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Acquired resistance to trastuzumab/pertuzumab or to T-DM1 in vivo can be overcome by HER2 kinase inhibition with TAS0728

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Abstract

HER2-targeting antibodies (trastuzumab, pertuzumab) and a HER2-directed antibody-drug conjugate (trastuzumab emtansine: T-DM1) are used for the treatment of HER2-overexpressing breast cancer. However, these treatments eventually become ineffective due to acquired resistance and there is an urgent need for alternative therapies. TAS0728 is a small-molecule, irreversible selective HER2 kinase inhibitor. In the present study, we established new in vivo models of cancer resistance by continuous exposure to a combination of trastuzumab and pertuzumab or to T-DM1 for evaluating the effect of TAS0728 on HER2 antibody-resistant populations. Treatment with trastuzumab and pertuzumab or with T-DM1 initially induced tumor regression in NCI-N87 xenografts. However, tumor regrowth during treatment indicated loss of drug effectiveness. In tumors with acquired resistance to trastuzumab and pertuzumab or to T-DM1, HER2-HER3 phosphorylation was retained. Switching to TAS0728 resulted in a significant anti-tumor effect associated with HER2-HER3 signal inhibition. No alternative receptor tyrosine kinase activation was observed in these resistant tumors. Furthermore, in a patient-derived xenograft model derived from breast cancer refractory to both trastuzumab/pertuzumab and T-DM1, TAS0728 exerted a potent anti-tumor effect. These results suggest that tumors with acquired resistance to trastuzumab and pertuzumab and to T-DM1 are still dependent on oncogenic HER2-HER3 signaling and are vulnerable to HER2 signal inhibition by TAS0728. These results provide a rationale for TAS0728 therapy for breast cancers that are refractory to established anti-HER2 therapies.

KEYWORDS
HER2 kinase inhibition, pertuzumab, TAS0728, T-DM1, trastuzumab

1 | INTRODUCTION

HER2 is an ErbB family kinase, and a well established therapeutic target in breast and gastric cancers.[1,2] In first-line treatment for HER2-positive breast cancer patients, a combination of trastuzumab, pertuzumab, and docetaxel is used.[3] Both trastuzumab and pertuzumab bind to the HER2 receptor on the cancer cells and exert significant anti-tumor effects.[3,4] The mode of action of trastuzumab is considered to be dependent not only on HER2 signal inhibition but also on ADCC, which is elicited by binding of Fcγ receptors (FcγR) in the host immune system. Evidence of this is provided by the drastic reduction of the anti-tumor effects of trastuzumab on BT474 tumors in FcγR-deficient nude mice compared with that in nude mice with intact FcγRs.[5] Pertuzumab binds to HER2 and also exerts its anti-tumor activity via ADCC, in addition to blocking HER2-HER3 dimerization.[4,6,7] Trastuzumab emtansine, which is used as second-line therapy against HER2-positive breast cancer, shows anti-tumor effects that are dependent on inhibition of microtubule assembly, on payloads conjugated to T-DM1, and by ADCC that is mediated through its HER2-antibody component.[8-10] Despite their initial clinical efficacy, most tumors subsequently become refractory to these HER2-targeting therapies, followed by disease progression. Therefore, alternative therapies that are effective against tumors with acquired resistance to HER2-targeting are required.

Lapatinib, a reversible HER2 and EGFR dual kinase inhibitor, was approved for use in combination with capecitabine for second-line treatment of HER2-overexpressing breast cancer.[11,12] However, the clinical benefits of lapatinib as a single agent for tumors that progress after first- and second-line treatments are still unclear.[13]

TAS0728 is a small-molecule, irreversible kinase inhibitor selective for HER2.[14] TAS0728 is orally available and its anti-tumor activity is based on selective HER2 kinase signal inhibition. It is still unclear whether TAS0728 can be an effective therapy for refractory tumors. Considering the different mode of action of TAS0728 compared with the HER2-targeting antibodies and ADCs, we hypothesized that TAS0728 monotherapy may be effective in treating cancers that have developed resistance to established HER2-targeting therapies.

To date, various preclinical cell-based cancer-resistance models have been developed via long-term continuous drug exposure,[15,16] and are used to elucidate the mechanisms of tumor resistance or for discovering new drug and/or combination therapy approaches to treat refractory tumors. Given that HER2-targeting antibodies and ADCs produce an ADCC effect that is dependent on the host immune system, HER2-targeting antibody and ADC resistance models will need to be established in vivo for clinically relevant evaluation of anti-tumor therapies.

In the present study, we developed novel in vivo cancer-resistance models by periodic administration of HER2-targeting antibodies or ADCs for evaluating the anti-tumor activity of TAS0728 against the efficacy of established HER2-targeting therapies.

2 | MATERIALS AND METHODS

2.1 | Compounds and antibodies

TAS0728 was synthesized at the Tsukuba Research Institute, Taiho Pharmaceutical Co., Ltd., by procedures described in our previous study.[14] Trastuzumab, pertuzumab, and T-DM1 were purchased from Chugai Pharmaceutical Co., Ltd. Lapatinib was purchased from LC Laboratories. For in vivo experiments, TAS0728 was dissolved in 0.1 N HCl solution or in 0.1 N HCl with 0.5% hydroxypropyl methylcellulose (HPMC). Trastuzumab, and T-DM1 were dissolved in distilled water and diluted with normal saline purchased from the Otsuka Pharmaceutical Factory, Inc. Pertuzumab was diluted with the normal saline. Lapatinib was suspended in a vehicle as described previously.[17]

The following antibodies were purchased from Cell Signaling Technology Japan, KK and used for western blotting: anti-phospho-HER2 (Tyr1196; #6942), anti-HER2 (#2165), anti-phospho-HER3 (Tyr1289; #4791), anti-HER3 (#12708), anti-phospho-AKT (Ser473; #4060), anti-AKT (#4685), anti-phospho-p44/42 MAPK (Thr202/Tyr204; #4370), and anti-p44/42 MAPK (#4695).

