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Original Article

Combined inhibition of PARP and ATR synergistically potentiates the antitumor activity of HER2-targeting antibody-drug conjugate in HER2-positive cancers

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Abstract: The therapeutic management of various HER2-positive malignancies involves the use of HER2-targeted antibody-drug conjugates (ADCs). The primary mechanism of action of ADCs is the release of cytotoxic chemicals, which leads to single- or double-strand DNA breaks and cell death. Since both endogenous and exogenous sources of DNA damage are unavoidable, cells have evolved DNA damage-repair mechanisms. Therefore, combining inhibitors of DNA damage repair and HER2-targeted ADCs may be a practical strategy for treating HER2-positive cancers. Effects of the HER2-targeted ADC, DS-8201, in combination with PARPi (AZD2281), a DNA damage repair inhibitor that targets poly(ADP-ribose) polymerase, and ATRi (BAY1895344), which inhibits the serine/threonine kinase ATR, were determined by assessing cell-growth inhibition, apoptosis and cell-cycle arrest, as well as using *in vivo* pharmacodynamic studies. Combined use of AZD2281 and BAY1895344 synergistically potentiated the inhibitory effects of DS-8201 on the growth of HER2-positive cancer cells, inducing DNA damage and apoptosis, but had no effect on HER2-negative MDA-MB-231 breast cancer cells. Our data demonstrate that DS-8201 and DNA damage repair inhibitors together have synergistic anticancer effects in NCI-N87 xenograft models, effects that may reflect upregulation of γ-H2AX protein in tumor tissues. Collectively, our results indicate that the combination of DS-8201, BAY1895344, and AZD2281 exerts significant synergistic antitumor activity, suggesting that DNA damage-repair inhibitors in combination with HER2-targeted ADCs is a potential approach for treating HER2-positive malignancies, offering a promising strategy for future clinical applications.

Keywords: Antibody-drug conjugate, DS-8201, PARP, ATR, synergy, DNA damage

Introduction

Human epidermal growth factor receptor 2 (HER2, Erbb2) is a receptor tyrosine kinase encoded by the *ERBB2* gene. It is a member of the HER (ErbB) receptor family, a subfamily of four closely related tyrosine kinases: HER1 (EGFR, ErbB-1), HER2 (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4) [1]. The HER2 extracellular domain has no known ligand and is activated by heterodimerization with other EGFR family members. Overexpression of HER2 leads to autophosphorylation of tyrosine residues in the cytoplasmic domain of heterodimeric HER receptors and initiates multiple signaling pathways that lead to cell proliferation and tumorigenesis [2, 3]. HER2 gene amplification or HER2 protein overexpression has been observed in HER2-positive cancers, including 15-20% of all breast cancers, 10% to 30% of all stomach and esophageal cancers, and a variety of other human malignancies, including gastric, lung, ovarian, bladder, and kidney carcinomas [4].

In recent years, the Food and Drug Administration (FDA) has approved a variety of HER2-targeted drugs for clinical use. These drugs, which are classified as monoclonal antibodies, small molecule chemicals and antibody-drug conjugates (ADCs) [5-7], include trastuzumab, lapatinib, pertuzumab, and T-DM1. Among these drugs, ADCs are considered a promising

and fast-growing targeted anticancer therapy [8], as evidenced by the more than 30 ADC programs currently in clinical trials [9, 10]. Notably, HER2-targeted therapy has dramatically improved survival in patients with HER2-positive cancer.

Trastuzumab deruxtecan (T-DXd, DS-8201, ENHERTU), a HER2-targeted ADC carrying a topoisomerase I inhibitor payload [8, 11], was approved by the FDA in 2019 to treat patients with HER2-positive, unresectable breast cancer or those with metastatic breast cancer who had previously received at least two anti-HER2-based therapies. It was subsequently approved for adult patients with locally advanced or metastatic HER2-positive gastric or gastroesophageal (GEJ) adenocarcinoma who had previously received trastuzumab therapy [12-14]. The antibody component of the drug can accurately identify and bind HER2-expressing tumor cells, even those with low HER2 expression, and internalizes the linked payload into target cells, which reduces the side effects associated with traditional chemotherapy drugs [15]. The linked cytotoxic drug inhibits the activity of topoisomerase I, as shown by its concentration-dependent inhibition of supercoiled DNA relaxation. Previous studies have shown that trastuzumab deruxtecan (hereafter DS-8201) induces DNA damage and apoptosis through phosphorylation of the histone, H2AX [16].

The maintenance of genomic stability requires a protective cellular response to DNA damage. The most common form of DNA damage is a single-strand break (SSB). When SSBs are not repaired in time, replication forks may collapse, causing double-strand breaks (DSBs)-a fatal type of injury that causes cell death. Different forms of DNA damage trigger responses through different repair mechanisms and signaling pathways. SSBs are repaired by the base excision repair (BER) pathway, whereas DSBs are mainly repaired by non-homologous end joining (NHEJ) or homologous recombination (HR), or other repair mechanisms. DNA damage-repair pathways encompass proteins that detect DNA damage, function in DNA repair pathways, and regulate the cell cycle. At present, research on small molecule inhibitors targeting key proteins that respond to DNA damage focuses mainly on poly(ADP-ribose) polymerase (PARP), ataxia telangiectasia and Rad3-related (ATR)-checkpoint kinase 1 (CHK1), ataxia telangiectasia mutated (ATM), checkpoint kinase 2 (CHK2), DNA-dependent protein kinase (DNA-PK), and the G2 checkpoint kinase WEE1, among others, and have made important progress in preclinical research and clinical applications [17-20].

PARP plays a crucial role in the repair of DNA SSBs and is involved in the monitoring and maintenance of genomic integrity [21]. PARP primarily binds to DNA damage sites and catalyzes the synthesis of poly(ADP-ribose) chains on protein substrates, and then co-repairs DNA damage by recruiting DNA repair proteins to the damage sites. PARP inhibitors suppress the growth of HR-deficient tumors through their synergistic lethal effects. Normally, DSBs in cells can be repaired through HR repair mechanisms, whereas they cannot be repaired in HR-deficient tumor cells and will thus be lethal [22, 23]. Accordingly, PARP inhibitors are actively employed therapeutically against a variety of tumors.

ATR protein is the core kinase of the DNA damage response, sensing replication stress and signaling it to S and G2/M checkpoints to facilitate repair [24]. In cancer, loss of G1 checkpoint control and activation of oncogenes that drive replication result in cancer cells being more likely to enter S phase with increased replication stress. These cancer cells become more reliant on their S and G2/M checkpoints, making these checkpoints attractive anticancer targets [25]. ATR inhibitors can interfere with DNA repair, causing cancer cells to accumulate errors in DNA that cannot be repaired, potentially giving rise to apoptosis.

A close relationship between cytotoxic drugs and DNA damage-repair mechanisms has been reported [26]. In this study, we explored the synergistic actions of HER2-targeted ADCs and DNA damage-repair inhibitors in improving treatment efficacy. We found that the combination of DS-8201 with the PARP inhibitor AZD2281 and ATR inhibitor BAY1895344 exerted significant synergistic inhibitory effects on cancer cell growth, inducing DNA damage and apoptosis in HER2-positive cancers. We further demonstrated that the three-drug combination increased DNA damage and had synergistic anticancer effects in NCI-N87 xenograft models. Thus, our data provide a basis for clini-

cal application of HER2-targeted ADC in combination with PARP and ATR inhibitors for the treatment of HER2-positive tumors.

