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Therapeutic potential of the dual EGFR/HER2 inhibitor AZD8931 in circumventing endocrine resistance

Gladys Morrison · Xiaoyong Fu · Martin Shea · Sarmistha Nanda · Mario Giuliano · Tao Wang · Teresa Klinowska · C. Kent Osborne · Mothaffar F. Rimawi · Rachel Schiff

Abstract Modest up-regulation of either HER-ligands or receptors has been implicated in acquired endocrine resistance. AZD8931, a dual tyrosine kinase inhibitor (TKI) of epithelial growth factor receptor (EGFR)/HER2, has been shown to more effectively block ligand-dependent HER signaling than the HER TKIs lapatinib or gefitinib. We therefore examined the effect of AZD8931 in ER-positive/HER2-negative breast cancer cells with acquired resistance to tamoxifen, where there is ligand up-regulation associated with HER pathway activation. RNA-seq ligand profiling and levels of HER receptors and signaling by western blotting were conducted in ER+ MCF7 and T47D parental cells and their Tam-resistant derivatives (TamRes). In vitro cell growth and apoptosis and HER ligand-stimulated signaling were measured in response to endocrine and HER TKIs. For studies in vivo, transplantable MCF7/TamRes xenografts were treated with tamoxifen or fulvestrant, either alone or in combination with AZD8931. AZD8931 only minimally enhanced endocrine sensitivity in MCF7 parental cells, but showed a greater effect in the T47D parental model. AZD8931 combined with either tamoxifen or fulvestrant inhibited cell growth more than lapatinib in T47D TamRes cells, and was also significantly, though modestly, more potent in MCF7 TamRes cells. In both TamRes models, AZD8931 significantly inhibited cell proliferation and induced apoptosis. Under ligand-stimulated conditions, AZD8931 more potently inhibited HER signaling than lapatinib or gefitinib. AZD8931 also significantly delayed the growth of MCF7 TamRes xenografts in the presence of tamoxifen or fulvestrant. The strongest inhibition was achieved with a fulvestrant and AZD8931 combination, though no tumor regression was observed. This study provides evidence that AZD8931 has greater inhibitory efficacy in tamoxifen-resistant settings than in an endocrine therapy naïve setting. The absence of tumor regression, however, suggests that additional escape pathways contribute to resistant growth and will need to be targeted to fully circumvent tamoxifen resistance.

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Introduction

Approximately, 70 % of breast cancers express estrogen receptor (ER) alpha and rely on this hormone receptor signaling pathway for tumor growth [1]. ER-targeting therapy represents the most effective and commonly used treatment strategy for ER-positive (+) breast cancer patients. The ER pathway can be effectively inhibited by using either selective ER modulators (SERMs) such as tamoxifen (Tam) or selective ER downregulators (SERDs)

such as fulvestrant (Ful), or by depriving the ER of its ligand estrogen (E_2) with aromatase inhibitors (AIs) in postmenopausal women or ovarian ablation in younger patients [2, 3]. However, many tumors exhibit de novo resistance to these endocrine treatments, while others acquire resistance after an initial response [4].

Compelling preclinical and clinical evidence suggests that in the presence of chronic ER blockade, the human epidermal growth (HER) factor receptor family plays a critical role in mediating endocrine therapy resistance [5–9]. It has been postulated that the up-regulation of HER receptor signaling, even when modest, modulates ER activity by enhancing ER's transcriptional activity, by causing ligand-independent receptor activation, by altering its transcriptional program, or by suppressing ER expression and its classic transcriptional function, thereby rendering the cells endocrine resistant [10–12].

The HER receptor tyrosine kinase family is comprised of four receptors: epidermal growth factor receptor (EGFR or HER1), HER2, HER3, and HER4 [13]. Activation of the HER pathway results from HER2 overexpression and HER2 homo-dimerization, or by hetero-dimerization of HER2 with other HER receptors after they are activated by ligand binding [14, 15]. After receptor dimerization, the HER tyrosine kinase domains are activated via phosphorylation which ultimately activates downstream pathways that regulate cell survival and proliferation as well as other biological processes important for breast tumor progression [16, 17].

About 10–15 % of ER+ tumors are HER2+, and patients with these tumors have a worse prognosis and higher risk of de novo resistance to endocrine therapies [7, 18, 19]. Furthermore, in ER+/HER2-negative tumors, compelling preclinical in vitro and in vivo studies have shown that acquired resistance to endocrine treatments is associated with an adaptive up-regulation of EGFR and HER2 as well as increased expression of HER-ligands [6, 20–22]. Thus, combined therapies that target both the HER receptor pathway and the ER pathway have recently been used to overcome endocrine resistance in clinical trial settings [23, 24]. Phase II clinical trials using the EGFR inhibitor gefitinib in combination with either anastrozole [25] or tamoxifen [26] in ER+ metastatic breast cancer patients showed that gefitinib enhanced the efficacy of anastrozole and tamoxifen at least in certain subsets such as those with tumors low in ER.

The dual EGFR/HER2 tyrosine kinase inhibitor (TKI) lapatinib was evaluated in combination with the AI letrozole in ER+ metastatic breast tumors [27]. This combination prolonged progression-free survival in patients with ER+/HER2+ tumors compared to letrozole treatment alone. It was, however, ineffective in patients with ER+/HER2− tumors [28].

