Cell Cycle 10:17, 2959-2966; September 1, 2011; © 2011 Landes Bioscience

Potent anti-proliferative effects of metformin on trastuzumab-resistant breast cancer cells via inhibition of ErbB2/IGF-1 receptor interactions

Bolin Liu,[1,*] Zeying Fan,[1] Susan M. Edgerton,[1] XiaoHe Yang,[3] Stuart E. Lind[1,2] and Ann D. Thor[1,*]

[1]Department of Pathology and [2]Medicine; University of Colorado Denver School of Medicine; Aurora, CO USA;
[3]Julius L. Chambers Biomedical/Biotechnology Research Institute; North Carolina Central University; Kannapolis, NC USA

Key words: metformin, erbB2, IGF-1 receptor, trastuzumab resistance, breast cancer

Abbreviations: AMPK, AMP-activated protein kinase; S6K1, S6 kinase 1; EGFR, epidermal growth factor receptor; FDA, food and drug administration; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PARP, poly(ADP-ribose) polymerase; PTEN, phosphatase and tensin homolog; PI-3K, phosphoinositide-3-kinase; RTK, receptor tyrosine kinase

We have shown that *erbB2* altered breast cancer cells are less sensitive to the anti-proliferative effects of metformin than triple negative cells, and have described the differences of molecular mechanisms of metformin action by tumor subtypes. We hypothesized that metformin may be more effective against trastuzumab-resistant erbB2-overexpressing breast cancer cells because it targets the critical signaling pathways that are altered with resistance. BT474, SKBR3 and derived trastuzumab-resistant sublines BT474-HR20 (HR20) and SKBR3-pool2 (pool2) were used to test this hypothesis. Metformin treatment resulted in significantly more inhibition of proliferation and clonogenicity in resistant sublines. It decreased erbB2/insulin-like growth factor-1 receptor (IGF-1R) complexes (present only in the resistant sublines) without altering erbB2 expression and reduced the expression and activity of erbB3 and IGF-1R in the trastuzumab-resistant but not parental cells. Trastuzumab-resistant sublines were resistant to rapamycin induced changes in mTOR activity and cell growth. In contrast, both BT474 and HR20 cells were highly sensitive to inhibitors of Src (Dasatinib) and PI-3K (LY294002). The pool2 cells showed higher sensitivity than SKBR3 cells to LY294002, but not Dasatinib. On the basis of these data, metformin appears to be significantly more effective against trastuzumab-resistant as compared with sensitive breast cancer cells. Metformin disrupts erbB2/IGF-1R complexes, erbB3 and IGF-1R expression and activity, as well as Src kinase and/or PI-3K/Akt signaling. This action appears to be independent of mTOR signaling. Our findings provide a rationale to study the effects of metformin on patients with erbB2 positive tumors treated with trastuzumab, with or without resistance.

Introduction

ErbB2 (*HER2*/*neu*) alterations occur frequently (25–30%) in invasive ductal carcinomas of the breast and are associated with a poor prognosis. The development of erbB2-targeted therapeutics like trastuzumab (Herceptin®),[1] provide significant benefit to breast cancer patients with this subtype, as a single agent or in combination with other drugs.[2-4] However, the majority of patients who achieve an initial response to trastuzumab develop resistance within one year.[5,6] A better understanding of the molecular mechanisms of resistance, identification of agents to delay or overcome trastuzumab resistance, or drugs that may be efficacious against resistant breast cancer may significantly benefit the breast cancer patients.

Metformin (1,1-dimethylbiguanide hydrochloride) is the most widely prescribed agent for patients with metabolic syndrome or type II diabetes. Obesity and its associated hyperinsulinemia/glucose intolerance and type II diabetes are each associated with a higher risk of breast cancer and reduction in survival for patients who develop the disease. Numerous studies have shown that the administration of metformin reduces breast cancer incidence and prolongs survival.[7-10] The beneficial anti-cancer activity of metformin appears to be unique to this drug, in that other anti-diabetic agents have not been associated with these benefits. The anti-cancer mechanisms of metformin action are not well understood.[11,12]

Metformin induces activation of AMP-activated protein kinase (AMPK), inhibits the mammalian target of rapamycin (mTOR) as well as S6 kinase, and is a general inhibitor of mRNA translation.[13] AMPK is widely regarded as a nutrient sensor critical to cell metabolism, whereas mTOR is a central

*Correspondence to: Bolin Liu and Ann D. Thor; Email: bolin.liu@ucdenver.edu and ann.thor@ucdenver.edu
Submitted: 05/05/11; Revised: 06/30/11; Accepted: 07/01/11
DOI: 10.4161/cc.10.17.16359

