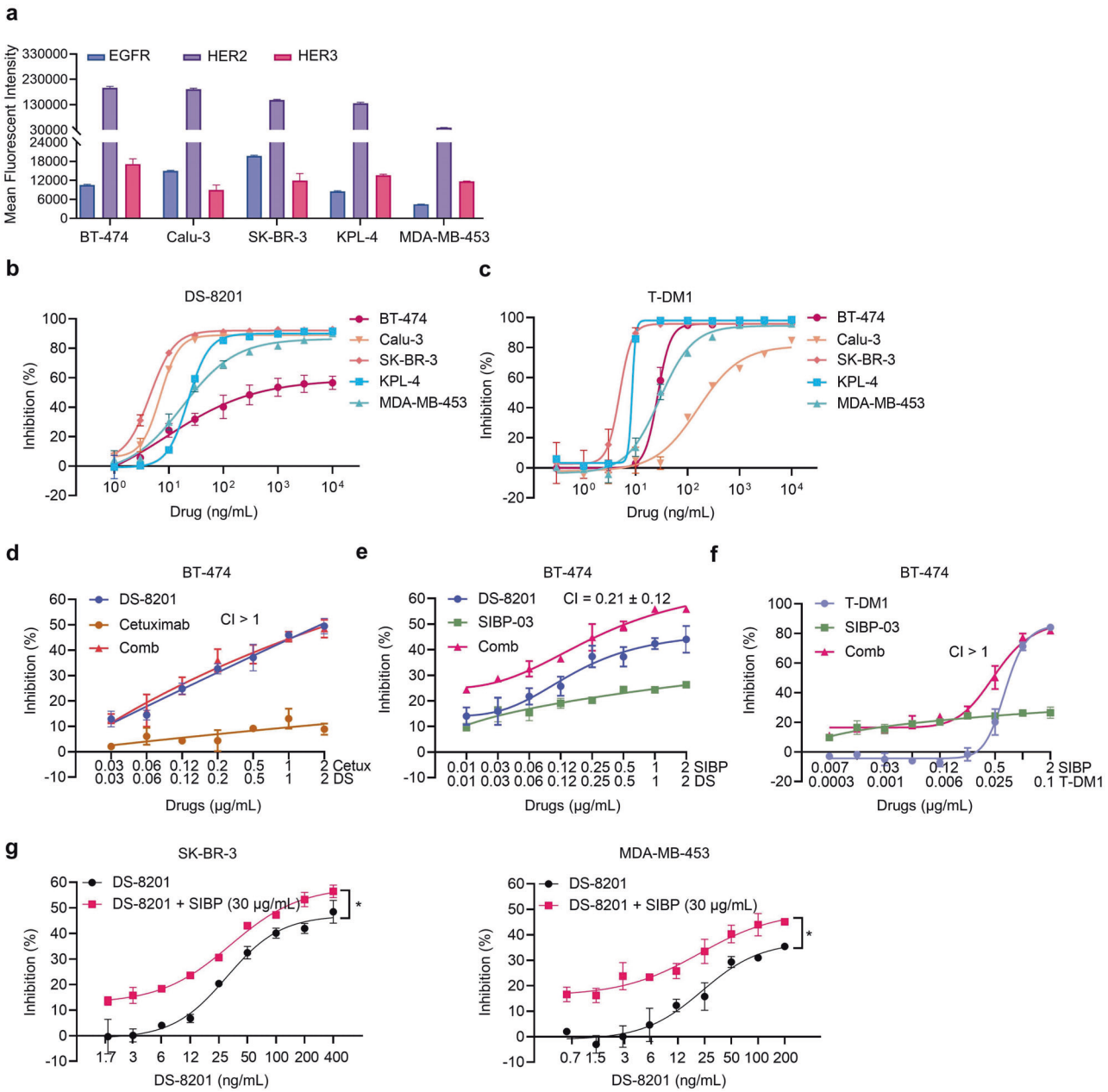

Fig. 1 HER3 inhibition potentiates DS-8201 efficacy in HER2-positive cancers. a Flow cytometric quantification of EGFR/HER2/HER3 surface expression. Cytotoxic effects evaluated by SRB assay after 144 h of treatment: DS-8201 (**b**), T-DM1 (**c**) alone or in combination with either cetuximab (**d**), or SIBP-03 (**e**–**g**). The combination index (CI) indicated synergistic effects (CI < 1). The data are shown as the means ± SDs ($n = 2$). *$P < 0.05$. Comb combination, Cetux Cetuximab, DS DS-8201, SIBP SIBP-03.

Therefore, we hypothesized that the increase in HER3 levels might be attributed to the activation of HER3 transcription factors. To this end, we focused on previously reported transcription factors of HER3 as well as potential transcription factors predicted by the JASPAR database (score: 650). Through this analysis, we identified several candidate transcription factors, including FoxA1, EHF, FoxO1, ZNF460, THRB, CTCF, and PATZ1. We then monitored their expression levels after DS-8201 treatment. Notably, the mRNA level of FoxO1 was significantly increased (~5-fold) after DS-8201 treatment, whereas the mRNA levels of other transcription factors were largely unchanged (Fig. 3c). We further investigated the dynamics of FoxO1 mRNA levels in BT-474 cells treated with DS-8201 for different durations. Consistent with the observed HER3 mRNA alterations, FoxO1 mRNA levels increased significantly after DS-8201 treatment (Fig. 3d). Specifically, the FoxO1 mRNA level also peaked at 8 h posttreatment. Moreover, DS-8201 and DXd treatment markedly enhanced FoxO1 protein expression in BT-474 cells (Fig. 3e), and ChIP–qPCR analysis demonstrated that DS-8201 (10 μg/mL) significantly enhanced the binding of FoxO1 to the *HER3* promoter region (Fig. 3f), indicating an association between the transcription factor FoxO1 and HER3 upregulation induced by DS-8201.

