HER2 Overexpression Renders Human Breast Cancers Sensitive to PARP Inhibition Independently of Any Defect in Homologous Recombination DNA Repair

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Abstract

HER2 overexpression in breast cancer confers increased tumor aggressiveness. Although anti-HER2 therapies have improved patient outcome, resistance ultimately occurs. PARP inhibitors target homologous recombination (HR)-deficient tumors, such as the BRCA-associated breast and ovarian cancers. In this study, we show that HER2+ breast cancers are susceptible to PARP inhibition independent of an HR deficiency. HER2 overexpression in HER2 negative breast cancer cells was sufficient to render cells susceptible to the PARP inhibitors ABT-888 and AZD-2281 both *in vitro* and *in vivo*, which was abrogated by HER2 reduction. In addition, ABT-888 significantly inhibited NF-κB (p65/RelA) transcriptional activity in HER2+ but not HER2 negative breast cancer cells. This corresponded with a reduction in phosphorylated p65 and total IKKα levels, with a concomitant increase in IκBα. Overexpression of p65 abrogated cellular sensitivity to ABT-888, whereas IκBα overexpression reduced cell viability to a similar extent as ABT-888. Therefore, susceptibility of HER2+ breast cancer cells to PARP inhibition may be because of inhibition of NF-κB signaling driven by HER2. Our findings indicate that PARP inhibitors may be a novel therapeutic strategy for sporadic HER2+ breast cancer patients. *Cancer Res; 72(18); 4796–806. ©2012 AACR.*

Introduction

The human epidermal growth factor receptor 2 (HER2) is a proto-oncogene that belongs to a family of 4 transmembrane receptor tyrosine kinases that mediate the growth, differentiation, and survival of cells. Overexpression of the HER2 protein, amplification of the *HER2* gene, or both, occur in approximately a 3rd of breast cancers and are associated with aggressive behavior in the tumor (1). This may be, in part, because of activation of the NF-κB signaling pathway by HER2, which enhances cell proliferation, invasion, and resistance to therapies (2–4). HER2 activation of NF-κB requires IKKα, and this activation leads to an increase in cytokine and chemokine expression, as well as an increase in invasive phenotype (3). In addition, activation of NF-κB depends on PARP (5, 6). Targeted therapy against HER2 has been shown to benefit patients with HER2+ breast cancer (1). However, a significant number of patients either do not respond or quickly relapse and exhibit resistance to therapy. Thus, novel therapeutic strategies are needed.

PARP inhibitors have shown initial promise in clinical trials for their single-agent activity in *BRCA*-associated breast and ovarian cancers, based on synthetic lethal interactions between PARP inhibition and homologous recombination (HR) repair defects (7–9). This is because of the conversion of single-strand DNA breaks into double-strand breaks in PARP inhibited cells, which is normally repaired by HR. However, because *BRCA*-associated tumors are deficient in HR, the double-strand break persists and is lethal to the tumor cell. Normal tissues still possess intact HR, which explains why minimal side effects have been observed in these patients. However, very few studies report the efficacy of PARP inhibition alone in sporadic cancers, presumably because of their intact HR repair. Instead, numerous trials incorporate PARP inhibitors because of their ability to enhance the action of other DNA damaging agents. Thus, the use of PARP inhibitors as part of novel therapeutic combinations is currently under extensive investigation.

In this study, we unexpectedly observed exquisite susceptibility of HER2+ breast cancer cells to PARP inhibitors alone independent of an inherent HR deficiency. HER2 overexpression itself was sufficient to render breast tumor cells susceptible to PARP inhibition. The mechanistic insight of this intriguing sensitivity is described in this report and involves attenuation of NF-κB signaling driven by HER2. Our results suggest that PARP inhibitors may be useful for sporadic HER2+ breast cancer patients.

Materials and Methods

Cell culture

The human breast cancer cell line MDA-MB-361 was obtained courtesy of Dr. Andra Frost (University of Alabama

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at Birmingham, Birmingham, AL), whereas SKBR3 and HCC 1954 were obtained courtesy of Dr. Donald Buchsbaum (University of Alabama at Birmingham). The human breast cancer cell lines BT-474 (HTB-20), MCF7 (HTB-22), and T47D (HTB-133) were obtained from American Type Culture Collection (ATCC). HER2-overexpressing MCF7 cells (MCF7 HER2) and its isogenic control (MCF7 NEO) were obtained courtesy of Drs. Rachel Schiff and Kent Osborne (Baylor College of Medicine, Houston, TX; refs. 10, 11). MCF7 DRGFP cells were a kind gift from Dr. Fen Xia (Ohio State University, Columbus, OH; ref. 12). The human breast cancer cell lines MDA-MB-361, SKBR3, and HCC 1954 were maintained in RPMI-1640 (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals). The human breast cancer cell line BT-474 (HTB-20) was cultured in RPMI-1640 supplemented with 10% FBS, 0.1% insulin (Sigma), and 4 mmol/L L-glutamine (Invitrogen). MCF7 (HTB-22) was maintained in DMEM (Invitrogen) supplemented with 10% FBS. MCF7 HER2 and MCF7 NEO were maintained in DMEM supplemented with 10% FBS, 0.4% G418 (Cellgro), and 15 μg/mL insulin. MCF7 DRGFP was cultured in DMEM supplemented with 10% FBS and 2 μg/mL puromycin (Sigma). T47D (HTB-133) was maintained in RPMI (Invitrogen) supplemented with 10% FBS. All cells were obtained in August 2010. The genetic background, including expression and function of key DNA repair and NF-κB signaling proteins, such as BRCA1, p53, p21, HER2, PAR, PARP, EGFR, p65, IκBα, and IKKα, as well as the growth characteristics and their response to genotoxic agents, was tested most recently on February 2012 using Western blot analysis, immunohistochemistry, and colony formation assays. All experiments were carried out within 10 passages, and no cell lines were kept in culture for more than 3 months after receipt or resuscitation. Cells obtained from the ATCC were also initially tested by ATCC via cytogenetic analysis and STR analysis.

Clonogenic survival assay

Cell survival was evaluated by the colony formation assay in the breast cancer cell lines as previously described (13, 14). Refer to Materials and Methods for details.

Cell viability

Cell viability was measured using the ATP Lite 1 step luminescence assay (Perkin Elmer) following the manufacturer's directions. Details are provided in Materials and Methods.

Immunofluorescence

To assay HR-mediated DSB repair capacity in breast cancer cell lines immunohistochemistry for radiation-induced rad51 foci was evaluated as previously described (13, 14). Immunofluorescence for HER2 expression in MCF7 HER2 tumor xenografts and H&E staining was carried out as previously described (15).