Materials and methods

Cell lines and cell culture

The human gastric carcinoma cell line NCI-N87, human lung adenocarcinoma cell line Calu-3, and the human breast adenocarcinoma cell lines BT-474, SK-BR-3, MDA-MB-453, and MDA-MB-231 were purchased from American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere.

Antibodies and chemicals

DS-8201 (ENHERTU) was purchased by Daiichi Sankyo Company Limited. BAY1895344 and AZD2281 were purchased from Selleck Co (Shanghai), and Dxd was purchased from MCE (Shanghai). Sulforhodamine B (SRB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against γ-H2AX, caspase 9, PARP, caspase 3, cleaved-caspase3, PARP, ATR, phospho-ATR, Chk1, phospho-Chk1, HER2, phospho-HER2, phospho-AKT, and β-tubulin were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Protein Tech (Rosemont, IL, USA).

Anti-proliferative effects assay

Cells were seeded in a 96-well plate at a density appropriate for each cell type. And cultured overnight at 37°C to allow cells to adhere. After treatment with DS-8201, the anti-proliferative effects of treatment for 120 h with AZD2281 and BAY1895344, alone or in combination, was assessed using sulforhodamine B (SRB; Sigma) assays, as described previously [27].

Western blotting

After drug treatment, cells were collected and lysed in sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 1 mM dithiothreitol). Equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, probed with primary antibodies, and then incubated with the appropriate secondary antibodies (Millipore). Immunoreactive bands were visualized using enhanced chemiluminescence reagents (Millipore).

Immunofluorescence

After treatment with drugs for 24 h, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 for 10 min. Cells were then incubated first with primary antibody for 1 h and then with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody for 30 min [28]. Finally, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with a Zeiss immunofluorescence microscope (LSM 710; Zeiss, Germany). The percentage of DAPI-stained nucleic containing γ-H2AX foci, was calculated based on an analysis of randomly chosen fields.

Cell cycle analysis

Cells were harvested post-treatment, fixed in ice-cold 70% (v/v) ethanol, and stored at -20°C. Prior to analysis, cells were washed with phosphate-buffered saline (PBS), resuspended in 500 μL PBS containing 50 μg/mL propidium iodide (Sigma-Aldrich) and 50 μg/mL RNase (Sigma-Aldrich), and incubated at 37°C for 30 min. Samples were analyzed on a BD Flow Cytometer (Thermo Fisher Scientific). Data were analyzed using Flow Jo Software (BD Biosciences, Wokingham, UK). Experiments were performed in at least triplicate.

In vivo study

Female Balb/cA-nude mice (5-6 wk old) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). Human tumor xenografts were prepared by subcutaneously implanting NCI-N87 cells into the right flank of the animal. Tumor diameter was measured by vernier caliper. Tumor volume was calculated as (length × width^2)/2. Body weight was monitored as an indicator of general health twice a week. When tumor volumes reached ~100 mm^3, mice were randomly assigned to treatment groups. Control groups were given vehicle, and treatment groups were given DS-8201

(0.5 mg/kg, IV), AZD2281 (30 mg/kg, PO), and BAY1895344 (10 mg/kg, PO), alone or in combination. The therapeutic effect of a given compound was expressed in terms of tumor growth inhibition (TGI), calculated as TGI = (Vt'-Vt)/(Vc'-Vc) × 100%, where Vt' and Vt are the tumor volumes in treatment groups on each day of treatment and on the day of initial treatment, respectively, and Vc' and Vc are tumor volumes in the vehicle control group on each day of treatment and on the day of initial treatment, respectively. Mice were euthanized by carbon dioxide at the end of the experiments. Tumor samples were analyzed by Western blotting. Animal experiments were carried out in accordance with guidelines of the Institutional Animal Care and Use Committee at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Pharmacodynamic studies

Female Balb/cA nude mice (5-6 wk old) were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai). Studies were conducted using an NCI-N87 xenograft model in nude mice. After administration of DS-8201 (0.5 mg/kg, IV, day 1), AZD2281 (30 mg/kg, BID × 8 d) and BAY1895344 (10 mg/kg, BID, days 5, 6, and 7), mouse tumor tissues were collected and γ-H2AX expression in the resulting tumors was analyzed by Western blotting.

Data analysis

Data were analyzed using GraphPad Prism software, and the results of repeated experiments were presented as means ± standard deviation (SD). Half-maximal inhibitory concentration (IC_{50}) values and dose-response curves were obtained by nonlinear regression analysis. A two-tailed Student's t-test was used to evaluate differences between groups, and $P < 0.05$ was considered statistically significant.

Results

DS-8201 exerts differential anti-proliferative effects in HER2-positive cancer cells

To investigate the efficacy of the HER2-targeted ADC, DS-8201, and combined inhibitory strategies, we first examined HER2 expression levels in various tumor cell lines by flow cytometry, measured as mean fluorescent intensity (MFI). We found that HER2 expression levels varied considerably among different tumor cell lines (**Figure 1A**), exhibiting the highest expression in the NCI-N87 gastric cancer cell line and BT-474 breast cancer cell line, with relative MFIs of 477,312 and 315,294, respectively. HER2 expression was moderate in the SK-BR-3 breast cancer cell line, Calu-3 lung cancer cell line and MDA-MB-453 breast cancer cell line, with relative MFIs of 229,555, 223098, 119,939 and 25,872, respectively. The HER2-negative MDA-MB-231 cell line [29] showed the lowest MFI (4857).

We then investigated the anti-proliferative effects of DS-8201 and its toxic moiety, DXd, against various cell lines. We found that the sensitivity to DS-8201 varied greatly among cell lines. Remarkable anti-proliferative effects of DS-8201 were observed in HER2-positive SK-BR-3, MDA-MB-453, NCI-N87 and Calu-3 cells, with IC_{50} values of 4.8, 11.5, 32.7 and 41.7 ng/mL, respectively. DS-8201 had lesser anti-proliferative effects in BT-474 cells, and showed no apparent anti-proliferative effects in HER2-negative MDA-MB-231 cells (IC_{50}> 3000 ng/mL). All seven cell lines were sensitive to the payload, DXd, although its anti-proliferative effects were weaker in BT-474 cells (IC_{50} = 54.5 nM) compared with other cells (IC_{50} = 0.7-14.5 nM) (**Figure 1B**).

Collectively, these results suggest that DS-8201 selectively inhibits the proliferation of HER2-positive cancer cells, and this inhibitory activity is affected by sensitivity to the payload, DXd.

Combined inhibition of PARP and ATR synergistically potentiates the anti-proliferative effects of DS-8201 against HER2-positive cancer cells

To improve the antitumor effects of DS-8201, we then explored the strategy of combining DS-8201 with other drugs. Because DS-8201 exerts its antitumor effects mainly by causing DNA damage [30], we tested the possibility of combining DS-8201 with agents that inhibit DNA damage-repair.

