

Ebastine targets HER2/HER3 signaling and cancer stem cell traits to overcome trastuzumab resistance in HER2-positive breast cancer

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Abstract. Despite advances in HER2-targeted therapy for HER2-positive breast cancer, resistance to trastuzumab and tumor recurrence remain major barriers to durable outcomes. The present study evaluated the therapeutic potential of ebastine, a second-generation H1-antihistamine, as a repurposing candidate to overcome trastuzumab resistance by targeting HER2 signaling and cancer stem cell (CSC)-associated phenotypes in HER2-positive breast cancer cells. Molecular docking studies revealed that ebastine bound to the ATP-binding site of the HER2 tyrosine kinase domain, thereby suppressing the phosphorylation of HER2, p95HER2 and HER3, as assessed by immunoblotting. Immunoprecipitation assay further demonstrated that this binding disrupted HER2/HER3 and HER2/EGFR heterodimerization, leading to reduced downstream AKT activation. Ebastine significantly decreased aldehyde dehydrogenase (ALDH)1 activity, decreased the CD44^{high}/CD24^{low} CSC-like population, as assessed by flow cytometry, and inhibited mammosphere formation. In a trastuzumab-resistant xenograft model, ebastine markedly suppressed tumor growth, decreased the Ki-67 proliferation index and angiogenesis and induced apoptosis. These effects were accompanied by decreased expression of HER2, HER3, ALDH1, CD44, and vimentin in tumor tissues, as

determined by immunohistochemistry. Furthermore, serum biochemical analyses revealed no significant hepatotoxicity or nephrotoxicity, indicating a favorable *in vivo* safety profile. These findings demonstrated that ebastine effectively disrupts key pathways involved in CSC-like traits and HER2 activity, even under trastuzumab-resistant conditions. Its multifaceted inhibitory effects support the repositioning of ebastine as a promising therapeutic strategy for treating refractory HER2-positive breast cancer.

Introduction

The human epidermal growth factor receptor 2 (HER2)-positive subtype accounts for 15-20% of breast cancer cases and is characterized by aggressive nature and poor clinical outcomes (1). Over the past two decades, the implementation of HER2-targeted therapeutics, including monoclonal antibodies, small molecule tyrosine kinase inhibitors and antibody-drug conjugates such as trastuzumab emtansine and trastuzumab deruxtecan, has markedly improved patient outcomes in patients with HER2-positive breast cancer (2). In the metastatic setting, these therapeutic advances are associated with a median overall survival exceeding 50 months, as demonstrated by clinical trials and real-world studies conducted in North America and Europe (3-5). Despite advances in HER2-targeted therapies, approximately 15-24% of patients with HER2-positive breast cancer develop metastatic disease, highlighting a persistent risk of disease progression (6). Therapeutic resistance remains a major limitation, underscoring the need for more effective and durable therapeutic strategies (2,7).

In breast cancer, cancer stem cells (CSCs) constitute a distinct subpopulation characterized by self-renewal capacity, multilineage differentiation potential and resistance to standard chemotherapy. These cells are commonly defined by specific phenotypic markers, most notably a CD44^{high}/CD24^{low} surface profile and elevated aldehyde dehydrogenase 1 (ALDH1) activity, both of which are associated with tumor initiation,

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progression, metastasis and poor clinical outcomes (8-10). HER2-positive breast cancer exhibits pronounced tumor heterogeneity and biological complexity, typically harboring increased CSC-like populations, which actively contribute to resistance mechanisms (11,12). In these resistant tumors, CSCs exhibit increased activation of survival pathways, including PI3K/AKT and Notch signaling, and CSC characteristics are further promoted by HER2/HER3-mediated signaling, creating a self-reinforcing cycle of resistance (13). Consequently, simultaneously targeting HER2/HER3 signaling and CSC pathways may offer an effective strategy to overcome trastuzumab resistance.

Recently, drug repurposing, which involves identifying novel anticancer indications for existing non-oncological agents, has emerged as a rational and cost-effective strategy in therapeutic development (14). Disulfiram, an ALDH inhibitor that targets CSC-like properties, has also been shown to inhibit HER2 signaling through proteasome inhibition and copper-dependent oxidative stress (15,16), and is being evaluated in a Phase II trial with copper in metastatic breast cancer (trial no. NCT03323346). Metformin, a widely used antidiabetic agent, improves clinical and pathological responses when combined with neoadjuvant doxorubicin and cyclophosphamide followed by a taxane, in breast cancer (trial no. NCT04170465) (17) and shows potential survival benefits in the HER2-positive subgroup in the large-scale MA.32 Phase III trial (trial no. NCT01101438) (18). Together, findings suggest that disulfiram and metformin are promising drug repurposing candidates for breast cancer therapy.

Ebastine is a second-generation antihistamine with favorable pharmacokinetic and safety profiles, including high oral bioavailability, minimal central nervous system (CNS) penetration and low systemic toxicity (19,20). Although primarily used for allergic conditions, recent *in vitro* and *in vivo* preclinical studies have shown its antitumor potential in various types of cancer through diverse mechanisms such as EZH2 (enhancer of zeste homolog 2) inhibition, autophagy induction and suppression of angiogenesis (21-23). Our previous study demonstrated that ebastine suppresses metastatic progression in triple-negative breast cancer by targeting focal adhesion kinase, leading to inhibition of STAT3/ERK signaling and decreased CSC-like properties (22). These findings highlight the potential of ebastine as a multi-targeted anticancer agent although its mechanism of action in the context of HER2-positive breast cancer remains to be fully elucidated. The present study investigated the potential of ebastine to overcome trastuzumab resistance by targeting HER2/HER3 heterodimerization and CSC-like properties *in vitro* and evaluated its antitumor efficacy *in vivo* using a trastuzumab-resistant xenograft model.

Materials and methods

Reagents and antibodies. Ebastine was purchased from Selleck Chemicals. Propidium iodide (PI), Triton X-100 and DMSO were obtained from Sigma-Aldrich (Merck KGaA). Phosphatase and protease inhibitor cocktail tablets were obtained from Roche Applied Sciences. RNase A was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Primary antibodies were as follows: Ki-67 (cat. no. ab16667), CD31 (cat. no. ab28364),

ALDH1A1 (Abcam; cat. no. ab52492), Bcl-2 (Abcam; cat. no. ab692) and CD44 (all Abcam; cat. no. ab254530); HER2 (Cell Signaling Technology, Inc.; cat. no. 2165), HER3 (Cell Signaling Technology, Inc.; cat. no. 12708), phosphorylated (p-)HER2 (Y1221/1222; Cell Signaling Technology, Inc.; cat. no. 2243), p-HER3 (Y1289; Cell Signaling Technology, Inc.; cat. no. 2842), Akt (Cell Signaling Technology, Inc.; cat. no. 9272), p-Akt (S473; Cell Signaling Technology, Inc.; cat. no. 4060), PARP (Cell Signaling Technology, Inc.; cat. no. 9542), cleaved PARP (Cell Signaling Technology, Inc.; cat. no. 5625), caspase-3 (Cell Signaling Technology, Inc.; cat. no. 7148), -7 (Cell Signaling Technology, Inc.; cat. no. 12827) and -8 (Cell Signaling Technology, Inc.; cat. no. 4790), cleaved caspase-3 (Cell Signaling Technology, Inc.; cat. no. 9664), -7 (Cell Signaling Technology, Inc.; cat. no. 8438) and -8 (Cell Signaling Technology, Inc.; cat. no. 9496), Bax (Cell Signaling Technology, Inc.; cat. no. 2772) and vimentin (Cell Signaling Technology, Inc.; cat. no. 5741); anti-intracellular domain (ICD) HER2 clone 4B5 (Ventana Medical Systems; cat. no. 790-4493) and GAPDH (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. MA5-15738). Secondary antibodies included HRP-conjugated anti-mouse (Bio-Rad Laboratories, Inc.; cat. no. 1721011) and anti-rabbit IgG (Bio-Rad Laboratories, Inc.; cat. no. 1706515), as well as Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A-11037), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A-11008), Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A-11032) and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A-11001).

Breast cancer cell lines. The human breast cancer cell lines SKBR3, BT474, MDA-MB-453 (American Type Culture Collection) and JIMT-1 (Leibnitz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) were cultured in DMEM, MEM or RPMI-1640 (all Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO₂. All cell lines were passaged for <6 months and were authenticated by short tandem repeat profiling performed by MacroGen, Inc. The study design is summarized in Fig. S1.