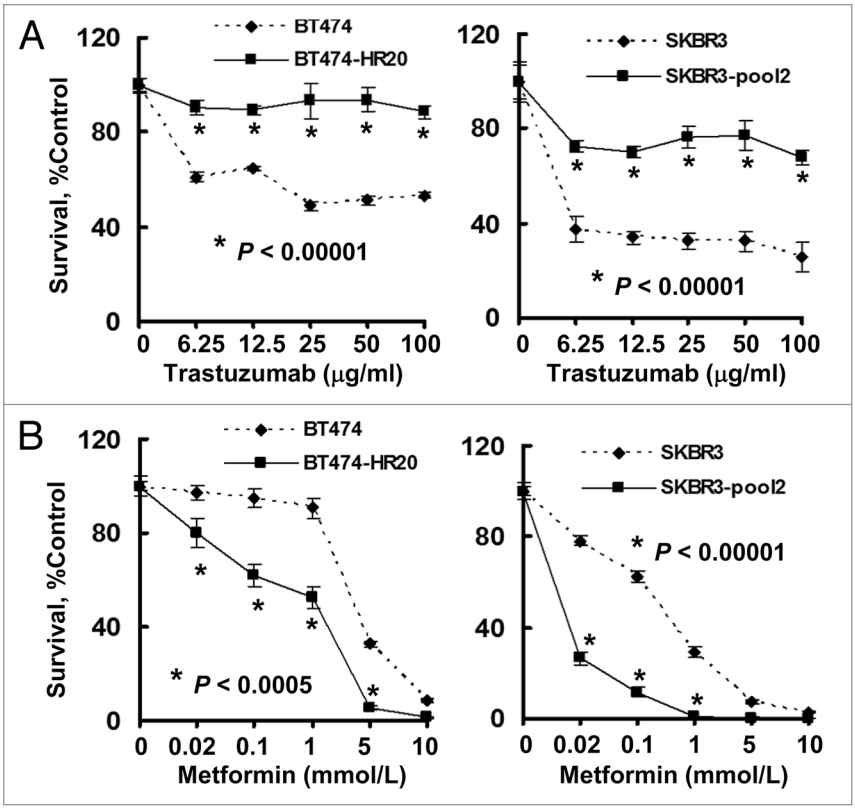

Figure 1. Trastuzumab-resistant breast cancer cells are more sensitive to metformin-induced growth inhibition. BT474, BT474-HR20, SKBR3 and SKBR3-pool2 human breast cancer cells were plated onto 96-well plates and incubated at 37°C with 5% CO_2. After 24 h, the culture medium was replaced with 0.1 ml fresh medium containing 0.5% FBS or the same medium containing the indicated concentrations of trastuzumab (A) or metformin (B) for another 72 h. The percentages of surviving cells from each cell line relative to control, defined as 100% survival, were determined by reduction of MTS. Bars, SD. Data shows a representative of three independent experiments.

regulator of cellular response to mitogenic stimuli that is frequently upregulated in breast cancers (especially in those with activating mutations of *PI3KCA* or a loss of *PTEN*).[14,15] We have reported that the biological and molecular actions of metformin against breast cancer vary by the molecular subtype of disease. Metformin induces S phase arrest and apoptosis in triple negative (basal-like) breast cancer cells, whereas it inhibits cell proliferation (G_1 arrest) through different molecular mechanisms without the induction of apoptosis in luminal A, B and erbB2 (HER2) breast cancer cells.[16,17] Metformin suppresses erbB2 expression and activation, which has been shown to be due to inactivation of mTOR and its downstream effector p70S6K1.[18] Interactions between metformin and erbB2 have been described in vitro and in vivo. Metformin treated genetically at risk female mice bearing the *erbB2/neu* transgene have shown a significant decrease in mammary tumorigenesis.[19]

In light of the aforementioned experimental evidence indicating that metformin has significant anti-cancer activity, clinical trials have been initiated in breast cancer patients. In a retrospective review of 2,592 patients with early or locally advanced breast cancer, the rate of pathologic complete response (pCR) following neoadjuvant chemotherapy was 24% in diabetic patients receiving metformin, 16% in non-diabetics and 8% in diabetic patients treated with other agents ($p < 0.001$).[20] Randomized, prospective trials of metformin treatment, with or without other drugs are actively enrolling patients.[21,22] None of these have restrictions based on the molecular subtypes of breast cancer, the presence or absence of metabolic dysregulation or disease.[21,22] Additional data suggests that metformin may interact with anti-cancer drugs or their associated toxicities. For example, metformin reportedly overcomes lapatinib resistance in breast cancer cells.[23] There are no reports of interactions between metformin and trastuzumab to date.

Results

Trastuzumab-resistant breast cancer cells are more sensitive to metformin-induced growth inhibition in vitro. Trastuzumab-resistant sublines have been developed by growing two trastuzumab sensitive erbB2-overexpressing breast cancer cell lines (BT474 and SKBR3) in the presence of progressively increasing concentrations of trastuzumab over several months.[24,25] We first compared the growth of parental and resistant sublines in culture, with and without trastuzumab, using a MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)-based cell proliferation assay. Trastuzumab resistance was significant in the HR20 and pool2 cells, whereas the parental BT474 and SKBR3 cells were sensitive to this agent (**Fig. 1A** and $p < 0.00001$ for each pair). We then compared the growth of these lines and sublines with and without metformin. Each of the trastuzumab-resistant sublines was significantly more sensitive to metformin-associated growth inhibition than its parental line (**Fig. 1B** and $p < 0.0005$ and $p < 0.00001$ respectively).