We first examined the anti-proliferative effects of the DNA damage-repair inhibitors AZD2281, a PARP inhibitor (PARPi), and BAY1895344, an ATR inhibitor (ATRi), in tumor cells. AZD2281

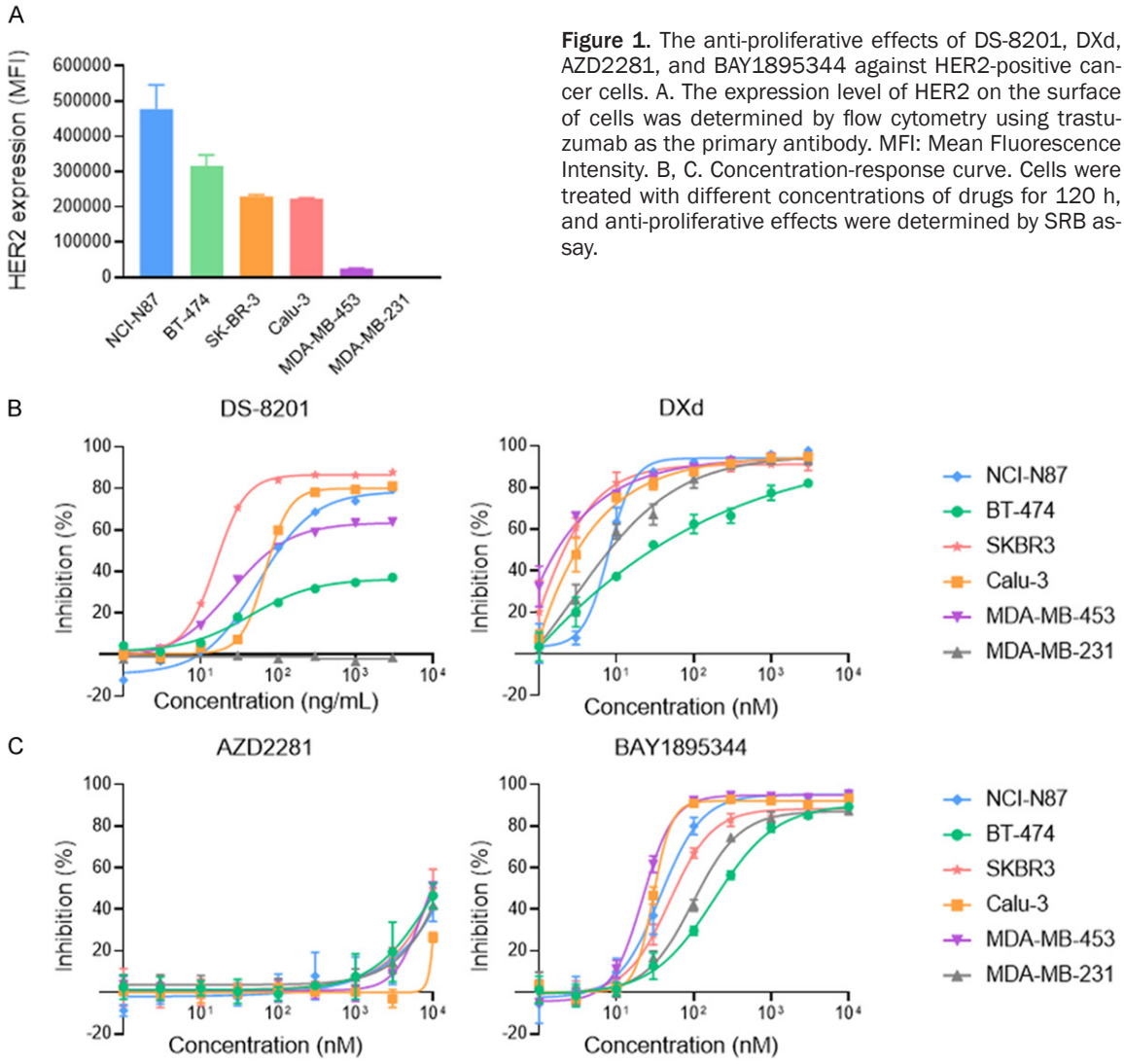

Figure 1. The anti-proliferative effects of DS-8201, DXd, AZD2281, and BAY1895344 against HER2-positive cancer cells. A. The expression level of HER2 on the surface of cells was determined by flow cytometry using trastuzumab as the primary antibody. MFI: Mean Fluorescence Intensity. B, C. Concentration-response curve. Cells were treated with different concentrations of drugs for 120 h, and anti-proliferative effects were determined by SRB assay.

Table 1. Anti-proliferative effects of DS-8201, AZD2281, BAY1895344, and Dxd against HER2-positive cancer cells

Drugs	IC_{50} (ng/mL or nM)					
	NCI-N87	BT-474	SK-BR-3	Calu-3	MDA-MB-453	MDA-MB-231
DS-8201 (ng/mL)	32.7±20.8	>3000	4.8±0.7	41.7±22.7	11.5±3.2	>3000
AZD2281 (nM)	>10000	>10000	>10000	>10000	>10000	>10000
BAY1895344 (nM)	37.0±5.1	185.9±29.6	48.4±2.9	29.6±1.6	21.8±1.6	97.8±15.4
Dxd (nM)	12.8±2.6	54.4±14.3	1.4±1.0	5.1±1.9	0.7±0.5	14.5±1.6

IC_{50} values were determined by sulforhodamine B assay in cells treated with different concentrations of drugs for 120 h. Data are presented as means ± S.D. of three independent experiments.

showed weak cell growth inhibitory activity, such that, at a concentration of 3 μM, it exhibited an inhibition rate of ~10% in most cell types. BAY1895344 showed more potent anti-proliferative activity, causing ~10% inhibition in most cell types at a concentration of 10 nM (**Figure 1C**; **Table 1**).

We then explored the strategy of co-treatment with these two DNA damage-repair agents to

enhance the efficacy of DS-8201. We first examined different combinations of DS-8201 (10 ng/mL) with AZD2281 (3 μM) and BAY-1895344 (10 nM) in NCI-N87 and BT-474-the two cell lines with the highest HER2 expression. In NCI-N87 and BT-474 cells, the inhibition rates of combined DS-8201 and AZD2-281 were 35.7% and 24.7%, respectively, and the corresponding rates for DS-8201 and BAY1895344 were 6.4% and 41.9%. These results suggest that both AZD2281 and BAY1895344 are capable of potentiating DS-8201 in these two cell lines. We further examined the three-drug combination strategy and found that it achieved remarkable inhibition rates in both NCI-N87 cells (75.5%) and BT-474 cells (56.4%). These results suggest that combining DS-8201 with PARP and ATR inhibitors improves anti-proliferative effects in cell lines expressing high levels of HER2 (**Figure 2A**).

To further investigate the efficacy of this combination strategy, we examined the anti-proliferative effects of the combination against different types of tumor cell lines. Consistent with our previous results, we found that DS-8201 combined with either AZD2281 or BAY1895-344 showed a synergistic effect, although DS-8201 combined with both AZD2281 and BAY1895344 exerted the strongest cell anti-proliferative effects (~6.7-fold sensitization of HER2-positive NCI-N87 cells); similar results were obtained in other HER2-positive cancer cell lines. In addition, although BT-474 cells were not very sensitive to DS-8201 alone, they showed greater susceptibility to the therapeutic effects of the three-drug combination. Unsurprisingly, no synergy was observed in HER2-negative MDA-MB-231 breast cancer cells (**Figure 2B**; **Table 2**).

In addition, we also explored the synergistic effects of DNA damage-repair inhibitors in combination with other ADCs. DS-7300 is a B7-H3 directed DXd ADC and DS-1062 is a TROP-2 directed DXd ADC. To investigate the synergy effects with DS-7300 and DS-1062, we use BxPC-3, a human pancreatic cancer cell line expressing B7-H3 and TROP-2, as a model. We found that combining the two DNA damage-repair inhibitors could potentiate DS-7300 as well as DS-1062 with similar synergistic effects (Supplementary Figure 1).