Cell viability assay. Cell viability was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer's instructions (Promega Corporation). The quantity of formazan product was determined by measuring absorbance at 490 nm using a SpectraMax® 190 microplate reader (Molecular Devices LLC).

Sub-G1 analysis and Annexin V/PI assay. For cell cycle analysis, JIMT-1, MDA-MB-453, SKBR3 and BT474 cells were fixed with pre-chilled 95% ethanol containing 0.5% Tween-20 at 4°C for 24 h, then incubated with PI and RNase A (both 50 µg/ml) at room temperature for 30 min. Apoptotic cell death was determined as the sum of early apoptotic (Annexin V⁺/PI⁻) and late apoptotic (Annexin V⁺/PI⁺) populations, using the FITC Annexin V Apoptosis Detection kit (BD Biosciences),

according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry using a BD LSRFortessa™ X-20 Cell Analyzer (BD Biosciences), and BD FACSDiva™ software (version 8.0.1; BD Biosciences).

Aldefluor-positivity assay and CD44^{high}/CD24^{low} staining. An Aldefluor™ assay kit (Stemcell Technologies, Inc.) was used to assess ALDH1 activity, according to the manufacturer's instructions. BT474 and SKBR3 cells were incubated for 45 min at 37°C in Aldefluor assay buffer containing the ALDH substrate BODIPY-aminoacetaldehyde (1 μM/0.5x10⁶ cells). The ALDH1-specific inhibitor diethylamino-benzaldehyde (50 mM) was used to define the baseline Aldefluor fluorescence. For CD44/CD24 analysis, cells (1x10⁶) were immunostained at 4°C for 30 min with FITC-conjugated anti-CD24 (cat. no. 555427), PE-conjugated anti-CD44 (BD Biosciences; cat. no. 555479), FITC-(cat. no. 553456) or PE-conjugated anti-mouse IgG (all 1:50; all BD Biosciences; cat. no. 555749), followed by flow cytometric analysis using a BD LSRFortessa X-20 flow cytometer (BD Biosciences). Data acquisition and analysis were performed using BD FACSDiva software (version 8.0.1; BD Biosciences).

Immunoblot analysis. JIMT-1, SKBR3 and BT474 cells were lysed in cold lysis buffer [0.5% Triton X-100, 30 mM NaCl, 50 mM Tris-HCl (pH 7.4)] containing protease and phosphatase inhibitor cocktail tablets. The supernatant was collected following centrifugation (14,000 x g at 4°C for 20 min), and protein concentrations were quantified using a Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Inc.). Equal amounts of protein (25 μg/lane) were separated by SDS-PAGE using 8-15% gradient gels and transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk for 30 min at room temperature and incubated overnight at 4°C with primary antibodies diluted in 5% BSA (Sigma-Aldrich; Merck KGaA; cat. no. A7906-100G) as follows: HER2 (1:2,000), p-HER2 (1:1,000), HER3, p-HER3, Akt, p-Akt, PARP, cleaved PARP, caspase-3, -7 and -8, cleaved caspase-3, -7 and -8, Bcl-2, Bax (all 1:2,000) or GAPDH (1:3,000). Following washing with 1X PBST, membranes were incubated with HRP-conjugated anti-mouse (1:3,000-1:10,000; cat. no. 1721011) or anti-rabbit secondary antibodies (1:3,000-1:5,000; both Bio-Rad Laboratories, cat. no. 1706515) for 1 h at room temperature. Signal intensity was detected using a Chemiluminescence kit (Thermo Fisher Scientific, Inc.) on X-ray film (AGFA HealthCare) and quantified using AlphaEaseFC software (version 4.0.0; Alpha Innotech).

Immunoprecipitation assay. A Dynabeads™ Protein G Immunoprecipitation kit (Thermo Fisher Scientific Inc.) was used to evaluate protein-protein interactions according to the manufacturer's instructions. Cells were lysed in Pierce® IP lysis buffer (Thermo Fisher Scientific, Inc.) supplemented with phosphatase and protease inhibitor cocktails. The supernatant was collected by centrifugation (14,000 x g at 4°C for 20 min) and equal amounts (1,000 μg) were incubated with 10 μg anti-HER2 antibody conjugated to Dynabeads Protein G at 4°C overnight. The protein complexes were eluted by boiling the beads in a mixture of SDS-PAGE sample buffer and elution buffer (1:1), followed by SDS-PAGE and immunoblotting as described above.

Cellular thermal shift assay (CETSA). CETSA was performed to evaluate the direct engagement of ebastine with endogenous HER2. 293T (American Type Culture Collection) cells were cultured overnight at 37°C in a humidified atmosphere with 5% CO₂, and treated with either DMSO (vehicle) or 30 μM ebastine for 1 h at 37°C. Cells were harvested, resuspended in PBS containing protease and phosphatase inhibitor cocktails (Roche Applied Science) and aliquoted into PCR tubes. Samples were heated at 45-63°C for 3 min using a thermal cycler, followed by cooling at room temperature for 3 min. The heated cells were lysed by three freeze-thaw cycles consisting of freezing in liquid nitrogen (-196 °C) for 3 min followed by thawing at room temperature for 1 min, and soluble fractions were obtained by centrifugation (14,000 x g at 4°C for 20 min). Supernatants were collected and analyzed by immunoblotting using anti-HER2 antibody, as aforementioned.

Mammosphere formation assay. BT474 (5x10⁴/ml) and JIMT-1 cells (1.5x10⁴/ml) were plated in ultra-low attachment dishes and cultured under serum-free suspension conditions in HuMEC basal serum-free medium (Gibco; Thermo Fisher Scientific, Inc.) for 5 days (BT474) or 8 days (JIMT-1) supplemented with B27 (1:50, Invitrogen; Thermo Fisher Scientific, Inc.), 20 ng/ml human EGF and basic fibroblast growth factor (both Sigma-Aldrich; Merck KGaA), 1% antibiotic-antimycotic (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 15240-062), 4 μg/ml heparin and 15 μg/ml gentamycin at 37°C in a 5% CO₂ atmosphere. The number and volume of mammospheres were assessed using an inverted light microscope, CKX53 (Olympus Corporation), and calculated using the formula: Volume=(4/3) x π x r³, where r is the radius.

Animals and in vivo xenograft experiment. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (approval no. KOREA-2021-0070-C1) of Korea University College of Medicine, Seoul, Republic of Korea. Female BALB/c nude mice (age, 5 weeks; n=10; initial body weight, 16-17 g) were purchased from NARA Biotech, housed under standard specific pathogen-free conditions (temperature, 22±2°C; humidity, 50±10%; 12-h light/dark cycle) and acclimated for 1 week with free access to food and water. JIMT-1 cells (3.5x10⁶) were inoculated into the fourth mammary fat pads of the mice. When the mean tumor volume reached 100 mm³, mice were randomized into two groups (n=5/group) and administered either a solvent control (DMSO/corn oil, 1:9) or ebastine (20 mg/kg body weight/day) via intraperitoneal injection every other day for 46 days. Tumor volume and body weight were measured twice/week. Tumor volume was calculated using the formula: Volume=(length x width²)/2. At study termination, the largest tumor measured 14.42 mm in diameter with a volume of 1,873.55 mm³, which was within the tumor-size limits permitted by the approved IACUC protocol. Mice were euthanized using a gradual-fill CO₂ method, starting at an initial concentration of 20% CO₂ and gradually increasing to 70% CO₂, followed by cervical dislocation to ensure complete euthanasia, in accordance with the IACUC-approved protocol. Death was confirmed by the absence of respiration and heartbeat.

Molecular modeling and docking analysis. Molecular docking studies were performed using publicly available platforms for protein-ligand virtual screening, including GalaxySagittarius (galaxy.seoklab.org/), DockThor (dockthor.lncc.br/) and CB-Dock2 (cadd.labshare.cn/cb-dock2/). Following completion of the docking simulations, 2D and 3D visualization of protein-ligand interactions, along with predicted binding affinities and binding energies, was performed using UCSF Chimera 1.16 (cgl.ucsf.edu/chimera/) and BIOVIA Discovery Studio 2021 (discover.3ds.com/discovery-studio-visualizer-download/).