2.2 | Cell line and cell culture

NCI-N87 cells were purchased from the American Type Culture Collection, and were authenticated by short tandem repeat profiling and tested negative for mycoplasma. The cell line was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

2.3 | Establishment of an in vivo trastuzumab- and pertuzumab-resistant tumor model to evaluate the anti-tumor efficacy of TAS0728

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of Taiho Pharmaceutical Co., Ltd. and carried out according to the guidelines for performing animal experiments of Taiho Pharmaceutical Co., Ltd. Nude mice (BALB/cAJcl-nu/nu, male) were purchased from CLEA Japan, Inc. Suspensions of NCI-N87 cells were implanted subcutaneously into the side flanks of 6-wk-old male nude mice. The TV (mm^3) was calculated as the length (mm) × width (mm^2)/2. During the treatment period, TV and BW were measured twice per week. When the final TV (TV$_{final}$) became smaller than the initial TV (TV$_{initial}$) in the treatment group, tumor regression was judged to have occurred. As an indicator of drug-related toxicity during the dosing period, the BW change (BWC; %) was calculated as 100 − ([BW on each measurement day] − [BW$_{initial}$])/(BW$_{initial}$) × 100, where BW$_{initial}$ is the initial body weight on the day of treatment allocation. The Dunnett test or Aspin-Welch t test was used as statistical methods to compare the TV data in the drug-treated and control groups.

The experiment was performed in two stages. In Stage I, tumor relapse was induced by long-term treatment with trastuzumab and pertuzumab; in Stage II, the efficacy of TAS0728 was evaluated against trastuzumab and pertuzumab-refractory tumors. Drug dosing was started on day 1 in both stages. In Stage I, resistance to the combined treatment with trastuzumab and pertuzumab was induced by intraperitoneal (ip) administration of trastuzumab and pertuzumab at doses of 20 mg/kg each once weekly for 8 wk to nude mice bearing NCI-N87 xenografts, because the dose was efficacious in the same model in a previous study.[7] The anti-tumor effect was evaluated on day 29 and day 57 after treatment. In Stage II, 20 animals bearing the largest TVs were selected and randomized to receive TAS0728 or trastuzumab and pertuzumab. The anti-tumor effects of TAS0728 (60 mg/kg/d, per oral [po]) or trastuzumab and pertuzumab (20 mg/kg each once weekly for 8 wk, ip) were compared on day 29 after the initiation of Stage II (85 d from the start of Stage I).

2.4 | Establishment of an in vivo T-DM1-resistance model for evaluating the anti-tumor efficacy of TAS0728

This experiment was carried out in two stages. In Stage I, tumor relapse was induced by long-term treatment with T-DM1 and, in Stage II, the efficacy of TAS0728 in xenografts pretreated with T-DM1 was evaluated. Drug dosing was started on day 1 in both stages. In Stage I, suspensions of NCI-N87 cells were implanted subcutaneously into the side flanks of 6-wk-old male nude mice and the anti-tumor effect of T-DM1 (10 mg/kg once every 3 wk, iv) was evaluated on d 22, 33, and 85. The dosage of T-DM1 was determined based on a previous study, which reported the efficacious doses of T-DM1.[18] In Stage II, 18 animals harboring the largest TVs were selected and randomized to receive TAS0728 or T-DM1. The anti-tumor effects of TAS0728 (60 mg/kg/d, po) and T-DM1 (10 mg/kg once every 3 wk, iv) were compared on day 43 after the initiation of Stage II (127 d from the start of Stage I).

2.5 | In vivo evaluation of TAS0728 and lapatinib efficacy on T-DM1 refractory tumor xenografts

Tumor fragments that were refractory to T-DM1 were collected from the T-DM1-resistant tumor model after evaluation on day 43 in Stage I, as described in the T-DM1-resistant tumor model above. The collected tumor fragments were passaged three times via subcutaneous implantation into the side flanks of the male nude mice, before final subcutaneous implantation into 6-wk-old male nude mice for drug treatments. When the tumors reached an average size of 100 mm³, the mice were randomized and separated into six groups that received no treatment, T-DM1 (10 mg/kg/d, iv), lapatinib (50 mg/kg/dose, bid, po) or three different doses of TAS0728 (15, 30, 60 mg/kg/dose, bid, po) for 21 d. During the treatment period, TV and BW were measured twice per week.

2.6 | Analysis of in vivo pharmacodynamics by western blotting, phospho-RTK array, and RNA sequence analyses

Tumors were collected from the animals, snap-frozen in liquid nitrogen, and stored at −80°C in a deep freezer prior to pharmacodynamic analysis. Frozen tumors were lysed in a lysis buffer containing Sample Diluent Concentrate 2 (R&D Systems, Inc), cOmplete Mini Protease Inhibitor Cocktail (Roche Diagnostics Japan), and PhosSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics), and processed for the phospho-RTK array (ARY001B, R&D systems, Inc) or western blot analysis. Chemiluminescent images were captured with an Amersham Imager 600 QC (GE Healthcare Japan Corporation). RNA sequence analysis was performed by TaKaRa-Bio Co. Ltd. using the NovaSeq 6000 instrument. Gene expression comparisons were conducted using the edgeR package in R.