Collectively, these results suggest that combined inhibition of PARP and ATR synergistically potentiates the anti-proliferative effects of DS-8201 as well as other DXd ADCs with different targets.

Combined inhibition of PARP and ATR synergistically enhances DS-8201-induced, HER2-mediated inhibition of downstream signaling and DNA damage in HER2-positive cancer cells

To explore the mechanisms underlying the synergistic effects of DS-8201 and DNA damage-repair inhibitors, we next examined the combined effects on HER2-mediated signaling and DNA repair pathways. Our data showed that DS-8201 caused downregulation of phosphorylated HER2, AKT and extracellular signal-regulated kinase (ERK) in HER2-positive NCI-N87 and BT-474 cells, and that the combination of DS-8201 with AZD2281 and BAY-1895344 caused enhanced suppression of HER2, AKT and ERK phosphorylation (**Figure 3A**). Moreover, we found that DS-8201 or AZD2281 induced phosphorylation of ATR and CHK1 in NCI-N87 and BT-474 cells, effects that were reversed by BAY1895344 in the three-drug combination (**Figure 3B**). Previous research has demonstrated that treatment with DS-8201, a PARP inhibitor or an ATR inhibitor leads to replication stress and DNA damage in tumor cells [16, 31]. Our results showed that the three-drug combination significantly increased levels of γ-H2AX, a surrogate indicator of replication stress and a marker of DNA double-strand breaks, in NCI-N87 and BT-474 cell lines (**Figure 3B**), indicating that the combination caused strong DNA damage effects. We further examined the formation of γ-H2AX foci in NCI-N87 cells by immunofluorescence assays. As shown in **Figure 3C**, DS-8201 induced formation of γ-H2AX foci, an effect that was enhanced by the combination with AZD2281 or BAY1895344 and was strongest with the three-drug combination (**Figure 4**).

Combined inhibition of PARP and ATR synergistically enhances DS-8201-induced G2/M arrest and apoptosis

Cells usually undergo cell-cycle arrest and subsequent apoptosis in response to DSBs [31]. Accordingly, we next investigated the influence of DS-8201, AZD2281 and BAY1895344, alone

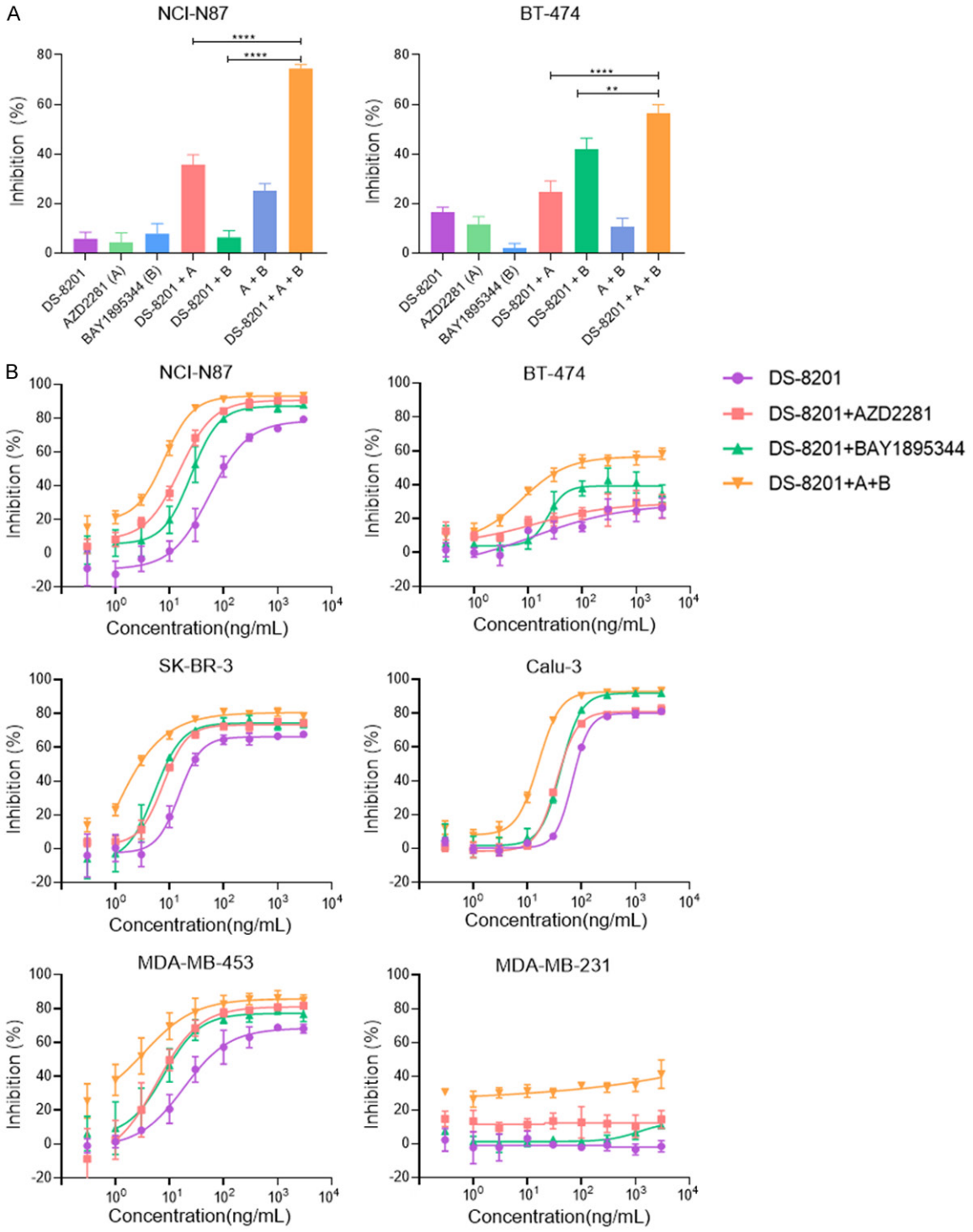

Figure 2. Combined inhibition of PARP and ATR synergistically potentiates the anti-proliferative effects of DS-8201 against HER2-positive cancer cells. A. Cells were treated with DS-8201 (10 ng/mL) in the absence or presence of AZD2281 (3 μM) and BAY1895344 (10 nM) for 120 h, and anti-proliferative effects were determined by SRB assay. B. Cells were treated with different concentrations of DS-8201 in the absence or presence of AZD2281 (3 μM) and BAY1895344 (10 nM) for 120 h, and anti-proliferative effects was determined by SRB assay. Note: The leftmost point represents a DS-8201 concentration of 0 ng/mL, an AZD2281 concentration of 3 μM, and a BAY1895344 concentration of 10 nM. Data shown represent means ± SD of three independent experiments. **P < 0.01, ****P < 0.0001.