To further validate the relationship between FoxO1 and HER3, we knocked out *FOXO1* in BT-474 cells (Fig. 3g) and examined whether DS-8201 treatment could impact HER3 expression. *FOXO1* knockout completely abolished the HER3 upregulation induced by DS-8201 and DXd (Fig. 3h), and similar effects were observed at the protein level (Fig. 3i). Moreover, *FOXO1* knockout shifted the effects of DS-8201 on HER3 expression from induction to inhibition, which is consistent with the results observed following Act D treatment.

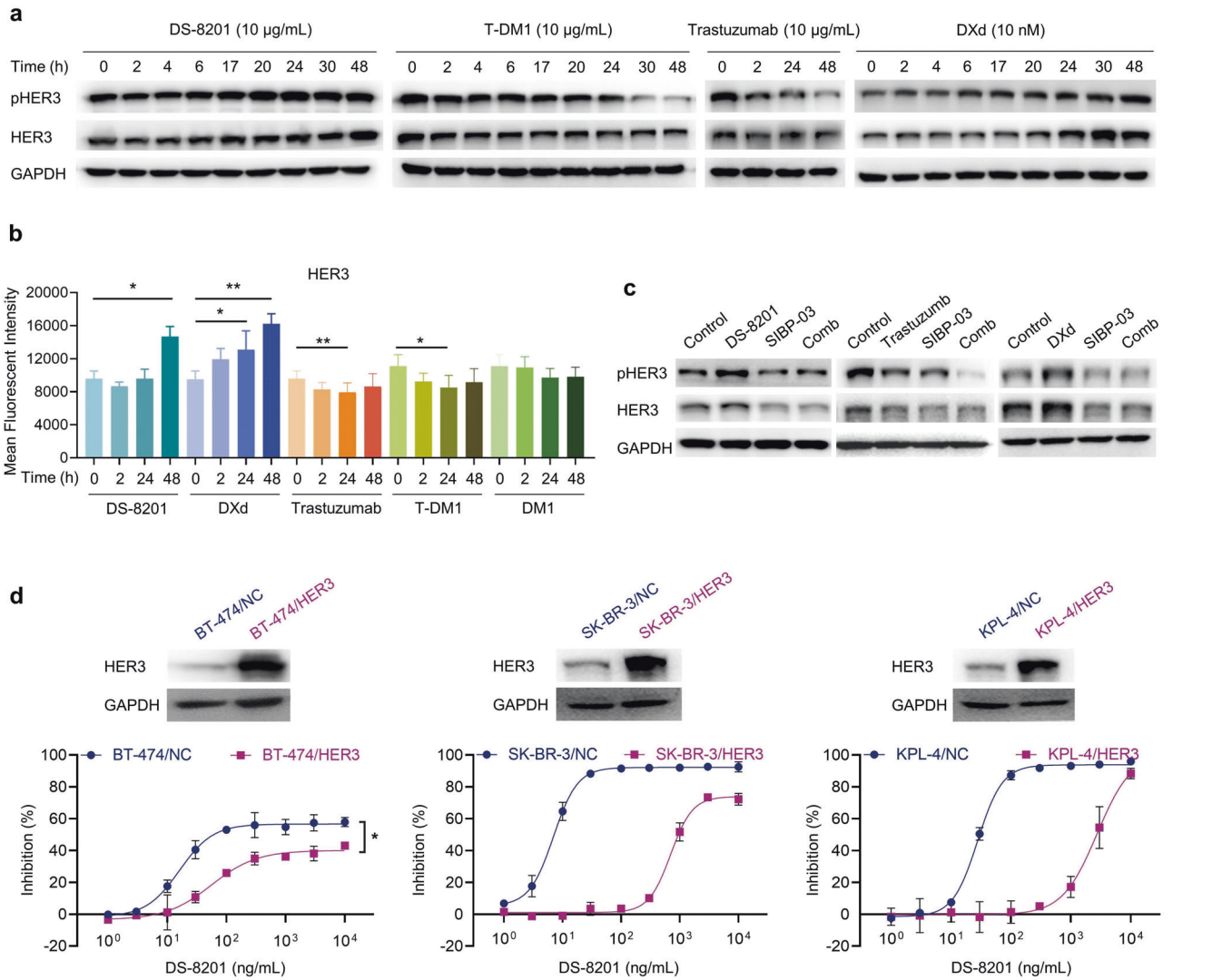

Fig. 2 HER3 is upregulated upon DS-8201 treatment. a Western blot analysis of phospho-HER3 and HER3 levels in BT-474 cells. **b** Flow cytometric quantification of cell-surface HER3 expression in BT-474 cells. The data shown are the means ± SDs ($n = 3$). *$P < 0.05$, **$P < 0.01$. **c** BT-474 cells were treated with DS-8201 (10 μg/mL), trastuzumab (10 μg/mL), or DXd (10 nM) alone or in combination with SIBP-03 (10 μg/mL) for 48 h. Comb: combination. **d** Cells were stably transfected with negative controls (BT-474/NC, SK-BR-3/NC, and KPL-4/NC) or human *HER3* (BT-474/HER3, SK-BR-3/HER3, and KPL-4/HER3). Cytotoxicity was determined by SRB assay following treatment with different concentrations of DS-8201 for 144 h. The data shown are the means ± SDs ($n = 2$). *$P < 0.05$.

These results demonstrate that FoxO1 plays a pivotal role in regulating HER3 transcription in response to DS-8201 exposure.