Drugs, plasmids, and transfection

ABT-888 was obtained from Enzo Life Sciences (catalog no. ALX-270-444), whereas lapatinib (catalog no. L-4804), iniparib (catalog no. I-5432) and olaparib (catalog no. O-9201) were obtained from LC Laboratories. The P65-DsRed and IκBα GFP

plasmids were obtained courtesy of Dr. Markus Bredel (University of Alabama at Birmingham). pDsRed and peGFP controls were obtained from Clontech. DR-GFP to measure chromosomal HR repair capacity, ISce-1 and the empty vector were gifts from Dr. Fen Xia (Ohio State University) and has been described previously (12). All transfections were carried out using Lipofectamine according to the manufacturer's recommendations (Invitrogen).

Chromosomal HR-mediated repair analysis

MDA-MB-361 cells were transfected with DRGFP substrate and stable integrant were selected with 2 μg/mL of puromycin (Sigma) for 3 weeks. Puromycin-resistant colonies were isolated and expanded. Chromosomal HR-mediated repair capacity was determined as described previously (12). Cells were transfected with either an empty vector, ISce-1 expression vector to measure HR-mediated repair capacity, or a GFP expression vector to measure transfection efficiency. %GFP+ cells were detected by flow cytometry. HR relative to total transfected cells was determined by division of the %GFP+ cells from each ISce-1 transfection by the % GFP+ cells from a parallel GFP transfection. Details are provided in Materials and Methods.

Immunoblotting

Immunoblotting was carried out as described previously (13). Antibodies used are described in Materials and Methods.

HER2 knockdown

Endogenous HER2 was knocked down using SignalSilence HER2/ErbB2 siRNA I (catalog no. 6283; Cell Signaling Technology). Scrambled siRNA (catalog no. 6568; Cell Signaling Technology) was used as a control.

Luciferase reporter assay

NF-κB transcriptional activity was measured using the NF-κB Secreted Luciferase Reporter System (catalog no. 631728; Clontech) according to the manufacturer's instructions.

Tumor growth delay

Three- to four-week-old female NOD.CB17-Prkdcscid/J mice were obtained from Jackson Labs and allowed a 1-week acclimatization period. Mice were anesthetized and supplemented with 0.72 mg 17β estradiol pellets from Innovative Research. After recovery from pellet implantation, 2.5×10^6 cells were injected into the mammary fat pad. Once the tumors were palpable (∼5 mm) mice were randomized into groups ($n = 8$): control and ABT-888 (100 mg/kg). ABT-888 was administered twice daily by oral gavage for 34 consecutive days. Tumor size was measured using digital calipers on alternate days and tumor volume was calculated using the following formula: $1/2 \times$ length $\times$ width^2. Tumor size was measured for 35 days after which mice were sacrificed. All animal procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (Animal protocol no. 101109241).

Statistical analysis

The data were analyzed via ANOVA followed by a Bonferroni post-test using GraphPad Prism version 4.02 (GraphPad Software). Data presented as average $\pm$ SEM.
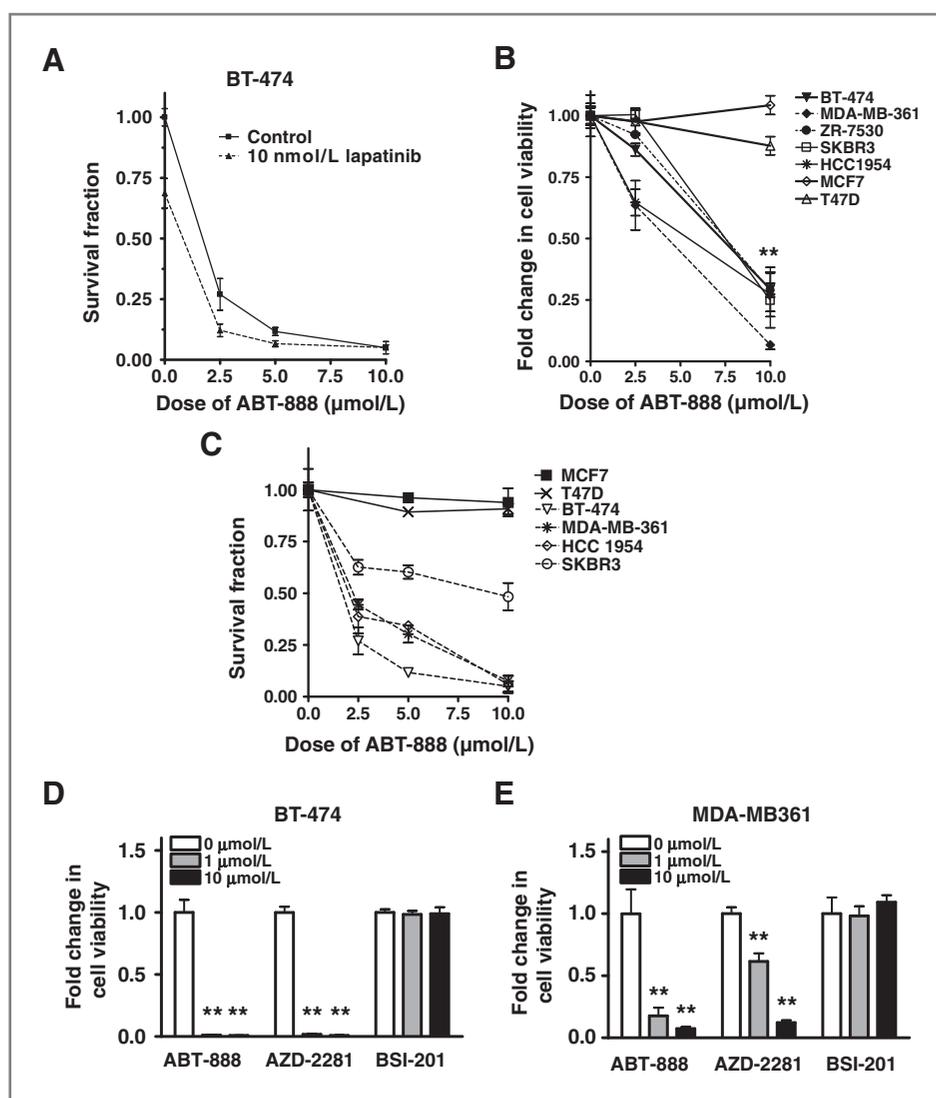
Figure 1. HER2-overexpressing breast cancer cells are susceptible to PARP inhibition alone. A, ABT-888 with or without lapatinib reduces the colony-forming ability of HER2-overexpressing BT-474 human breast cancer cells. Cells were seeded for colony formation assay and treated with 10 nmol/L lapatinib or vehicle control. Sixteen hours after initial treatment, the cells were exposed to different doses of ABT-888 or vehicle control. Shown is the mean survival fraction ($\pm$SEM) from at least 3 independent experiments ($P < 0.01$). B and C, other HER2-overexpressing breast cancer cells are also exquisitely susceptible to ABT-888. Breast cancer cell lines were seeded for ATP lite 1 step luminescence assay (B) and colony formation assays (C) and exposed to various doses of ABT-888 or vehicle control. As expected, the HER2 negative MCF7 and T47D cell lines failed to exhibit cytotoxicity to ABT-888. Shown is the mean survival fraction ($\pm$SEM) from at least 3 independent experiments ($P < 0.01$). D and E, HER2-overexpressing cells are susceptible to ABT-888 and AZD-2281 but not BSI-201. Cell viability was measured using ATP Lite assay after 24 hours treatment with various doses of ABT-888, AZD-2281, BSI-201, or vehicle control. Both BT-474 (D) and MDA-MB-361 (E) were susceptible to ABT-888 or AZD-2281 alone but not BSI-201. Shown is the average fold change in cell viability ($\pm$SEM) from at least 3 independent experiments carried out in quadruplicate (**$P < 0.01$).