Table 2. Combined inhibition of PARP and ATR synergistically potentiates the anti-proliferative effects of DS-8201 against HER2-positive cancer cells

Drugs	IC_{50} (ng/mL)					
	NCI-N87	BT-474	SK-BR-3	Calu-3	MDA-MB-453	MDA-MB-231
DS-8201	54.7±19.7	>3000	15.0±0.3	68.4±3.1	27.5±13.1	>3000
+ 3 μM AZD2281	15.7±2.9 (3.5)[a]	>3000 (N/A)	7.6±0.2 (2.0)	34.8±0.4 (2.0)	6.1±2.7 (4.5)	>3000 (N/A)
+ 10 nM BAY1895344	24.9±7.5 (2.2)	>3000 (N/A)	5.3±0.2 (2.8)	41.8±4.4 (1.6)	9.0±1.6 (3.0)	>3000 (N/A)
+ 3 μM AZD2281+10 nM BAY1895344	8.1±1.3 (6.7)	7.6±1.7 (N/A)	0.7±0.5 (21.4)	15.1±1.5 (4.5)	5.1±1.0 (5.4)	>3000 (N/A)

IC_{50} values were determined by sulforhodamine B assay in cells treated with different concentrations of drugs for 120 h. Data are presented as means ± S.D. of three independent experiments. [a]Value in parentheses indicates fold-sensitization, determined as IC_{50} (combination)/IC_{50} (DS-8201 alone).

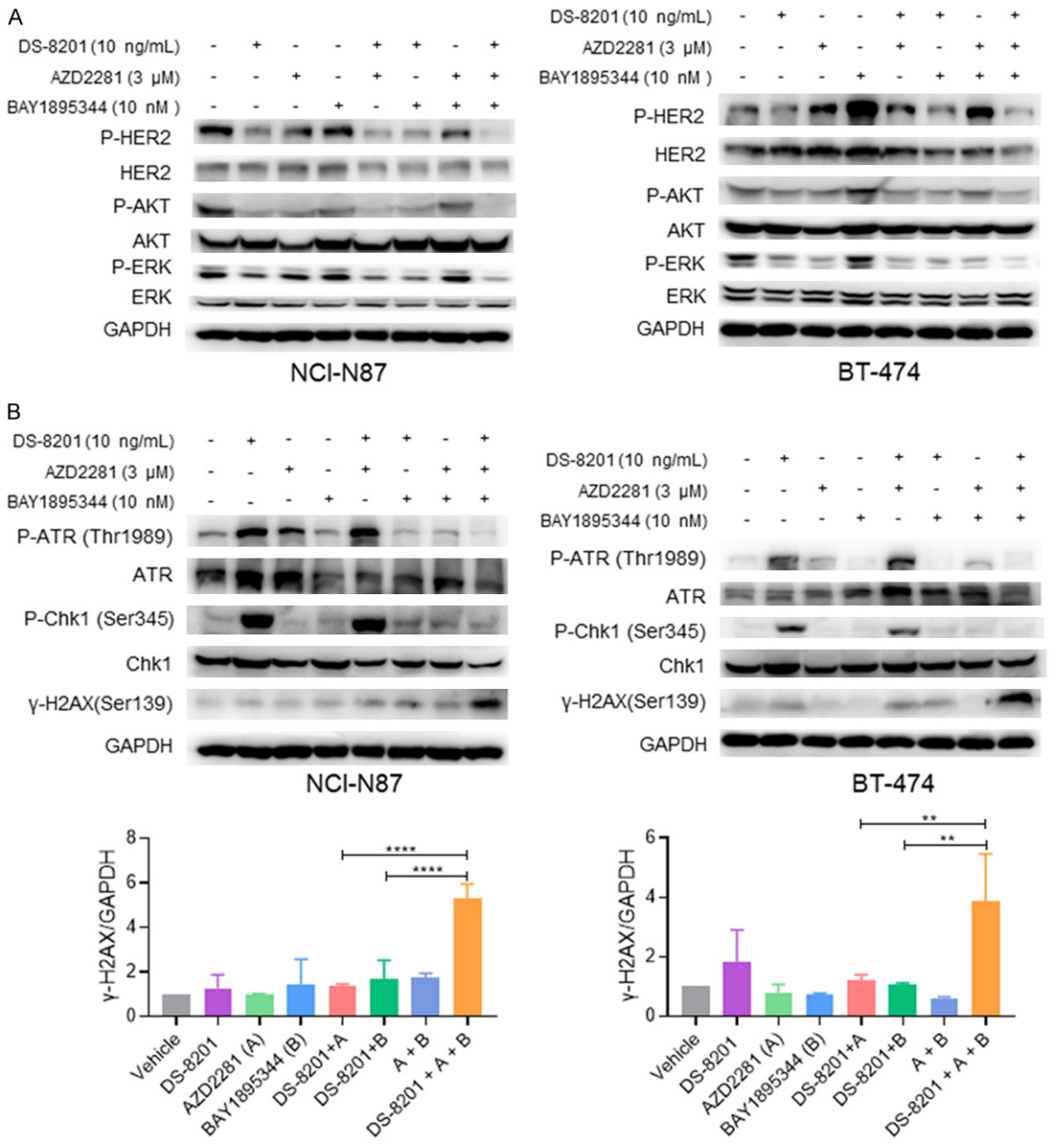

Figure 3. Combined inhibition of PARP and ATR synergistically enhances DS-8201-induced inhibition of HER2 downstream signaling and DNA damage in HER2-positive cancer cells. (A, B) Cells were treated with DS-8201(10 ng/mL) in the absence or presence of AZD2281 (3 μM) and BAY1895344 (10 nM) for 72 h. Whole-cell lysates were analyzed for HER2 downstream signaling molecules by Western blotting using the indicated antibodies (A), and DNA damage was assessed by quantifying γ-H2AX levels using Image J software using GAPDH as an internal reference (B). Data are presented as means ± S.D. of three independent experiments. **P < 0.01, ****P < 0.0001.