We next analyzed the growth of these cell lines with and without metformin over a wide range of concentrations and calculated the EC_{50} for each. The EC_{50} of the HR20 cells was approximately 4-fold less (2.6 mmol/L) than the parental BT474 cells (10 mmol/L). The EC_{50}s of SKBR3 and pool2 cells were also significantly different, at 5.6 mmol/L and 0.062 mmol/L, respectively. Clonogenic assays showed significantly greater metformin-associated inhibition in the resistant sublines as compared with parental cells (**Fig. 2** and $p = 0.01$ and $p = 0.007$ respectively). We also compared metformin and trastuzumab effects using clonogenic assays. Metformin was associated with significantly greater inhibition of clonogenicity, as compared with trastuzumab, in the resistant cell lines (**Fig. 2B**). We then used metformin and trastuzumab in combination. When these drugs were given together, both the parental and resistant lines showed similar inhibition in colony formation and there was significantly greater inhibition

than the administration of either agent alone (**Fig. 2B** and p = 0.003 and p < 0.006 respectively). These data suggest that the administration of metformin with trastuzumab, in patients who have not yet developed resistance, should be explored.

Metformin inactivates erbB3, IGF-1R and their downstream signaling with a reduction of erbB2/IGF-1R interactions in trastuzumab-resistant cells. Receptor dimerization between erbB2 and other RTK, such as erbB3,[26] EGFR,[27] or IGF-1R[25] has been shown to play an important role in trastuzumab resistance. We have recently reported that erbB3 and IGF-1R work cooperatively with erbB2, and that these three RTKs can interact with each other as a complex in trastuzumab-resistant lines to promote downstream signaling.[24] Using two-directional immunoprecipitation assays followed by western blots, we observed strong interactions between erbB2 and IGF-1R only in the trastuzumab-resistant HR20 and pool2 cells (**Fig. 3A**). These findings are consistent with the work of Nahta et al.[24,25] The administration of metformin to resistant cells significantly decreased erbB2/IGF-1R interactions in a time-dependent manner (**Fig. 3B**). Metformin reduced erbB3 and IGF-1R protein levels, without a significant change in erbB2 expression (**Fig. 4A**). Metformin also significantly reduced the level of both activated (phosphorylated) erbB3 (P-erbB3) and IGF-1R (P-IGF-1R) only in the resistant sublines (**Fig. 4A**). Metformin reduced the phosphorylation levels of only one receptor per line in the parental controls (P-IGF-1R in BT474; P-erbB3 in SKBR3). Metformin dramatically inhibited Akt activity in HR20 cells, and inactivated both Akt and MAPK in pool2 cells. There was significantly less inhibition of these signaling pathway intermediates in the parental cell lines (**Fig. 4B**).

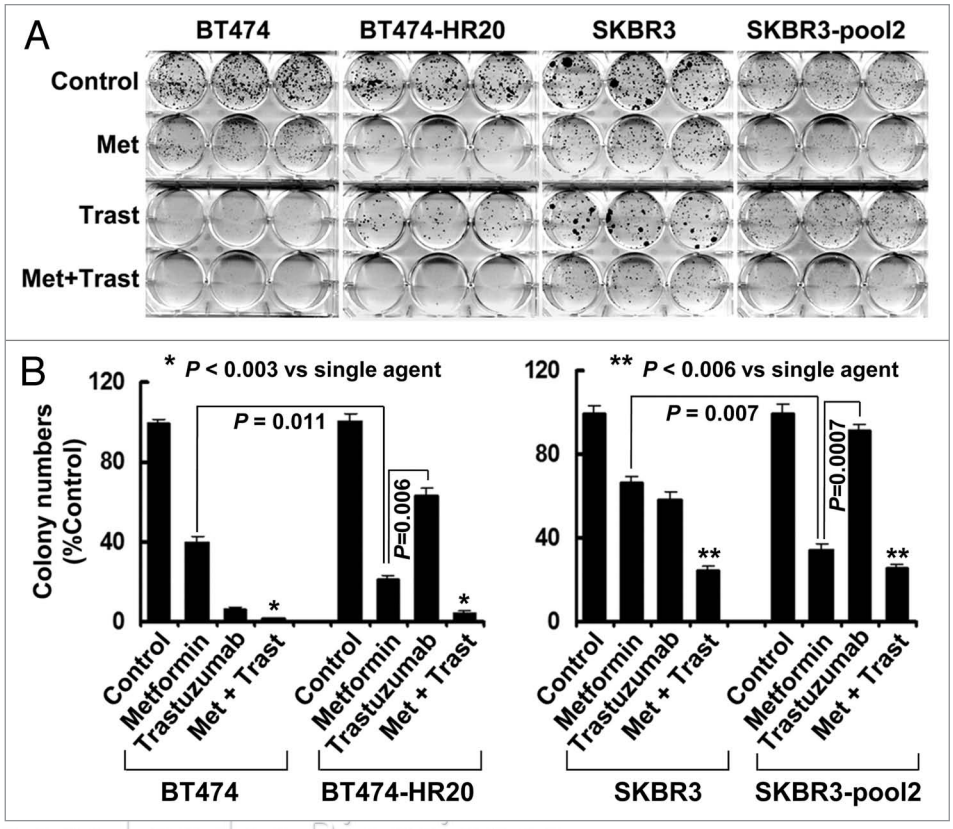

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Figure 2. Metformin has greater inhibitory effects on colony formation of trastuzumab-resistant breast cancer cells. BT474, BT474-HR20, SKBR3 and SKBR3-pool2 cells were grown in six-well plates (1,000 cells/well) in triplicates. After 24 h, the culture medium was replaced with fresh medium containing 2.5% FBS as control, or same medium containing either metformin (2 mmol/L) or trastuzumab (10 μg/ml) alone, or their combinations. The culture medium was changed once every three days for four weeks. The pictures of six-well plates with colonies were taken by a digital camera on day 28 and the bar graph was obtained by calculating the percentages of colony numbers from each cell line relative to control, defined as 100%, measured by the Bio-Rad Molecular Imager Gel Doc XR+ System. Bars, SD. Data shows the representative of three independent experiments.