Results

HER2+ breast cancers are susceptible to PARP inhibitors alone independent of steroid receptor or p53 status

We recently reported that cetuximab, which inhibits the EGFR (HER1) signaling pathway, can generate a DNA repair defect in head and neck cancer cells and subsequently induce a contextual synthetic lethality with the PARP inhibitor ABT-888 (veliparib; ref. 13). We thus hypothesized that lapatinib, a dual tyrosine kinase inhibitor that interrupts the HER1/HER2 growth receptor pathways, would generate a similar DNA repair deficit and induce susceptibility to ABT-888 in human HER2+ breast cancer cells. Consistent with our hypothesis, lapatinib indeed significantly reduced HR-mediated repair capacity in the well-characterized BT-474 human HER2+ breast cancer cells (Supplementary Fig. S1A; ref. 16). However, unexpectedly, ABT-888 alone caused similar levels of cytotoxicity as the combination treatment of lapatinib and ABT-888 (Fig. 1A). A subtherapeutic dose of lapatinib (10 nmol/L) alone, which was chosen to test for synergy with PARP inhibition, produced a 30% reduction in the survival fraction of these cells.
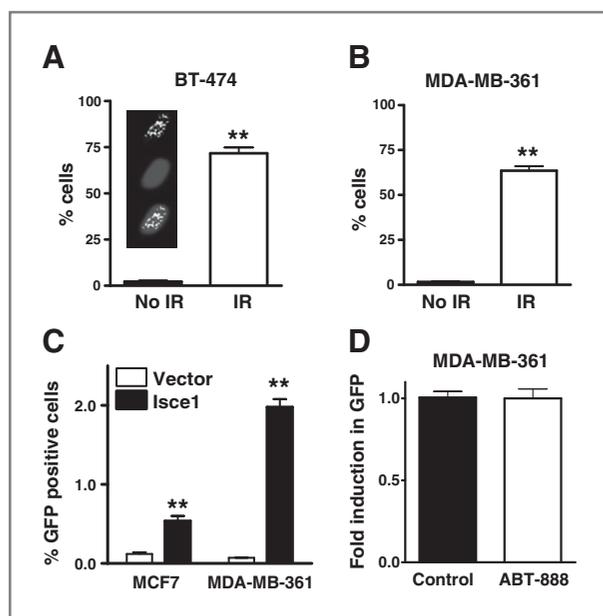
Figure 2. Susceptibility of HER2+ breast cancer cells to PARP inhibition is independent of a homologous recombination (HR) defect. HR repair capacity was measured in BT-474 (A) and MDA-MB-361 (B) human HER2+ breast cancer cell lines by assessing radiation-induced rad51 foci, a well-characterized marker for HR repair. Briefly, cells were exposed to mock or 4-Gy irradiation (IR) and subsequently subjected to immunofluorescence staining for rad51 foci. Shown is the representative data of 3 independent experiments the % of cells (mean ± SEM) with rad51 foci (**, $P < 0.01$ compared with vehicle). Inset, representative staining of a cell exhibiting rad51 foci (top) with the nucleus stained with DAPI (middle). A merged image is also shown (bottom). C, chromosomal HR repair capacity was directly measured in MCF7 and MDA-MB-361 cells stably expressing the DRGFP repair substrate. Forty-eight hours after transfection with ISce-1 or control vector, cells were subjected to flow cytometry for GFP expression. Shown is the representative data of 3 independent experiments the %GFP+ cells (mean ± SEM) (**$P < 0.01$ compared with vector control). D, ABT-888 does not induce an HR defect. MDA-MB-361 cells expressing DRGFP were treated with 10 μmol/L of ABT-888 or vehicle control. Eight hours after the treatment period, cells were transfected with ISce-1 or control vector. Forty-eight hours after transfection, cells were subjected to flow cytometry for GFP expression. Shown is the representative fold induction in GFP (mean ± SEM) from at least 3 independent experiments.

To further confirm our observations that ABT-888 alone was cytotoxic to HER2+ breast cancer cells, the effects of ABT-888 on cell viability (Fig. 1B) were assayed using ATP Lite 1 step luminescence assay on four other HER2-overexpressing cell lines of various steroid receptor (estrogen and progesterone, ER/PR, respectively) and p53 status (Supplementary Table S1), including MDA-MB-361, HCC1954, SKBR3, and ZR-7530 (16). Intriguingly, all HER2+ breast cancer cell lines showed exquisite susceptibility to ABT-888.

Because assays of cell viability are often a measure of cellular metabolic activity and not necessarily cytotoxicity, and because PARP activity can change cellular levels of NAD+ and ATP (17), the effects of PARP inhibition in our assays may be because of alterations in metabolic processes rather than cell death itself. Thus, to validate PARP inhibitor-mediated susceptibility of HER2+ breast cancer cells, we used the gold

standard cytotoxicity assay, the colony formation assay. As shown in Fig. 1C, ABT-888 also significantly reduced the survival fraction of all HER2-overexpressing cells tested in a dose-dependent manner but, as expected, showed minimal cytotoxicity in non-HER2-overexpressing MCF7 and T47D cells ($P < 0.01$). These results confirmed our cell viability data that HER2+ breast cancer cells are susceptible to PARP inhibition. Thus, for the remainder of our susceptibility experiments, we utilized the ATP Lite 1 step luminescence cell viability assay.

To further assess whether susceptibility of HER2+ breast cancer cells to ABT-888 can be generalized to other PARP inhibitors, we next tested the efficacy of BSI-201 (iniparib) and AZD-2281 (olaparib), currently used in clinical trials, in BT-474 and MDA-MB-361 HER2+ breast cancer cells. Similar to results observed with ABT-888, BT-474 (Fig. 1D), and MDA-MB-361 (Fig. 1E) cells were susceptible to AZD-2281 alone. However, consistent with recent reports that BSI-201 is not a bona fide PARP inhibitor, HER2+ breast cancer cells did not exhibit significant cytotoxicity after BSI-201 treatment (18, 19). These novel and intriguing results suggested that indeed, human HER2+ breast cancer cells are susceptible to PARP inhibitors alone independent of ER, PR, and p53 status.