The ATR/CHK1 axis mediates DS-8201-induced FoxO1/HER3 upregulation

Since FoxO1 and HER3 upregulation induced by DS-8201 is mediated mainly by the payload DXd, a topoisomerase 1 inhibitor that exerts antitumor effects mainly through DNA damage [24, 25], we sought to investigate whether the induction of HER3 and FoxO1 was caused by the DNA damage process. In response to DNA damage, cells activate the DNA damage response (DDR), which involves the transcriptional and translational regulation of numerous repair factors [26]. Therefore, we systematically examined the effects of DS-8201 and DXd on DDR-related pathways. We found that both DS-8201 and DXd elevated the DNA damage marker γH2A.X and activated the ATR, ATM, DNA-PK, and PARP pathways (Fig. 4a). To determine whether the activation of these DDR-related pathways was responsible for the upregulation of HER3, we treated BT-474 cells with the ATM inhibitor AZD1390 (3 μM), the DNA-PK inhibitor AZD7648 (3 μM), the ATR inhibitor BAY1895344 (0.03 μM), the CHK1/2 inhibitor AZD7762 (0.3 μM), and the PARP inhibitor AZD2281 (10 μM) in combination with DXd (10 nM) or DS-8201 (10 μg/mL) and assessed the changes in HER3 expression (Fig. 4b, c). Notably, specific inhibition of ATR or CHK1/2, but not other pathways, reversed the HER3 upregulation induced by DS-8201 and DXd. We subsequently knocked out *ATR* in BT-474 cells and found that *ATR* knockout prevented DS-8201 or DXd from upregulating HER3 (Fig. 4d) and FoxO1 (Fig. 4f). These results suggest that the ATR/CHK1 pathway plays a critical role in the DS-8201-induced upregulation of HER3 and FoxO1. We further evaluated whether the inhibition of ATR could enhance the antiproliferative effects of DS-8201. We found that the ATR inhibitor BAY1895344 significantly sensitized BT-474 cells to DS-8201 treatment (Fig. 4e). Taken together, these observations indicate that the ATR/CHK1/FoxO1 axis mediates DS-8201-induced HER3 upregulation and that the inhibition of key factors in this axis significantly potentiates the antiproliferative effects of DS-8201.