Several studies have shown that expression of HER-ligands and activation of HER receptors and downstream pathways can modulate ER transcriptional activity resulting in endocrine resistance [22, 29]. Several reports have shown that lapatinib is less effective in the presence of HER-ligands, in ER+/HER2+ and ER+/HER2− tumors [30, 31]. This suggests that in scenarios of acquired endocrine resistance, where ligand expression is still critical to activate the HER pathway, there is a need to develop new and more potent anti-HER treatment strategies with more efficacy in inhibiting HER ligand-dependent signaling.

AZD8931 is a novel dual EGFR and HER2 TKI that targets EGFR, HER2, and HER3. Its mechanism of action is distinct from current anti-HER therapies, and under ligand-stimulated conditions, AZD8931 is more potent than lapatinib [32]. Its ability to delay or overcome acquired resistance in ER+/HER2− breast tumors, however, has not yet been evaluated. Herein, we show that AZD8931 is significantly more effective than lapatinib in tamoxifen resistance that is mediated by increased HER-ligands and receptors, using both in vitro and in vivo experimental models of tamoxifen resistance.

Materials and methods

Cell lines and establishment of resistant lines and reagents

MCF7 (MCF7L, originally from Dr. Marc Lippman's lab) and T47D breast cancer cells were grown in RPMI-1640 medium (Lonza, Walkersville, MD) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin and glutamine. To establish tamoxifen-resistant cell lines (TamRes), MCF7 and T47D cells that had already been selected for resistance to estrogen deprivation (ED) were continuously treated with tamoxifen (Tam, 10^{-7} M, >6 months), and the resistant derivatives were selected when the initially sensitive cells resumed comparable growth to the parental cells. The TamRes cell lines were maintained as previously described [5]. Lapatinib (GlaxoSmithKline, Research Triangle Park, NC), AZD8931, or gefitinib (AstraZeneca, UK) was dissolved in DMSO and added to cell cultures at 1 µM concentration. 17β estradiol (E2), 4-hydroxy tamoxifen (for all in vitro studies) from Sigma (St Louis, MO), and ICI 182,780 (Fulvestrant, AstraZeneca, UK), were dissolved in ethanol. Tamoxifen citrate (Sigma) was used in all in vivo treatments as previously described [5].

Cell growth assays

Parental and TamRes cells were starved in medium with 5 % charcoal-stripped FBS for 48 h, then plated in 96-well plates (5,000 cells per well) for a further 48 h before beginning additional treatments. The different treatment groups were

estradiol (E2, 10^{-9} M), ED, Tam (10^{-7} M), or Ful (10^{-7} M), alone or in combination with either lapatinib (1 μM) or AZD8931 (1 μM). The number of cells at days 0 and 6 was assessed using an in situ cell cytometer (Celigo, Brooks Life Sciences Systems, Chelmsford, MA). Growth experiments: all treatment groups were plated in quadruplicate. Experiments in all cell models were repeated and analyzed three separate times. A representative experiment is shown.

Immunoblotting assays

Cells were plated in 6-well plates and treated as previously described in cell growth assays. Cells were lysed in RIPA buffer and equivalent amounts of protein (15 μg) from each sample were used as previously described [33]. Primary antibodies were phospho-EGFR-Tyr1173, EGFR, phospho-HER2-Tyr1221, phospho-HER2-Tyr1248, HER2, phospho-HER3-Tyr1289, phospho-AKT-Ser473, AKT, phospho-p44/42 MAPK-Thr202/Tyr204, p44/42 MAPK, β-actin, cleaved PARP, (Cell Signaling Technology, Beverly, MA), ERα 6F11 (Abcam, Fremont, CA), and progesterone receptor (PR) (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were performed at least three independent times.

RNA interference

SMARTpool small interfering RNAs (siRNAs) against EGFR, HER2, HER3, and scrambled nonspecific siRNA (control) were purchased from Dharmacon (Lafayette, CO). Cells were transfected following the manufacturer's instructions (Dharmacon). Transfection protocol for the growth assay used was previously described [33]. For parallel protein expression, parental and TamRes cells were plated into 24-well plates and subjected to the transfection protocol as above.

Ligand stimulation

Cells were plated in 6-cm dishes for 3 days in their growth media, and serum-starved overnight. Cells were hormonally treated (E2) or treated with Tam, or Ful, alone or in combination with the TKIs (lapatinib, AZD8931, and gefitinib, all at 1 μM concentration) for 90 min followed by stimulation with 20 μM HRG or EGF for 10 min. Lysates were collected and protein levels were detected by immunoblotting as indicated above.

In vitro cell proliferation and apoptosis assays

The cell proliferation assay was performed using the Click-iT EdU (5-ethynyl-2′-deoxyuridine) Microplate Assay (Invitrogen, Carlsbad, CA), and apoptosis assays were performed using the Annexin V-FITC Apoptosis Detection

Kit (Abcam, Cambridge, MA) following 48 h treatment, as described in cell growth assays. Assays were performed and analyzed as described previously [33]. All measurements were performed in quadruplicate. T47D apoptosis under our experimental conditions was not associated with elevated annexin V levels and was therefore measured by c-PARP expression after 48 h of treatment as described in cell growth and immunoblotting assays.