Serum biochemistry for liver and renal injury biomarkers. At the time of sacrifice, blood samples (0.5–1.0 ml) were collected from each animal, and serum was obtained by centrifugation at 1,100 × g for 20 min at 4°C. Serum biochemical markers of liver and renal function, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBL), blood urea nitrogen (BUN) and creatinine, were evaluated using a serum biochemistry profiling service by DKKorea Inc.

Immunohistochemistry with apoptosis in-situ localization (TUNEL). Tumors were fixed in 4% paraformaldehyde at room temperature for 24 h and embedded in paraffin. Tissue sections (5 µm thickness) were mounted on positively charged glass slides, deparaffinized with xylene and rehydrated through a graded ethanol series to water. For antigen retrieval, sections were boiled in citric acid buffer (pH 6.0) at ~95–100°C for 20 min. The sections were incubated overnight at 4°C with primary antibodies diluted in antibody diluent [Ki-67, CD31, HER2, 4B5, HER3, ALDH1A1, CD44 (all 1:100) and vimentin (1:300)], followed by incubation with Alexa Fluor® 488- or 594-conjugated secondary antibodies at room temperature for 2 h. Slides were mounted with ProLong™ Gold Antifade Reagent with DAPI at room temperature for 1 h. *In situ* TUNEL assays were performed on tissue sections using a TUNEL kit (Roche Applied Sciences; cat. no. 11684795910), according to the manufacturer's instructions. Briefly, sections were incubated with the TUNEL reaction mixture at 37°C for 1 h, and fluorescence signals were evaluated in at least five randomly selected fields of view per section. All images were acquired using a confocal microscope, and fluorescence intensity was analyzed using the histogram tool in ZEN Blue software (version 3.2; Carl Zeiss Microscopy GmbH).

Hematoxylin and eosin (H&E) staining. Organs (kidney, liver and lung) were excised, fixed in 10% neutral-buffered formalin at room temperature for 24–48 h, embedded in paraffin, and sectioned at 4 µm thickness. Sections were deparaffinized in xylene, rehydrated through graded ethanol to water, and stained with hematoxylin for 3 min followed by eosin for 1 min at room temperature, followed by dehydration, clearing and mounting. H&E-stained sections were examined under a light microscope to assess histopathological abnormality. Whole-slide images were acquired using a slide scanner (Axio Scan, Z1; Carl Zeiss Microscopy GmbH), and digital images were used for histopathological evaluation and quantitative analysis.

Public datasets and bioinformatics analysis. For survival analyses, mRNA expression data and clinical information of patients with breast cancer were obtained from the UCSC

Xena TCGA-BRCA cohort (tcga.xenahubs.net; dataset IDs: TCGA.BRCA.sampleMap/HiSeqV2 and TCGA.BRCA.sampleMap/BRCA_clinicalMatrix) (24) and the GENT2 database (gent2.appex.kr/gent2/; GEO-derived; GPL570 and GPL96) (25). Patients were stratified into high- and low-expression groups based on the median expression of each gene (TCGA: CD44=13.023, ALDH1A1=8.5464, ERBB2=12.707; GENT2: CD44=9.894818, ALDH1A1=7.870365, ERBB2=9.210671). Survival regression curves were generated using GraphPad Prism 9.0 software (Dotmatics) and overall survival was analyzed for up to 200 months using the log-rank test.

Statistical analysis. All data were analyzed using GraphPad Prism 9.0 statistical software (Dotmatics). All data are presented as the mean ± SD from at least three independent experiments. Statistical comparisons were performed using unpaired Student's t-test or one- or two-way ANOVA. For multiple group comparisons, the Bonferroni post hoc test was applied. Spearman's rank correlation coefficient was calculated to determine the association between ALDH1A1 and CD44 expression in patients with HER2-positive breast cancer. P<0.05 was considered to indicate a statistically significant difference.

Results

Ebastine induces apoptosis in trastuzumab-resistant HER2-positive breast cancer cells. Ebastine is a piperidine derivative containing a diphenylmethoxy group, connected via a butan-1-one chain to a 4-tert-butylphenyl group. This structural configuration enhances membrane permeability and metabolic stability (Fig. 1A) (19,26). To evaluate its therapeutic potential in overcoming trastuzumab resistance, the present study examined whether ebastine inhibited the viability of HER2-positive breast cancer cells with established trastuzumab resistance. The JIMT-1 cell line, derived from a patient with intrinsic resistance to trastuzumab, and the MDA-MB-453 cell line, established from the pleural effusion of a patient with metastatic breast cancer and known to be insensitive to trastuzumab, were used as representative models (27–29). Compared with trastuzumab-sensitive BT474 and SKBR3 cells, these resistant lines showed notably lower basal HER2/HER3 expression and phosphorylation, consistent with their decreased responsiveness to trastuzumab (Fig. S2).

Ebastine (0–100 µM, 24–72 h) decreased viability in JIMT-1 and MDA-MB-453 cells in a time- and dose-dependent manner (Fig. 1B). To determine whether this inhibition was associated with apoptosis, the present study performed DNA content analysis to quantify the sub-G1 population. A significant accumulation of HER2-positive breast cancer cells in the sub-G1 phase was occurred following ebastine treatment (10 µM, 48 h, Fig. 1C). Ebastine treatment led to an increase in early and late apoptotic JIMT-1 and MDA-MB-453 cells (Fig. 1D). Consistent with these findings, ebastine-treated JIMT-1 cells exhibited characteristic morphological features of apoptosis, including nuclear condensation, cytoplasmic shrinkage and detachment from the culture substrate (Fig. 1E). Mechanistically, ebastine-induced apoptosis involved caspase activation, evidenced by the cleavage of caspase-8 and