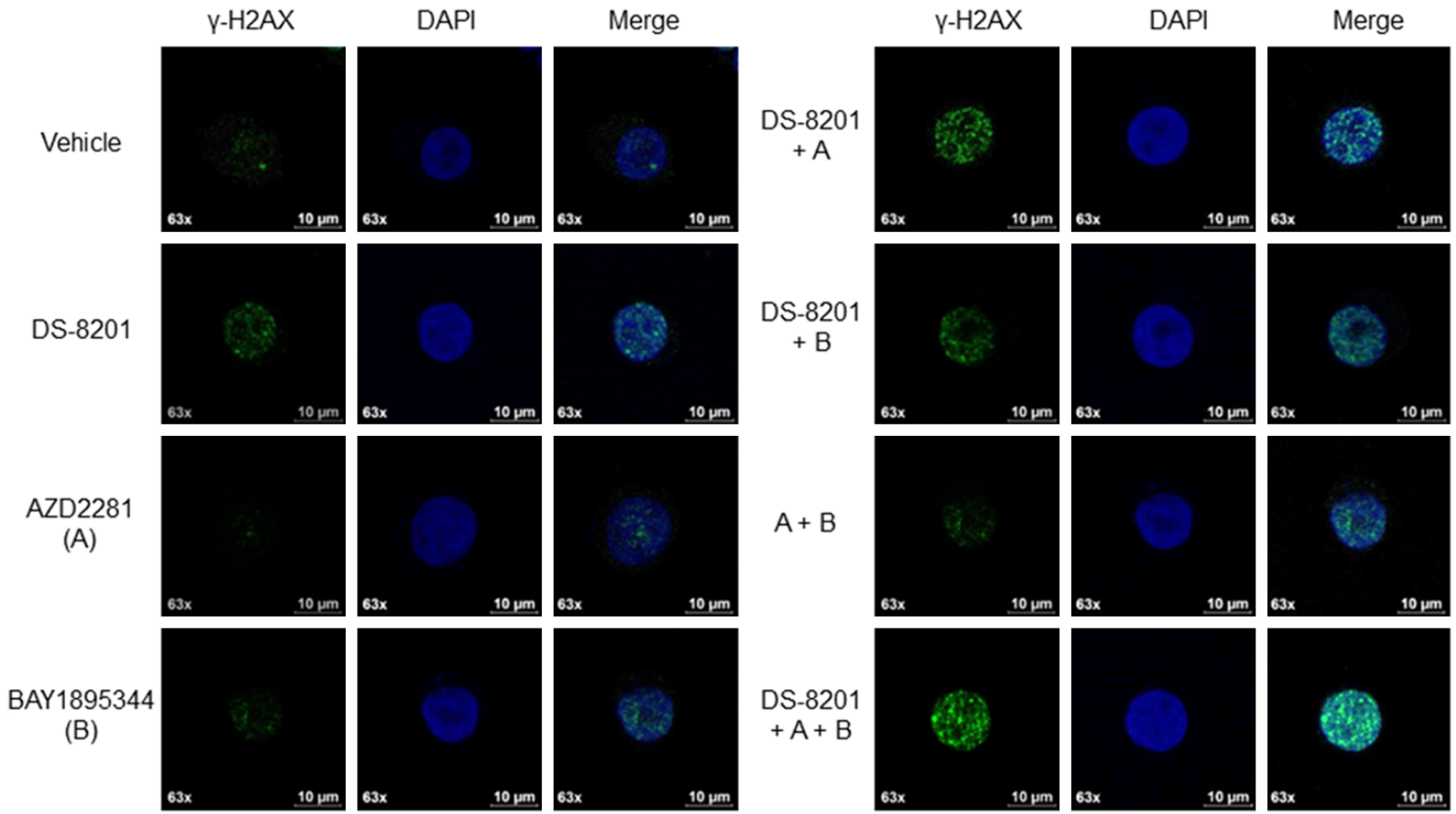

Figure 4. Combined inhibition of PARP and ATR synergistically enhances DS-8201-induced γ-H2AX foci formation. DNA damage was detected by immunofluorescence and visualized by confocal microscopy following treatment of NCI-N87 cells with DS-8201 (10 ng/mL), AZD2281 (3 μM), and BAY1895344 (10 nM), alone or in combination, for 24 h.

or in various combinations, on the cell cycle and DNA damage. We found that DS-8201 and AZD2281 alone increased G2/M-arrested cells from 5.63% to 12.31% and 23.50%, respectively, and increased the percentage of cells in S phase from 34.92% to 45.88% and 40.91%, respectively. BAY1895344 alone decreases G2/M phase-arrested cells from 5.63% to 1.06% and increased S-phase cells from 34.92% to 46.73%. Combining the three drugs increased both G2/M phase-arrested cells (from 5.63% to 37.27%) and S-phase cells (34.92% to 56.70%), and reduced G1-phase cells to 6.03% (**Figure 5A**, **5B**), indicating that most proliferating cells were arrested. We also detected apoptosis-related proteins, with different combinations of DS-8201 with these inhibitors increasing the cleavage of PARP and caspase-3. The highest level of apoptosis was observed in cells treated with the combination of DS-8201, AZD2281, and BAY1895344 (**Figure 5C**).

DS-8201 combined with PARP and ATR inhibitors exerts enhanced antitumor efficacy against NCI-N87 xenografts

Finally, to extend these *in vitro* findings to an *in vivo* setting, we investigated the antitumor effects of DS-8201, AZD2281 and BAY189-5344, alone or in combinations, in nude mice bearing NCI-N87 xenografts. As shown in **Figure 5A**, monotreatment with DS-8201 (0.5 mg/kg, n = 6), AZD2281 (30 mg/kg, n = 6) or BAY1895344 (10 mg/kg, n = 6) led to only modest inhibition of tumor growth (50.1%, 16.8% and 17.0%, respectively). Combining DS-8201 with AZD2281 or BAY1895344 slightly enhanced these inhibition effects (56.9% and 57.7%, respectively), whereas the three-drug combination led to a significant and striking shrinkage of tumors (84.4%). Moreover, no apparent toxicity was observed in either group (**Figure 6A**). We further assessed γ-H2AX levels in NCI-N87 xenografts. Consistent with our *in vitro* results, we found that the combination of DS-8201, AZD2281 and BAY1895344 significantly increased γ-H2AX levels in xenografts (**Figure 6B**). Taken together, these findings suggest that combining DS-8201 with AZD2281 and BAY1895344 produces significant antitumor effects in HER2-positive NCI-N87 gastric cancer xenografts that may reflect the synergistic induction of DNA damage, as illustrated by the enhanced γ-H2AX levels.

Discussion

HER2-targeted ADC is currently attracting considerable research attention owing to its promising results in various cancers. DS-8201 loaded with a topoisomerase I inhibitor acts as a HER2-targeted ADC that causes DNA damage. However, DNA damage repair mechanisms in tumor cells will inevitably repair some damage caused by DS-8201, which may lead to less effective therapeutic outcomes [32]. Therefore, combining DS-8201 with drugs that inhibit DNA repair may be a practical strategy. Previous studies have shown that DNA damage-repair inhibitors can be used in combination with a variety of DNA-damaging chemotherapeutic agents, such as alkylating agents, platinum-based agents and topoisomerase I inhibitors, among others [33-39]. However, few studies have examined the efficacy of HER2-targeted ADC drugs in combination with DNA damage-repair inhibitors. In this study, we found that the combination of DS-8201 with a PARP inhibitor (AZD2281) and ATR inhibitor (BAY1895344) showed significant synergistic antitumor effects both *in vitro* and *in vivo*. Accordingly, this report provides a promising strategy for the treatment of HER2-positive cancer.

In this study, we found that DS-8201 has a significant inhibitory effect on a variety of tumor cells from different sources, not only cell lines with high HER2 expression, but also low HER2-expressing cell lines, such as the MDA-MB-453 breast cancer cell line, where it exerts a certain inhibitory effect. The inhibition of proliferation induced by DS-8201 was further increased when combined with DNA damage-repair inhibitors, offering new hope for the treatment of patients with low HER2-expressing cancers. At the same time, DS-8201 exerts clear antiproliferative activity in various types of solid tumors, suggesting that our combined drug regimen is not limited to the treatment of breast and gastric cancer, but may also be useful for the treatment of lung cancer and other cancers. Moreover, the synergistic effects are not limited to specific target of ADCs. We found that DNA damage-repair inhibitors also potentiated DS-7300 (B7-H3 directed DXd ADC) and DS-1062 (TROP-2 directed DXd ADC) with simi-

