

Trastuzumab-Resistant HER2-Driven Breast Cancer Cells Are Sensitive to Epigallocatechin-3 Gallate

Sean F. Eddy,¹ Susan E. Kane,² and Gail E. Sonenshein¹

¹Department of Biochemistry and Women's Health Interdisciplinary Research Center, Boston University School of Medicine, Boston, Massachusetts and ²Division of Molecular Medicine, Beckman Research Institute of the City of Hope, Duarte, California

Abstract

Overexpression of the epidermal growth factor receptor family member HER2 is found in ~30% of breast cancers and is a target for immunotherapy. Trastuzumab, a humanized monoclonal antibody against HER2, is cytostatic when added alone and highly successful in clinical settings when used in combination with other chemotherapeutic agents. Unfortunately, HER2 tumors in patients develop resistance to trastuzumab or metastasize to the brain, which is inaccessible to antibody therapy. Previously, we showed that the green tea polyphenol epigallocatechin-3 gallate (EGCG) inhibits growth and transformed phenotype of Her-2/*neu*-driven mouse mammary tumor cells. The different modes of action of EGCG and trastuzumab led us to hypothesize that EGCG will inhibit HER2-driven breast cancer cells resistant to trastuzumab. We studied trastuzumab-resistant BT474 human breast cancer cells, isolated by chronic trastuzumab exposure, and JIMT-1 breast cancer cells, derived from a pleural effusion in a patient who displayed clinical resistance to trastuzumab therapy. EGCG treatment caused a dose-dependent decrease in growth and cellular ATP production, and apoptosis at high concentrations. Akt activity was suppressed by EGCG leading to the induction of FOXO3a and target cyclin-dependent kinase inhibitor p27^{Kip1} levels. Thus, EGCG in combination with trastuzumab may provide a novel strategy for treatment of HER2-overexpressing breast cancers, given that EGCG can cross the blood-brain barrier. [Cancer Res 2007;67(19):9018–23]

Introduction

The *HER2* (*neu* or *c-ErbB2*) oncogene encodes a 185-kDa transmembrane tyrosine kinase receptor belonging to the epidermal growth factor receptor family that is amplified or overexpressed in ~30% of breast cancers and is a poor prognostic factor (1). Overexpression of *Her-2/neu* in mammary cancer cells activates the Akt kinase (2), which has been linked to enhanced survival and chemoresistance (3). Targeted expression of *Her-2/neu* in the mammary epithelia of mice resulted in mammary tumor formation and metastasis (4). Trastuzumab is a humanized monoclonal antibody directed against HER2, known commercially as Herceptin, which inhibits growth and proliferation of cancer cells overexpressing HER2 (5). Administration of trastuzumab caused complete or partial remission in ~12% of HER2-overexpressing breast cancer cases where chemotherapy was previously unsuccessful (6). When given as a single agent in the first line of

treatment in HER2-positive breast cancers, trastuzumab provided an objective response rate of 40%, which included stabilization and, occasionally, regression of the disease (7). When used after adjuvant therapy with chemotherapeutic drugs, such as paclitaxel and doxorubicin, clinical outcomes improve substantially with upwards of 60% of HER2-overexpressing breast cancers responding (8). Furthermore, metastases rates were ~50% lower in patients treated with trastuzumab after initial adjuvant chemotherapy (9). However, many patients respond only poorly to trastuzumab. In addition, many other patients with tumors that initially showed a response subsequently developed metastases to the central nervous system (10), in part because antibody molecules, such as trastuzumab, are inefficient in crossing the blood-brain barrier (11).

Our laboratory has shown that green tea extracts, which have been found to be extremely safe in clinical settings, exhibit potent antitumor effects in carcinogen-derived mammary tumors in rats and epigallocatechin-3 gallate (EGCG), the major polyphenol of green tea, reduces transformed phenotype of estrogen receptor α -negative breast cancer cells in culture, as evidenced by inhibition of growth and induction of the p27^{Kip1} cyclin-dependent kinase (CDK) inhibitor (12). EGCG also potently inhibited anchorage-independent growth in soft agar of the *Her-2/neu*-driven murine breast cancer cell line NF639, which correlated with reduced autophosphorylation of the *Her-2/neu* receptor and decreased Akt-mediated signaling (13). Similarly, EGCG inhibited growth of HER2-driven BT474 and SKBR3 cells.³ Akt has been implicated in the control of cellular proliferation, in part via repression of the forkhead box O transcription factor FOXO3a. Under conditions where cells are proliferating, FOXO3a is phosphorylated by Akt and exported to the cytoplasm (14). On inhibition of Akt activity, FOXO3a becomes hypophosphorylated and translocates to the nucleus activating target genes, such as the CDK inhibitor p27^{Kip1}, whose product controls G₁-S phase progression (12). Considering that mechanisms of trastuzumab resistance include sustained Akt activity and loss of p27^{Kip1} expression (15), here we investigated whether HER2-driven breast cancer cells resistant to trastuzumab would retain sensitivity to EGCG. We show that EGCG inhibits proliferation of trastuzumab-resistant human breast cancer cells and show that these effects are mediated by the ability of EGCG to reduce Akt activity and induce FOXO3a and p27^{Kip1}.