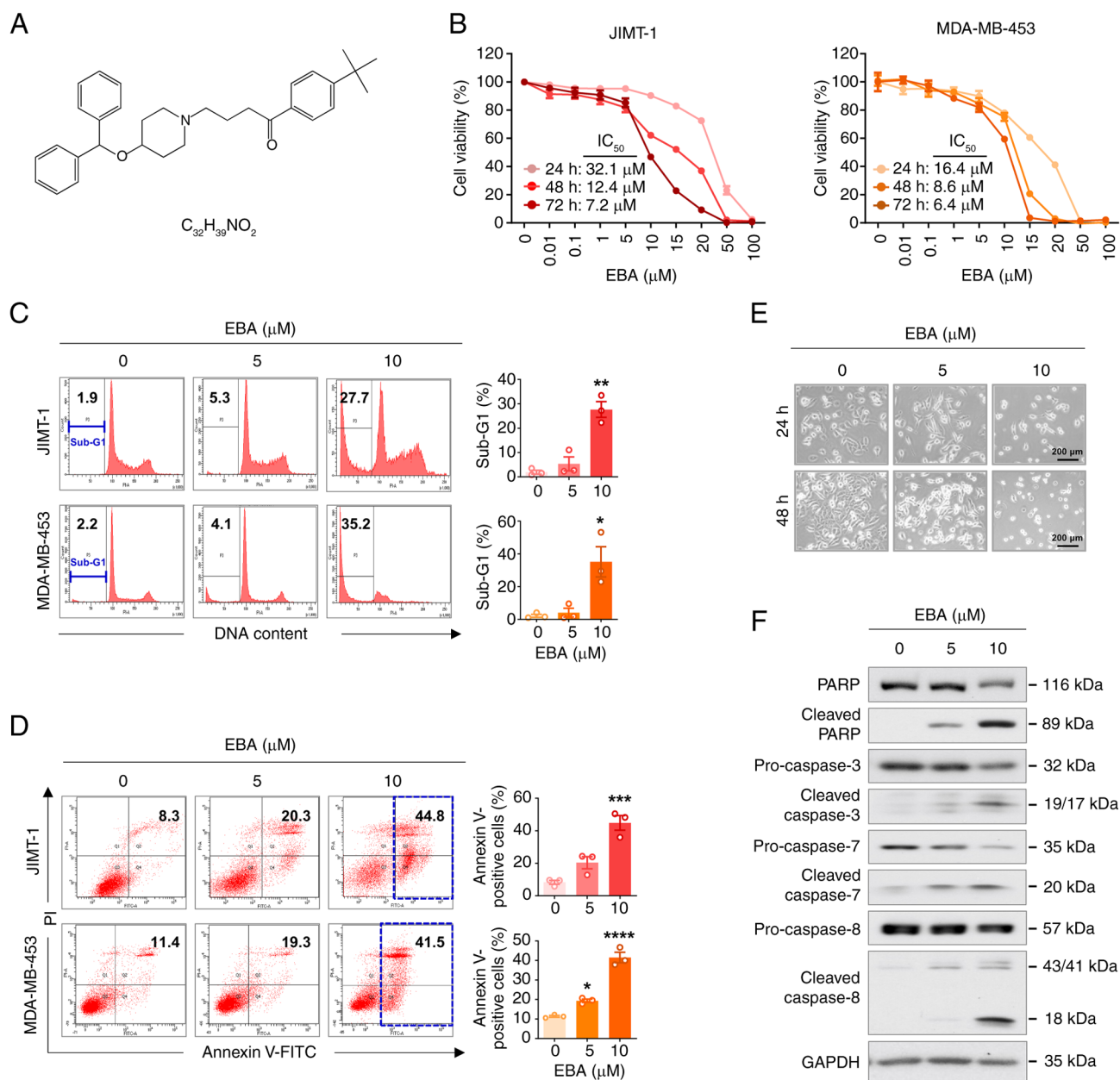


Figure 1. EBA induces apoptosis in trastuzumab-resistant breast cancer cells. (A) Chemical structure of EBA. (B) Cell viability and IC₅₀ values were determined using the MTS assay. JIMT-1 and MDA-MB-453 cells were treated with EBA (48 h) and analyzed by flow cytometry to assess (C) sub-G1 phase populations and (D) early and late apoptotic cells. (E) Morphological changes in JIMT-1 cells following EBA treatment, observed by phase-contrast microscopy. Magnification, x200. (F) Effect of EBA on the expression of PARP, cleaved PARP, caspase-3, -7 and -8 and cleaved caspase-3, -7 and -8 in JIMT-1 cells. GAPDH was used as a loading control. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. vehicle-treated control (0 μM EBA). EBA, ebastine.

activation of executioner caspases-3, -7, and -8 (Fig. 1F). Notable PARP cleavage indicated that cell death occurred through a caspase-dependent pathway.

The present study evaluated the effect of ebastine on trastuzumab-sensitive HER2-positive breast cancer BT474 and SKBR3 cells. Ebastine (0-100 μM , up to 72 h) decreased cell viability in a dose- and time-dependent manner (Fig. S3A and B). The apoptotic features included notable morphological changes (Fig. S3C), a marked increase in the sub-G1 population (Fig. S3D), and an elevated proportion of Annexin V-positive cells (Fig. S3E) following ebastine treatment (5-10 μM , 48 h). These effects coincided with activation of caspases-3 and -7, increased PARP cleavage (Fig. S3F and G)

and increased p18Bax levels without altering Bcl-2 expression (Fig. S3H and I).

Ebastine downregulates HER2/HER3 and truncated-p95HER2. Trastuzumab resistance is primarily driven by HER2, HER3 and the truncated isoform p95HER2, which maintain pro-survival PI3K/AKT signaling through persistent HER2/HER3 heterodimerization (30). Ebastine (5-10 μM , 48 h) notably decreased total and p-HER2 (Y1221/1222), as well as HER3 and p-HER3 (Y1289) in JIMT-1 (Fig. 2A and B) cells. p95HER2, which lacks the extracellular domain required for trastuzumab binding, retains HER2 kinase activity and contributes to therapeutic resistance (31,32). Ebastine also

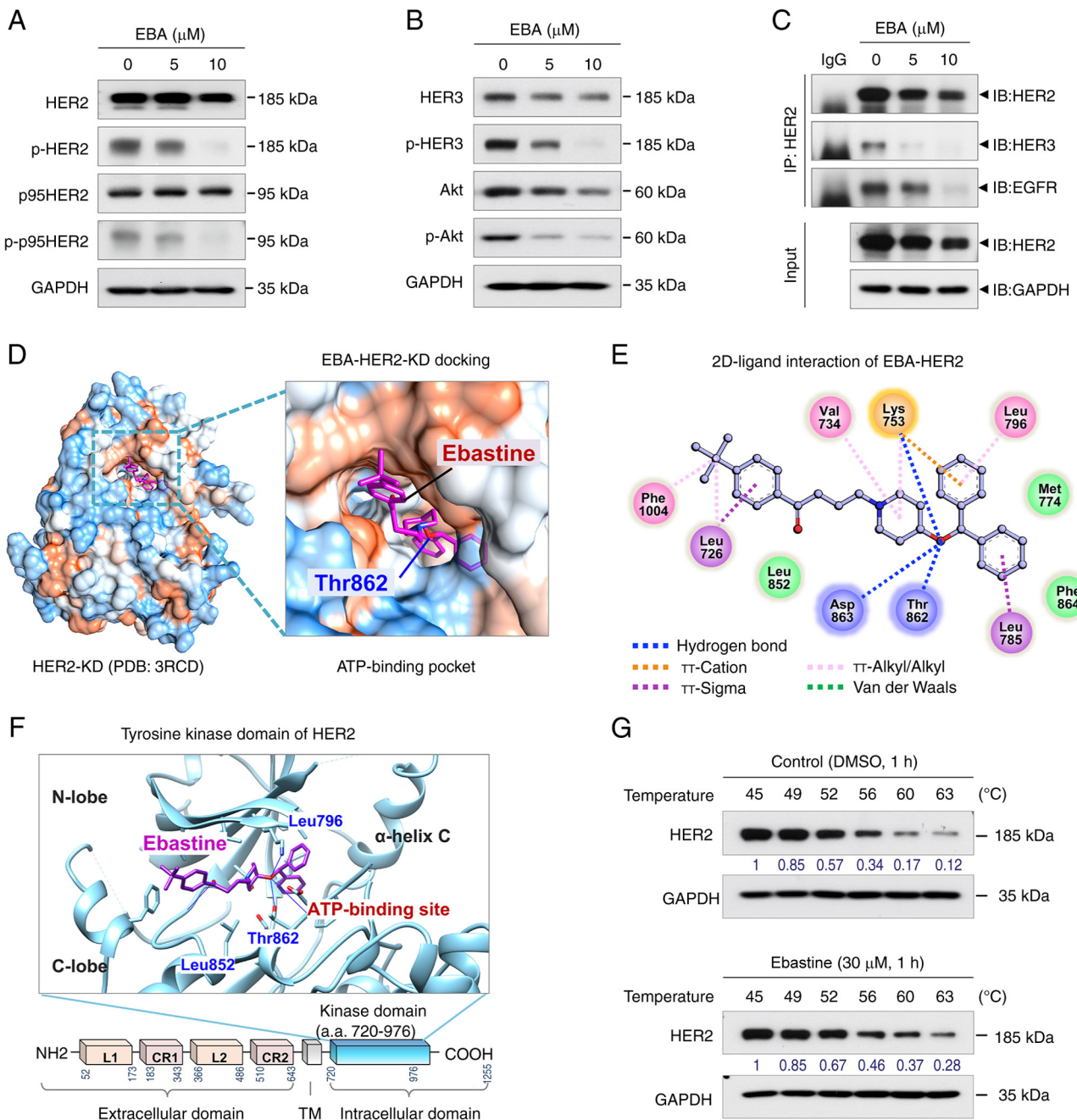


Figure 2. EBA downregulates HER2, p95HER2, HER3 and AKT expression. (A) Immunoblot analysis of HER2, p95HER2 and p-HER2 (Y1221/1222) in JIMT-1 cells treated with EBA for 48 h. (B) Immunoblot analysis of HER3, p-HER3 (Y1289), AKT and p-AKT following treatment with EBA (48 h) in JIMT-1 cells. (C) Immunoblot analysis of HER2, HER3 and EGFR following IP with anti-HER2 antibody in JIMT-1 cells treated with EBA. *In silico* molecular docking of EBA with the crystal structure of HER2-KD. (D) Surface map of lipophilic and hydrophilic properties at the ATP-binding site of HER2-KD (red, hydrophobic; blue, hydrophilic). (E) 2D interaction diagram showing intermolecular interactions between EBA and HER2-KD. Key amino acid residues within the binding pocket are shown. (F) Predicted binding pose of EBA (purple stick model) within the tyrosine kinase domain of HER2 (blue ribbon). (G) 293T cells were treated with DMSO or EBA for 1 h at 37°C, followed by heating for 3 min. Soluble fractions were collected following centrifugation and analyzed by immunoblotting using an anti-HER2 antibody. EBA, ebastine; p-, phosphorylated; IP, immunoprecipitation; IB, immunoblotting; KD, kinase domain PCB, protein complex binding; TM, transmembrane; a.a., amino acid.