2.7 | In vivo evaluation of TAS0728 in a patient-derived xenograft model

The anti-tumor effect of TAS0728 was evaluated in a "Low-Passage Champions Tumor Graft" model (CTG1171) by Champions Oncology, Inc. Tumor fragments generated from the model were passaged subcutaneously into the left flank of immune-compromised female athymic nude *Foxn1nu* mice. When average TVs reached approximately 150-300 mm³, 10 mice per group were treated once per day for 28 d with vehicle control or TAS0728 at 30 mg/kg/d by oral gavage. Tumor growth was monitored twice a week using digital calipers and the TV was calculated using the formula (0.52 × [length × width²]). All experimental procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Champions Oncology, Inc.

3 | RESULTS

3.1 | Tumors with acquired resistance to combination treatment with trastuzumab and pertuzumab were vulnerable to TAS0728

To generate tumors that were resistant to combination treatment by trastuzumab and pertuzumab in vivo, mice bearing NCI-N87 xenografts were treated by both drugs. The NCI-N87 tumors grew slowly in response to single-agent treatment with trastuzumab or pertuzumab when compared with non-treatment, and tumors treated with a combination of trastuzumab and pertuzumab gradually regressed. Maximal shrinkage of tumors treated with both drugs was observed on day 25, after which the tumor began to grow gradually to reach more than twice its minimal tumor size by day 57. Once the tumor regrew, the tumors were no longer sensitive to trastuzumab and pertuzumab treatment (Figure 1A).

We next evaluated the anti-tumor effect of TAS0728 in tumors that had been treated with trastuzumab and pertuzumab for 56 d. TAS0728 treatment at 60 mg/kg/d exerted a significant anti-tumor effect, while treatment with trastuzumab and pertuzumab was not effective (Figure 1B). During treatment, none of the mice showed a significant decrease in BW. Further, the efficacious dose of TAS0728 tested in this study was comparable with that in a treatment-naïve NCI-N87 model.[14]

3.2 | HER2-HER3 signals were observed in tumors with acquired resistance to combination treatment with trastuzumab and pertuzumab

To investigate the mechanism of TAS0728 effectiveness in tumors that had become refractory to combination treatment with trastuzumab and pertuzumab, pharmacodynamic evaluations were performed. Signals of HER2 phosphorylation remained in the refractory tumors for 85 d, although a slightly decreased level of HER2 phosphorylation was noted (Figure 2A); whereas phosphorylation levels of HER3 after 85 d of combined treatment was almost equivalent to levels in non-treated tumors. In contrast, in TAS0728-treated tumors, phosphorylation of HER2 and HER3 was inhibited. These data suggest that tumors with acquired resistance to trastuzumab and pertuzumab still possess detectable HER2-HER3 activation signals and may continue to be dependent on HER2-HER3 signaling for malignant growth. To test our hypothesis that trastuzumab and pertuzumab-refractory tumors are dependent on HER2-HER3 signaling, we analyzed the phosphorylation status of various RTKs, as previous studies suggested that alternative RTK activation can be a mechanism of resistance to targeted therapy.[16] A phospho-RTK array was used to analyze refractory tumors and non-treated control tumors. The 49 tested RTKs are listed in Table S1. In the
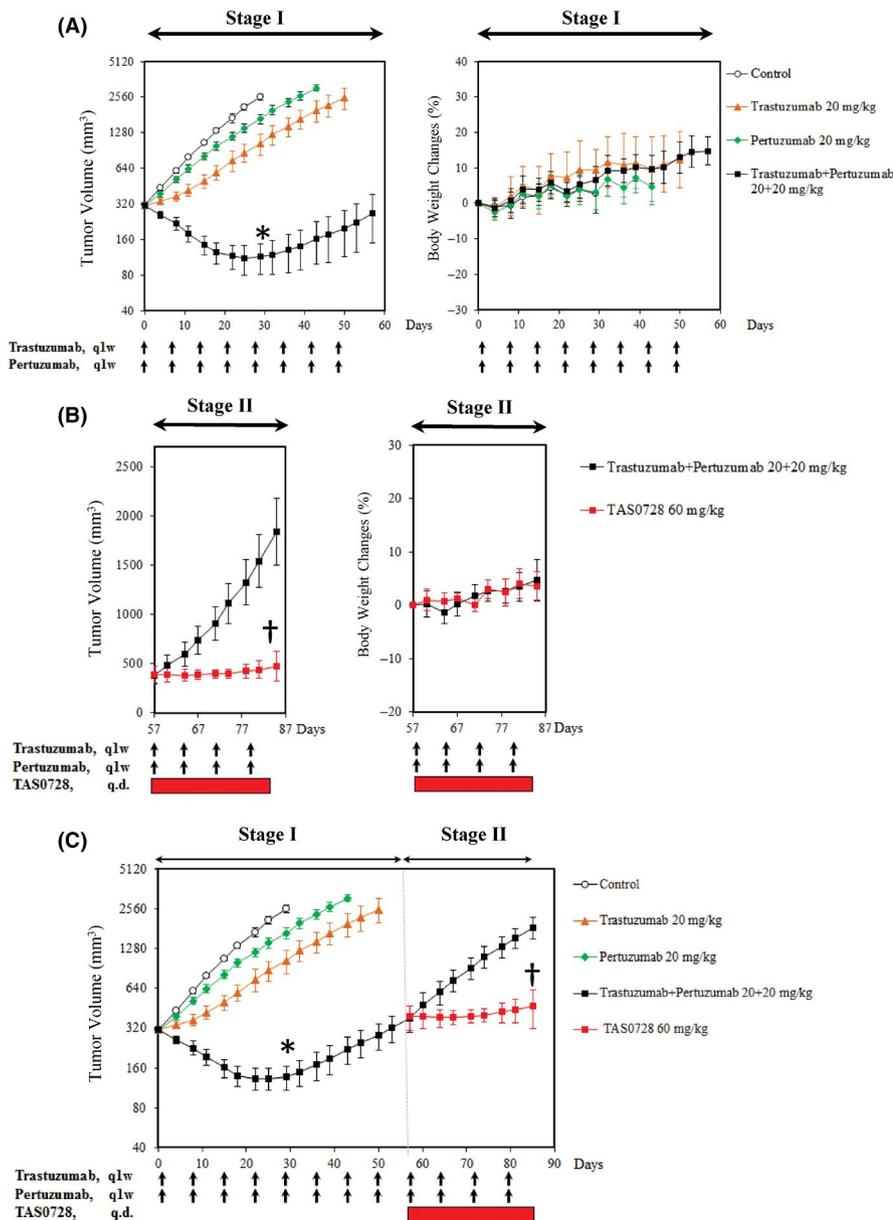