Materials and Methods

Cell culture and treatment conditions. Trastuzumab-resistant human BT474 breast cancer clones were isolated during a 5-month selection process in the continuous presence of 0.2 or 1 μ mol/L trastuzumab, BT/Her^R 0.2 clone D and BT/Her^R 1.0 clone E, respectively (16). Resistant cells displayed high levels of HER2 and Akt, and Akt activity (16). JIMT-1 cells

Requests for reprints: Gail E. Sonenshein, Department of Biochemistry, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118. Phone: 617-638-4120; Fax: 617-638-4252; E-mail: gsonensh@bu.edu.

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³ S.F. Eddy, unpublished observation.

were isolated from a pleural effusion in a patient with metastatic HER2-driven breast disease that was clinically resistant to trastuzumab therapy. JIMT-1 cells, which are epithelial in origin, displayed overexpression of wild-type HER2, formed xenograft tumors in nude mice, and retained resistance to trastuzumab and a second HER2 targeting drug, pertuzumab (17). JIMT-1 cells were purchased from DSMZ and grown according to the supplier's protocols. EGCG (LKT Laboratories) was dissolved in DMSO and diluted in DMSO to maintain a constant DMSO volume in samples. Controls were incubated in a volume of carrier DMSO equivalent to the highest dose of EGCG. For cell counting studies, cells were plated in triplicate and grown for 24 h. Following addition of EGCG, cells were grown for 72 h and counted, and total cell numbers were plotted as a mean \pm SD.

CellTiter 96 Aqueous proliferation assay. Cells were grown, in quadruplicate, in the presence of carrier DMSO (0 μ g/mL EGCG) or EGCG (40, 80, or 160 μ g/mL). At the indicated times, cells were incubated in the presence of 20 μ L CellTiter 96 Aqueous One Reagent (Promega) per 100 μ L medium for 1 h and the reaction was measured colorimetrically at 490 nm. Background levels were determined in medium supplemented with EGCG at the appropriate dose and subtracted from experimental values and findings were graphed as mean \pm SD.

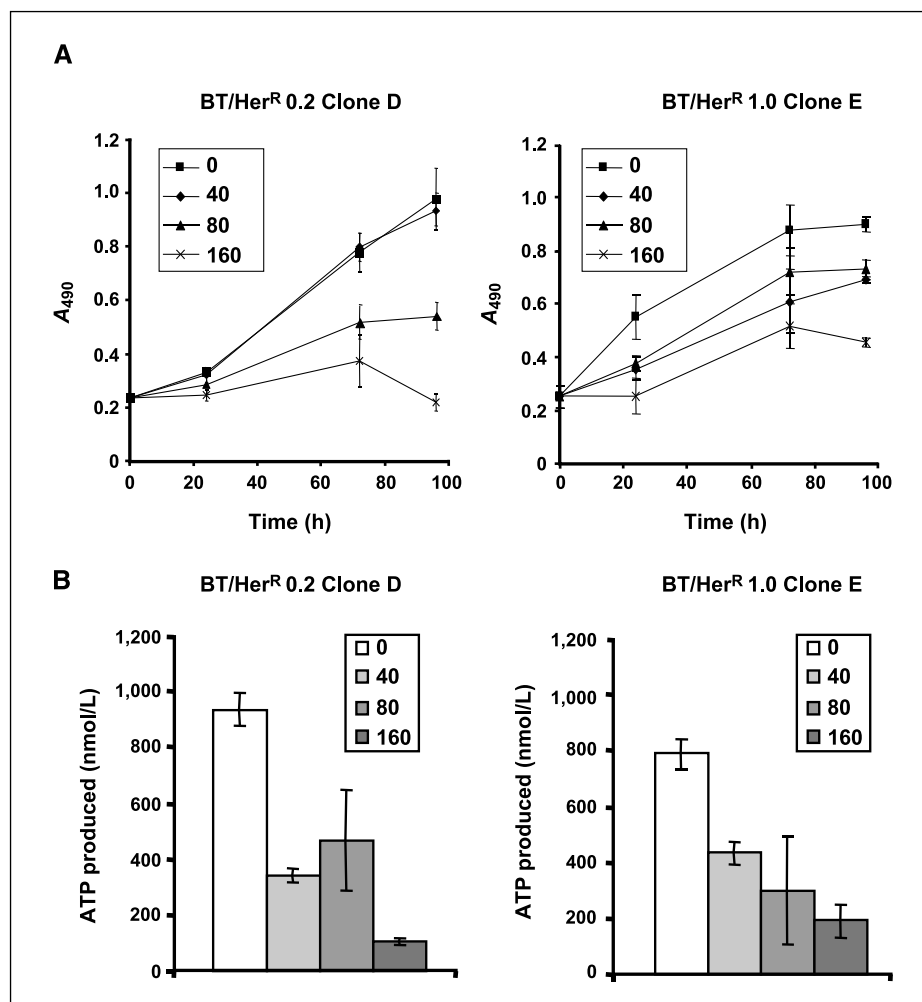
CellTiterGlo ATP production assay. Cells, plated in quadruplicate in 96-well plates, were grown in the presence of carrier DMSO or EGCG for 72 h. An equal volume of CellTiterGlo (Promega) reagent was added to each well and luciferase activity was measured in a 96-well luminometer. A standard curve was prepared to determine ATP production in cells. Background luciferase levels of medium with or without EGCG were negligible.