downregulated cleaved p95HER2 and suppressed the expression of AKT and p-AKT (Fig. 2A and B). Since AKT functions downstream of the HER2/HER3 signaling pathway, the present study examined its phosphorylation status as an indicator of downstream signaling (33,34). Immunoprecipitation analysis using anti-HER2 revealed that ebastine substantially impaired the heterodimerization of HER2 with both HER3 and EGFR (Fig. 2C). Similarly, in trastuzumab-sensitive BT474 and SKBR3

cells, ebastine decreased the phosphorylation of HER2, HER3, and p95HER2, leading to decreased AKT activation (Fig. S4). The present study performed molecular docking simulation to determine whether this was due to the binding of ebastine to HER2 (Fig. 2D-F). Docking studies using the crystal structure of the HER2 kinase domain retrieved from the Protein Data Bank revealed that ebastine fit into the ATP-binding pocket near the hinge region between the N- and C-lobe of the

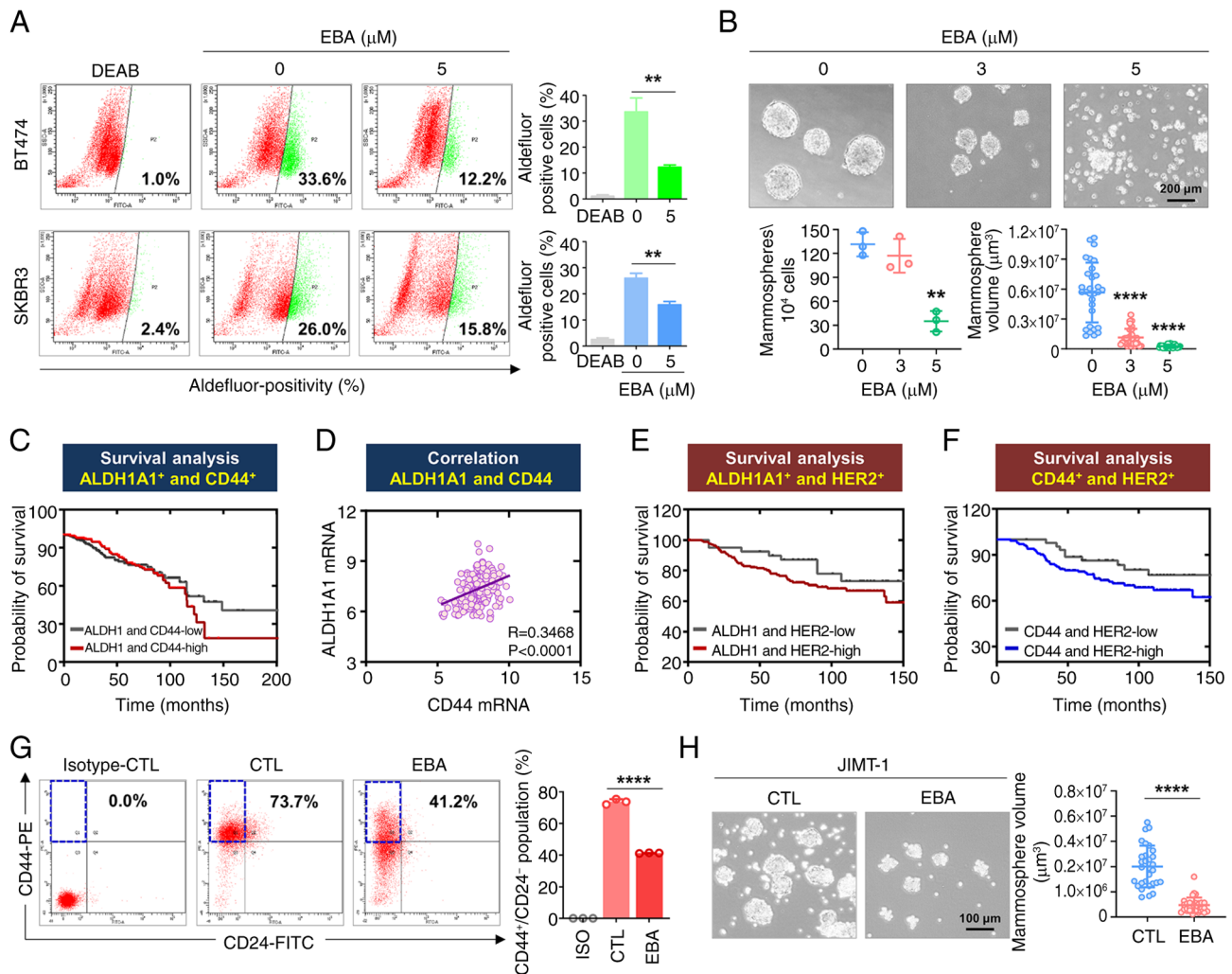


Figure 3. EBA impairs cancer stem cell-like properties. (A) BT474 and SKBR3 cells were treated with EBA for 48 h, and ALDH1 activity was assessed by flow cytometry using the Aldefluor assay. DEAB was used to define the baseline of Aldefluor-positive fluorescence. (B) BT474 cells (5×10^4 cells/ml) were plated in ultra-low attachment dishes and cultured in the presence or absence of EBA for 5 days. The number and volume of mammospheres were measured by microscopy. (C) Overall survival of patients with breast cancer stratified by the co-expression of ALDH1A1 and CD44. (D) Spearman correlation analysis of ALDH1A1 and CD44 mRNA levels in patients with HER2-positive breast cancer from The Cancer Genome Atlas cohort ($n=76$). Kaplan-Meier survival analyses of patients with HER2-overexpressing breast cancer stratified by (E) ALDH1A1 and (F) CD44 expression. Patients were divided into high- and low-expression groups based on the median gene expression. Statistical significance was determined using the log-rank test. (G) JIMT-1 cells were treated with EBA (3μ M) for 48 h and the CD44^{high}/CD24^{low} cell populations were identified by flow cytometry. (H) JIMT-1 cells (1.5×10^4 cells/ml) were cultured under serum-free suspension conditions in the presence of EBA (3μ M) for 8 days. Mammosphere number and volumes were quantified. ** $P < 0.01$ and **** $P < 0.0001$ vs. vehicle-treated control (0μ M EBA). EBA, ebastine; ALDH, aldehyde dehydrogenase; DEAB, diethylaminobenzaldehyde; CTL, control; ISO, isotype.

tyrosine kinase domain (Fig. 2D and F). This interaction was stabilized by three hydrogen bonds, several π -interactions and hydrophobic contacts. Ebastine formed hydrogen bonds with key residues within the ATP-binding site of HER2-KD (HER2 kinase domain), including Thr862, Asp863 and Lys753 and a π -cation interaction occurred between the active site residue Lys753 and a phenyl ring of ebastine (Fig. 2E). To validate this predicted interaction, CETSA was performed using 293T cells. Ebastine (30μ M, 1 h) markedly enhanced the thermal stability of endogenous HER2 compared with DMSO-treated controls. In DMSO-treated samples, the soluble fraction of HER2 was largely lost at temperatures $>60^\circ\text{C}$, whereas ebastine-treated cells demonstrated detectable HER2 up to $\sim 63^\circ\text{C}$ (Fig. 2G). These results indicated that ebastine stabilized HER2 in cells, supporting the hypothesis of a physical interaction between the proteins. Collectively, molecular docking and CETSA data suggested that ebastine may interact with the HER2 kinase

domain and was associated with decreased HER2 phosphorylation, which may overcome trastuzumab resistance in HER2-positive breast cancer.