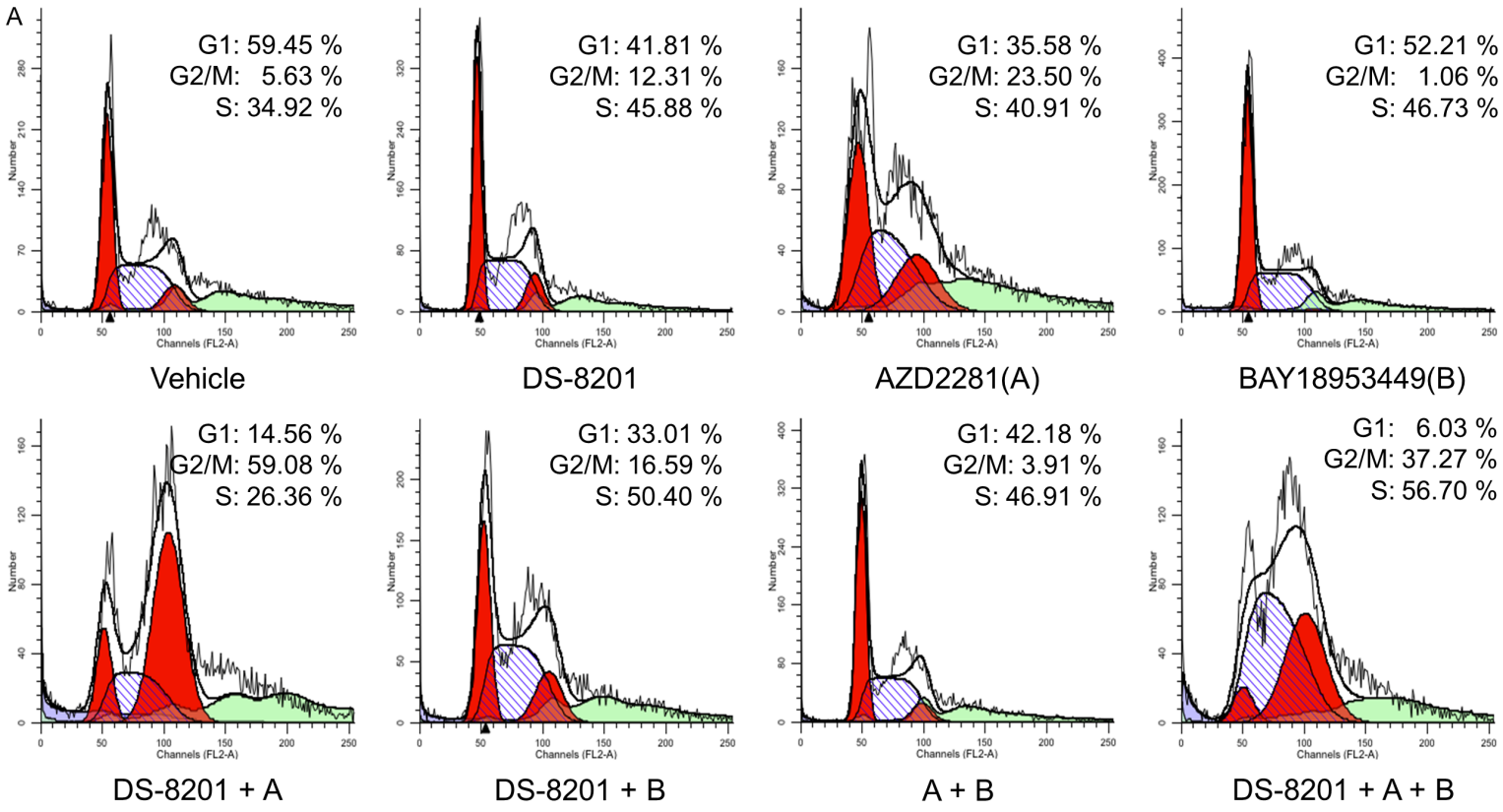
A
G1: 59.45 %
G2/M: 5.63 %
S: 34.92 %
Vehicle
G1: 41.81 %
G2/M: 12.31 %
S: 45.88 %
DS-8201
G1: 35.58 %
G2/M: 23.50 %
S: 40.91 %
AZD2281(A)
G1: 52.21 %
G2/M: 1.06 %
S: 46.73 %
BAY18953449(B)
G1: 14.56 %
G2/M: 59.08 %
S: 26.36 %
DS-8201 + A
G1: 33.01 %
G2/M: 16.59 %
S: 50.40 %
DS-8201 + B
G1: 42.18 %
G2/M: 3.91 %
S: 46.91 %
A + B
G1: 6.03 %
G2/M: 37.27 %
S: 56.70 %
DS-8201 + A + B
Number
Channels (FL2-A)
B
Cells (%)
S
G2/M
G0/G1
control
DS-8201
AZD2281 (A)
BAY1895344 (B)
DS-8201+A
DS-8201+B
A+B
DS-8201+A+B
C
DS-8201 (10 ng/mL)
AZD2281 (3 μM)
BAY1895344 (10 nM)
PARP
Cleaved-PARP
caspase3
Cleaved-caspase3
β-Tubulin

Figure 5. ATR inhibitor reverses G2/M arrest induced by DS-8021 and PARP inhibitor, and combined inhibition of PARP and ATR synergistically enhances DS-8201-induced cell apoptosis. A, B. NCI-N87 Cell were treated with DS-8201 (10 ng/mL) in the absence or presence of AZD2281 (3 μM) and BAY1895344 (10 nM) for 48 h and the cell cycle was analyzed by flow cytometry. A. Representative flow histogram. B. Cell cycle analysis. Data are presented as means ± S.D. of three independent experiments. C. Cells were treated with different concentrations of drugs for 72 h, and then apoptosis-related proteins were analyzed by Western blotting using the indicated antibodies.