Xenograft studies

The MCF7 TamRes xenograft tumors were generated and maintained by continuously harvesting and transplanting TamRes tumors that have reached 1,000 mm^3 in the presence of Tam from donors to recipients as previously described [34]. Studies were conducted using ovariectomized 5–6 week-old athymic mice (Harlan Sprague–Dawley, Madison, Wisconsin). Tumors were then transplanted into Tam-treated mice, and when tumors reached 150–200 mm^3 (1–2 weeks), the mice bearing these MCF7 TamRes xenografts were randomly allocated to four treatment groups: continued Tam, Tam+AZD8931 (100 mg/kg by oral gavage daily), ICI 182,780 (Ful (100 μg once a week)), and Ful+AZD8931. Each treatment group contained a minimum of 8 mice. Tumor volumes were measured weekly as previously described [5]. All tumors were harvested 4 h post treatment, after 11 days of treatment (short-term) or after longer treatment (1,000 mm^3). Each tumor included in the statistical analysis came from a different mouse.

Statistical analysis

Cell growth, proliferation, and apoptosis experiments were analyzed within each E2-stimulated or endocrine therapy group using one-way ANOVA with the Bonferonni post hoc test. Error bars on plots represent ± standard error of the mean (SEM). Kaplan–Meier estimators and Wilcoxon tests were used for in vivo xenograft tumor analysis. Pairwise comparisons with p value adjustment were performed to compare the difference between treatments. Statistical analyses were done using SAS 9.2 (SAS Institute Inc., Cary, NC).

Results

HER ligand expression is up-regulated in cells acquiring tamoxifen resistance

Several studies have shown that increased expression of HER-ligands and/or their receptors activates downstream pathways that can modulate ER function resulting in

endocrine therapy resistance [21, 22, 29]. We have used RNA-Seq to investigate more thoroughly the change in expression of several HER-ligands upon the acquisition of tamoxifen resistance by comparing MCF7 and T47D parental cell lines with their tamoxifen-resistant derivatives (Fig. 1a). We found that EGFR ligands were up-regulated in MCF7 TamRes cells compared with parental cells. T47D parental cells show higher expression of several ligands in comparison to MCF7 parental cells, and the ligand expression was further increased in the T47D TamRes cells.

We next investigated the levels of ER and HER and downstream signaling in our cell models (Fig. 1b). In MCF7 cells, levels of HER2, EGFR, and downstream effectors were up-regulated in resistant compared to parental cells. In the T47D model, both parental and TamRes cells expressed moderate levels of EGFR, but HER2 was up-regulated only in the TamRes cells. Although HER2 was increased at the time of endocrine resistance in both of these cell lines, the levels were still substantially less than those observed in HER2-gene-amplified BT474 cells. Overall, these results are consistent with previous studies [5, 35, 36] and suggest that
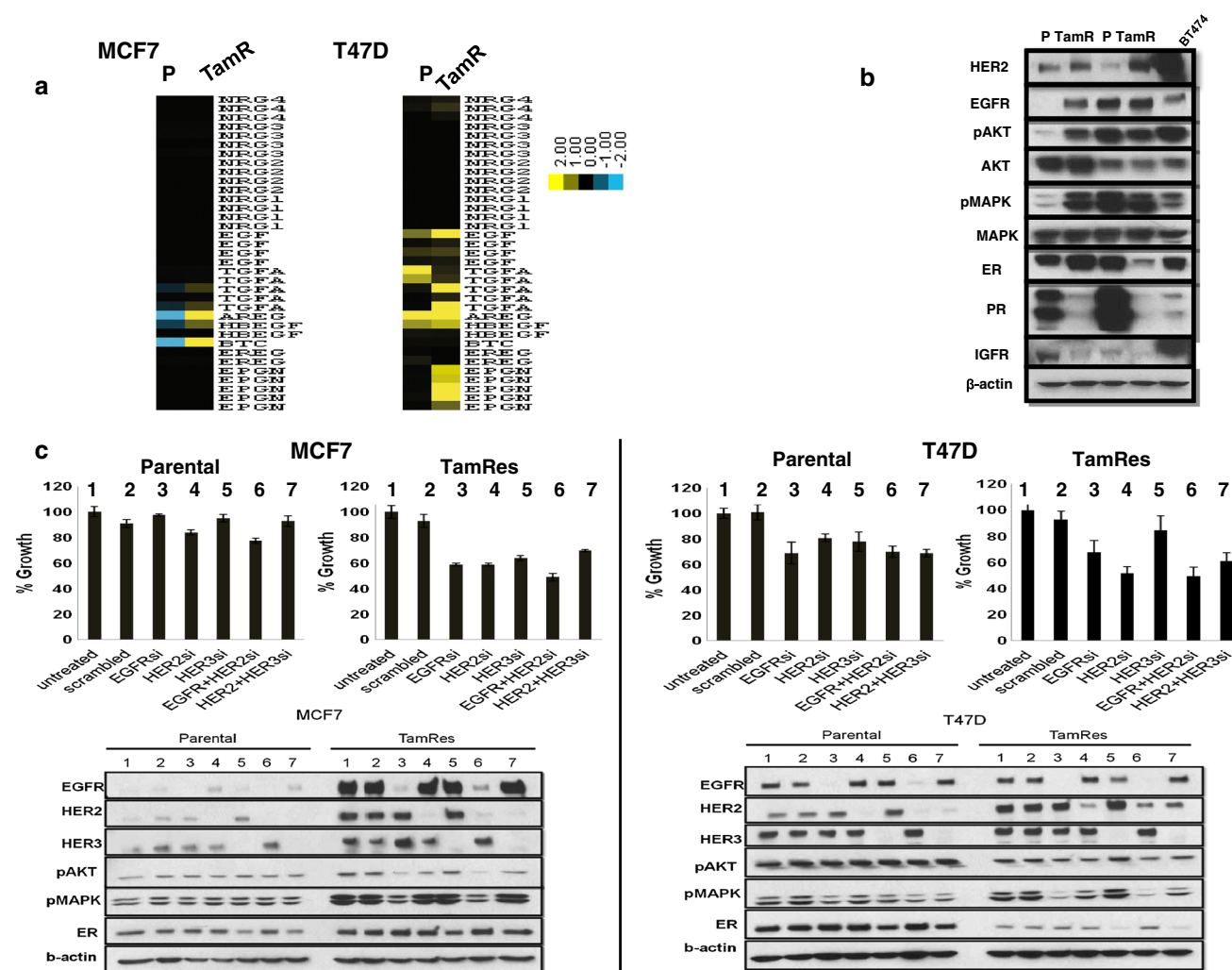