HER2+ breast cancer cells do not possess an inherent or induced HR-mediated repair deficiency

PARP inhibitors have been previously shown to target HR-deficient cells (20–22). Our unanticipated and novel results of susceptibility of HER2-overexpressing breast cancer cells to ABT-888 and AZD-2281 as a single agent put forth the intriguing hypothesis that HER2+ breast cancer cells may possess an inherent HR-mediated DNA repair defect despite not harboring BRCA mutations. Thus, to assess the inherent HR repair capacity of HER2+ cells, we first analyzed rad51 foci, a well-established functional marker of HR repair activity (13, 14). Because basal levels of HR repair are typically very low, radiation was used to create additional DNA damage and amplify the HR response. As shown in Fig. 2, a robust induction in rad51 levels was observed in both BT-474 (72% after radiation treatment vs. 2.1% in control; Fig. 2A) and MDA-MB-361 (64% after radiation treatment vs. 1.6% in control; Fig. 2B) cells ($P < 0.01$). As a positive control, the HER2 negative MCF7 cells, which are repair proficient, also exhibited robust induction in rad51 after radiation (70% after radiation treatment vs. 6.8% in control; Supplementary Fig. S2). This suggested that HER2+ breast cancer cells do not harbor defects in HR-mediated repair.

To further substantiate intact HR-mediated repair in these cells, we also directly measured HR using a GFP-based chromosomal HR repair assay (12). MDA-MB-361 cell lines stably expressing the DRGFP HR repair substrate were generated and transiently transfected with the ISce-1 endonuclease, which induces a DNA double-strand break. Forty-eight hours after transfection, GFP+ cells, indicative of HR-mediated repair of the ISce-1–induced DSB, were sorted by flow cytometry. As shown in Fig. 2C, a significant increase in GFP+ cells was observed in ISce-1–transfected cells compared with vector alone. As a positive control, the well-characterized MCF7 DRGFP cells (12), which possesses only a single integrated copy of DRGFP, were also analyzed for chromosomal HR repair
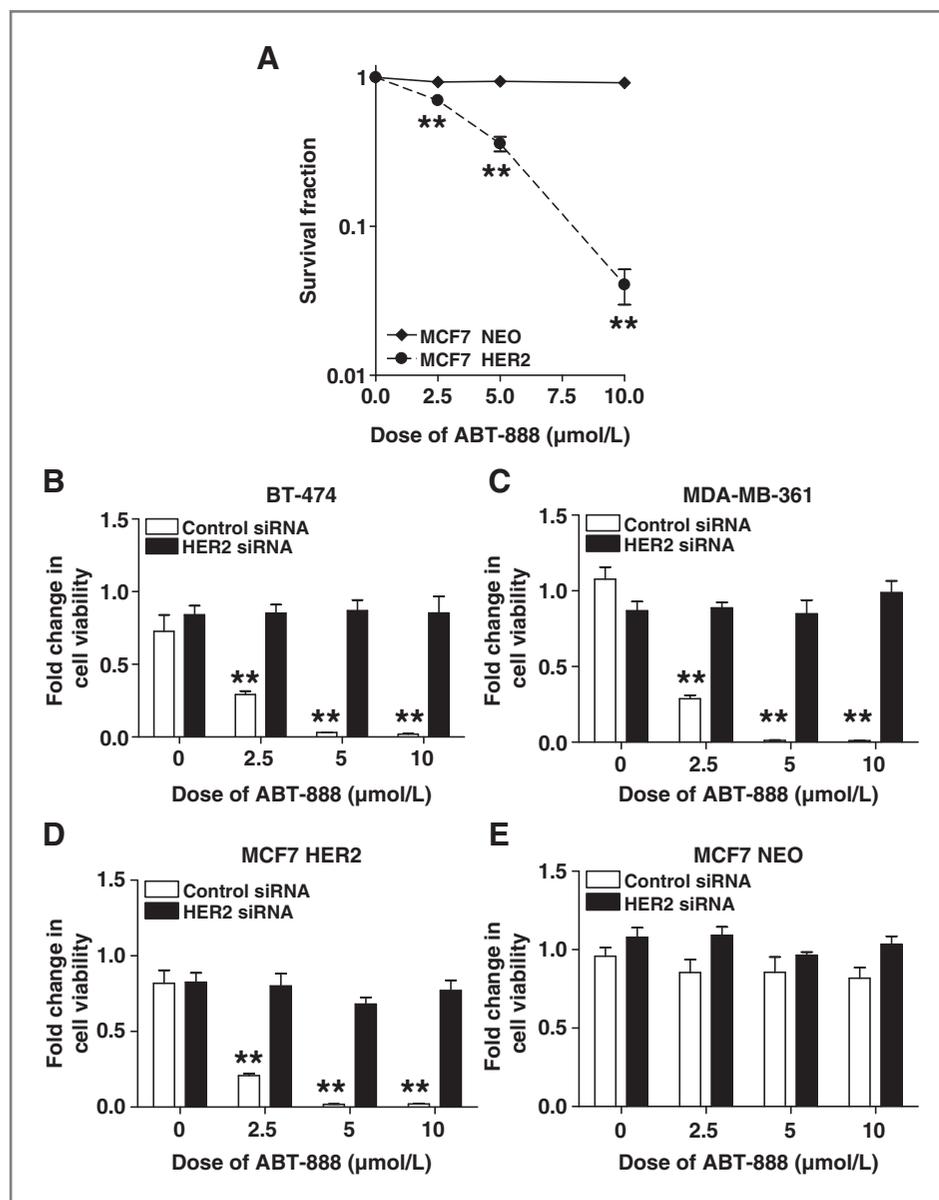
Figure 3. HER2 overexpression is sufficient to confer susceptibility to ABT-888 while silencing HER2 expression induces cellular resistance to ABT-888. A, MCF7 cells stably expressing HER2 (MCF7 HER2) and its isogenic control were seeded for colony formation assay and exposed to different doses of ABT-888 or vehicle control and left until colonies formed. Shown is the mean survival fraction ($\pm$SEM) from at least 3 independent experiments. B–E, HER2+ breast cancer cells were transfected with HER2 or scrambled siRNA. Twenty-four hours after transfection, cells were exposed to various doses of ABT-888 or vehicle control. Cell viability of BT-474 (B), MDA-MB-361 (C), MCF7 HER2 (D), and MCF7 NEO (E) cells was subsequently measured 24 hours after ABT-888 using the ATP Lite 1 step luminescence assay. Shown is the average fold change in cell viability ($\pm$SEM) from at least 3 independent experiments carried out in quadruplicate (**$P < 0.01$).

capacity. These results reaffirmed that HER2-overexpressing cell lines do not possess an inherent repair deficiency.

We next hypothesized that it may be possible that ABT-888 induced an HR deficit as previously reported (21). As shown in Fig. 2D, no significant difference in HR capacity as measured by the %GFP+ cells was observed after ABT-888. Thus, the vulnerability of HER2+ breast cancer cells to PARP inhibitors alone appeared to also be independent of an induced HR repair deficiency.