Ebastine targets breast CSC (BCSC)-like properties in HER2-positive breast cancer. BCSCs are defined by their self-renewal and tumor-initiating capacity and characterized by distinct molecular markers and functional attributes (35). ALDH1, a detoxifying enzyme for intracellular aldehydes, contributes to maintaining the stem-like state in tumor cells with activated cancer hallmarks (10). HER2-positive breast cancer exhibits the highest ALDH1 activity among all subtypes (15). Ebastine significantly decreased ALDH1 activity in BT474 and SKBR3 cells (Fig. 3A). To evaluate self-renewal, the present study performed 3D mammosphere assay, which functionally assess the tumor-initiating potential of BCSCs (36). Ebastine significantly decreased mammosphere number and

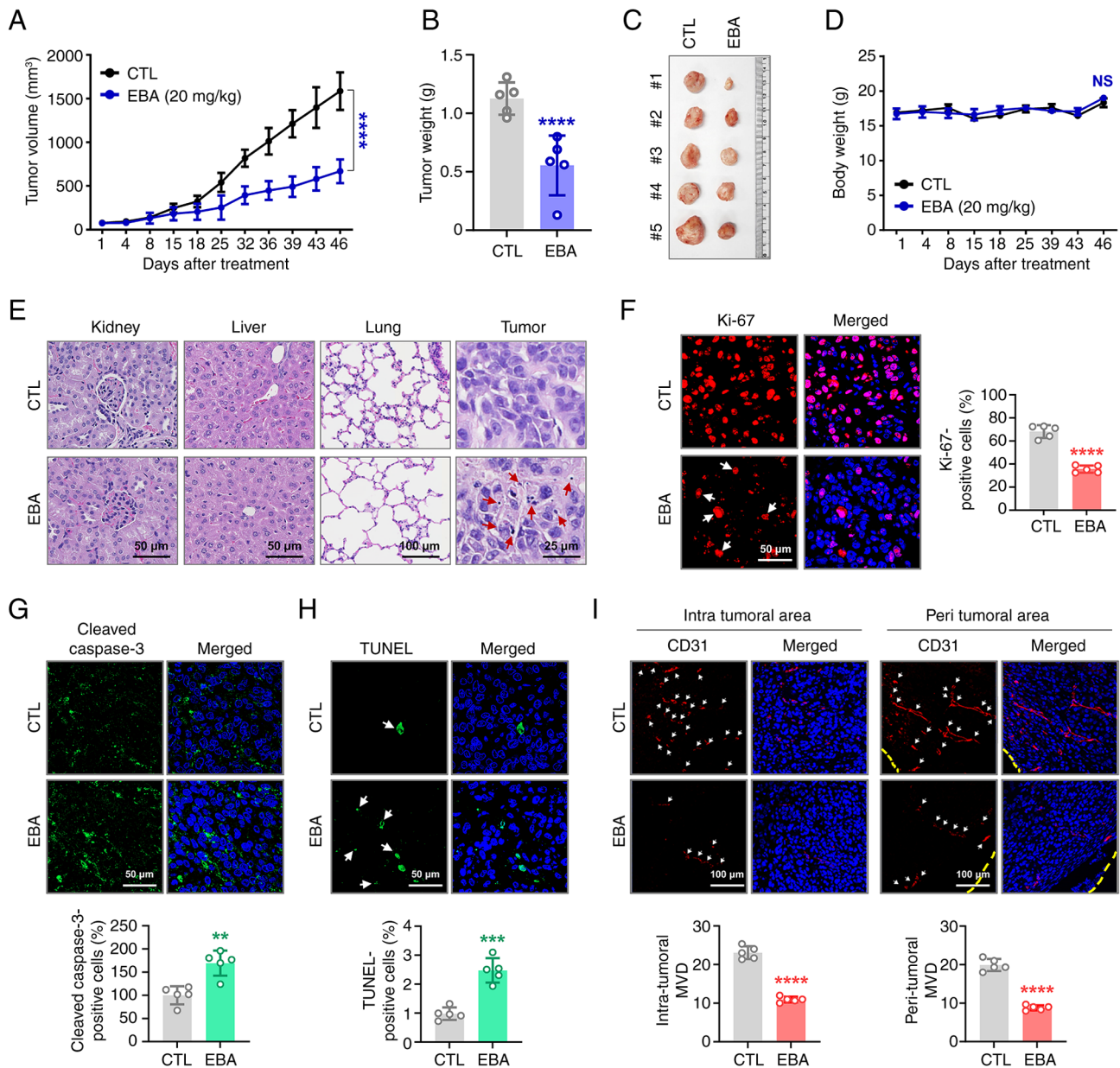


Figure 4. EBA inhibits tumor growth in trastuzumab-resistant JIMT-1 xenografts. JIMT-1 cells (3.5×10^6) were injected into the fourth mammary fat pad of BALB/c nude mice. Mice were administered EBA intraperitoneally (20 mg/kg body weight) every other day for 46 days. Tumor (A) volume and (B) weight were measured. ****P<0.0001 vs. CTL (vehicle-treated group). (C) Representative xenograft tumors. (D) No significant alteration in body weight between the EBA-treated and CTL groups was observed (n=5). (E) Representative hematoxylin and eosin staining of kidney, liver, lung and tumor tissue. Red arrows indicate the apoptotic cells with cell shrinkage and nuclear condensation. (F) Tumor sections were immunostained for Ki-67 (red) and DAPI (blue). Ki-67-positive cells were quantified. Apoptosis was assessed by (G) immunostaining for cleaved caspase-3 and (H) TUNEL assay. Arrows indicate TUNEL-positive nuclei. (I) Tumor angiogenesis was assessed by immunostaining with the endothelial cell marker CD31 (red) and DAPI (blue). CD31-positive microvessels in intra- and peritumoral regions were quantified. CD31-positive staining within the tumor is indicated by white arrows. The tumor-stroma interface is indicated by a yellow dashed outline. **P<0.01, ***P<0.001, ****P<0.0001 vs. CTL. EBA, ebastine; CTL, control; NS, not significant; MVD, microvessel density.

volume in BT474 cells (P<0.01, Fig. 3B), indicating impaired self-renewal.

The CD44^{high}/CD24^{low} phenotype marks a CSC-like population in breast cancer and is associated with increased invasiveness, a key feature of early metastasis (10,37). Tumorigenesis can be initiated by a limited number of cells, requiring ~500 Aldefluor-positive cells or 20 ALDH⁺/CD44⁺/CD24⁻ cells (10). Kaplan-Meier analysis showed that patients with breast cancer with high expression of ALDH1A1 and CD44 had a shorter overall survival (Fig. 3C). Gene expression analysis of GENT2 dataset showed a

significant positive correlation between CD44 and ALDH1A1 in patients with HER2-high breast cancer (Fig. 3D). High expression of either ALDH1A1 (Fig. 3E) or CD44 (Fig. 3F) was associated with poor prognosis in this HER2-overexpressing patient group. These findings indicated that CSC markers were clinically associated with poor outcomes in patients with HER2 overexpression, supporting the mechanistic relevance of ebastine CSC-targeting effects observed *in vitro*. CD44-associated gene expression is positively associated with trastuzumab response, suggesting CD44 as a potential biomarker (38). Ebastine treatment significantly decreased the