Fig. 1 Side by side comparison of ligand and HER receptor levels and pathway activation in parental and TamRes cells. **a** RNA-Seq data were used to analyze HER ligand expression across HER2-low models. Expression patterns are represented using heat maps (*yellow* high expression, *blue* low expression). Each *column* represents a cell model. *P* parental, *TamR* tamoxifen-resistant. **b** HER and ER expression and signaling were compared by western blotting in low-HER cells MCF7 and T47D (P and TamRes) and high-HER BT474 cells. **c)** MCF7 parental and resistant cells, T47D parental and resistant cells were treated with non-targeting control siRNA

(scrambled), EGFR, HER2, HER3 siRNA, and their combination, for 6 days to evaluate effect of down-regulation on growth. To confirm protein knockdown, cells were treated with siRNA for 72 h and downregulation of EGFR, HER2, and HER3 was detected by Western blot. Whole-cell extracts were analyzed with the indicated antibodies, including downstream signaling markers. Unlike MCF7 parental cells, T47D parental cells were slightly sensitive to EGFR and EGFR+HER2 inhibition. Both MCF7 and T47D TamRes cells were sensitive to EGFR and EGFR+HER2 siRNA and showed down-regulation of downstream effectors such as pAKT and pMAPK

Fig. 2 Effects of AZD8931 or lapatinib alone or in combination with endocrine therapy in TamRes MCF7 and T47D cells. **a, b** Cell growth assays: prestarved parental and TamRes MCF7 and T47D cells were treated with either estrogen (E2), estrogen deprivation (ED), tamoxifen (Tam), or fulvestrant (Ful), all in the presence of vehicle (DMSO), lapatinib, or AZD8931. Cells were counted using the Celigo in situ cell cytometer at day 6, post treatment. **c** Proliferation assays: cells were treated under conditions defined in **a**. After 48 h of treatment, proliferation was measured using the Click-iT EdU (5-ethynyl-2'-deoxyuridine) Microplate Assay. **d** Apoptosis assays: MCF7 TamRes cells were incubated with Annexin V-FITC after 48 h of treatment as in **a**. Positive cells from **c** and **d** were visualized and quantitated by the Celigo cytometer. Graphs show mean ± SE of quadruplicate wells and are representative of three independent experiments. In the T47D parental model, cell lysates were collected after 48 h of treatment. c-PARP levels were assessed by western blotting and normalized to β-actin using Image J. Graphs show mean ± SE of three independent experiments. *$p < 0.01$ comparing DMSO with either lapatinib or AZD8931 treatment groups; #$p < 0.01$ comparing lapatinib with AZD8931