Metformin-associated inhibition of Src kinase and/or PI-3K/Akt signaling, but not mTOR, is critical in trastuzumab-resistant breast cancer cells. Metformin's anti-cancer activity has been generally attributed to its inhibition of mTOR in the literature.[28,29] Because our rapamycin data suggested that mTOR was likely not a major factor in metformin action in the resistant lines, we conducted several additional experiments. We first compared expression levels of mTOR and phosphorylated mTOR (P-mTOR) levels in trastuzumab-resistant and -sensitive cells, and found no significant differences (**Fig. 5A**). We next treated each of these cells with metformin and examined mTOR and P-mTOR levels. In the BT474 parental and HR20 resistant cells, metformin reduced mTOR expression and activity to the same extent. In pool2 cells, however, metformin inhibited mTOR activity much more than it did in the parental SKBR3 cells (**Fig. 5A**). We also studied the response of these cells to rapamycin. Both of the trastuzumab-resistant sublines were more resistant to increasing doses of rapamycin than their parental lines (**Fig. 5B**). These data suggest that the metformin-associated potent anti-proliferative activity in the trastuzumab-resistant cells is not due to inhibition of mTOR signaling.

We have previously reported significant activation of Src kinase and Akt signaling in HR20 and pool2 cells as compared with the parental lines.[24] We therefore examined the effects of metformin on Src and Akt signaling pathways, comparing its effects with those of the specific Src inhibitor (Dasatinib) and the PI-3K inhibitor (LY294002). Metformin more effectively reduced phosphorylated Src (P-Src) levels in the trastuzumab resistant as compared with parental lines, whereas Dasatinib reduced P-Src in both the sensitive and resistant sublines (**Fig. 6A**). SKBR3 cells were more sensitive to growth inhibition with Dasatinib at

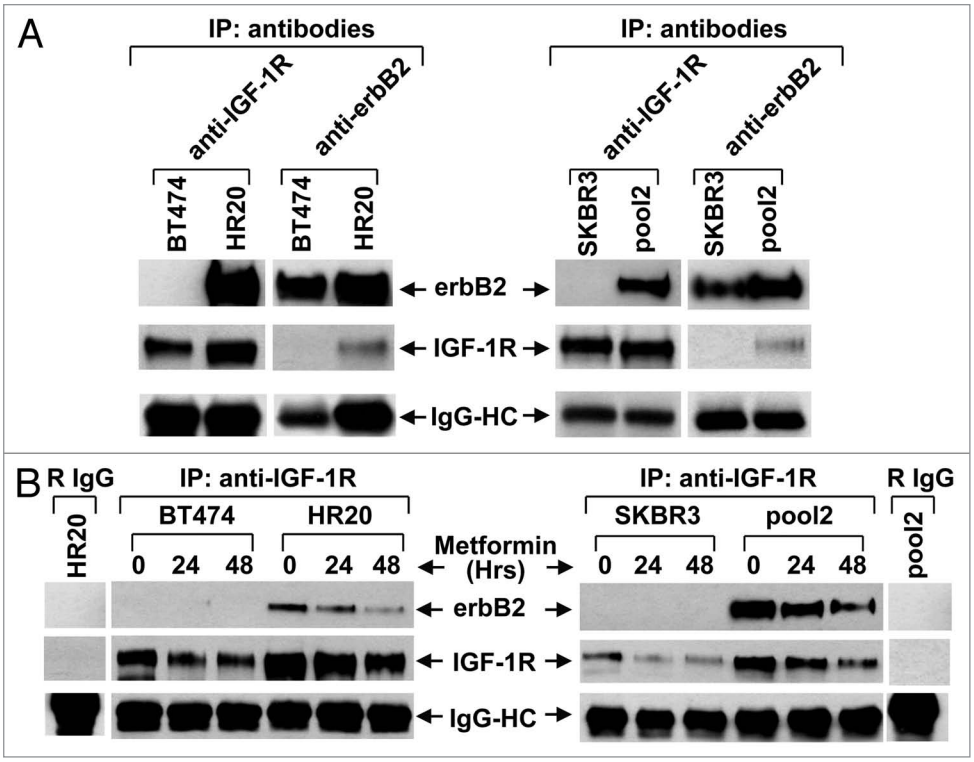

Figure 3. Metformin decreases erbB2/IGF-1R complexes in trastuzumab-resistant breast cancer cells. Co-immunoprecipitation assays were performed to detect the protein-protein interactions. (A) BT474, BT474-HR20, SKBR3 and SKBR3-pool2 cells were cultured at normal condition in the absence of trastuzumab for 24 h. Equal amount of total cell lysate was subjected to immunoprecipitation (IP) with an anti-IGF-1R or anti-erbB2 antibody. The immunoprecipitates were then analyzed by western blot assays with specific antibody directed against erbB2 or IGF-1R. (B) The same cells were grown in DMEM/F12 with 0.5% FBS and untreated (0) or treated with metformin (5 mmol/L) for 24 or 48 h. Equal amount of total cell lysate was subjected to IP with a rabbit IgG (R IgG) or the rabbit anti-IGF-IR antibody, and then followed by western blot analyses with specific antibodies directed against erbB2 or IGF-1R.