HER2 overexpression itself is sufficient to confer susceptibility to ABT-888

Because the susceptibility of HER2+ breast cancer cells to PARP inhibitors was not because of an inherent or induced HR-mediated repair deficiency, we postulated that one mechanism may involve HER2 itself, because HER2 negative cells maintain

resistance to PARP inhibition alone. To test our hypothesis, we assessed cellular susceptibility to PARP inhibitors in MCF7 cells (non–HER2-overexpressing) engineered to stably over-express HER2 (MCF7 HER2; refs. 10 and 11) and compared with its isogenic control (MCF7 NEO; courtesy of Drs. K. Osborne and R. Schiff). These cells have been previously shown to express functional HER2 levels comparable to BT-474 cells and, as typical of HER2+ cells, are sensitive to HER inhibition with lapatinib and trastuzumab (Herceptin; refs. 10 and 11).

Similar to our previous results with "native" HER2-over-expressing cells, MCF7 HER2 showed exquisite susceptibility to ABT-888 in a dose-dependent manner (Fig. 3A; $P < 0.01$). However, ABT-888 showed no effect on MCF7 NEO breast cancer cells. Similar results were obtained with AZD-2281 (Supplementary Fig. S3A and S3B). HER2 overexpression was thus sufficient to convert cellular susceptibility to these agents.
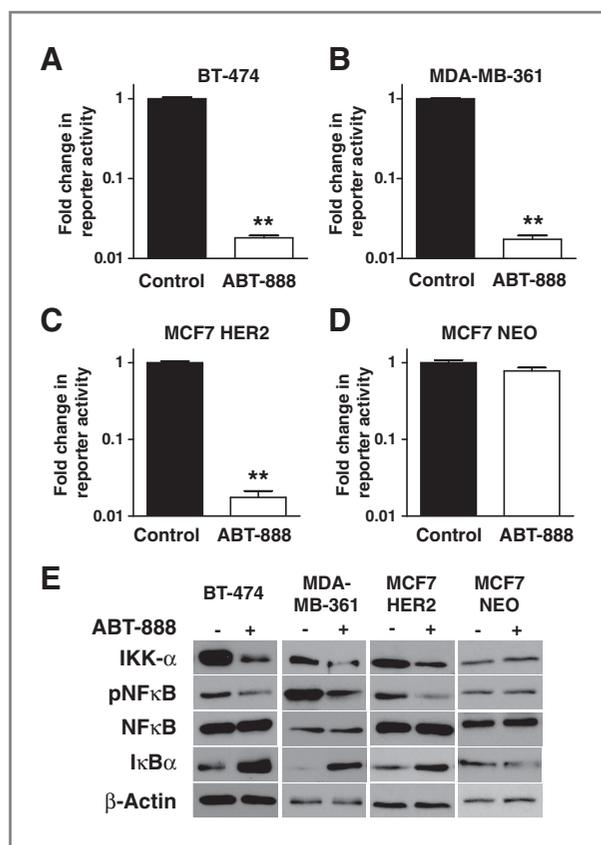
Figure 4. ABT-888 abrogates NF-κB transcriptional activity in HER2+ but not HER2 negative breast cancer cells. A–D, cells were seeded and transfected with the NF-κB–driven luciferase plasmid NF-κB-MetLuc2 or its vector control MetLuc2. Twenty-four hours after transfection, cells were exposed to 10 μmol/L ABT-888 or vehicle control. Twenty-four hours later, luciferase activity was assayed in BT-474 (A), MDA-MB-361 (B), MCF7 HER2 (C), and MCF7 NEO (D) cell lines. Shown is the average fold change in reporter activity (±SEM) from at least 3 independent experiments carried out in quadruplicate (**, $P < 0.01$). E, ABT-888 alters levels of proteins involved in NF-κB–mediated signaling. Exponentially growing breast cancer cells were seeded and subjected to either 10 μmol/L ABT-888 or vehicle control. Protein lysates were harvested 24 hours after the treatment and levels of IKKα, phospho p65, total p65, and IκBα were detected by immunoblotting. A dramatic reduction in IKKα and phospho P65 levels with a concomitant increase in IκBα levels and no alteration in total P65 levels was observed in the HER2-overexpressing BT-474, MDA-MB-361, and MCF7 HER2 cells. No alternations were observed in the non-HER2-overexpressing MCF7 NEO cells.

To further validate our hypothesis that HER2 overexpression conferred susceptibility to ABT-888, we transiently silenced HER2 expression using siRNA and subsequently assessed ABT-888–mediated cytotoxicity. As shown in Supplementary Fig. S3C, a significant (but incomplete) reduction in HER2 levels was observed 24 hours after HER2 siRNA transfection in BT-474, MDA-MB-361, and MCF7 HER2 cells, but not in control scrambled siRNA transfected cells. Similar to our results with a subtherapeutic dose of the HER inhibitor lapatinib, HER2 knockdown mildly reduced cell viability of HER2+ breast cancer cells. Importantly and consistent with our hypothesis, HER2 knockdown induced resistance of BT-474 (Fig. 3B), MDA-MB-361 (Fig. 3C), and

MCF7 HER2 (Fig. 3D) cells to ABT-888. No effect was observed in non-HER2–overexpressing MCF7 NEO control cells (Fig. 3E). Taken together, our findings support that HER2 itself was sufficient to confer susceptibility to ABT-888 in breast cancer cells.

NF-κB signaling is significantly inhibited by ABT-888

NF-κB (p65/RelA), a key transcription factor that can initiate prosurvival signals, is activated in many cancer cells overexpressing HER2 (4, 23). A possible cooperation between HER2 and NF-κB signaling in HER2+ breast tumor formation and resistance has also been previously reported (23, 24). Moreover, PARP and NF-κB have also been linked. Specifically, PARP1 is a coactivator of NF-κB and is required for its activity (5, 6). Thus, we hypothesized that 1 mechanism of ABT-888–mediated cell death may involve abrogation of NF-κB–mediated transcriptional activity. To test this hypothesis, we utilized an NF-κB–driven luciferase reporter assay. As shown in Fig. 4, addition of ABT-888 significantly reduced luciferase activity by >95% in the HER2-overexpressing cell lines BT-474 (Fig. 4A), MDA-MB-361 (Fig. 4B), and MCF7 HER2 (Fig. 4C) but not in the HER2 negative MCF7 NEO cell line (Fig. 4D). These results confirmed our hypothesis that indeed, ABT-888–mediated cytotoxicity corresponds to a reduction in NF-κB–mediated transcriptional activity. Further support of PARP inhibitor-mediated suppression of NF-κB–mediated transcription was found with reduction of levels of the NF-κB–regulated protein c-Myc (Supplementary Fig. S4; ref. 25).