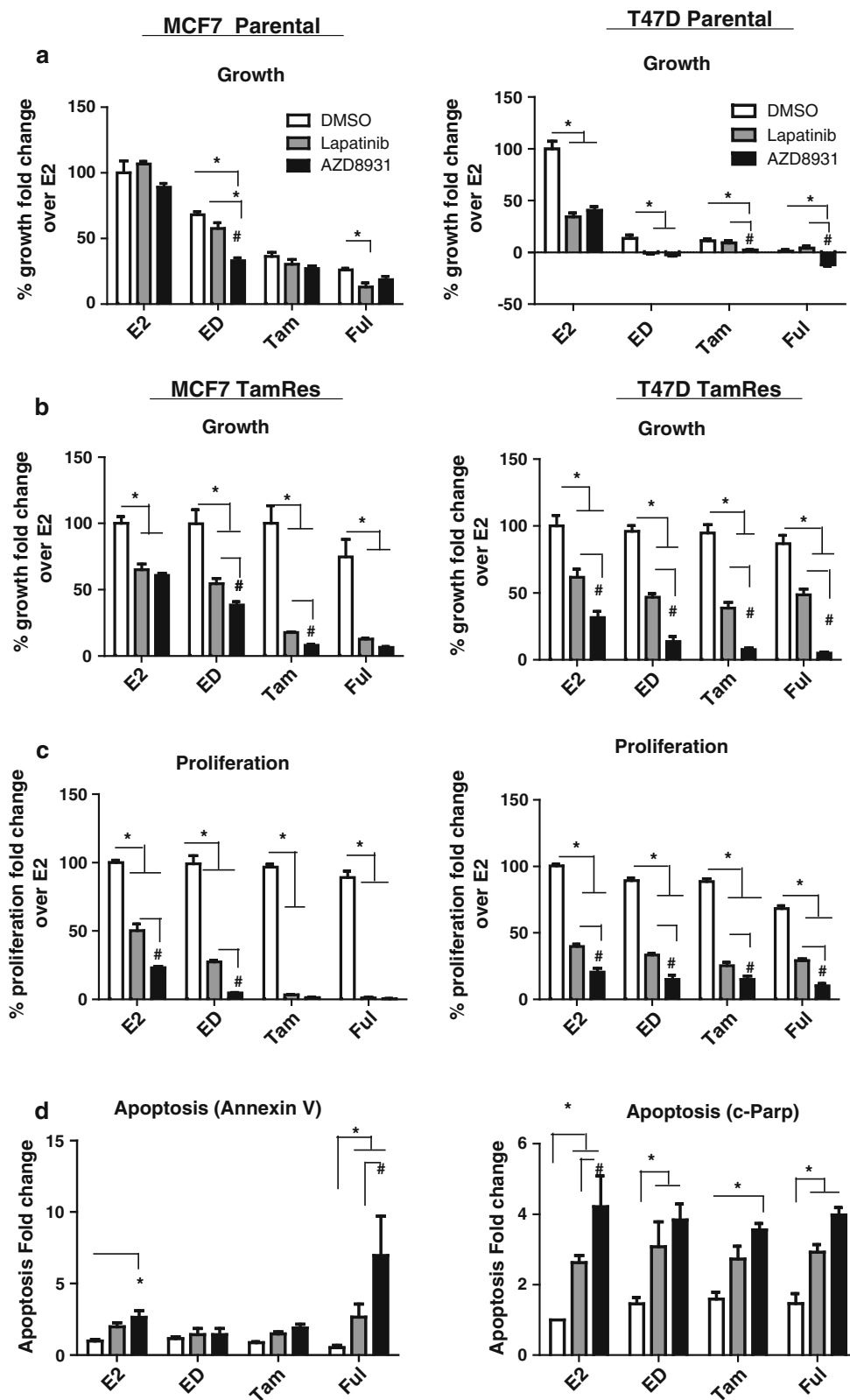
the up-regulation of HER-ligands and receptors and the concomitant decrease in classic ER activity may contribute to endocrine resistance. Using siRNA inhibition, (Fig. 1c),

we find that both TamRes models were more sensitive to EGFR and HER2 siRNA than parental cells, indicating their partial dependence on the HER pathway for their growth.

AZD8931 and lapatinib inhibit tamoxifen-resistant growth in vitro

We next investigated whether AZD8931 combined with endocrine therapy is more effective than either treatment alone in inhibiting growth of parental cells (Fig. 2a). As expected, MCF7 parental cell growth was significantly inhibited by endocrine therapy. With the exception of ED, neither AZD8931 nor lapatinib enhanced the effect of endocrine therapy. Using a second ER+ breast cancer cell model that expresses higher levels of EGFR, we found that the T47D parental cells were dependent on both the ER and HER pathways, and thus sensitive to each of these treatments alone under E2 treatment.

We next assessed the effect of AZD8931 in the TamRes models that are characterized by a modest up-regulation of HER-ligands and HER receptors (Fig. 1a, b). Although the combination of lapatinib with endocrine therapy significantly inhibited MCF7 TamRes cell growth, AZD8931 combinations were slightly more effective than lapatinib when combined with ED and Tam, with a similar trend in combination with Ful ($p < .07$) in MCF7 (Fig. 2b). Likewise, in the T47D TamRes model, AZD8931 was superior to lapatinib when combined with endocrine therapy. AZD8931 inhibited cell proliferation more effectively than lapatinib in MCF7 E2 and ED combinations and in all combinations in the T47D TamRes models (Fig. 2c). Ful in combination with AZD8931 significantly induced apoptosis in MCF7 TamRes cells to a greater extent than lapatinib. In the T47D TamRes model, lapatinib significantly induced apoptosis in combination with only ED or Ful, whereas AZD8931 induced apoptosis in combination with all 3 endocrine treatments (Fig. 2c). Taken together, these data suggest that in parental models, where the cells are less dependent on the HER pathway, AZD8931 does not enhance endocrine therapy activity. On the other hand, AZD8931 shows significant activity in the TamRes cell models, where the modestly increased levels of HER receptors and/or ligands contribute to endocrine resistance.