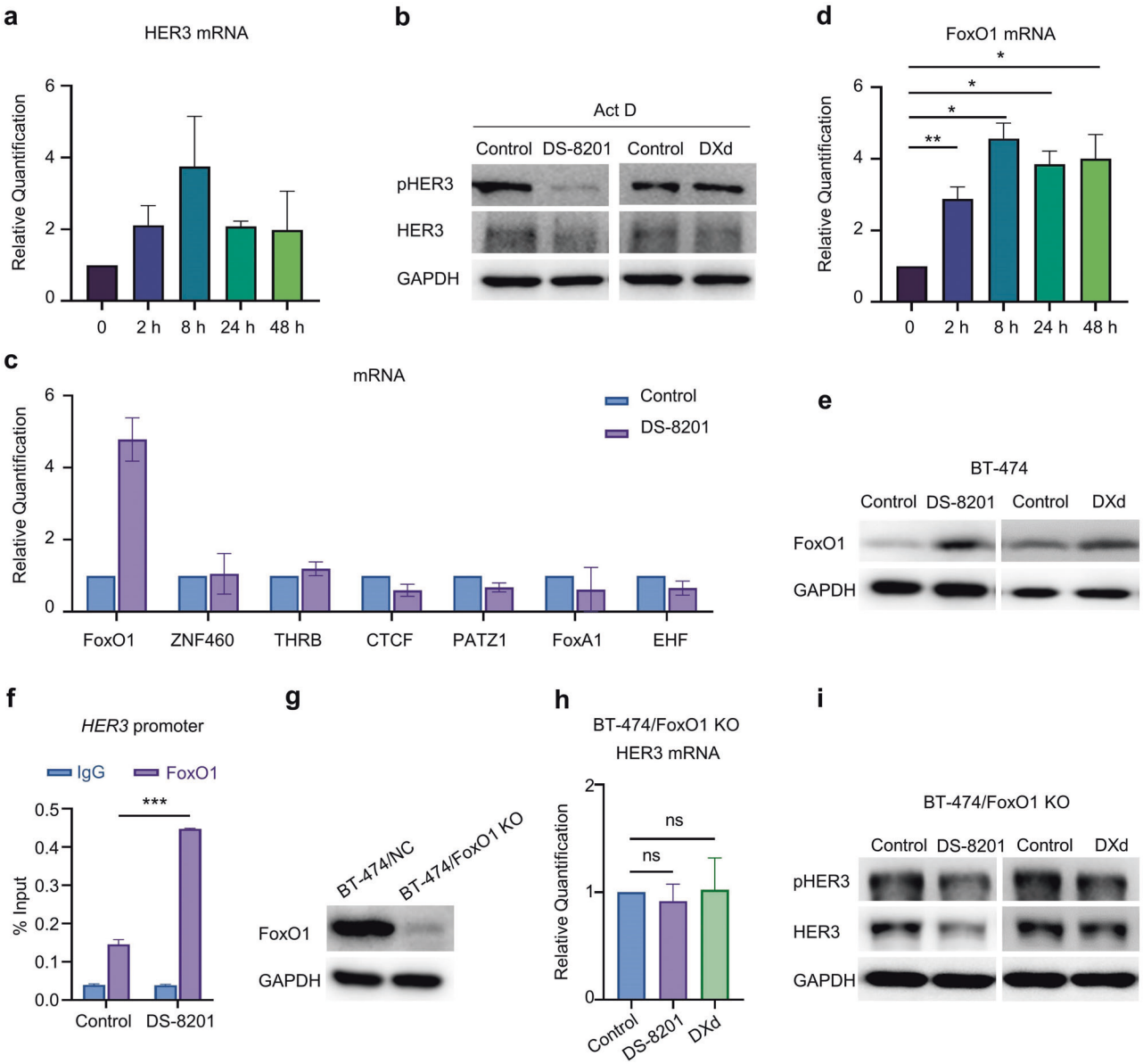

Fig. 3 DS-8201-induced HER3 expression is mediated by FoxO1. **a** HER3 mRNA in BT-474 cells treated with DS-8201 (10 μg/mL) for the indicated times. The data shown are the means ± SDs ($n = 2$). **b** Phospho-HER3 and HER3 levels in BT-474 cells treated with the transcription inhibitor actinomycin D (Act D, 2 μg/mL) along with DS-8201 (10 μg/mL) or DXd (10 nM) for 48 h. **c** Changes in the mRNA levels of transcription factors in BT-474 cells treated with DS-8201 (10 μg/mL) for 8 h. The data shown are the means ± SDs ($n = 2$). **d** FoxO1 mRNA in BT-474 cells treated with DS-8201 (10 μg/mL) for the indicated times. The data shown are the means ± SDs ($n = 3$). *$P < 0.05$, **$P < 0.01$. **e** FoxO1 protein levels in BT-474 cells treated with DS-8201 (10 μg/mL) or DXd (10 nM) for 48 h. **f** ChIP–qPCR analysis of FoxO1 binding to the *HER3* promoter in BT-474 cells treated with DS-8201 (10 μg/mL) for 8 h. The data shown are the means ± SDs ($n = 2$). ***$P < 0.001$. **g** CRISPR/Cas9-generated *FOXO1* knockout (FoxO1 KO) and control (NC) cell lines. **h** HER3 mRNA in BT-474/FoxO1 KO cells treated with DS-8201 (10 μg/mL) or DXd (10 nM) for 8 h. The data shown are the means ± SDs ($n = 2$). Ns: no significance. **i** HER3 protein levels in cells treated with DS-8201 (10 μg/mL) or DXd (10 nM) for 48 h. Relative quantitative analysis of the mRNA and protein levels was determined by qPCR and Western blotting, respectively.

Inhibition of ATR/HER3 broadly enhances the activity of DS-8201

As noted above, our study demonstrated that HER3 upregulation mediated by the ATR/FoxO1 pathway was associated with the therapeutic insensitivity of DS-8201 in BT-474 cells. To determine whether this mechanism is broadly applicable among other HER2-positive tumor cells, we extended our investigation to examine the impact of DS-8201 on HER3 levels in a panel of HER2-positive tumor cell lines. Consistently, we observed the activation of ATR, an increase in the HER3 transcription factor FoxO1, and elevated HER3 levels in these cell lines after DS-8201 treatment (Fig. 5a). In addition, the extracellular HER3 levels in these cells consistently increased following treatment with DS-8201 (Fig. 5b) or DXd (Fig. 5c), ranging from 22% to 46%. However, the magnitude of HER3 upregulation was less pronounced than that observed in BT-474 cells (Fig. 2b), suggesting a potential inverse correlation between the extent of HER3 upregulation and sensitivity to DS-8201. Additionally, the increase in HER3 levels triggered by DS-8201 (Fig. 5b) or DXd (Fig. 5c) could be effectively suppressed by the ATR inhibitor BAY1895344. Furthermore, BAY1895344 enhanced the antiproliferative effects of DS-8201 on HER2-positive cells in vitro (Fig. 5d). Taken together, these findings indicate that DS-8201 commonly induces varying degrees of HER3 upregulation by the ATR/FoxO1 pathway in HER2-positive tumor cells and that targeting this pathway enhances the therapeutic efficacy of DS-8201 in vitro.