Because NF-κB transcriptional activity was significantly inhibited by ABT-888, we proceeded to verify whether key proteins of the NF-κB–mediated growth pathways (p65, IKKα, IκBα) were also altered by ABT-888. As shown in Fig. 4E, the reduced NF-κB transcriptional activity corresponded with reduced levels of phosphorylated p65, decreased total IKKα, and a concomitant increase in the NF-κB inhibitor IκBα in HER2-overexpressing BT-474, MDA-MB-361, and MCF7 HER2 cells. Importantly, no changes in the protein levels were detected in MCF7 NEO control cells, further verifying the effect of ABT-888 on NF-κB signals only in the HER2-overexpressing cells.

If inhibition of NF-κB is an important mediator in HER2+ breast cancer cellular susceptibility to ABT-888, we hypothesized that overexpression of p65 would induce resistance to ABT-888. As shown in Supplementary Fig. S5A to S5C, transient overexpression of p65 resulted in increased NF-κB–driven luciferase activity in HER2+ breast cancer cells. Consistent with our hypothesis, this ectopic overexpression of p65 abrogated sensitivity to ABT-888 in BT-474 (Fig. 5A), MDA-MB-361 (Fig. 5B), and MCF7 HER2 (Fig. 5C). Interestingly, increased NF-κB expression had minimal effects on cell viability or NF-κB–driven luciferase activity in MCF7NEO controls (Fig. 5D and Supplementary Fig. S5D).

Conversely, we hypothesized that overexpression of IκBα, a regulatory protein that inhibits NF-κB, would reduce cell viability of HER2+ breast cancer cells to a similar extent as ABT-888. As expected, transient transfection of IκBα reduced NF-κB–mediated luciferase activity (Supplementary
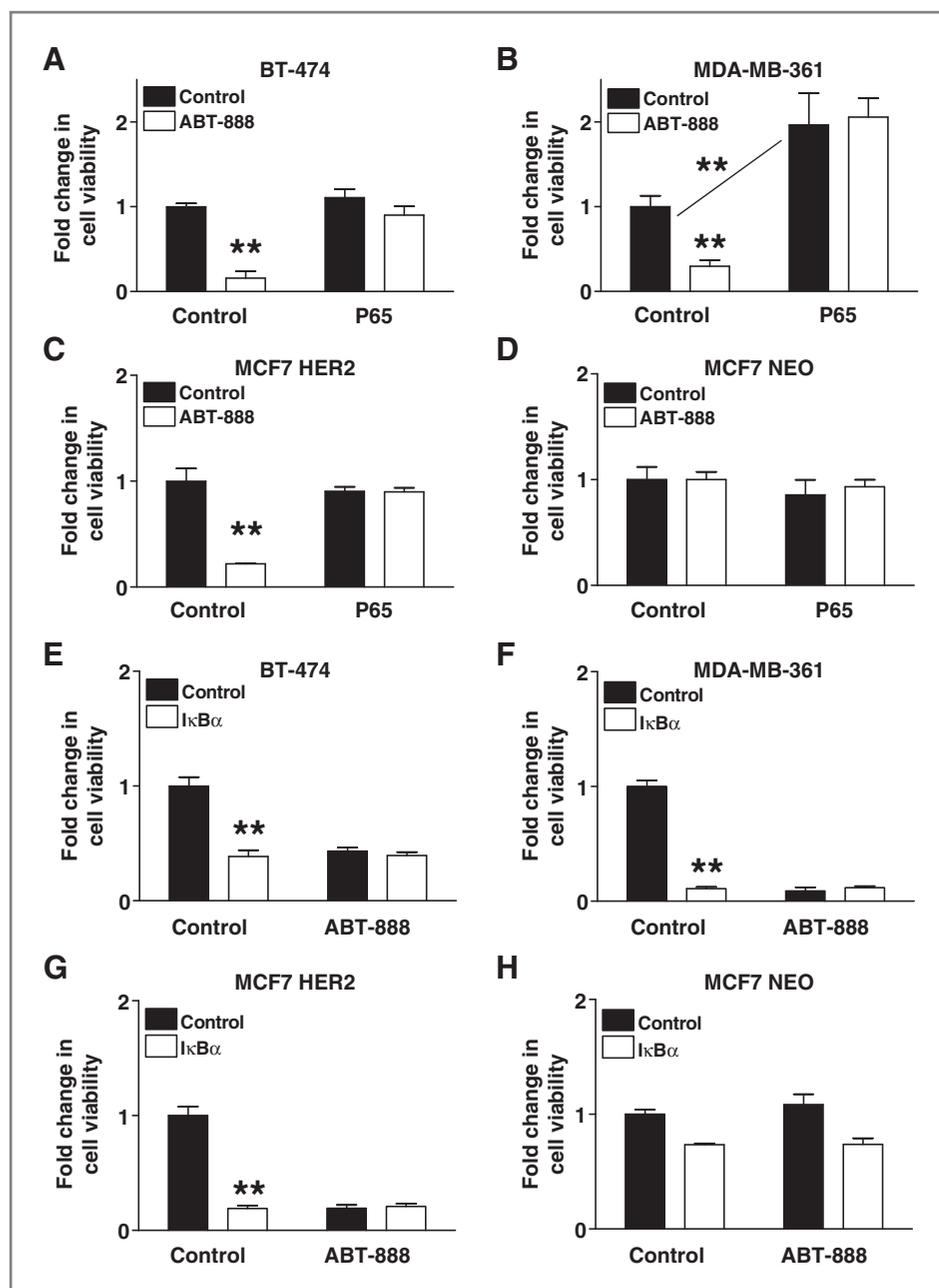
Figure 5. Overexpression of p65 induces ABT-888 resistance in HER2-overexpressing breast cancer cells, whereas overexpression of IκBα reduces cell viability to similar extent as ABT-888–treated cells. A–D, breast cancer cells were seeded and transfected with p65 DsRed or vector control. Twenty-four hours after transfection, cells were exposed to 10 μmol/L ABT-888 or vehicle control. Cell viability of BT-474 (A), MDA-MB-361 (B), MCF7 HER2 (C), and MCF7 NEO (D) cells was subsequently measured 24 hours later using ATP Lite 1 step luminescence assay. Shown is the average fold change in cell viability (±SEM) from at least 3 independent experiments carried out in quadruplicate (**$P < 0.01$). E–H, breast cancer cells were seeded and transfected with IκBα GFP or vector control. Twenty-four hours after transfection, cells were exposed to 10 μmol/L ABT-888 or vehicle control. Cell viability of BT-474 (E), MDA-MB-361 (F), MCF7 HER2 (G), and MCF7 NEO (H) cells was measured 24 hours later using ATP Lite assay. Shown is the average fold change in cell viability (±SEM) from at least 3 independent experiments carried out in quadruplicate (**, $P < 0.01$).