Hoechst staining of cells. Breast cancer cells were plated on coverslips in DMEM containing 10% fetal bovine serum (FBS). After 24 h, EGCG was added to the medium at the indicated concentrations, and the cells were incubated an additional 72 h. Cultures were then washed three times with PBS and fixed in 3.7% formaldehyde solution. Following treatment with 0.5% Triton X-100, DNA was stained with 5 ng/mL Hoechst dye for 5 min. Coverslips were then mounted on slides and dried overnight at 4°C in the dark before visualization at $\times 10$ magnification using a Zeiss Axiovert microscope.

Apoptosis assay. Cells were grown, in quadruplicate, in the presence of carrier DMSO (0 μ g/mL EGCG) or high-dose EGCG (80 or 160 μ g/mL). After 72 h, cells were lysed and the level of histone cleavage from nucleosomes was measured using the Cell Death Detection^{plus} kit (Roche). Negative controls were run in medium supplemented with EGCG at the appropriate dose and subtracted from experimental values and findings were graphed as mean \pm SD.

Green fluorescent protein growth assay. BT/Her^R 0.2 clone D and JIMT-1 cells were plated in duplicate in 12-well dishes. Cells were cotransfected with 1 μ g pRES-GFP and 1 μ g of either a vector expressing myristylated Akt (Myr-Akt) or a parental empty vector DNA (a kind gift of Z. Lou, Boston University School of Medicine, Boston, MA). After an 8-h incubation period, control carrier DMSO (0 μ g/mL) or EGCG was added at doses of 40 and 80 μ g/mL and cells were incubated for a further 72 h. Green fluorescent protein (GFP)-positive cells and total cells were counted under $\times 10$ magnification. The ratio of GFP-positive cells/total cells are presented as a mean \pm SE normalized to empty vector untreated control cells (set to 1.0).

Figure 1. EGCG reduces proliferation and ATP production of trastuzumab-resistant BT474 breast cancer cells. **A**, BT/Her^R 0.2 clone D (left) and BT/Her^R 1.0 clone E (right) cells were plated, in quadruplicate, at a density of 8×10^3 cells/mL. After overnight incubation, cells were treated for the indicated periods with 40, 80, or 160 μ g/mL EGCG dissolved in DMSO or DMSO alone (0 μ g/mL EGCG) as control. Points, mean (A_{490}); bars, SD. **B**, BT/Her^R 0.2 clone D (left) and BT/Her^R 1.0 clone E (right) cells were plated, in quadruplicate, at a density of 8×10^3 cells/mL. After overnight incubation, cells were treated with carrier DMSO (0 μ g/mL) or with 40, 80, or 160 μ g/mL EGCG. After 72 h, cellular ATP production was quantified using the CellTiterGlo ATP production assay and measured in a 96-well luminometer. Columns, mean; bars, SD. ANOVA analysis was used to determine significance for both cell lines ($P < 0.001$).