AZD8931 is a potent inhibitor of EGF- and HRG-mediated signaling and growth

We next explored the conditions under which AZD8931 manifests its greatest effects to inhibit HER signaling in the context of endocrine therapy. AZD8931 has been shown to block ligand-induced activation of HER receptors in several solid tumor cell models [32]. We evaluated AZD8931 effects on both EGF-and HRG-mediated signaling, since members of both ligand families were up-regulated in our TamRes models (Fig. 1a), and since overexpression of HRG results in de novo endocrine resistance [22]. As expected, EGF treatment increased phosphorylation of HER2 and EGFR, AKT

and MAPK in both MCF7 and T47D TamRes models (Fig. 3a). In EGF-mediated signaling, which induces EGFR homo and hetero-dimerization, gefitinib in combination with Tam decreased the levels of pAKT and pMAPK much more effectively than lapatinib, a weaker EGFR inhibitor. Importantly, AZD8931 combined with Tam or Ful consistently showed stronger inhibition of downstream signaling than either gefitinib or lapatinib. As expected, HRG also strongly activated AKT and MAPK signaling in both MCF7 and T47D TamRes cells (Fig. 3b). AZD8931 was the most potent agent in blocking phosphorylation of HER2 and HER3, as well as downstream effectors AKT and MAPK, in both Tam and Ful conditions, although both lapatinib and, to a much lesser extent, gefitinib inhibited AKT and MAPK activation as well. Finally, AZD8931 significantly inhibited cell growth under ligand-stimulated conditions in a dose-dependent manner (Fig. 3c). These data suggest that AZD8931 is a more effective inhibitor of ligand-dependent HER receptor signaling and growth in combination with endocrine therapy than lapatinib and gefitinib. The results also suggest that both EGFR and HER2 need to be blocked for maximal growth inhibition.

AZD8931 significantly delays Tam-resistant tumor growth in vivo

While our data clearly indicate that AZD8931 can inhibit TamRes breast cancer cells in vitro, it is still unclear whether AZD8931 treatment can circumvent tamoxifen resistance in vivo. Therefore, we investigated whether AZD8931 treatment could delay the growth of MCF7 TamRes xenografts where resistance is associated with up-regulation of the HER pathway [36]. We transplanted established MCF7 TamRes tumor xenografts into nude mice in the presence of Tam. When the tumors reached 200 mm^3, mice were randomized to continued Tam, Tam+AZD8931, Ful, or Ful+AZD8931. In accordance with our in vitro results, the addition of AZD8931 to Tam significantly delayed TamRes tumor growth. Ful treatment alone was more potent than Tam+AZD8931, but the combination of AZD8931 plus Ful was most effective in delaying tumor growth (Fig. 4a). It should be emphasized, however, that although these treatments slowed tumor growth, none of them resulted in significant tumor regression, suggesting that other survival pathways must also be functioning to cause tamoxifen resistance in these models.

To evaluate the mechanisms by which AZD8931 delays tamoxifen resistance, tumors harvested after short (11 days) or long (when tumors reached 1,000 mm^3) term treatment were subjected to western blot analysis (Fig. 4b). Levels of pEGFR and HER2, as well as their downstream effectors pMAPK, pAKT, pFOXO3a, and Cyclin D1, were analyzed. AZD8931+Tam inhibited pHER2, pMAPK, and pAKT
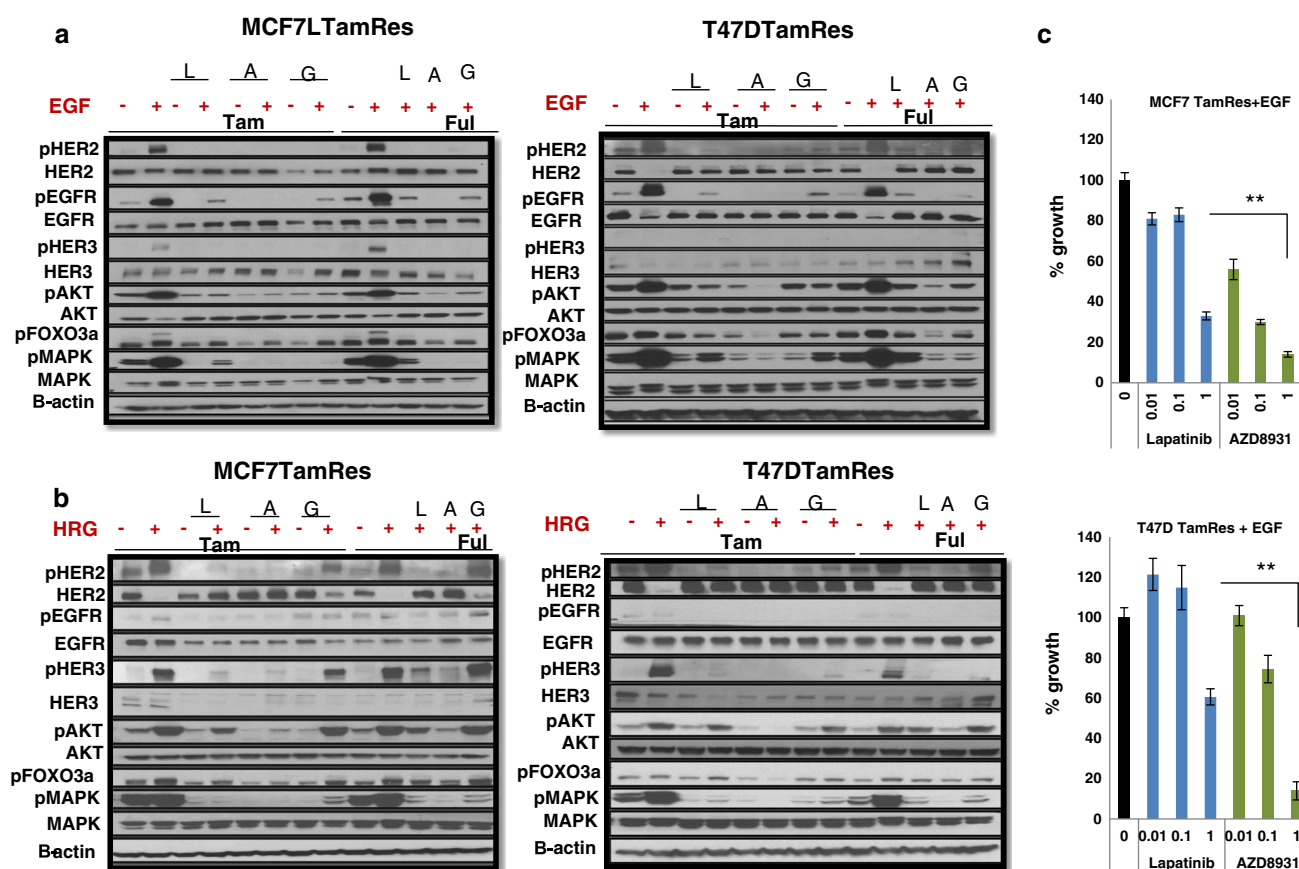
Fig. 3 Effects of anti-HER inhibitors on EGF and HRG-stimulated HER signaling. **a** MCF7 and T47D TamRes cells were serum-starved for 24 h and treated with endocrine therapy, with and without lapatinib (L), AZD8931 (A), or gefitinib (G) as described in Materials and Methods. Cells were treated with EGF for 10 min, and effects of treatment on HER signaling were assessed by western blotting. **b** MCF7 and T47D TamRes cells were serum-starved for 24 h and treated as described in "Materials and methods". Cells were treated with HRG for 10 min, and effects of treatment on HER signaling were assessed by western blotting. Both EGF and HRG stimulation further increased the expression of phospho-HER receptors as well as their downstream signals (pAKT and pMAPK). AZD8931 treatments more potently inhibited the levels of phospho-(HER2, EGFR, HER3, AKT, and MAPK) than lapatinib in both TamRes models. **c** Both cell line models were starved and treated with different concentrations of lapatinib or AZD8931 under EGF stimulation. Effect on growth was analyzed after 6 days. ******<.001 lapatinib compared to AZ8931