Fig. S5E–S5G). Supporting a role of NFκB suppression in susceptibility of HER2+ breast cancer cells to PARP inhibition, ectopic overexpression of IκBα induced similar levels of cytotoxicity as ABT-888 alone in BT-474 (Fig. 5E), MDA-MB-361 (Fig. 5F), and MCF7 HER2 (Fig. 5G). Again, minimal effects on cell viability or luciferase activity were observed in MCF7NEO controls (Fig. 5H and Supplementary Fig. S5H). Interestingly, the combination of IκBα overexpression and ABT-888 failed to augment cytotoxicity, further supporting the notion that ABT-888–mediated cell death involved inhibition of NF-κB. Taken together, susceptibility of HER2+ breast cancer cells to PARP inhibition may be, in part, because of inhibition of NF-κB that is driven by HER2.

ABT-888 delays growth of HER2-overexpressing tumors *in vivo*

To validate our observed effects *in vivo*, we assessed tumor growth delay in mice bearing orthotopic xenografts of the isogenic pair of MCF7 HER2 or MCF7 NEO. This pair was specifically chosen because our *in vitro* data not only point to HER2+ breast cancer cellular susceptibility to PARP inhibitors, but also that HER2 itself was sufficient to confer this cytotoxic response. Representative hematoxylin and eosin (H&E) stained images of MCF7 HER2 and MCF7 NEO tumor xenografts are shown in Fig. 6A. HER2 expression in MCF7 HER2 xenografts was also verified by immunofluorescence (Fig. 6B). As shown in Figs. 6A and C and similar to other reports (11), HER2
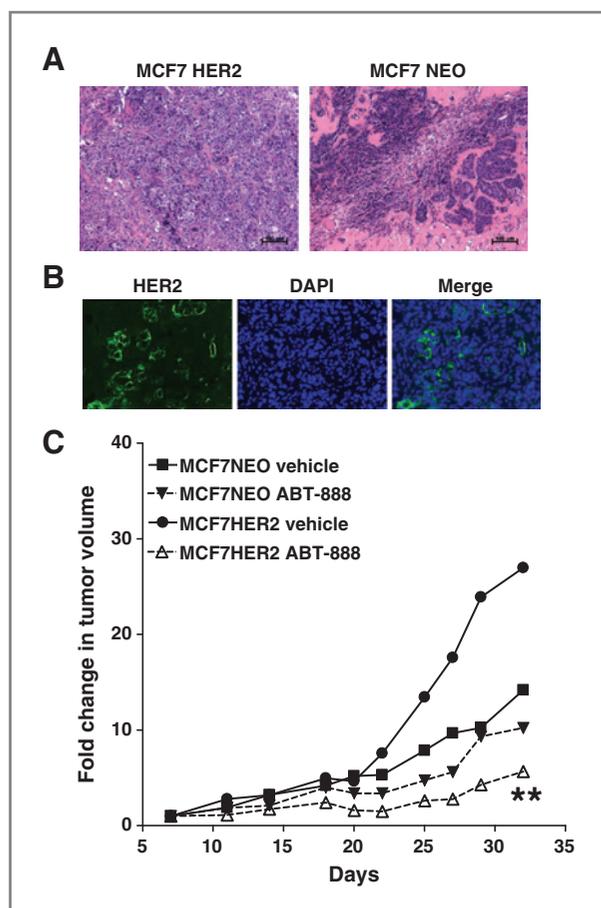
Figure 6. ABT-888 delays growth of orthotopically implanted HER2-overexpressing breast tumors *in vivo*. A, representative H&E staining of MCF7 HER2 and MCF7 NEO orthotopic tumor xenografts. Original magnification, ×100. B, HER2 expression was verified in MCF7 HER2 tumor xenografts. Original magnification, ×400. C, tumor growth delay of orthotopic breast tumor xenografts of the isogenic pair of MCF7 HER2 or MCF7 NEO. Mice were randomized into groups ($n = 8$ per group) and oral gavaged 100 mg/kg ABT-888 or vehicle control twice daily. Tumor volume was measured on alternate days. Shown is the mean fold change in tumor volume (**, $P < 0.01$).

overexpression itself conferred increased tumor aggressiveness as reflected by a more disaggregated and invasive appearance on histology as well as the faster growth of MCF7 HER2 tumors in mice compared with MCF7 NEO. In addition, administration of ABT-888 significantly delayed tumor growth of MCF7 HER2 xenografts (5-fold tumor growth delay in ABT-888 treated, $P < 0.01$). No significant tumor growth delay was observed in MCF7 NEO after ABT-888 treatment. Thus, these results validated the significant cytotoxic response of HER2+ breast tumors *in vivo* to ABT-888.

Discussion

PARP inhibitors have gained recent press because of their unique selectivity against HR-mediated DNA repair deficient tumors, although maintaining minimal toxicity in HR-proficient normal tissues (7–9). Despite the fact that the enrolled patients were heavily pretreated and had poor prognoses, the

tumor response rates were significantly better than the expected 20% or less with cytotoxic chemotherapy (8, 9). Importantly, these agents were well tolerated and produced minimal adverse events. However, this approach is only applicable to a small percentage of cancers, and much effort has been undertaken to expand the utility of PARP inhibitors beyond the realm of BRCA-associated tumors by combining with agents that alter the DNA damage/repair pathways. Indeed, PARP inhibitors have been reported to enhance cytotoxicity in sporadic tumors when combined with other DNA damaging agents, such as with platinum-based chemotherapy in breast cancer or with temozolamide in prostate and brain cancers (26–28). However, our results from this study implicate possible novel interactions of the PARP inhibitors ABT-888 and AZD-2281 with HER2 and NF-κB signaling that may have important therapeutic implications. Specifically, PARP inhibitors may be effective for HER2+ breast cancer patients to not only improve outcomes but also maintain patient quality of life.

There have been several reports that suggest sporadic tumor susceptibility to PARP inhibition alone (29, 30). The tumor suppressor phosphatase and tensin homolog (*PTEN*) is frequently lost in cancer cells resulting in genomic instability and subsequent altered radiation and drug sensitivity. It was shown that tumors harboring *PTEN* mutations are susceptible to PARP inhibition both *in vitro* and *in vivo* because of generation of a DNA repair deficiency (29). However, the role of PTEN in DNA repair is controversial. Recent studies in prostate cancer cells indicate that PTEN did not regulate rad51 expression or HR-mediated repair and did not confer susceptibility to PARP inhibition. Thus, it seems that *PTEN* mutation may not be an optimal biomarker for tumor sensitivity to PARP inhibition (31). Our results are likely unrelated to *PTEN* mutations, because the cells tested do not harbor *PTEN* mutations and were HR proficient (32–34). In addition, our results reveal for the first time a DNA repair independent mechanism of susceptibility to the PARP inhibitors ABT-888 and AZD-2281.

The micromolar doses of PARP inhibitors used in our study are compatible with those in other published reports (35–37). Specifically, for ABT-888, although pharmacokinetic studies in a Phase 0 trial revealed that a single 50 mg dose yielded a peak plasma concentration of 1 μmol/L (38), current clinical trials are now utilizing ABT-888 at 400 mg twice daily. In addition, our *in vivo* results in mice using 100 mg/kg twice daily suggest the tumor effects of ABT-888 are achievable. Thus, we believe the doses used in our studies remain clinically relevant.