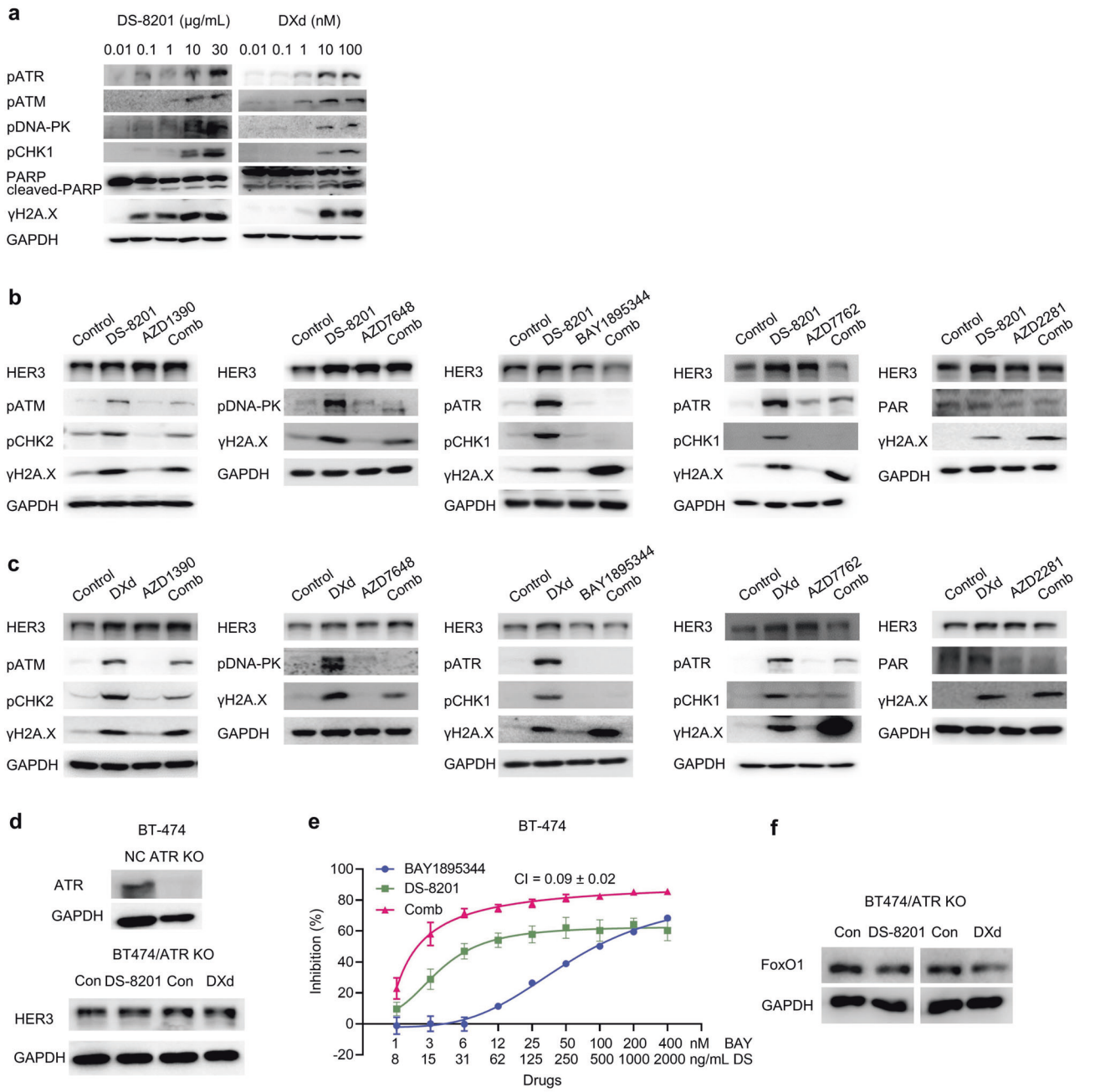

Fig. 4 The ATR/CHK1 axis mediates DS-8201-induced FoxO1/HER3 upregulation. a Activation of DNA damage repair pathway signaling molecules in BT-474 cells following 48 h of treatment with DS-8201 or DXd. (**b**, **c**) HER3 expression in BT-474 cells treated with DS-8201 (10 μg/mL) (**b**) or DXd (10 nM) (**c**) in combination with AZD1390 (3 μM, an ATM inhibitor), AZD7648 (3 μM, a DNA-PK inhibitor), BAY1895344 (0.03 μM, an ATR inhibitor), AZD7762 (0.3 μM, a CHK1/2 inhibitor), or AZD2281 (10 μM, a PARP inhibitor) for 48 h. Comb: combination. **d** CRISPR/Cas9-generated *ATR* knockout (ATR KO) and control (NC) cell lines. HER3 levels were determined in BT-474/ATR KO cells treated with DS-8201 (10 μg/mL) or DXd (10 nM) for 48 h. **e** Cytotoxic effects evaluated by SRB assay after 144 h of treatment. The data are presented as the means ± SDs ($n = 3$). **f** FoxO1 in BT-474/ATR KO cells treated with DS-8201 (10 μg/mL) or DXd (10 nM) for 48 h. Con control, Comb combination, BAY BAY1895344, DS DS-8201.

Both an anti-HER3 antibody and an ATR inhibitor potentiate the antitumor activity of DS-8201 in vivo

Having demonstrated that the inhibition of ATR/FoxO1/HER3 enhances the therapeutic efficacy of DS-8201 in vitro, we next investigated whether this strategy could be applied in vivo. In the BT-474 xenograft models, DS-8201 (1.5 mg/kg) alone showed a TGI of 62%, and SIBP-03 (15 mg/kg) alone had almost no antitumor growth effect; however, the combination of SIBP-03 and DS-8201 resulted in significant tumor growth inhibition (TGI = 81%) (Fig. 6a–c). This combination treatment was well tolerated and produced no significant toxicity. In addition, Western blotting analysis of BT-474 tumor tissues revealed that DS-8201 significantly induced the upregulation and activation of HER3, which could be blocked by the addition of SIBP-03, thereby explaining the synergistic antitumor effect (Fig. 6d). Similarly, the combination of DS-8201 and the ATR inhibitor BAY1895344 resulted in a TGI of 99%, which was markedly greater than that caused by either single agent alone (TGI = 62%, 47%) (Fig. 6a–c). Additionally, BAY1895344 potently inhibited the activation of ATR induced by DS-8201 and significantly increased the accumulation of the