FIGURE 1 Tumors with acquired resistance to combination treatment with trastuzumab and pertuzumab were vulnerable to TAS0728. A, Inhibition of tumor growth in mice bearing NCI-N87 xenografts treated with trastuzumab, pertuzumab, or a combination of trastuzumab plus pertuzumab (n = 5 each in the control, trastuzumab-, and pertuzumab-treated group, n = 50 in the group treated with a combination of trastuzumab and pertuzumab). *P < .001 compared with the control group by Dunnett test. B, Anti-tumor effect of trastuzumab and pertuzumab or TAS0728 in mice bearing NCI-N87 xenografts after treatment with trastuzumab and pertuzumab for 56 d. After treatment for 56 d, 20 out of 50 mice were selected and randomly assigned to the trastuzumab and pertuzumab group or the TAS0728 group (n = 10 per group). †P < .0001 compared with the trastuzumab and pertuzumab group and the TAS0728 group by the Aspin-Welch t test. C, Combined time-courses of the mean TV in each treatment
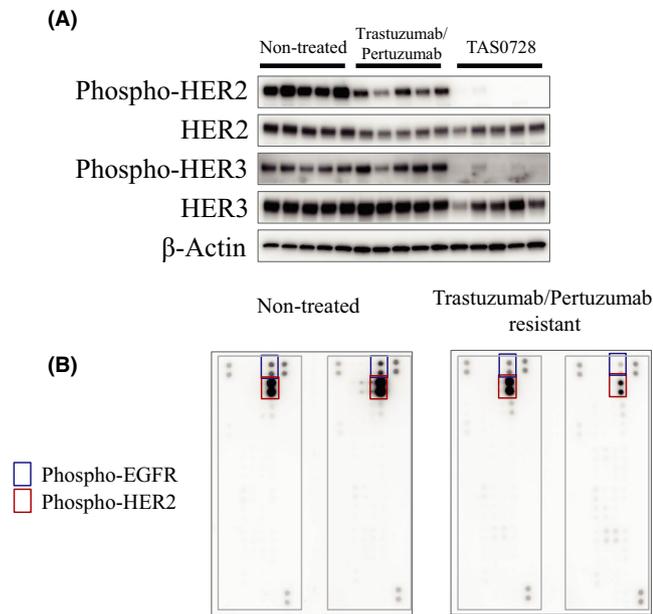
(A)

(B)

□ Phospho-EGFR
□ Phospho-HER2

FIGURE 2 Molecular profiling of tumors with in vivo resistance to trastuzumab and pertuzumab. A, Pharmacodynamic activity of trastuzumab and pertuzumab or TAS0728 on HER2-HER3 in xenograft tumors. At 1 or 3 h after administration of TAS0728 or trastuzumab and pertuzumab on day 85 in the trastuzumab- and pertuzumab-resistance model as described in Figure 1B, xenograft tumors were collected. Phosphorylation of HER2-Tyr1196 and HER3-Tyr1289 were determined by western blotting. B, The xenograft tumors treated by trastuzumab and pertuzumab collected on day 85, or non-treated control tumors collected on day 29 were analyzed using the human phospho-RTK array kit

refractory tumors, there was no increase in the phosphorylation levels of the 49 RTKs. Instead, pEGFR was decreased (Figure 2B). HER2 phosphorylation levels were also slightly decreased, but still detectable in refractory tumors, consistent with results from the western blot analyses. To characterize the molecular profiles of the tumors, RNA sequence analysis was conducted to identify differentially expressed genes between refractory and non-treated tumors. RTKs and the drug efflux transporters were not differentially expressed (Table S2), suggesting that the tumors did not employ alternative signaling pathways or activate drug efflux mechanisms to develop resistance. Further, the tumors with acquired resistance to trastuzumab and pertuzumab still expressed detectable HER2-HER3 activation signals, suggesting that the resistant tumors may be addicted to HER2-HER3 signaling.