We also report that HER2 overexpression at a level that does not cause oncogene-induced senescence was sufficient to confer this susceptibility independent of HR defects (10, 11). As HER2 overexpression is also seen in ovarian, stomach, and aggressive forms of uterine cancer, such as uterine serous endometrial carcinoma, it will be interesting to assess susceptibility to PARP inhibitors in these HER2-overexpressing tumors (39–41). We are currently actively investigating this question.

Our results also reveal that estrogen receptor or progesterone receptor status likely does not play a role in the sensitivity to PARP inhibition, because susceptibility to PARP inhibition was observed in cells with various receptor status
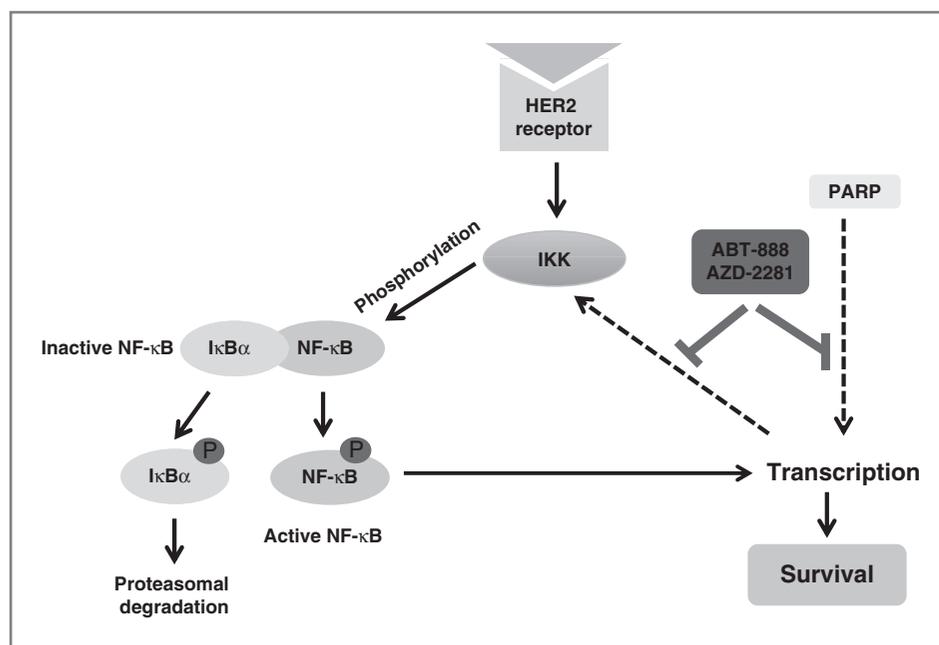
Figure 7. A simplified model of the proposed mechanism of HER2+ breast cancer susceptibility to PARP inhibition. HER2 overexpression confers oncogenic addiction to NF-κB–mediated signaling pathway. HER2 activates IKKα, which in turn phosphorylates and dissociates the NF-κB and IκBα complex. IκBα is subsequently degraded by the proteasome, whereas NF-κB translocates to the nucleus to activate prosurvival pathways. PARP is a coactivator of NF-κB and thus inhibition of PARP abrogates NF-κB–mediated transcription, which subsequently inhibits the oncogenic pathway to which HER2+ breast cancer cells may be addicted.

(Supplementary Table S1). The outcomes seem to also be irrespective of p53 status. This is not unexpected given the recent report of both p53 dependent and independent mechanisms of cell death caused by PARP inhibition (42).

Intriguingly, sensitivity may be, in part, because of inhibition of NF-κB–driven by HER2. Figure 7 depicts a simplified overview of the proposed pathway. HER2 activates NF-κB through the canonical pathway involving IKKα, and knockdown of IKKα has been reported to significantly decrease transcription levels of multiple NF-κB–regulated cytokine and chemokine genes (3, 23). In addition, IKKα knockdown reduced formation of HER2+ tumors in mice (4). These results suggest that HER2+ tumors may possess an oncogenic addiction to NF-κB signals for proliferation and survival. This is further supported by sensitivity of HER2+ breast cancer cells to the NF-κB inhibitor velcade (43). These observations may then explain our observation that HER2+ breast cancer cells are exquisitely sensitive to PARP inhibition, because PARP inhibition also attenuates NF-κB pathways. Studies to examine the mechanisms by which PARP regulates NF-κB–mediated transcription are currently ongoing. In addition, a role of altered DNA damage response cannot be ruled out, because an interaction between ATM and NF-κB has been previously reported (44, 45). However, our data supports an HR-independent mechanism, because the HER2+ breast cancer cells used in our study are HR proficient as evidenced by robust induction of radiation-induced rad51 foci and by the GFP-based HR repair assay.

Interestingly, activation of NF-κB has been shown to block apoptosis in HER2 expressing cells and thus may contribute to tumor resistance. Specifically, therapeutic resistance to HER2-targeted agents may be because of upregulation of NF-κB–induced genes (2, 24). Thus, the combination of PARP inhibition with HER2-targeted therapies may delay the onset of resistance and warrants further investigation.

Recent negative results in clinical trials with BSI-201 (iniparib) have raised concerns about the usefulness of inhibiting PARP as a therapeutic strategy (26). BSI-201 was initially reported to possess PARP inhibitory activity by covalently binding to the zinc finger of PARP1 (46). However, recent reports suggest that its effects are unlikely to reflect PARP inhibition and should not be used to guide decisions about other PARP inhibitors. This is supported by our current results of BSI-201 not having any effect on HER2+ cancer viability, although the other 2 bona fide inhibitors, AZD-2281 and ABT-888, caused significant cytotoxicity.

In summary our intriguing and novel results point to a broader utility of PARP inhibitors in the treatment of breast cancer beyond hereditary BRCA1- and BRCA2-deficient types. In addition, these observations suggest a DNA repair independent action of PARP inhibition. Further mechanistic studies are thus needed to understand the full spectrum of cancer types for which chemical inhibition of PARPs might be therapeutically beneficial. Biomarkers to predict therapeutic response to PARP inhibitors are also warranted, and HER2 overexpression may be one such marker. In addition, clinical studies will be required to assess the efficacy *in vivo*. Nonetheless, inhibition of PARP is indeed a promising therapeutic approach that may ultimately maximize the therapeutic ratio.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Nowsheen, J. A. Bonner, E. S. Yang
Development of methodology: S. Nowsheen, E. S. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Nowsheen, J. A. Bonner, E. S. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Nowsheen, J. A. Bonner, E. S. Yang
Writing, review, and/or revision of the manuscript: S. Nowsheen, T. Cooper, J. A. Bonner, A. F. LoBuglio, E. S. Yang

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HER2 Overexpression Renders Human Breast Cancers Sensitive to PARP Inhibition Independently of Any Defect in Homologous Recombination DNA Repair

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