Immunoblotting. Whole-cell protein extracts were prepared in extraction buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L β -glycerophosphate, 10 mmol/L *p*-nitrophenyl phosphate, 10 mmol/L NaF, 1 mmol/L DTT, 1 mmol/L EDTA, 300 μ mol/L Na_3VO_4 , 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin, 5 μ g/mL aprotinin]. Samples (40 μ g) were electrophoresed and subjected to immunoblotting, as described (2). Nuclear extracts were prepared from isolated nuclei by lysis in extraction buffer and samples (15 μ g) were subjected to immunoblotting. Antibodies for Akt and phosphorylated Ser⁴⁷³ Akt (p-Ser⁴⁷³ Akt), and for p27^{Kip1} were purchased from Cell Signaling and Santa Cruz Biotechnology, respectively. Antibodies for FOXO3a and β -actin (AC-15) were purchased from Upstate Biotechnology and Sigma, respectively.

Statistics. Where indicated, ANOVA analysis was done to determine significance.

Results and Discussion

EGCG slows growth of trastuzumab-resistant human BT474 breast cancer cells. To assess the effects of EGCG on growth of BT/Her^R 0.2 clone D and BT/Her^R 1.0 clone E BT474 cells, which displayed resistance to the presence of 0.2 and 1.0 μ mol/L trastuzumab, respectively, cultures were incubated either with carrier DMSO alone (0 μ g/mL EGCG) or with 40, 80, or 160 μ g/mL EGCG for 24, 48, or 96 h and proliferation was monitored using the CellTiter 96 Aqueous One Reagent cell proliferation assay (Fig. 1A). For both clones, a dose-dependent response was observed. To obtain a second measure of cell metabolism and viability, cellular ATP was measured using the CellTiterGlo luminescence assay. This assay assesses ATP production as a measure of cellular metabolism and an approximate measure of cell number. BT/Her^R 0.2 clone D and BT/Her^R 1.0 clone E cells were plated in quadruplicate and treated with 0, 40, 80, or 160 μ g/mL EGCG, as above. After 72 h, cells were assayed for ATP production. EGCG treatment caused a significant dose-dependent decrease in ATP production in BT/Her^R clone 0.2 clone D and BT/Her^R 1.0 clone E cells (Fig. 1B). Thus, EGCG reduces the ability of trastuzumab-resistant BT474 breast cancer cells to proliferate and to produce ATP.

EGCG reduces total numbers of trastuzumab-resistant breast cancer cells. The effects of EGCG treatment on total BT/Her^R 0.2 clone D and BT/Her^R 1.0 clone E cell numbers were determined. BT/Her^R clones, plated at a concentration of 7.5×10^4 cells/mL (Fig. 2A and B, *Dashed line*), were allowed to recover for 24 h before initiating treatment with EGCG. After 72 h, total cell numbers were determined (Fig. 2). Control DMSO-treated cells almost doubled in number during the 96 h in culture, consistent with the \sim 100-h doubling time for the BT474 breast cancer cell line. Treatment with 80 μ g/mL EGCG resulted in a decrease below the original numbers of BT/Her^R 0.2 clone D (Fig. 2A) and BT/Her^R 1.0 clone E (Fig. 2B) cells plated. Further decreases were seen with 160 μ g/mL EGCG treatment. If EGCG were cytostatic, it would be expected that the cell density after treatment would be similar to the starting number. The decrease below 7.5×10^4 cells suggests that cell death has occurred as a result of EGCG treatment.

JIMT-1 trastuzumab-resistant breast cancer cells were isolated from a pleural effusion of a patient who was clinically resistant to trastuzumab therapy (17). To assess the effects of EGCG on the JIMT-1 breast cancer line, cells were plated at a density of 1.0×10^5 /mL (Fig. 2C, *dashed line*) and treated as described above. Consistent with the \sim 40-h doubling time, control DMSO-treated JIMT-1 breast cancer cells nearly quadrupled over the full time