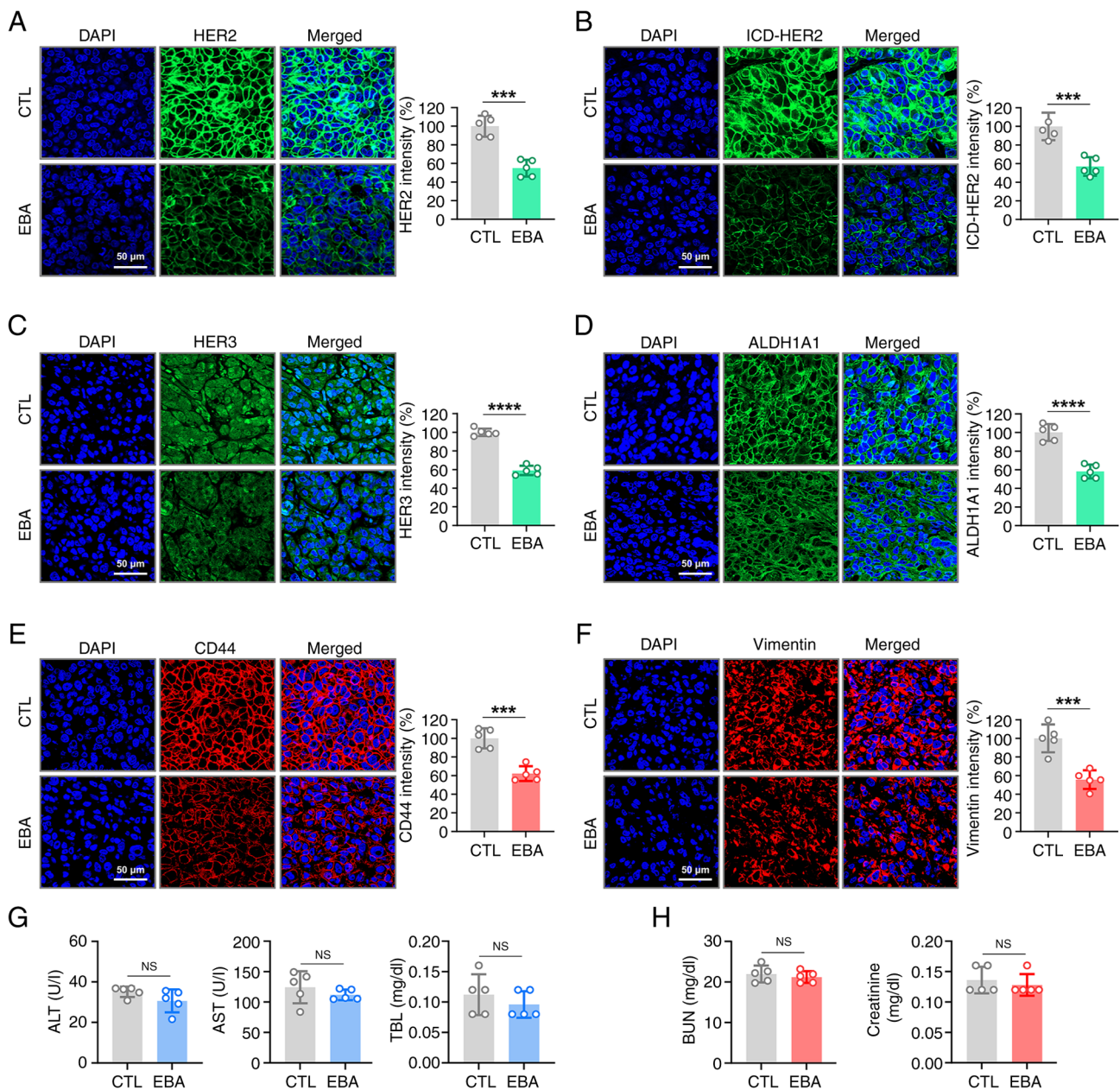


Figure 5. EBA downregulates HER2, ICD-HER2, HER3, ALDH1A1, CD44 and vimentin in JIMT-1 xenograft tumors. Immunofluorescence staining of JIMT-1 xenograft tumor tissue for (A) full-length HER2 (green), (B) ICD-HER2 (green) and (C) HER3. Immunohistochemical analysis of (D) ALDH1A1 (green) and (E) CD44 (red) in tumor tissue. (F) Tumor sections were immunostained for vimentin (red). Magnification, x500. Fluorescence intensities were quantified. Serum biochemical analysis for (G) liver and (H) kidney function in EBA-treated or CTL mice (n=5). Serum levels of ALT, AST, TBL, BUN and creatinine were assessed. ***P<0.001, ****P<0.0001. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; EBA, ebastine; ICD, intracellular domain; ALDH, aldehyde dehydrogenase; CTL, control; NS, not significant.

CD44^{high}/CD24^{low} stem-like population (Fig. 3G) and impaired mammosphere formation in trastuzumab-resistant JIMT-1 cells (Fig. 3H), demonstrating that ebastine effectively targeted CSC-like properties in trastuzumab-resistant contexts.

Ebastine exerts anti-tumor activity in trastuzumab-resistant HER2-positive breast cancer *in vivo*. To assess the physiological relevance of the *in vitro* findings, the present study established a trastuzumab-resistant JIMT-1 xenograft model to evaluate the antitumor effect of ebastine *in vivo* (Fig. 4A). When tumor volumes reached ~50 mm³, mice were administered ebastine (20 mg/kg, every other day) or vehicle control. At day 46, ebastine led to a significant suppression of tumor volume (Fig. 4A)

and tumor weight (Fig. 4B and C), without affecting body weight (Fig. 4D). Histopathological examination using hematoxylin and eosin (H&E) staining revealed no observable abnormality in the kidney, liver or lung tissue of ebastine-treated mice compared with controls. Tumor tissue from the ebastine-treated group exhibited a notable number of cells with nuclear condensation and fragmentation, indicative of treatment-induced apoptosis (Fig. 4E). Immunohistochemical analysis revealed a significant reduction in the Ki-67 proliferation index following ebastine treatment (Fig. 4F), supporting its antiproliferative activity. Apoptosis induction was evidenced by a significant increase in DNA fragmentation, as determined by the *in situ* TUNEL assay (Fig. 4H), accompanied by enhanced caspase-3 cleavage

(Fig. 4G). The antiangiogenic potential of ebastine was assessed by quantifying microvessel density using the endothelial marker CD31. A significant decrease in the number of CD31-positive microvessels was observed in both intratumoral and peritumoral (Fig. 4I) regions following ebastine treatment.

Ebastine downregulates HER2/HER3 expression and CSC-associated markers *in vivo*. To validate the *in vitro* observations, the present study examined the expression of full-length HER2, ICD-HER2 and HER3 in trastuzumab-resistant xenograft tumors using immunohistochemistry. The present study assessed ICD-HER2 expression with 4B5, an FDA-approved antibody that specifically recognizes an epitope within the HER2 intracellular domain. Tumors from ebastine-treated mice exhibited significantly decreased expression of full-length HER2 (Fig. 5A), ICD-HER2 (Fig. 5B) and HER3 (Fig. 5C) relative to control tumors. Consistent with the *in vitro* abrogation of the CSC-like phenotype, ebastine administration significantly decreased the expression of ALDH1A1 (Fig. 5D) and CD44 (Fig. 5E) in tumor tissues. Expression of the mesenchymal marker vimentin was also significantly decreased in the ebastine-treated group (Fig. 5F).

Ebastine exhibits no significant hepatotoxicity or nephrotoxicity *in vivo*. To evaluate the potential hepatotoxicity of ebastine, serum samples from treated mice were analyzed for AST, ALT and TBL. No significant alterations were detected in these hepatic toxicity markers (Fig. 5G). BUN and creatinine levels, key indicators of renal function, remained within the normal range (Fig. 5H). These biochemical results indicated that ebastine did not cause hepatic or renal dysfunction, even at doses that produce anticancer efficacy.

Discussion

Repurposing existing drugs for new therapeutic indications has attracted attention in oncology as a cost-effective and time-efficient alternative to traditional drug development (14,39,40). As these agents already possess established safety and pharmacokinetic profiles, they can be more rapidly transitioned into clinical use, offering a practical strategy to address issues such as treatment resistance and tumor heterogeneity (14,40). The present study investigated the anticancer potential of ebastine, an FDA-approved second-generation antihistamine, in the context of trastuzumab-resistant HER2-positive breast cancer. First-generation antihistamines, such as diphenhydramine, often cause CNS side effects due to their ability to cross the blood-brain barrier (41). By contrast, ebastine has minimal CNS penetration, high oral bioavailability and low toxicity, making it more suitable for long-term use (19,42). These advantages highlight its potential as a safe and effective anticancer agent for drug repurposing in oncology.

In ebastine, the diphenylmethoxy-substituted piperidine and 4-tert-butylphenyl group enhance flexibility and hydrophobic interactions at protein binding sites. Docking simulations showed that ebastine fit into the ATP-binding pocket of the HER2 kinase domain. Its aromatic rings form π - π and π -cation interactions, while the piperidine group forms hydrogen bonds with key residues such as Thr862, Asp863 and Lys753. This interaction likely facilitates the stable binding of ebastine

to HER2, notably downregulating p-HER2 and HER3, as well as trastuzumab resistance-associated p95HER2. This results in the disruption of HER2/HER3 and HER2/EGFR heterodimerization, a key mechanism underlying persistent downstream signaling in HER2-positive tumors refractory to standard therapy. Trastuzumab and HER2-targeted tyrosine kinase inhibitors (TKIs; such as lapatinib, neratinib and tucatinib) differ mechanistically, with trastuzumab targeting the extracellular HER2 domain and TKIs inhibiting intracellular kinase activity, however, these agents typically share common mechanisms of therapeutic resistance (43). A notable mechanism involves HER2/HER3 heterodimerization, where HER3, despite lacking intrinsic kinase function, serves as a potent co-activator by recruiting PI3K to its docking sites. This interaction activates downstream PI3K/AKT signaling, enabling tumor cells to bypass HER2-targeted inhibition (2,43). Additionally, genetic alterations such as activating mutations in PIK3CA or loss of PTEN limit the efficacy of these targeted agents (44).