3.3 | Tumors with acquired resistance to T-DM1 were vulnerable to TAS0728

T-DM1 is used as therapy for patients with HER2-overexpressing metastatic breast cancer who have progressive disease after receiving combination therapy with trastuzumab and pertuzumab. However, some patients subsequently acquire resistance to T-DM1.[8,9,19] To explore the possibility of TAS0728 as monotherapy

for T-DM1-resistant tumors, we first generated resistant tumors in vivo by long-term treatment with T-DM1. All of the NCI-N87 tumors engrafted in mice initially responded to T-DM1 with substantial tumor shrinkage, with maximal levels of shrinkage observed on day 47; the mean TV at this time-point corresponded to approximately 27% of the mean TV recorded on day 0. However, the tumors began to grow again after day 47, reaching sizes that were an approximately 70% increase of the mean TV by day 85. Once the tumors regrew, they were no longer sensitive to T-DM1 treatment (Figure 3A).

To investigate whether T-DM1-refractory tumors were sensitive to TAS0728, 18 mice bearing tumors of high TVs were selected and re-randomized to receive TAS0728 or T-DM1. TAS0728 treatment led to tumor shrinkage, while T-DM1 treatment was ineffective (Figure 3B). During the treatment, none of the mice in both groups showed significant decreases in BW. These results suggest that TAS0728 could be effective against HER-2 positive tumors that have become refractory to T-DM1 treatment.

3.4 | HER2-HER3 signals were observed in tumors with acquired resistance to T-DM1

To determine the mechanism of action of TAS0728 on T-DM1 resistant tumors, pharmacodynamic evaluations were performed. Western blot analysis revealed that phosphorylated HER2 signals remained in the refractory tumors, although at slightly lower levels. Phosphorylation levels of HER3 were almost equivalent to that observed in non-treated control tumors (Figure 4A). In contrast, in TAS0728-treated tumors, there was a profound decrease in phosphorylation of HER2 and HER3. These results suggest that the anti-tumor of TAS0728 was mediated via HER2-HER3 signal inhibition, and that the tumors with acquired resistance to T-DM1 were still dependent on HER2-HER3 signaling.

To test our hypothesis that T-DM1-refractory tumors require HER2-HER3 signals, we compared the activation status of RTKs in refractory and non-treated tumors using a phospho-RTK array. No increase in phosphorylation levels of the 49 RTKs tested was observed in refractory tumors. Instead, levels of phosphorylated EGFR and phosphorylated HER2 were reduced in these tumors (Figure 4B). RNA sequence analysis was also conducted, and differentially expressed genes between T-DM1 refractory tumors and control tumors were identified. RTKs were not differentially expressed (Table S3) suggesting that the tumors did not employ alternative signaling pathways to develop T-DM1 resistance, and that they still rely on HER2-HER3 signaling. In contrast, the gene expression levels of drug efflux transporters, including ABCC2, ABCG2, and ABCB1 were significantly increased in the resistant tumor compared with non-treated control tumor ($P < .05$). However, expression of the genes was very low in the resistant and control tumors (Table S3). Taken together, these data suggest that an increased expression of the transporters in a T-DM1-treated tumor may not have a significant effect on the development of drug resistance.
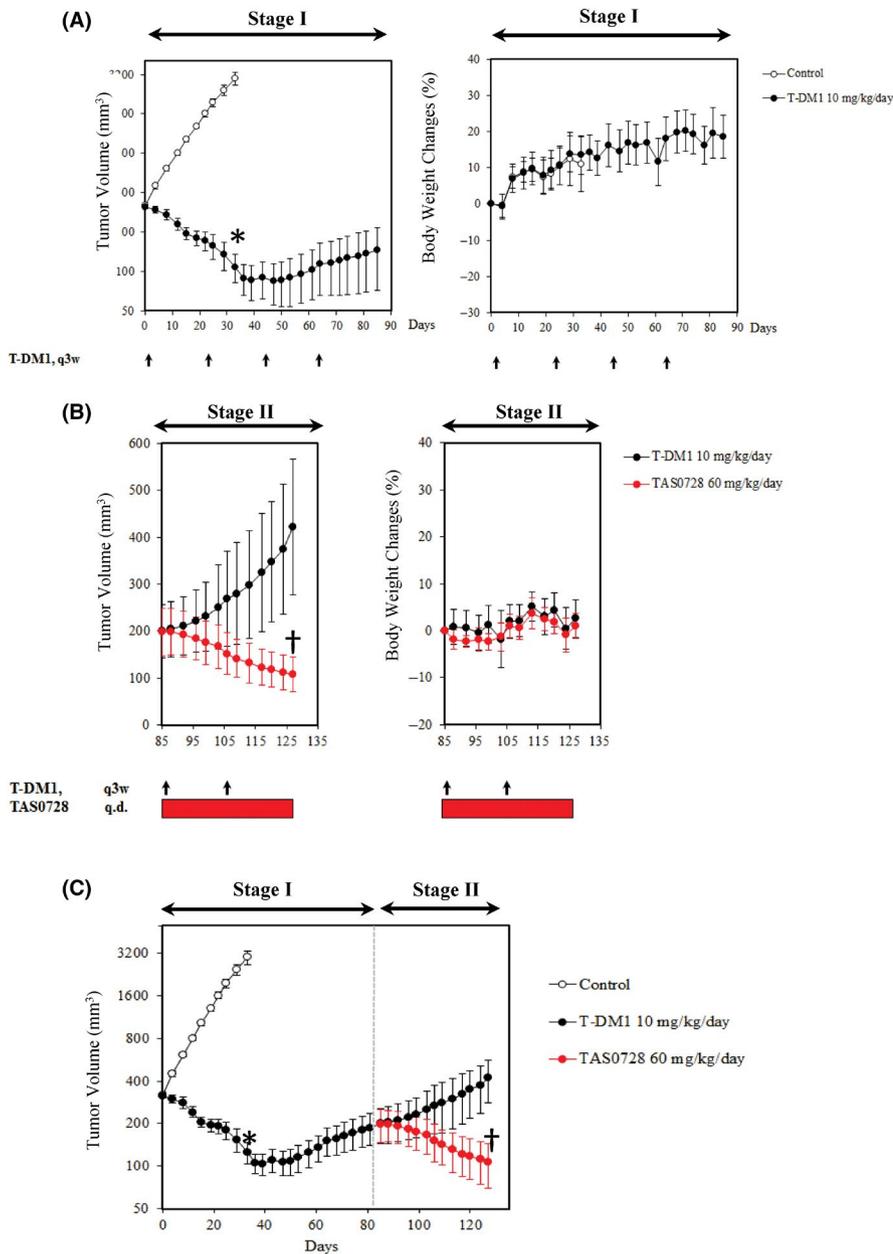
FIGURE 3 Tumors with acquired resistance to T-DM1 were vulnerable to TAS0728. A, Inhibition of tumor growth in mice bearing NCI-N87 xenografts treated with T-DM1 (n = 6 in the control group, n = 36 in the T-DM1 treatment group). *$P < .0001$ compared with the control group and the T-DM1 group by the Aspin-Welch t test. B, Anti-tumor effect of T-DM1 or TAS0728 in mice bearing NCI-N87 xenografts after treatment with T-DM1 for 85 d. After T-DM1 treatment for 85 d, 18 out of 36 mice were selected and randomly assigned into the T-DM1 group or the TAS0728 group (n = 9 per group). †$P < .0001$ compared with the T-DM1 group and the TAS0728 group by the Aspin-Welch t test. C, Combined time-courses of the mean tumor volume in each treatment