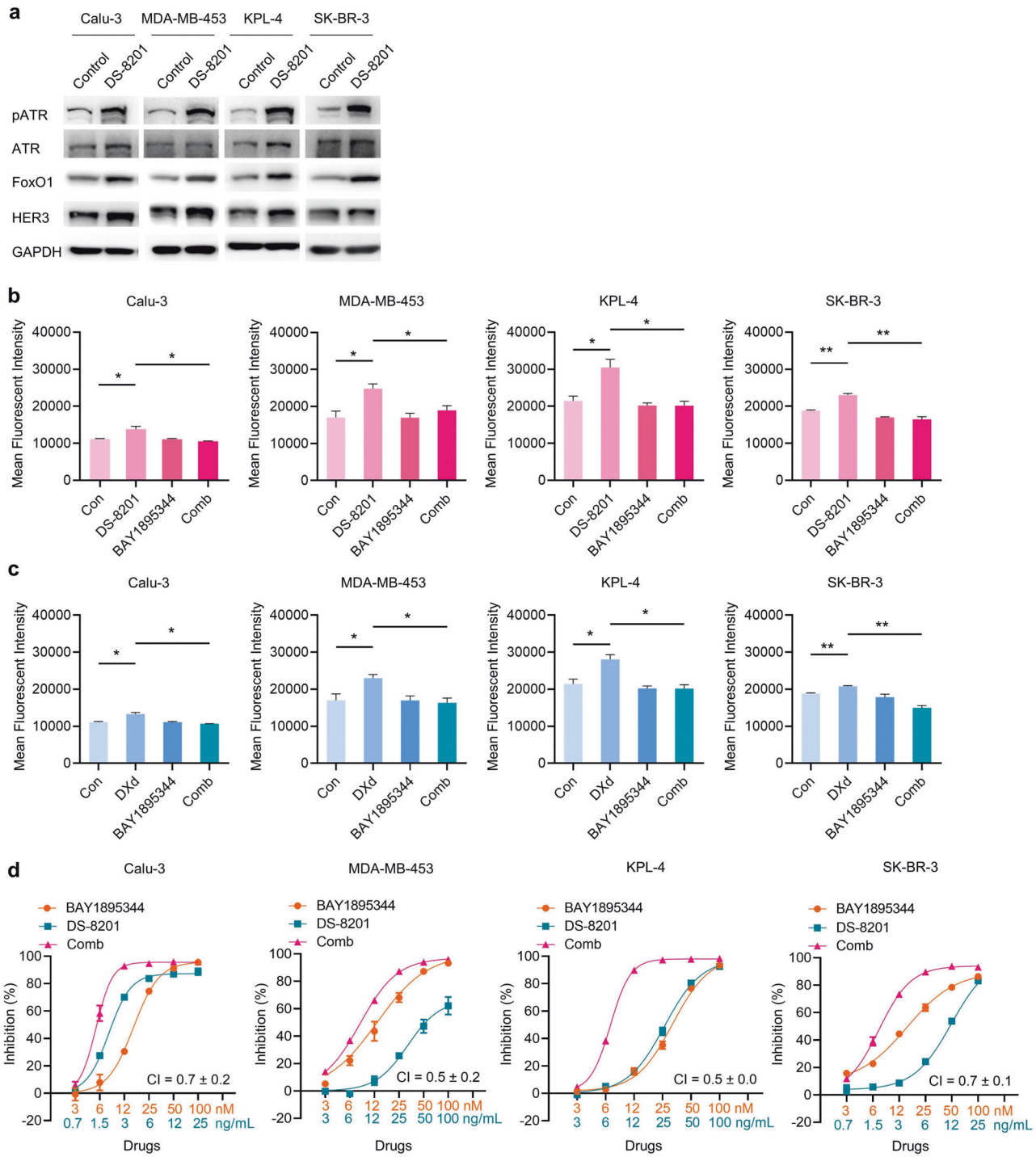

Fig. 5 Inhibition of ATR/HER3 enhances the activity of DS-8201 in HER2-positive cancer cells. a Phospho-ATR, ATR, FoxO1, and HER3 levels in HER2-positive cancer cells treated with DS-8201 for 48 h. Flow cytometric quantitative analysis of cell-surface HER3 expression in HER2-positive cancer cells treated with DS-8201 (10 μg/mL) (**b**) or DXd (10 nM) (**c**) in combination with BAY1895344 (0.03 μM) for 48 h. The data shown are the means ± SDs ($n = 2$). $^{*}P < 0.05$, $^{**}P < 0.01$. (**d**) Cytotoxic effects evaluated by SRB assay after 144 h of treatment. The data shown are the means ± SDs ($n = 3$). Con control, Comb combination.

DNA damage marker γH2A.X in BT-474 tumor tissues (Fig. 6e), which explained the synergistic antitumor effect of the combination therapy. Moreover, the inhibition of ATR effectively prevented the increase in and activation of HER3 induced by DS-8201 in tumor tissues, confirming the inductive effect of ATR on HER3 (Fig. 6e). Collectively, these results demonstrate that both the anti-HER3 antibody and the ATR inhibitor significantly enhance the antitumor activity of DS-8201 in vivo.

DISCUSSION

The HER2-targeting ADC DS-8201 has emerged as a groundbreaking therapeutic option for patients with advanced HER2-positive tumors. However, clinical challenges remain: nearly half of patients exhibit a poor response to treatment, and severe adverse reactions, including potentially fatal interstitial lung disease and organ failure, have been reported. These limitations hinder the broader clinical application of DS-8201. Furthermore, optimal