levels greater than 0.2 μmol/L, whereas pool2 cells were more resistant to its growth inhibitory effects even at higher drug concentrations. In BT474 and HR20 cells, Dasatinib induced similar growth inhibition (**Fig. 6B**). Hence, the cell responses to the specific Src inhibitor showed some similarity to metformin in one resistant subline (HR20), but close similarities were not observed in the pool2 cells. A similar strategy was employed to study the effects metformin and a specific PI-3K inhibitor LY294002 on trastuzumab-resistant and -sensitive lines. LY294002 and metformin reduced phosphorylated Akt (P-Akt) levels similarly in pool2 cells (**Fig. 6C**). Pool2 cells also showed significantly more growth inhibition than their parental SKBR3 cells in response to LY294002 (**Fig. 6D**). In contrast, HR20 cells showed no significant inhibition of P-Akt in response to metformin with 6-h treatment, whereas the P-Akt levels were dramatically decreased by LY294002 (**Fig. 6C**). However, with 24-h treatment, metfromin did significantly reduce the levels of P-Akt in both HR20 and pool2 cells (**Fig. 4B**). In the parental line BT474, both metformin and LY294002 reduced P-Akt levels (**Fig. 6C**). This parental and derived subline exhibited an equal responsiveness to LY294002-induced growth inhibition (**Fig. 6D**). Collectively, our data suggest that metformin exhibits potent inhibitory effects on trastuzumab-resistant breast cancer cells via inactivation of Src kinase and/or PI-3K/Akt pathway, but independent of mTOR signaling.

Discussion

Metformin is generally non-toxic, available worldwide and it is remarkably inexpensive. Although it is best known as an anti-diabetic drug, metformin has been shown to have significant and diverse anti-cancer activity. This is widely believed to be due to a reduction in insulin/insulin like growth factor signaling and suppression of the mTOR/p70S6K1 axis.[30,31] Because of its potent activation of the energy sensor AMPK and AMPK-activating kinase LKB1,[32,33] metformin has also been shown to have similar biological effects as calorie restriction (CR) in mammals to increase life span and reduce the incidence of human cancer.[34] Metformin is currently being used in combination with other agents in randomized trials for breast and other cancer patients (www.clinicaltrials.gov). Preclinical, clinical and epidemiologic study data for the use of metformin against breast cancer is promising.[21,22] In a phase II trial of non-diabetic early breast cancer patients (of all molecular subtypes) who had completed primary therapy, metformin significantly reduced both insulin levels (by 22%) and body mass index (BMI).[35] These factors have long been associated with a worse outcome for breast cancer patients.

Therapeutic potential of metformin for women with erbB2-overexpressing breast cancers has been suggested by in vitro data showing a reduction of erbB2 tyrosine kinase activity, even at low concentrations of the drug.[16,18,36] A recent in vitro study has suggested that metformin may also interact significantly with other agents, modifying toxicity or resistant patterns.[23] Because the mechanisms of therapeutic resistance for lapatinib and trastuzumab are different,[37] we sought to explore the effects of metformin on trastuzumab-resistant breast cancer cells in vitro. To explore these interactions in vivo, a clinical trial of neo-adjuvant chemotherapy plus trastuzumab, with or without metformin, has recently been initiated in erbB2 positive breast cancer patients.[38] This trial may provide important clinical data as to whether metformin enhances survival or modifies trastuzumab sensitivity (or alternatively the emergence of resistance). Because of the study design, this study will not likely address a possible role for metformin in patients with trastuzumab resistance.

In aggregate, our data shows that trastuzumab resistant lines are relatively insensitive to the mTOR inhibitor rapamycin, even though resistant and parental lines expressed similar levels of P-mTOR and mTOR. Both resistant sublines expressed higher levels of activated Src and Akt when compared with the parental controls. Metformin significantly reduced activation of both Src and Akt, especially in the resistant lines. The resistant cells were variably sensitive to specific inhibitors of Src and PI-3K/Akt signaling. One resistant subline HR20 responded similarly as compared with its parental control BT474 to Dasatinib and LY294002, suggesting that inactivation of both Src kinase and PI-3K/Akt signaling contribute to its enhanced sensitivity to metformin. In contrast, the pool2 cells were resistant to Dasatinib, but exhibited a much greater sensitivity to LY294002 than the respective parental SKBR3 cells. Thus in the pool2 cells, PI-3K/Akt signaling likely plays the pivotal role in the trastuzumab-resistance and metformin sensitivity. Although we expected to show that metformin acted through mTOR via AMPK-dependent[39] or -independent[40] mechanisms, our data opposes this assumption. We showed that resistant cells had activation of erbB3 and IGF-1R, formed erbB2/IGF-1R complexes (**Figs. 3 and 4**), and other downstream signaling pathways were active, such as Src kinase and/or PI-3K/Akt.[24] Our data are therefore consistent with other reports that have shown that PI-3K/Akt and Src activation are critical mechanisms of trastuzumab resistance.[41,42] Of particular translational importance, our data suggest that the anti-diabetic agent metformin may be particularly effective in the resistant cells due to its strong inhibition on Src and Akt.