3.5 | Superior anti-tumor efficacy of TAS0728 compared with lapatinib in a T-DM1 refractory NCI-N87 xenograft model

In a previous study, we had shown that TAS0728 had superior anti-tumor activity compared with lapatinib in a treatment-naïve HER2-positive breast cancer xenografts model.[14] In the present study, we compared the efficacy of TAS0728 and lapatinib in T-DM1-refractory tumors that had been established via long-term T-DM1 exposure in vivo, and passaged three times before subcutaneous implantation into male nude mice. Subsequent T-DM1 treatment did not induce tumor shrinkage, indicating that the tumors retained T-DM1 resistance, even after in vivo passage (Figure 5A). Both doses of TAS0728 treatment (30 or 60 mg/kg/d) induced tumor regression. In contrast, tumor shrinkage was not observed in the lapatinib-treated group.

Pharmacodynamic analysis of tumors treated with 60 mg/kg/d of TAS0728 revealed that phosphorylation of HER2, HER3, AKT, and ERK was decreased when compared with that in the control tumors at 1 h after administration. In contrast, T-DM1 and lapatinib did not decrease phosphorylation of HER2 or its downstream signaling molecules (Figure 5B).

3.6 | TAS0728 was effective in treating patient-derived xenografts originating from HER2-positive breast cancer that was refractory to trastuzumab, pertuzumab, T-DM1, and chemotherapies

To investigate the therapeutic potential of TAS0728 against heavily pretreated tumors that had become refractory to trastuzumab and

pertuzumab as well as T-DM1, we examined the anti-tumor activity of TAS0728 in a patient-derived xenograft model where tumors originated from a breast cancer patient. The tumor engrafted was a
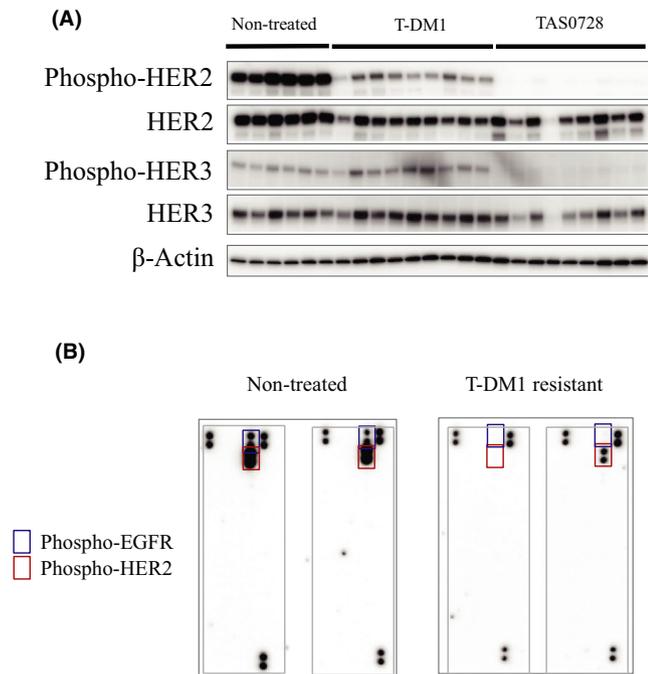