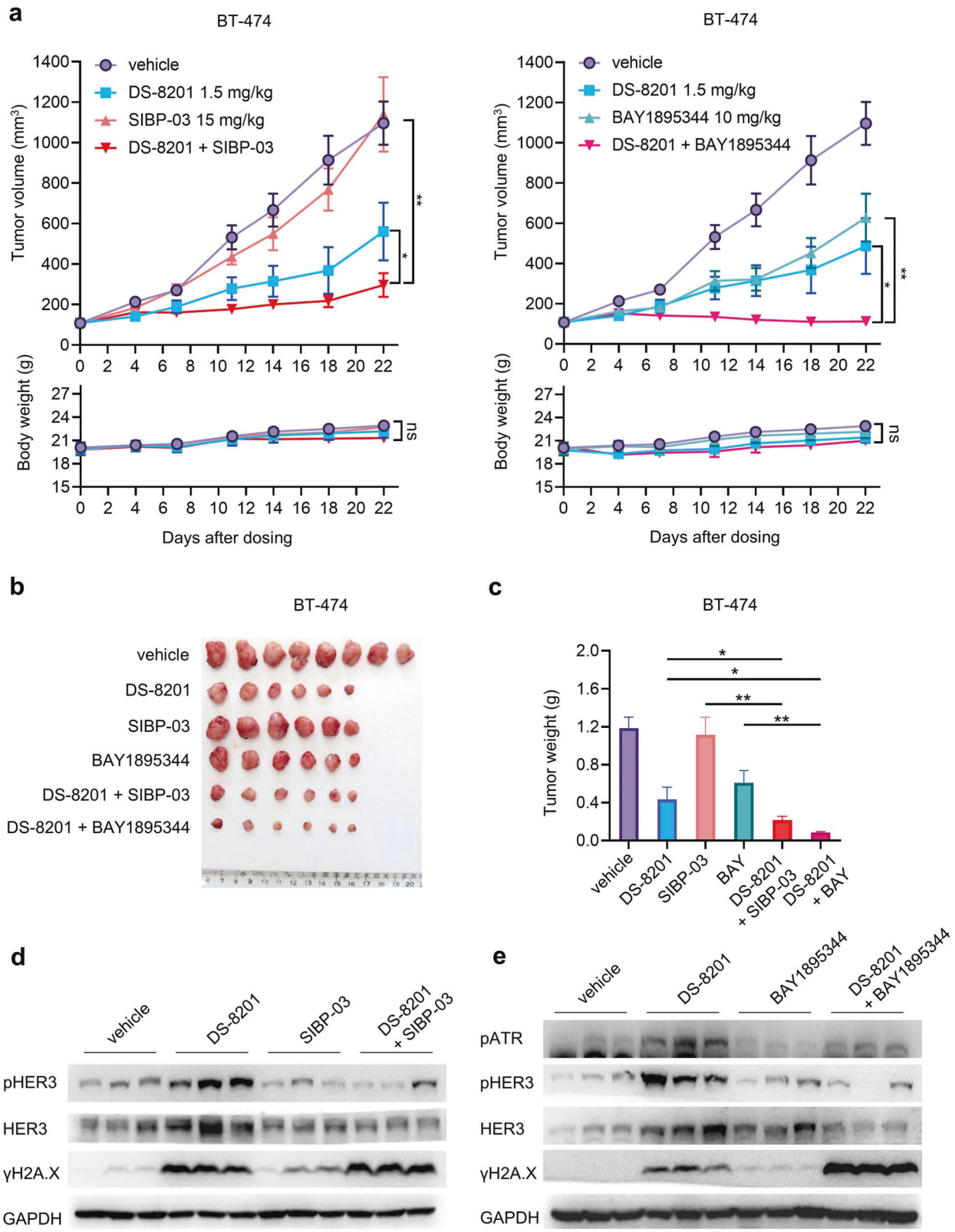

Fig. 6 Both HER3 blockade and ATR inhibition enhance DS-8201 efficacy in vivo. a Tumor volume and body weight in BT-474 tumor-bearing mice administered vehicle (intravenously, dosed on day 0), DS-8201 (1.5 mg/kg, intravenously, dosed on day 0), SIBP-03 (15 mg/kg, intraperitoneally, twice a week), BAY1895344 (10 mg/kg, intragastric, twice daily), or a combination. Vehicle: $n = 8$ mice; treatment groups: $n = 6$ mice. The data are presented as the means ± SEMs. $^{*}P < 0.05$, $^{**}P < 0.01$. Ns: no significance. Macroscopic tumor morphology (**b**) and final tumor weight (**c**) at the endpoint. The data are presented as the means ± SEMs. $^{*}P < 0.05$, $^{**}P < 0.01$. **d**, **e** Western blot analysis of γH2A.X and HER3-related signaling molecules in tumor tissues collected at the endpoint.

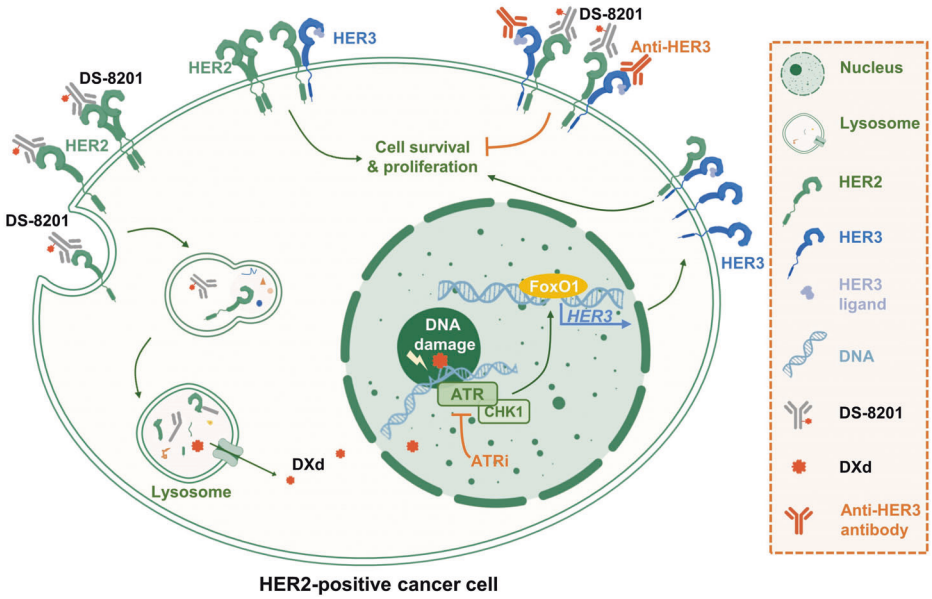

Fig. 7 Mechanism underlying HER3-mediated insensitivity to DS-8201. Upon DS-8201 treatment, HER2-positive cancer cells activate DNA damage repair responses, in which the ATR kinase pathway induces the expression of the HER3 transcription factor FoxO1, leading to increased HER3 levels.

strategies to increase its efficacy while minimizing adverse effects in patients with HER2-positive tumors remain poorly defined. Here, we discovered a novel mechanism underlying the insensitivity of HER2-positive breast cancer to DS-8201: significant upregulation of HER3 following DS-8201 treatment (Fig. 7). Mechanistically, we demonstrate that DS-8201 triggers a DDR response, in which the ATR kinase pathway induces the expression of the HER3 transcription factor FoxO1, leading to the upregulation of HER3. This process is driven by the payload component of DS-8201, DXd, rather than the antibody component. On this basis, we propose a novel combinatorial therapeutic strategy: combining HER3-targeting antibodies or ATR inhibitors with DS-8201. This approach demonstrates remarkably significant synergistic efficacy both in vitro and in vivo, without inducing intolerable toxicity. Our findings not only elucidate a key resistance mechanism to DS-8201 but also provide a promising therapeutic strategy to improve outcomes for patients with HER2-positive cancers. This work paves the way for further clinical investigations into optimizing DS-8201-based therapies.

Several preceding studies have demonstrated an association between EGFR family proteins and the DDR response in tumor cells [27, 28]. For example, in human breast cancer cells, the loss of HER2 function impairs the activation of the DDR-related kinases ATM/ATR, thereby disrupting cell cycle checkpoints [29]. Similarly, in human head and neck tumor models, the pan-EGFR inhibitor pan-HER was found to interfere with DDR processes, leading to increased programmed cell death [30]. These studies demonstrate that EGFR family proteins can regulate kinase activity in DNA damage repair. However, it remains unclear whether the DDR pathway exerts an inverse regulatory effect on EGFR family proteins, such as modulating their expression or activity. Our study revealed that DS-8201-induced DNA damage triggers the upregulation of HER3. Mechanistically, the ATR/CHK1 pathway was activated and mediated the FoxO1-dependent transcriptional upregulation of HER3, which subsequently affected therapeutic sensitivity to DS-8201. These findings suggest that HER3-targeted therapies hold promise as a strategy to augment the antitumor efficacy of DNA-damaging drugs. This reciprocal regulatory interplay between the DDR pathway and EGFR family proteins opens new avenues for optimizing combination therapies for cancer.