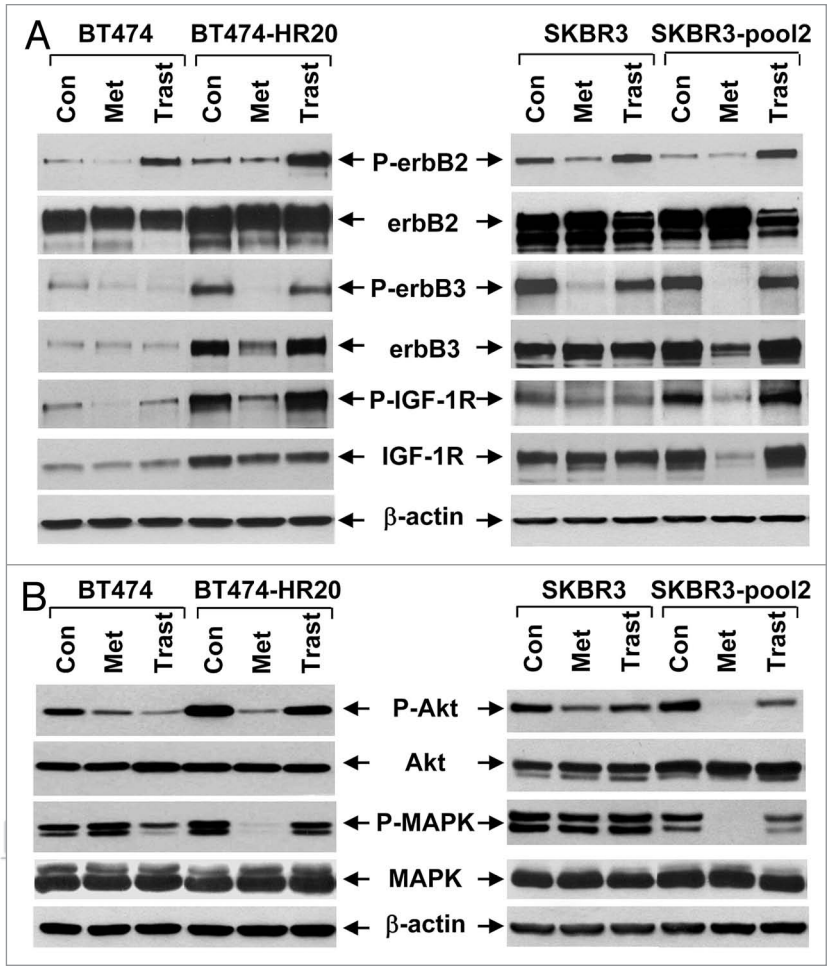

Figure 4. Metformin dramatically reduces the levels of both P-erbB3 and P-IGF-1R, and inactivates their downstream signaling in trastuzumab-resistant cells. BT474, BT474-HR20, SKBR3 and SKBR3-pool2 cells were untreated or treated with either metformin (2 mmol/L) or trastuzumab (10 μg/ml) for 48 h. Cells were collected and lysed. (A) Equal amount of total cell lysate was subjected to western blot analyses with specific antibody directed against P-erbB2, erbB2, P-erbB3, erbB3, P-IGF-1R, IGF-1R or β-actin. (B) The same lysates were subjected to western blot analyses with specific antibodies directed against P-Akt, Akt, P-MAPK, MAPK or β-actin.

IGF-1/IGF-1R signaling appears to be important in the development of trastuzumab resistance.[43-45] We and others have shown that crosstalk occurs between IGF-1R and erbB2 signaling, and that IGF-1R physically interacts with erbB2 in trastuzumab-resistant breast cancer cells.[24,25] Although one might expect metformin to only prevent activation of the IGF-1R signaling in vivo, due to a reduction in circulating insulin and IGF-1 in cancer patients,[22,35] our studies indicated that metformin also significantly reduced the erbB2 and IGF-1R interactions in a time-dependent manner in both HR20 and pool2 cells (**Fig. 3**). Further detailed analyses and clinical trials will be necessary to identify the specific molecular mechanisms, and to determine whether metformin would have similar effects in vivo. We are excited by the recent reports suggesting that metformin disrupts crosstalk between insulin/IGF-1 receptors and G protein-coupled receptors in pancreatic cancer cells.[46,47] This mechanism should be explored in patients or models of breast cancer as well.

Another important receptor tyrosine kinase, the erbB3 receptor plays a critical role in breast carcinogenesis driven by *erbB2* amplification/overexpression.[48] A recent report suggests that ligand-induced dimerization between erbB3 and erbB2 is one likely mechanism of trastuzumab resistance.[26] We have reported that both erbB3 and IGF-1R work cooperatively with erbB2, and these three RTKs interact with each other by forming multi-receptor complexes. These appear to induce activation of downstream signaling in trastuzumab-resistant breast cancer cells.[24] Considering the importance of both IGF-1R and erbB3 signaling in this process, it is conceivable that the broad effects of metformin may include prevention or delay of the development of trastuzumab resistance.