HER2-positive, ER/PR-negative Stage IV breast tumor. Prior to collection, the tumor initially responded to combinatorial treatment with trastuzumab, carboplatin, and docetaxel, followed by treatment with trastuzumab, vinorelbine, and 5-fluorouracil. However, subsequent treatments including liposomal doxorubicin, paclitaxel, and bevacizumab, and combinatorial trastuzumab, pertuzumab, and carboplatin, and T-DM1, were ineffective. Details of the tumor sample are shown in Table S4. Animals engrafted with the patient-derived tumor were administered vehicle control or TAS0728 at 30 mg/kg by oral gavage for 28 d. The doubling time of the tumor in the vehicle control group was 19.9 d. Treatment of 10 animals with TAS0728 at 30 mg/kg significantly decreased the TVs compared with that of the vehicle control group at day 27 (Figure 6). The mean animal BW of control animals and TAS0728-treated animals increased at a similar rate (Figure S1). There were also no mortalities or moribund animals in both groups, indicating that TAS0728 was well tolerated in this study. These results demonstrated the potential efficacy of TAS0728 against HER2-positive tumors that had been heavily pretreated with trastuzumab, pertuzumab, T-DM1, and chemotherapies.

FIGURE 4 Molecular profiling of tumors with in vivo resistance to T-DM1. A, Pharmacodynamic activity of T-DM1 or TAS0728 on HER2 and HER3 in xenograft tumors. At 1 h after administration of TAS0728 or T-DM1 on day 127 in T-DM1-resistance model as described in Figure 3B, xenograft tumors were collected. Phosphorylation of HER2-Tyr1196, HER3-Tyr1289 was determined by western blotting. B, The xenograft tumors treated by T-DM1 for 127 d or the non-treated control tumors were collected on day 127 or day 85 and were analyzed using the human phospho-RTK array kit

4 | DISCUSSION

Although established HER2-targeting therapies with anti-HER2 antibodies or an ADC initially exerted significant clinical responses, increasing evidence suggests that patients who initially respond to these drugs subsequently develop resistance. Therapy resistance is an important clinical challenge in the management of cancer patients, and alternative effective therapies to overcome the resistant tumors are urgently required. In this study, we aimed to investigate whether TAS0728, a small-molecule, irreversible, selective HER2
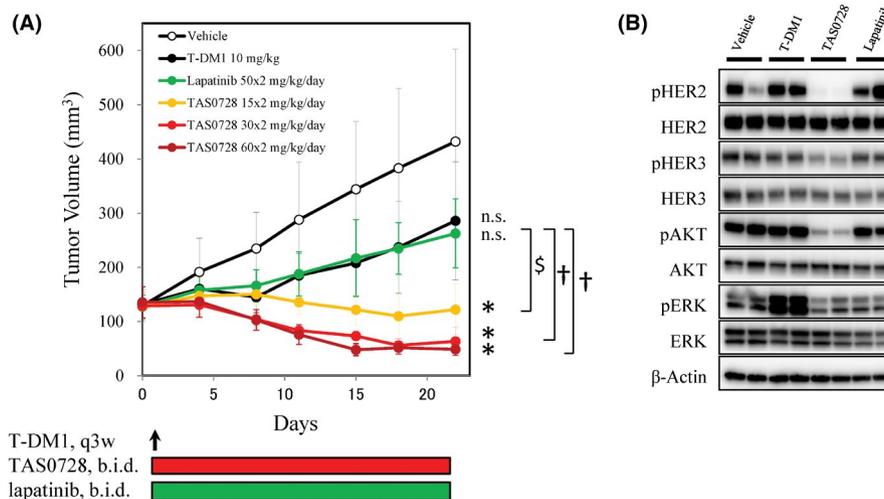

FIGURE 5 In vivo comparison of the anti-tumor activity of T-DM1, lapatinib and TAS0728. A, Inhibition of tumor growth in mice bearing NCI-N87 xenografts treated with T-DM1, TAS0728, or lapatinib for 21 d (n = 6 per group). n.s.: not significant or *P < .001 according to Aspin-Welch t test, compared with the control group. $^{†}P$ < .001 or $^{\$}P$ < .01 compared with the lapatinib group and the TAS0728 group by Aspin-Welch t test. B, Pharmacodynamic activity of T-DM1, TAS0728, and lapatinib on HER2 and downstream molecules in xenograft tumors. Mice bearing NCI-N87 xenografts were administered T-DM1, TAS0728, or lapatinib, and the xenograft tumors were collected at 1 h after administration. Phosphorylation of HER2-Tyr1196, HER3-Tyr1289, AKT-Ser473, and ERK1/2-Thr202/Tyr204 were determined using western blot analysis
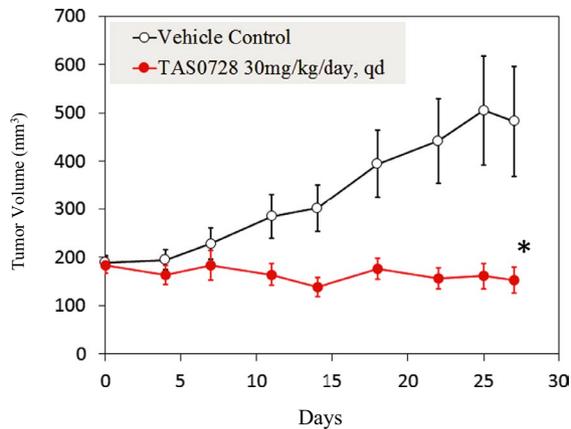
FIGURE 6 In vivo anti-tumor activity of TAS0728 on a PDX model derived from a breast cancer patient who did not respond to the combination therapy of trastuzumab, pertuzumab, and carboplatin or to T-DM1. Inhibition of tumor growth in mice bearing CTG-1171 PDX xenografts treated TAS0728 (n = 10 per group). *P < .001 compared with the control group and the TAS0728 group by Dunnett test. The tumor profile of the model and the treatment history before collecting samples are available in Table S4