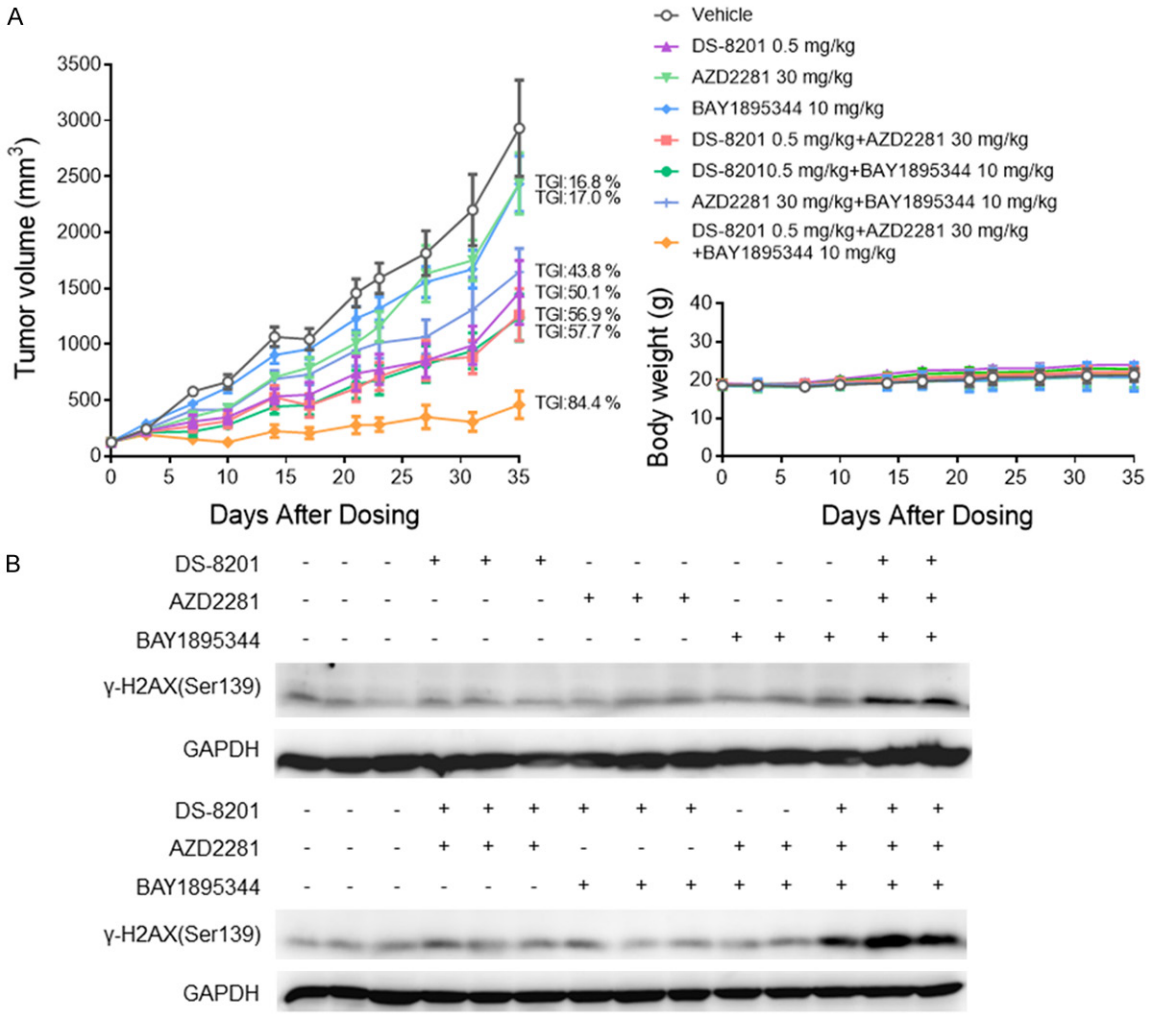

Figure 6. Combined inhibition of PARP and ATR synergistically enhances the antitumor effects of DS-8201 against NCI-N87 xenografts. A. NCI-N87 tumor-bearing mice received vehicle, the indicated dosages of DS-8201, AZD2281 or BAY1895344, or a combination of drugs. Tumor volumes were measured and body weights were determined. Data are presented as means ± SEM *P < 0.05. TGI: Tumor Growth Inhibition value. B. Photographs of tumors on the day mice were sacrificed. C. NCI-N87 tumor-bearing mice received vehicle or DS-8201 (0.5 mg/kg, i.v.) in the absence or presence of AZD2281 (30 mg/kg, i.g.) and BAY1895344 (10 mg/kg, i.g.). Mice were sacrificed 24 h after the last dose, and tumors were removed and analyzed by Western blotting. Data are presented as means ± SEM *P < 0.05.

lar synergistic effects, offering the opportunity for the extensive application of this combined drug regimen.

The combination of DS-8201 with PARP and ATR inhibitors led to increased γ-H2AX levels, a hallmark of DNA damage, most likely owing to reduced DNA damage-repair capacity [40-42]. We found that the combination of DS-8201 and AZD2281 resulted in G2/M cycle arrest, suggesting that intracellular activation of ATR recruits cell cycle-related proteins that arrest

cell cycle progression and prevent cells from entering mitosis and causing mitotic disaster, thus buying time for other DNA repair pathways [43, 44]. When combined with DS-8201 and AZD2281, BAY1895344 reversed the G2/M cycle arrest caused by DS-8201 and AZD2281, leading to the entry of cells with greater DNA damage into mitosis, causing a mitotic catastrophe and apoptosis. Our study also showed that the combination of these drugs synergistically inhibited phosphorylation of HER2, AKT, and ERK. The induction of DNA damage and inhibition of HER2 signaling pathways may be the main mechanisms of the observed synergistic antitumor activity [31].

Previous studies showed that DS-8201 significantly improves response and overall survival in patients with HER2-positive gastric cancer, but also noted severe toxic effects, including myelosuppression and interstitial lung disease. Anemia and nausea are the main toxic effects in patients treated with AZD2281, whereas the most common side effect of BAY1895344 is anemia [16, 45-47]. In our study, we found no significant toxicity in a mouse transplantation tumor model. In this context, the combination strategy is expected to reduce the dose of each drug used in cancer patients and thus reduce toxic effects to a greater extent, providing a rational basis for combining drugs in a clinical setting.

Our studies demonstrate that the HER2-targeting ADC, DS-8201 in combination with AZD2281 and BAY1895344 exert synergistic antitumor activity against HER2-positive cancer cells *in vitro* and *in vivo* by enhancing inhibition of DNA damage-repair pathways. Patients may benefit from this strategy as a result of both enhanced efficacy and reduced drug toxicity. This report provides a strong case for investigating the efficacy of DS-8201 in combination with AZD2281 and BAY1895344 in the treatment of most HER2-positive cancers in future clinical trials.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

PARP, Poly (ADP-ribose) Polymerase; ATR, Ataxia Telangiectasia and Rad3-related; DDR, DNA-Damage Response; SSB, Single-Strand Break; DSB, Double-Strand Break.

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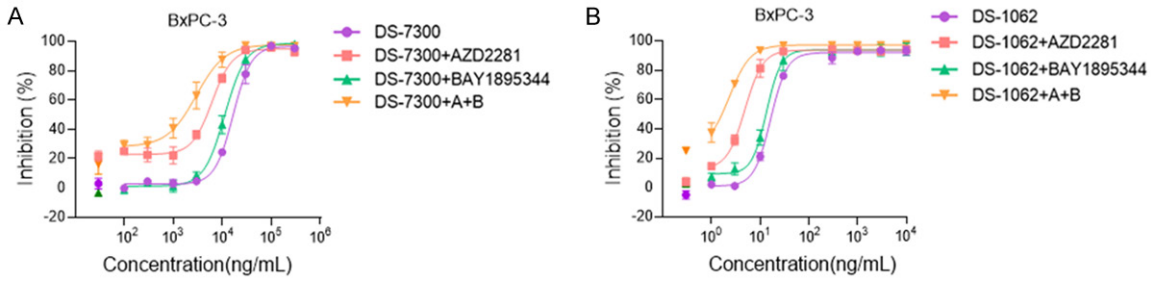

Drugs	BxPC-3 (IC_{50}, ng/mL)
DS-7300	16802.0 ± 2047.8
+ 3 μM AZD2281	6261.5 ± 577.7(2.7)[a]
+ 3 nM BAY1895344	11514.0 ± 1518.9(1.5)
+ 3 μM AZD2281+ 3 nM BAY1895344	3119.5 ± 1523.8(5.4)

Drugs	BxPC-3 (IC_{50}, ng/mL)
DS-1062	16.4 ± 0.5
+ 3 μM AZD2281	4.9 ± 0.7(3.3)
+ 3 nM BAY1895344	13.6 ± 1.4(1.2)
+ 3 μM AZD2281+ 3 nM BAY1895344	2.3 ± 0.1(7.1)

Supplementary Figure 1. Combined inhibition of PARP and ATR synergistically potentiates the anti-proliferative effects of other DXd-ADC. (A, B) Cells were treated with different concentrations of DS-7300 (A)/DS-1062 (B) in the absence or presence of AZD2281 (3 μM) and BAY1895344 (3 nM) for 120 h, and anti-proliferative effects was determined by SRB assay. Note: The leftmost point represents a DS-7300 (A)/DS-1062 (B) concentration of 0 ng/mL, an AZD2281 concentration of 3 μM, and a BAY1895344 concentration of 3 nM. Data shown represent means ± SD of three independent experiments. [a]Value in parentheses indicates fold-sensitization, determined as IC_{50} (combination)/IC_{50} (DS-7300 or DS-1062 alone).