In summary, *erbB2* altered breast cancer cells develop trastuzumab resistance as a result of complex and interactive molecular escape mechanisms. Because metformin induces a wide range of molecular changes in the cancerous cells, and many of these include mechanisms of trastuzumab resistance, its use in patients who develop resistant disease may be beneficial. We have shown

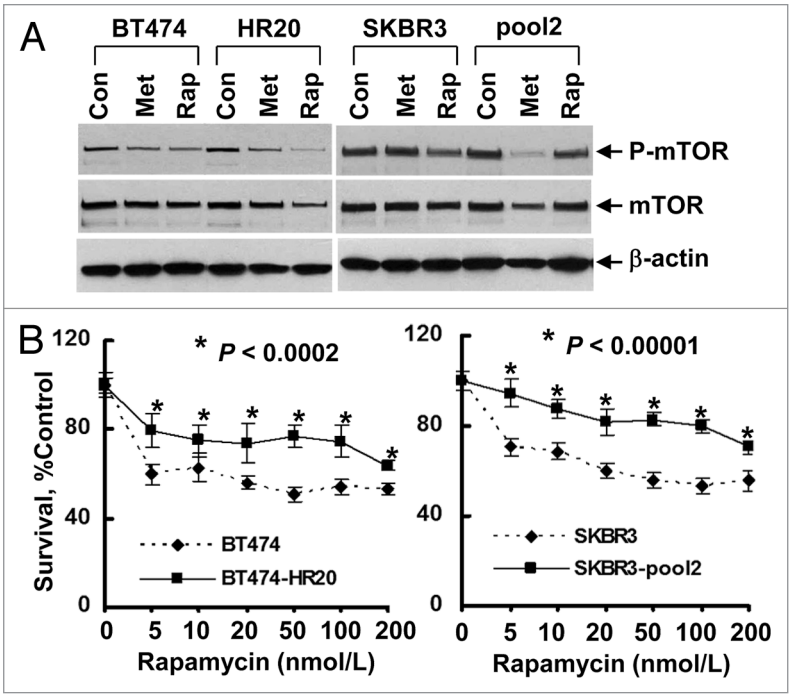

Figure 5. Trastuzumab-resistant breast cancer cells remain insensitive to mTOR inhibitor rapamycin. (A) BT474, BT474-HR20, SKBR3 and SKBR3-pool2 cells were untreated or treated with either metformin (10 mmol/L) or rapamycin (200 nmol/L) for 24 h. Cells were collected and subjected to western blot analyses with specific antibodies directed against P-mTOR, mTOR or β-actin. (B) BT474, BT474-HR20 or SKBR3, SKBR3-pool2 cells were plated onto 96-well plates and incubated at 37°C with 5% CO_2. After 24 h, the culture medium was replaced with 0.1 ml fresh medium containing 0.5% FBS or the same medium containing the indicated concentrations of rapamycin for another 72 h. The percentages of surviving cells from each cell line relative to control, defined as 100% survival, were determined by reduction of MTS. Bars, SD. Data shows a representative of three independent experiments.

that it can inactivate erbB3 and IGF-1R, reduce complex formation between erbB2 and IGF-1R, and deactivate downstream signaling involving Src kinase and/or the PI-3K/Akt pathways. Our studies provide a rationale to study the activity of metformin in patients with trastuzumab-resistant breast cancers in vivo.

Materials and Methods

Reagents and antibodies. Metformin (1,1-dimethylbiguanide hydrochloride) was purchased from MP Biomedicals, LLC (Solon, OH), and dissolved in sterile water to make a 1 mol/L stock solution. Trastuzumab (Herceptin®) was obtained from Genentech Inc., (South San Francisco, CA) under a research agreement. Dasatinib was from LC Laboratories (Woburn, MA). LY294002 was from EMD Biosciences (Gibbstown, NJ). Rapamycin was from Sigma Corp., (St. Louis, MO). Antibodies were obtained from the following sources: P-erbB2 (Tyr1248), erbB3 (2C3) (Lab Vision, Fremont, CA), erbB2 (Ab-3) (Oncogene Research Products, San Diago, CA), P-erbB3 (Tyr1289), P-Akt (Ser473), Akt, P-MAPK (Thr202/204), MAPK, P-mTOR (Ser2448), mTOR, P-Src (Tyr416), Src, P-IGF-1R (Tyr1131/Tyr1146), IGF-1Rβ (Cell Signaling Technology Inc., Danvers, MA). All other reagents were purchased from Sigma unless otherwise specified.

Cells and cell culture. ErbB2-overexpressing human breast cancer cell lines BT474 and SKBR3 were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in Dulbecco's Modified Eagle's Medium: Nutrient Mix F-12 (D-MEM/F-12 1:1) (Invitrogen Corp., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). Both cell lines and their trastuzumab-resistant sublines, BT474-HR20 (HR20) and SKBR3-pool2 (pool2),[24] were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO_2 and split twice a week. Both HR20 and pool2 were maintained with 20 μg/ml of trastuzumab added to the media. The identity of all the cell lines was recently confirmed with DNA profiling by our Cancer Center's DNA Sequencing and Analysis Core facility.

Cell proliferation assays. A CellTiter96™ AQ non-radioactive cell proliferation kit (Promega Corp., Madison, WI) was used to determine cell viability as described previously in reference 16 and 17. In brief, cells were plated onto 96-well plates. After 24 h incubation in complete media (10% FBS in DMEM/F12), the cells were then grown in either DMEM/F12 medium with 0.5% FBS as control, or the same medium containing different concentrations of metformin and/or trastuzumab, and incubated for another 72 h. After reading all wells at 490 nm with a micro-plate reader, the percentages of surviving cells from each group relative to controls, defined as 100% survival, were determined by reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt).