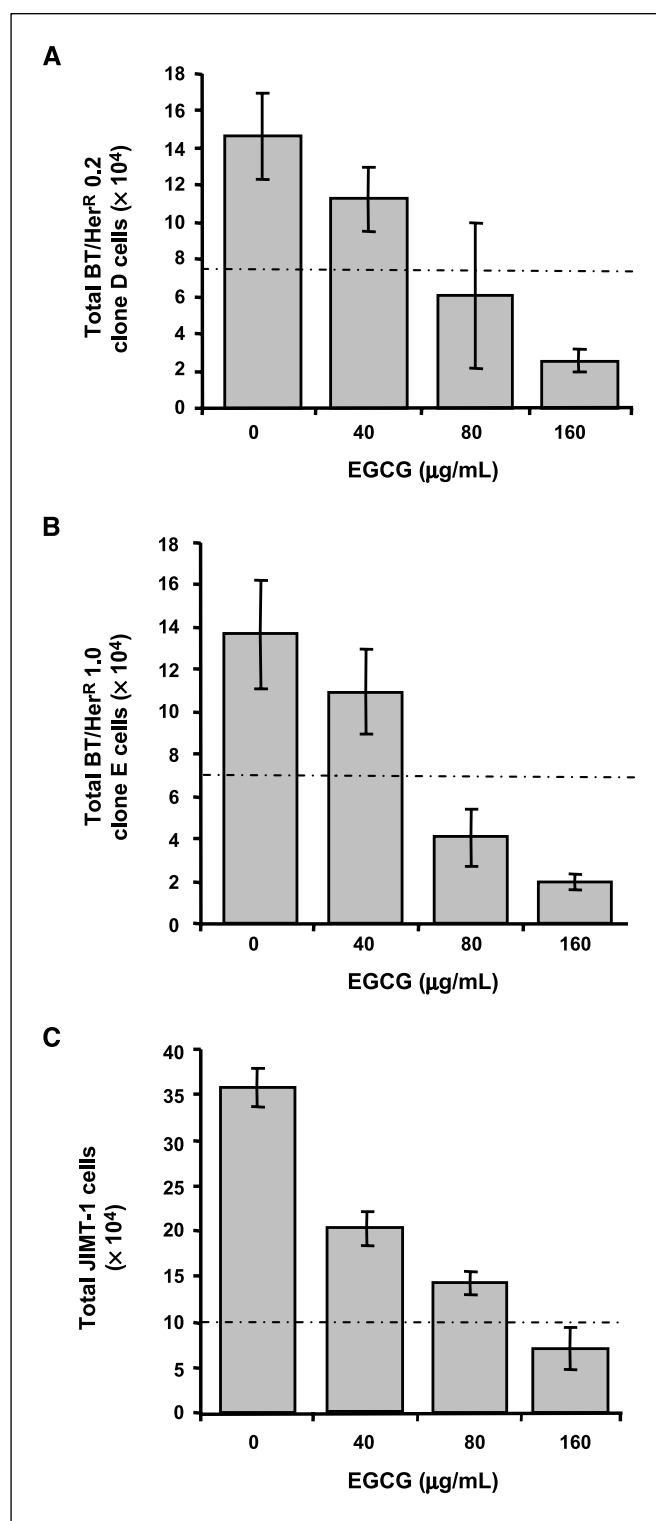


Figure 2. EGCG reduces the total cell number of trastuzumab-resistant breast cancer cells. BT/Her^R 0.2 clone D (A) and BT/Her^R 1.0 clone E (B) breast cancer cells were plated, in triplicate, at a density of 7.5×10^4 /mL and JIMT-1 trastuzumab-resistant breast cancer cells (C) were plated, in triplicate, at a density of 1.0×10^5 /mL in 12-well plates. After overnight incubation, cells were treated with either control DMSO (0 μ g/mL) or EGCG at the indicated concentration. Total cell numbers were counted after 72 h. *Dashed lines*, starting numbers of cells. Significance between final cell counts of untreated (0 μ g/mL) and treated (40, 80, or 160 μ g/mL EGCG) samples was confirmed using ANOVA ($P < 0.001$) for all three cell lines. The experiment was repeated with similar results.

course. Treatment with EGCG caused a significant dose-dependent decrease in cell growth and at a dose of 160 $\mu\text{g}/\text{mL}$ EGCG, cell numbers decreased. Thus, EGCG slows growth of trastuzumab-resistant breast cancer cells at lower doses and may promote death at higher doses.