To evaluate the *in vivo* relevance of the present findings, a trastuzumab-resistant xenograft model was established using JIMT-1 cells, which were originally derived from a patient exhibiting intrinsic resistance to trastuzumab (27). Notably, JIMT-1 cells display resistance not only to trastuzumab but also to numerous HER2-targeted TKIs (45), rendering them a representative preclinical model for studying refractory HER2-positive breast cancer. In the xenograft model, ebastine treatment resulted in a significant decrease in the expression of full-length HER2, ICD-HER2 and HER3 within tumor tissues, further substantiating its ability to interfere with key therapeutic resistance pathways.

As a transmembrane receptor for hyaluronic acid, CD44 binds to its polymerized form, contributing to the formation of a physical barrier that obscures HER2 and diminishes the efficacy of HER2-targeted antibody therapy (46). CD44 is notably overexpressed in HER2-positive breast cancer cells exhibiting trastuzumab resistance compared with sensitive cell lines (47). Consistently, the trastuzumab-resistant cell line JIMT-1 exhibited a high proportion (~50%) of CD44^{high}/CD24^{low} cells, compared with the low frequencies observed in sensitive cell lines such as SKBR3 (0.03%) and BT474 (0.36%) in our previous studies (15,47). Suppression of CD44 enhances trastuzumab sensitivity and decreases the invasive behavior and anchorage-independent proliferation of resistant cells (38). Furthermore, CD44 activates Rho GTPases, PI3K/AKT and MAPK/ERK signaling pathways, which collectively promote cell survival, proliferation and invasion, thereby contributing to the aggressive phenotype of tumor cells (48,49). In this context, ebastine treatment significantly decreased the CD44⁺/CD24⁻ subpopulation *in vitro* and decreased CD44 expression in trastuzumab-resistant JIMT-1 xenograft tumors *in vivo*, highlighting its potential to overcome resistance by targeting CD44. In parallel, ebastine also effectively attenuated stem-like phenotypes in HER2-positive breast cancer by decreasing ALDH1 enzymatic activity and impairing the mammosphere-forming capacity. Elevated expression of ALDH1A1 and CD44 is associated with shorter overall survival and poor prognosis in HER2-overexpressing breast cancer, highlighting their potential as biomarkers for trastuzumab resistance. These

findings indicate that ebastine may improve therapeutic outcomes by targeting CD44⁺/ALDH1⁺ stem-like tumor populations.

Vimentin, a key indicator of epithelial-mesenchymal transition, promotes tumor progression in breast cancer by enhancing cell plasticity and motility through cytoskeletal remodeling (50,51). Vimentin is frequently upregulated in aggressive breast cancer subtypes, such as triple-negative breast cancer, and is highly expressed in HER2-positive breast cancer exhibiting resistance to trastuzumab (52,53). Its expression is associated with the acquisition of stem-like characteristics, including the CD44⁺/CD24⁻ phenotype (54,55). In addition to its role in promoting stemness, vimentin enhances endothelial cell motility and structural organization and promotes tumor angiogenesis by regulating VEGF-dependent signaling pathways (56,57). Although HER2 is typically associated with an epithelial phenotype, the trastuzumab-resistant JIMT-1 cell line exhibits mesenchymal features marked by elevated vimentin expression during tumor progression. Here, ebastine administration markedly decreased vimentin levels, which may contribute to suppression of tumor angiogenesis and growth *in vivo*.

Although HER2-targeted TKIs effectively inhibit HER2 signaling, several studies have reported that TKI exposure can enrich CSC-like populations (58-60). For example, lapatinib has been shown to increase mammosphere formation in HER2-overexpressing breast cancer cells, suggesting CSC enrichment (58,61). By contrast, the present data showed that ebastine decreased ALDH activity and the CD44^{high}/CD24^{low} fraction and impaired mammosphere formation, indicating suppression of CSC-like traits. Several drug-repurposing candidates, including disulfiram, metformin and niclosamide, have also been explored for their potential to modulate CSC characteristics by targeting pathways involved in self-renewal, metabolism and therapeutic resistance (14,61,62). Disulfiram exerts its anticancer and CSC-suppressive effects primarily via ALDH inhibition and proteasome-mediated cytotoxic mechanisms (15,16,63). However, disulfiram has limited clinical success because its anticancer activity is highly copper-dependent, resulting in unstable and inconsistent therapeutic efficacy *in vivo* (64,65). By contrast, ebastine offers advantages for drug repurposing due to its metal-independent mechanisms, stable pharmacokinetic profile and predictable formation of the active metabolite carebastine (19,66). Metformin is a promising drug repurposing candidate because it has a long-established safety profile and low cost, while exerting anticancer effects partly via AMPK activation, mitochondrial complex I inhibition and suppression of CSCs (67,68). Although metformin downregulates HER2 expression through inhibition of the mTOR effector p70S6K1 (69), evidence for its direct targeting of HER2 signaling remains limited, whereas ebastine decreases both full-length HER2 and p95HER2 expression and modulates key signaling nodes associated with trastuzumab resistance. Niclosamide suppresses key oncogenic pathways, including Wnt/ β -catenin and STAT3 signaling, demonstrating broad antitumor potential. However, its poor oral bioavailability necessitates the use of salt forms, prodrugs or nano-based delivery systems to achieve adequate systemic exposure (61,70). By

contrast, ebastine shows high oral bioavailability, predictable pharmacokinetics and reliable *in vivo* exposure, making it a more practical and clinically feasible option for drug repurposing (19,26,66).

Ebastine may offer strategic therapeutic advantages by concurrently attenuating the HER2/HER3 signaling pathway, one of the key drivers of trastuzumab resistance, and suppressing CSC-associated phenotypes, thereby exhibiting multifaceted anticancer activity. Taken together, the present findings demonstrate that ebastine inhibited HER2 kinase activation and HER2/HER3 heterodimerization, induced caspase-mediated apoptosis and downregulated CSC-associated markers such as CD44, ALDH1 and vimentin, supporting its potential as a promising drug-repurposing candidate for trastuzumab-resistant HER2-positive breast cancer.

While the present study provided evidence that ebastine inhibited HER2 signaling and overcomes trastuzumab resistance, it has several limitations. Although the molecular docking and CETSA results support a cellular interaction between ebastine and HER2, these findings do not constitute definitive biochemical evidence of direct HER2 inhibition. Additional biochemical and biophysical analyses, such as *in vitro* kinase assay, surface plasmon resonance and isothermal titration calorimetry, are required to confirm direct binding and quantify binding affinity. Furthermore, site-directed mutagenesis of key residues (Lys753, Thr862 and Asp863) will be necessary to validate the predicted binding sites. Future studies should also focus on evaluating the pharmacokinetic and pharmacodynamic profiles of ebastine in tumor-bearing models, exploring its combinatorial efficacy with trastuzumab or HER2-targeted tyrosine kinase inhibitors and conducting early-phase clinical investigations to assess its translational potential. Collectively, these approaches are essential to elucidate the molecular mechanism of the ebastine-HER2 interaction and facilitate its clinical development as a repurposed anticancer agent.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

EJ, YJK, JYK and JHS conceived and designed the study. EJ, DK, JS, SL, DL, SK, KL, YJK and JYK performed the experiments. EJ, DK, JS, MP, SK, SP, YKK, KDN, YJK and JYK analyzed the data. EJ, YJK and JYK wrote the manuscript. JYK and JHS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed in strict accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Korea University College of Medicine, Seoul, Republic of Korea (approval no. KOREA-2021-0070-C1; approved on May 23, 2022). All procedures were conducted in compliance with the Animal Research: Reporting of *In Vivo* Experiments guidelines and relevant national regulations for the care and use of laboratory animals. The study did not involve human participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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