levels in both short and long-term treated tumors compared to tumors treated with Tam alone. Ful reduced ER levels particularly in long-term treated tumors. Ful alone in long-term treated tumors reduced pHER2, but had little effect on pMAPK and pAKT compared to Tam in long-term treated tumors. AZD8931 combined with Ful reduced the levels of pHER2, pMAPK, pAKT, and pFOX03a. Furthermore, AZD8931 in combination with Ful inhibited cyclin D1 levels more than the other treatment groups.

Discussion

Crosstalk between ER and HER2 pathways has been shown to play an important role in endocrine resistance [20, 37] and it is known that HER2+ tumors are more intrinsically resistant to endocrine therapy [19, 20]. Similarly, in ER+HER2− (HER2-low) tumors, modestly elevated levels of the EGFR and HER2 receptors and/or their ligands upon chronic endocrine treatment have been suggested to play an important role in acquired endocrine resistance [5, 6, 23, 38]. Previous studies showed that both the EGFR TKI gefitinib and the dual EGFR/HER2 TKI lapatinib only modestly and/or transiently delayed the development of endocrine resistance [25, 26, 39–41]. This modest response to HER inhibitors may at least partly be due to the incomplete blockade of the HER receptor layer by gefitinib (EGFR inhibitor), and because lapatinib is most effective in a HER2-amplified breast cancer and is less effective in inhibiting EGFR and ligand-dependent activation of the HER pathway [5, 32]. Here, we used two parental ER+/HER2-negative (or HER2-low) cell line models and their corresponding tamoxifen-resistant derivatives and compared the therapeutic effect of lapatinib with the equipotent
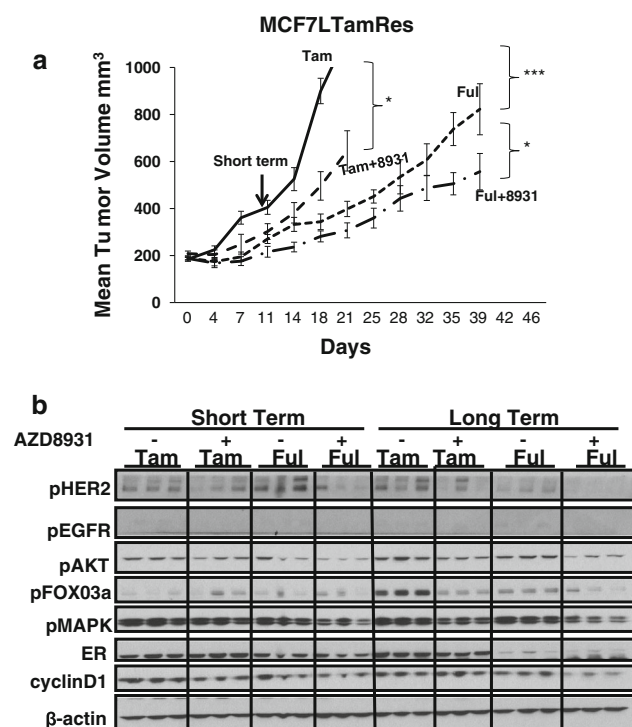
Fig. 4 Effects of AZD8931 in overcoming endocrine resistance in vivo. **a** TamRes tumor xenografts were transplanted into nude mice and continuously grown with Tam. After reaching 200 mm³, tumors were randomized to continued Tam, Tam+AZD8931, Ful, or Ful+AZD8931. Tumor size tripling from the randomization was considered as the survival event of tumor progression.*$p < 0.05$ (Tam vs Tam+8931, Tam+8931 vs Ful+8931), ***$p < 0.0001$ (Tam vs Ful). **b** Immunoblotting analysis of phospho-HER receptors and their downstream effectors, phospho-(MAPK, AKT and FOXO3a) in three representative individual tumor extracts per group, treated by Tam or Ful alone or in combination with AZD8931 at 11 days of treatment (short-term), or after long-term treatment (harvested at 1,000 mm³). All tumors were harvested 4 h. post treatment and extracts were on the same gel, and ß-actin is shown as the control for equivalent loading