kinase inhibitor is effective for treating tumors that are resistant to established HER2-targeting therapies. We used three different in vivo models, including new models of tumors that are refractory to trastuzumab in combination with pertuzumab, or to T-DM1, and a PDX model derived from a HER2-positive breast cancer patient who showed resistance to trastuzumab and pertuzumab, T-DM1, and chemotherapeutic agents. We demonstrate that the tumors that have become refractory to HER2-targeting therapies still retain detectable HER2-HER3 signals, and inhibition of the signals with TAS0728 produced significant anti-tumor effects.

Combinatorial treatment with trastuzumab and pertuzumab is used as a first-line therapy against HER2-positive metastatic breast cancer. While a significant outcome is observed with the combination of these HER2-targeting antibodies, most patients ultimately become resistant to the treatment.[3] However, little information is known concerning the underlying mechanisms of resistance, and models of acquired resistance to both trastuzumab and pertuzumab are limited. Given that the combination of HER2-directed antibodies may have various modes of actions that are dependent on host physiology and immunity, including ADCC,[4,6,7,20] in vivo models are required to recapitulate tumor responses to treatment, the tumor microenvironment, and tumor-host interactions. Therefore, we established a new in vivo model of trastuzumab and pertuzumab resistance, in which tumors initially responded to trastuzumab and pertuzumab, but eventually became treatment resistant after a period of tumor regression. We detected residual HER2-HER3 signals in these refractory tumors, contrary to the reports that trastuzumab and pertuzumab inhibit HER2-HER3 signaling.[20,21] We further showed in this model that TAS0728 inhibits tumor growth associated with HER2-signal inhibition, and

that TAS0728 may inhibit residual HER2-HER3 signaling. Although the mechanism of resistance to trastuzumab and pertuzumab is still unclear, and likely includes complex and multiple molecular pathways, molecular profiling of the resistant tumor tissue did not implicate activation of alternative signaling pathways, which has been reported as a mechanism of resistance to trastuzumab.[16] We found that TAS0728 was also effective in a PDX model derived from a breast cancer patient who had stopped responding to trastuzumab and pertuzumab (as well as T-DM1). Taken together, these results show that TAS0728 may be beneficial for the tumors that are refractory to the combination of trastuzumab and pertuzumab.

In the present study, we also showed that HER2-kinase inhibition by TAS0728 was effective against tumors refractory to T-DM1, which is used as a second-line treatment in HER2-positive breast cancer patients, where T-DM1 initially exerts significant clinical responses but treatment resistance eventually develops. To date, many T-DM1-resistant cell lines have been established to investigate the mechanisms of resistance. However, most of these have been established in vitro. Given that T-DM1's anti-tumor effects depend on both ADCC and cytotoxicity, an in vivo model is more appropriate for recapitulating the tumor microenvironment and tumor-host interactions. We showed in our model that, after treatment with T-DM1, tumors still had the detectable levels of HER2 phosphorylation, and were therefore vulnerable to HER2-signal inhibition. Indeed, although T-DM1 was no longer effective once tumors started to regrow during treatment, switching to TAS0728 effectively induced tumor regression. TAS0728 was also effective in a PDX model derived from a breast cancer patient who did not respond to T-DM1 (as well as trastuzumab and pertuzumab). While we did not evaluate the potential of TAS0728 as a third-line therapy in a NCI-N87 xenograft model sequentially treated with the first- and second-line standard therapies against HER2-positive metastatic breast cancer, we did show that TAS0728 as a monotherapy was effective in the PDX model that was derived from tumors pretreated with both first- and the second-line treatments in the patient. Taken together, TAS0728 may be beneficial not only for tumors refractory to T-DM1 but also for tumors treated with third-line HER2-targeting therapies.

Despite some preclinical studies on drug resistance, the detailed mechanisms of resistance to established HER2-targeting therapies are still unclear. Moreover, it is also unclear whether TAS0728 has a clinical benefit for patients who have acquired resistance to trastuzumab and pertuzumab as well as to T-DM1. Currently, a Phase 1 study of TAS0728 in patients with solid tumors is ongoing, and data describing its efficacy are awaited. We have not arrived at a conclusive mechanism of resistance in our model but our results provide an improved understanding of the molecular mechanisms of acquired resistance to HER2 antibodies and ADCs, and may provide valuable leads to enhance treatment efficacy.

In conclusion, we successfully established, for the first time, in vivo tumor models of trastuzumab and pertuzumab or T-DM1 resistance, and TAS0728 monotherapy was found to be effective against tumors resistant to these therapies. These observations imply that

the tumors that are resistant to trastuzumab and pertuzumab or to T-DM1 do not show cross-resistance to TAS0728, and that acquired resistance to these therapies can be overcome by HER2 kinase inhibition with TAS0728 in vivo. Therefore, TAS0728 is a potentially useful treatment option for patients with tumors refractory to established anti-HER2 antibodies and HER2-targeting ADC.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.