Higher doses of EGCG induce cell death. Because the decrease in cell numbers at the higher doses of EGCG suggested that cell death was occurring, nuclear morphology and DNA staining were investigated using Hoechst 33258. In healthy cells, Hoechst 33258 stains DNA that is condensed and localized tightly in the nucleus; however, in cells undergoing stress or dying, DNA becomes fragmented and is dispersed throughout the cytoplasm. In BT/Her^R 0.2 clone D, BT/Her^R 1.0 clone E, and JIMT-1 cells exposed to carrier DMSO for 72 h, Hoechst 33258 staining was quite visible and confined to the nuclear region (Fig. 3A, left). However, on treatment with 80 $\mu\text{g}/\text{mL}$ EGCG for 72 h, dispersal of DNA throughout the cytoplasm and loss of nuclear integrity was observed in BT/Her^R 0.2 clone D and BT/Her^R 1.0 clone E cells, whereas JIMT-1 cells showed lower but detectable levels of cytoplasmic DNA staining (Fig. 3A, middle). At a dose of 160 $\mu\text{g}/\text{mL}$ EGCG, all three cell lines seemed to substantially lose their nuclear integrity and Hoechst staining was quite broadly distributed (Fig. 3A, right). As another measure of the effects of high-dose EGCG on apoptosis, we used the Cell Death Detection^{Plus} kit, which detects cleaved nucleosomal components. BT/Her^R 0.2 clone D, BT/Her^R 1.0 clone E, and JIMT-1 cells were plated in quadruplicate and incubated in either carrier DMSO (0 $\mu\text{g}/\text{mL}$) or EGCG at doses of 80 or 160 $\mu\text{g}/\text{mL}$ for 24 h. Background levels were determined in medium supplemented with DMSO or EGCG. In all three cell lines, DNA fragmentation was increased at 80 $\mu\text{g}/\text{mL}$ EGCG and even further at 160 $\mu\text{g}/\text{mL}$ (Fig. 3B). Of note, JIMT-1 cells seemed more resistant to apoptosis from EGCG, as was seen in Hoechst staining (Fig. 3A) and cell numbers (Fig. 2). Thus, EGCG induces death of trastuzumab-resistant breast cancer cell lines at higher doses.

EGCG inhibits constitutive Akt signaling and induces FOXO3a activity and levels of p27^{Kip1}. BT/Her^R 0.2 clone D

and BT/Her^R 1.0 clone E were found to retain sensitivity to the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, suggesting the importance of PI3K-Akt signaling to trastuzumab resistance (16). Previously, we showed that EGCG treatment down-regulated Akt activity in Her-2/*neu*-driven NF639 mouse mammary tumor cells (13). To determine whether EGCG treatment of the trastuzumab-resistant lines similarly leads to inhibition of Akt activity, BT/Her^R 0.2 clone D, BT/Her^R 1.0 clone E, and JIMT-1 cells were treated with 0, 40, or 80 $\mu\text{g}/\text{mL}$ EGCG for 72 h. Phosphorylation of Akt at Ser⁴⁷³ was reduced in a dose-dependent fashion with EGCG treatment in all three lines (Fig. 4A). Because the effects at 40 $\mu\text{g}/\text{mL}$ were not as strong in JIMT-1 cells, doses of 60 and 80 $\mu\text{g}/\text{mL}$ EGCG were used next to characterize the effects on FOXO3a, which is negatively regulated upon phosphorylation by Akt (14). At these concentrations, EGCG treatment enhanced nuclear expression of FOXO3a (Fig. 4B), which varied inversely with Akt activity in the three trastuzumab-resistant lines, as expected. JIMT-1 had the lowest levels of FOXO3a when untreated and showed a modest increase on treatment with EGCG, which was not quantifiable due to undetectable levels in the control DMSO sample. The p27^{Kip1} gene is a major growth regulatory target of FOXO3a (18). Because loss of p27^{Kip1} expression has been correlated with trastuzumab resistance whereas induction of p27^{Kip1} confers sensitivity to trastuzumab in HER2-positive breast cancer cells (15), we next determined whether levels of p27^{Kip1} were affected by EGCG. Expression of p27^{Kip1} in all three trastuzumab-resistant breast cancer cell lines correlated quite closely with the nuclear expression of nuclear FOXO3a.

To verify if the effects of EGCG were mediated via reduction in Akt signaling, we asked whether a constitutively active form of Akt can override the EGCG-induced growth arrest. BT/Her^R 0.2 clone D and JIMT-1 breast cancer cells were cotransfected with a GFP-expressing vector and either constitutively active Myr-Akt or parental empty vector DNA. After 8 h, both empty vector- and Myr-Akt-transfected cells were incubated in the presence of control carrier DMSO (0 $\mu\text{g}/\text{mL}$) or EGCG at doses of 40 or 80 $\mu\text{g}/\text{mL}$. After 72 h, GFP-positive cells were counted