dual EGFR/HER2 TKI AZD8931, which more effectively blocks EGFR and ligand-dependent HER signaling, when both are combined with endocrine therapies (32).

Our in vitro data show that AZD8931 does not enhance endocrine therapy sensitivity in the parental cells with low-HER ligand and signaling (i.e., MCF7-like). In contrast, both the MCF7 and T47D tamoxifen-resistant models were characterized by up-regulation of HER-ligands and receptors. In this setting, with increased dependence on the HER pathway for growth and survival, AZD8931 was slightly more effective than lapatinib in enhancing the response of these cells to multiple endocrine therapies. Fulvestrant, which results in ER degradation and would be expected to inhibit ligand-dependent and independent ER activity, had the most synergistic effect. In our in vivo model of tamoxifen resistance, we also found that AZD8931 in combination with tamoxifen significantly slowed growth.

Fulvestrant alone also significantly slowed tamoxifen-resistant tumor growth in vivo as has been previously shown [42]. This suggests that in tamoxifen-resistant breast cancer cells, ER still plays a role in promoting growth. As previously shown, increased HER signaling can either modify ER activity to promote endocrine resistance [5, 29], or can promote additional alternative signaling to bypass ER [43]. Indeed, we found that the combination of fulvestrant with AZD8931 inhibited tamoxifen-resistant tumor growth significantly better than either treatment alone, emphasizing the importance of dual-pathway blockade in overcoming endocrine resistance. Of note, although AZD8931 in combination with fulvestrant inhibited tumor growth, tumor regression did not occur. Western blot analyses of xenografts harvested at time of tumor progression on fulvestrant combined with AZD8931, showed that even with potent and sustained inhibition of pHER2; downstream pAKT and pMAPK levels were still maintained, suggesting the presence of additional compensatory pathways contributing to endocrine resistance in these cells.

AZD8931 remains effective in the presence of HER-ligands, distinguishing itself as a more potent inhibitor of HRG and EGF-induced activation of HER signaling, while the anti-tumor effects of other inhibitors such as lapatinib [31, 44] and gefitinib [45] are decreased by these HER-ligands. Previous data showed that the drug lapatinib effectively blocks HER signaling and tumor growth in HER2+ tumors [41]. Our data suggest that in ER+ settings of acquired tamoxifen resistance, where tumors express increased levels of HER-ligands and receptors, a TKI such as AZD8931 may be a better choice, as it is more effective in global inhibition of ligand-dependent activation of the HER pathway. Importantly, our in vitro and in vivo data suggest that an effective combination to study is a HER inhibitor such as AZD8931 with fulvestrant, which degrades ER and would block ER that has been activated by growth factor signaling in an estrogen-independent way.

A Phase II clinical study, the MINT trial, with AZD8931 in ER+ breast cancer has recently been reported [46]. In this trial, AZD8931 was combined with anastrozole in women with endocrine therapy-naive advanced breast cancer where growth factor signaling would be expected to be less, and overall there was no evidence of significant therapeutic benefit. It is interesting to speculate based on our preclinical data whether the use of AZD8931 in a different group of patients might have resulted in greater clinical benefit. Indeed, a recently published study correlated levels of ER to response in a trial of letrozole versus letrozole plus lapatinib in metastatic ER-positive, HER2-negative breast cancer patients. That study showed that the subgroup of patients with lower levels of ER benefited significantly from the addition of lapatinib to letrozole,

while those with high-ER content did not [28]. This demonstrates the importance of patient selection when designing trials studying the role of drugs targeting pathways associated with endocrine therapy resistance. Patients who are likely to be resistant to endocrine-therapy-alone should be selected for these trials, or we would risk having negative trial results driven by the inclusion of patients with endocrine therapy-sensitive tumors and diluting out the benefit experienced in patients with resistant tumors.

Our data also suggest that resistance to endocrine therapy in a tumor is likely to be multi-factorial and might require inhibition of multiple signaling pathways in addition to ER and HER2.

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