The dimerization-driven activation of EGFR family receptors tends to generate bypass signals that compensate for single-target inhibition by drugs [31], potentially resulting in an inadequate drug response [32]. Although the HER2 monoclonal antibody trastuzumab has been shown to induce HER2/HER3 internalization with short-term use [18], long-term use inevitably leads to the development of drug resistance, which is associated with the upregulation of HER3 [33, 34]. Our previous study demonstrated that anti-HER3 therapy synergizes effectively with trastuzumab, relying on the synergistic inhibition of relevant signaling pathways and the enhancement of antibody-dependent cellular cytotoxicity and phagocytosis (ADCP) effects [18]. Although HER3 inhibition has been recognized as a promising strategy to increase the efficacy of HER2-targeted antibodies, its potential benefits in the context of HER2 ADCs remain underexplored.

Previous studies in HER2-positive breast cancer have demonstrated that prolonged T-DM1 treatment induces significant HER3 upregulation in tumor tissues and trastuzumab-resistant cell lines [33, 35]. Importantly, these effects were reversible through HER3 blockade or inhibition of HER2/HER3 dimerization [35], indicating trastuzumab-mediated regulation of HER3 expression. In contrast, our findings revealed that DS-8201 induces HER3 upregulation primarily through its payload (DXd) by the activation of DNA damage response pathways rather than through trastuzumab-mediated mechanisms. This distinction may reflect differences in treatment duration (long-term T-DM1 administration versus short-term DS-8201 exposure) or payload-specific effects. Nevertheless, these collective findings underscore the critical role of HER3 in mediating resistance to HER2-targeted ADCs.

As a well-established and successful ADC payload, DXd has been incorporated into several ADCs for different targets, such as Dato-DXd (TROP2 ADC), DS-7300 (B7-H3 ADC), and DS-6000 (CDH6 ADC) [36–40]. Notably, HER3 is frequently overexpressed in lung cancer, with approximately 83% of NSCLC cases exhibiting HER3 expression [41]. This finding raises the possibility that HER3 inhibition could enhance the antitumor efficacy of these "X-DXd" ADCs, potentially expanding their clinical utility and benefits. Additionally, similar to DXd, other topoisomerase I inhibitors (exatecan and SN-38) also induced the activation and upregulation of ATR/FoxO1/HER3 pathway proteins (Fig. S3). The

development of ADCs incorporating these inhibitors as payloads, including IBI343 [42] and IMMU-132 [43], is advancing rapidly. Thus, targeting HER3 or ATR has the potential to enhance the clinical benefit of these drugs in the future.

Previous studies have shown that DS-8201 significantly improves survival in patients with advanced HER2-positive tumors [44]. However, it is worth noting that this treatment is associated with serious adverse effects, including ILD, hematological toxicity, and cardiotoxicity [10, 11]. The pathogenesis of DS-8201-induced ILD involves multiple factors, including off-target ADC uptake, bystander effects, and systemic payload (DXd) release [45–47]. While these mechanisms have been characterized, the potential involvement of HER3/ATR signaling pathways in ILD development remains unclear and warrants further investigation. The ATR kinase plays a dual role in both DNA damage repair and maintenance of lung homeostasis [48, 49]. Recent studies have demonstrated that YTHDC1 modulates ATR signaling through the formation of the TopBP1-MRE11 complex, which is essential for DNA repair in alveolar type II cells [49]. This biological context raises theoretical concerns that ATR inhibition might compromise pulmonary repair mechanisms and thereby potentiate DS-8201-associated ILD risk. However, clinical evidence from phase I (NCT04514497) [50, 51] and phase II (NCT03896503) [52] trials evaluating topoisomerase I inhibitor/ATR inhibitor combinations has not shown an increased incidence of ILD compared with monotherapy. Notably, the phase II trial NCT03896503 demonstrated that, compared with monotherapy, combination therapy increased the incidence of myelosuppression by only 3%, with comparable or reduced rates of other toxicities [52]. Consistent with these clinical observations, our preclinical studies revealed no significant tolerability issues when DS-8201 was combined with ATR inhibition. While these data suggest that the combination may not substantially increase toxicity, careful monitoring for pulmonary toxicity and myelosuppression remains essential in clinical implementation.

In conclusion, our study revealed that the HER2-targeting ADC DS-8201 induces FoxO1-dependent upregulation of HER3 through the ATR-mediated DDR pathway, which ultimately impacts the therapeutic efficacy of DS-8201 in HER2-positive cancers. We demonstrate that combining DS-8201 with either an ATR inhibitor or a HER3-targeting antibody significantly enhances its antitumor efficacy without inducing notable toxicity both in vitro and in vivo. This combination strategy holds substantial promise for patients with advanced HER2-positive tumors, offering the dual benefits of improved therapeutic outcomes and reduced adverse effects. Collectively, our findings provide a novel and promising approach for the treatment of HER2-positive tumors, paving the way for future clinical applications and the optimization of ADC-based therapies.

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AUTHOR CONTRIBUTIONS

LGL conceived and designed the research. WJL performed experiments and prepared the figures and manuscript. KGK and YXZ performed part of the cell experiments. WJL, KGK, YXZ, and XXZ contributed to the acquisition and analysis of the data. XZ, JT, and YPL helped with the data analysis and validation. HYF and QY participated in the mouse experiments. LW and LGL revised and approved the manuscript. All authors approved the final manuscript.

ADDITIONAL INFORMATION

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