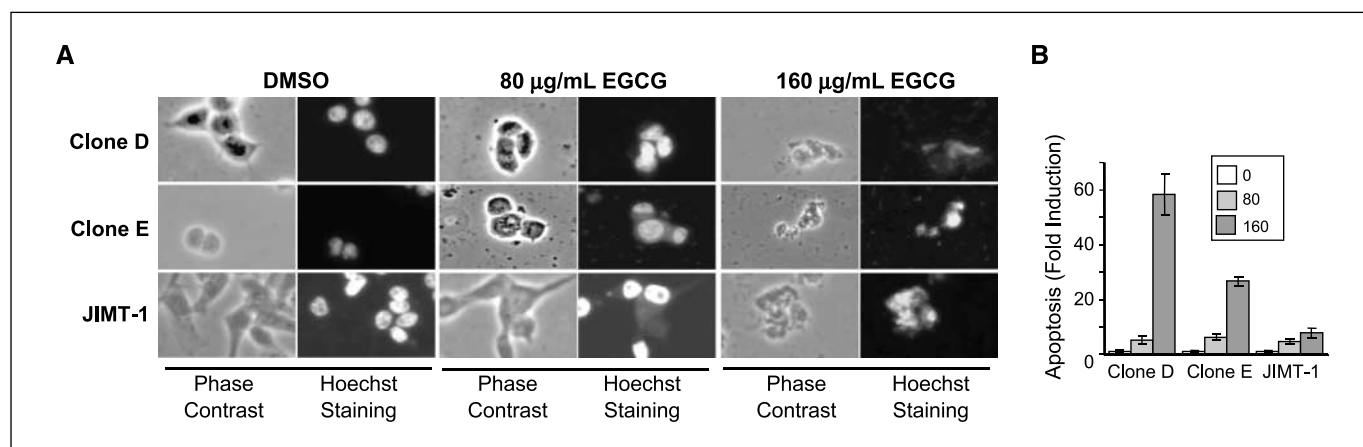


Figure 3. EGCG induces death of trastuzumab-resistant breast cancer cells. **A**, BT/Her^R 0.2 clone D, BT/Her^R 1.0 clone E, and JIMT-1 trastuzumab-resistant breast cancer cells were plated on coverslips in DMEM + 10% FBS. After an initial 24-h period of incubation in complete medium, cells were treated with either DMSO (0 $\mu\text{g}/\text{mL}$) or EGCG (80 or 160 $\mu\text{g}/\text{mL}$), as above. After incubation, the washed cells were fixed in 3.7% formaldehyde solution, extracted in 0.5% Triton X-100, and stained with 5 ng/mL Hoechst dye for 5 min. Coverslips were then mounted on slides and dried overnight before visualization using a Zeiss Axiovert microscope under $\times 10$ magnification. Both phase-contrast and fluorescent micrographs are shown. **B**, BT/Her^R 0.2 clone D, BT/Her^R 1.0 clone E, and JIMT-1 cells were plated, in quadruplicate, at a density of 1.5×10^4 cells/mL in 96-well plates. After overnight incubation, cells were treated either with control DMSO (0 $\mu\text{g}/\text{mL}$) or with 80 or 160 $\mu\text{g}/\text{mL}$ EGCG, as indicated. After 24 h, cells were quantified for apoptosis using the Cell Death Detection^{Plus} system. Columns, mean; bars, SD.

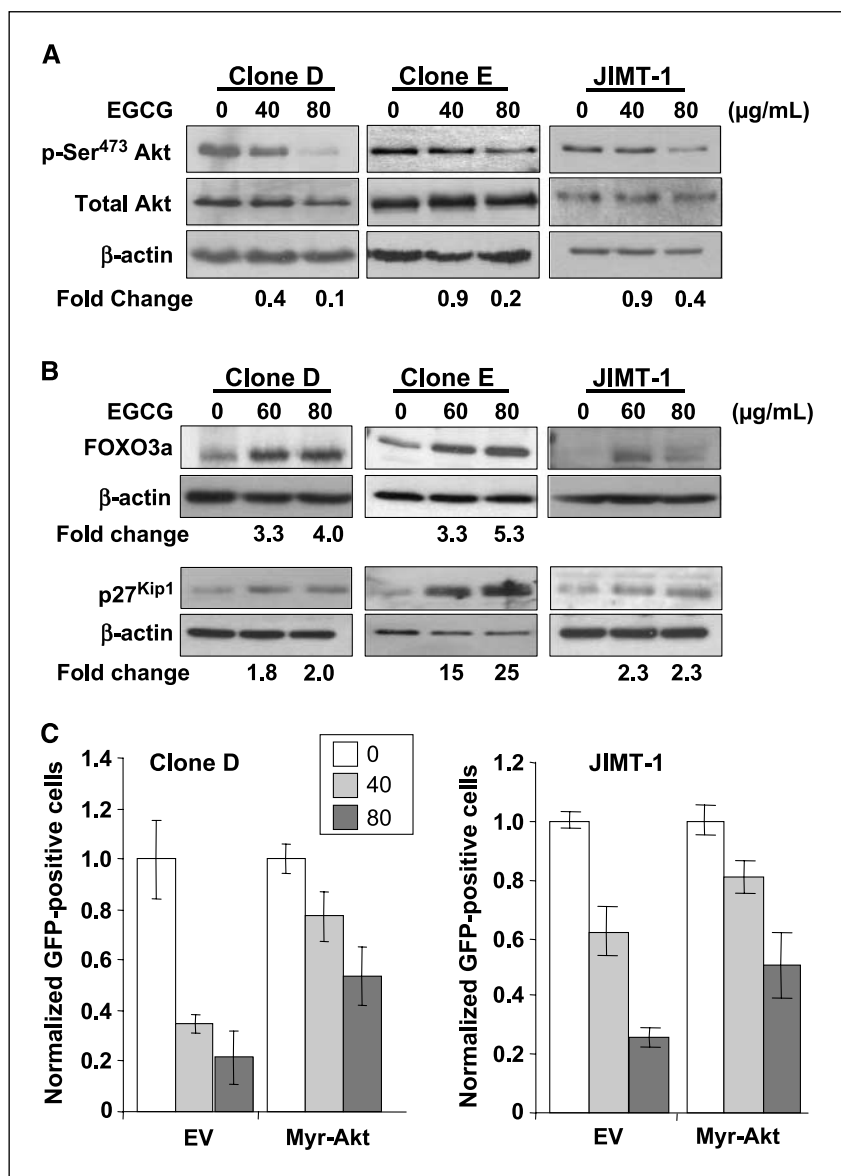


Figure 4. EGCG reduces Akt signaling in trastuzumab-resistant breast cancer cells and induces functional FOXO3a and expression of p27^{Kip1}. BT/Her^R 0.2 clone D, BT/Her^R 1.0 clone E, and JIMT-1 breast cancer cells were incubated in the presence of DMSO (0 μg/mL) or the indicated dose of EGCG. **A**, whole-cell protein extracts were subjected to immunoblotting for p-Ser⁴⁷³ Akt, total Akt, and β-actin, to control for loading. **B**, *top*, nuclear extracts were subjected to immunoblotting for FOXO3a; *bottom*, whole-cell protein extracts were immunoblotted for p27^{Kip1} expression. Densitometric analysis was done on immunoblots using the Kodak Digital Science 1D 2.0 system. Fold changes, normalized to β-actin, are presented relative to the levels in the control (DMSO) cells. The experiment was repeated with similar results. **C**, BT/Her^R 0.2 clone D and JIMT-1 breast cancer cells were plated, in duplicate, at a density of 1.5×10^4 /mL in 12-well plates. After 24 h, cells were cotransfected with 1 μg pIRES-GFP and 1 μg of either Myr-Akt or empty parental vector (EV) DNA. After an 8-h incubation, EGCG was added to the medium at the indicated concentration for 72 h. GFP-positive and total cell numbers were counted. The ratio of GFP-positive cells/total cells are presented as a mean ± SE normalized to empty vector untreated control cells (set to 1.0). *Columns*, mean; *bars*, SE. The experiment was repeated with similar results.

and expressed as a percentage of total cells per field of view. These levels were then normalized to empty vector control levels, which were set to 1 (Fig. 4C). Transfection of Myr-Akt conferred resistance to the effects of EGCG as measured by the presence of a higher number of GFP-positive cells, suggesting that down-regulation of Akt activity by EGCG is critical to growth arrest of trastuzumab-resistant cells. Similar data were obtained with BT/Her^R 1.0 clone E cells (data not shown). Thus, EGCG effectively reduces Akt activity and induces downstream targets FOXO3a and p27^{Kip1} in trastuzumab-resistant HER2-overexpressing breast cancer cells.

Our findings are the first to indicate that trastuzumab-resistant breast cancer cells display sensitivity toward EGCG. In particular, EGCG treatment reduced cell proliferation, ATP production, and Akt activity. Concomitantly, EGCG treatment induced nuclear FOXO3a and p27^{Kip1} levels. At higher doses, EGCG induced apoptosis. Pharmacokinetic studies of bioavailability of EGCG in mice showed that it is widely distributed in all of the major

organs tested after a single i.v. dose (19). When EGCG was administered through a gastric tube, the polyphenol or its metabolites were detectable in brain after only 1 h and levels continued to increase over a 24-h time course (20). Because induction of p27^{Kip1} confers sensitivity to trastuzumab and because EGCG can cross the blood-brain barrier, the sensitivity of trastuzumab-resistant breast cancer cells to EGCG suggests that preclinical animal testing of combinatorial therapeutic strategies is warranted.

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