Clonogenic assays. Clonogenic assays were performed as previously described in reference 16 and 17. In brief, cells were seeded into 6-well plates in triplicate at a density of 1,000 cells/well with 2 ml of complete medium containing 10% FBS. After 24 h, cultures were replaced with fresh medium containing 2.5% FBS or the same medium containing metformin, trastuzumab or a combination of metformin and trastuzumab. Cells were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 for 4 weeks. The cell clones were stained for 15 min with a solution of 0.5% crystal violet and 25% methanol, followed by three rinses with tap water to remove excess dye. Colony numbers were counted by a gel documentation system (Bio-Rad Laboratories, Hercules, CA).

Immunoprecipitation and western blot analyses. Immunoprecipitation and western blot assays were performed as described previously in reference 24 and 49. Equal amounts of cell lysates were incubated with primary antibody for 2 h at 4°C, followed by incubation with protein A or G-agarose (Roche Diagnostics Corp., Indianapolis, IN) at 4°C overnight. The immunoprecipitates or equal amounts of total cell lysates were boiled in SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose (Bio-Rad Laboratories), and probed with primary antibodies as described in the figure legends. After the blots

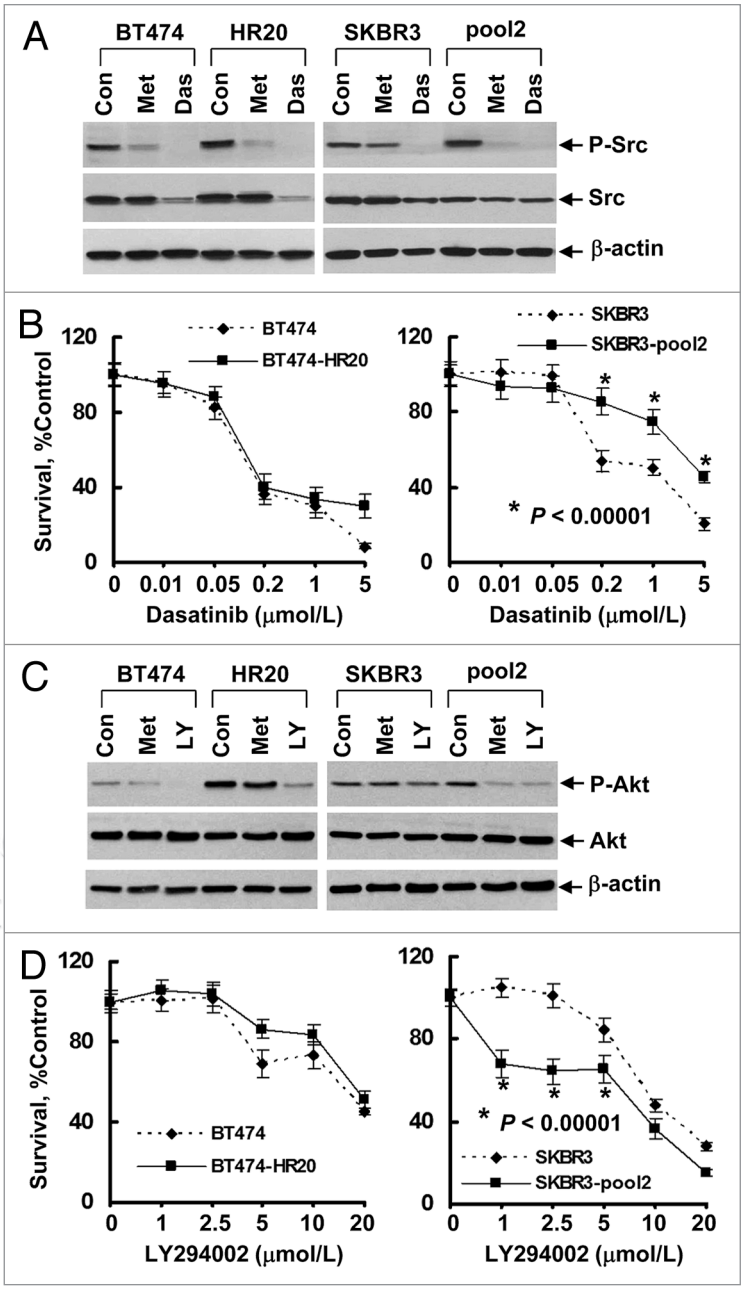

Figure 6. Dasatinib or LY294002 inhibits proliferation of trastuzumab-sensitive and -resistant breast cancer cells. (A) BT474, BT474-HR20, SKBR3 and SKBR3-pool2 cells were untreated or treated with either metformin (10 mmol/L) or Dasatinib (0.2 μmol/L) for 24 h. Cells were collected and subjected to western blot analyses with specific antibodies directed against P-Src, Src or β-actin. (B) BT474, BT474-HR20 or SKBR3, SKBR3-pool2 cells were plated onto 96-well plates and incubated for 24 h. The culture medium was then replaced with fresh medium containing 0.5% FBS or the same medium containing the indicated concentrations of Dasatinib for another 72 h incubation. The percentages of surviving cells from each cell line relative to controls, defined as 100% survival, were determined by reduction of MTS. Bars, SD. Data shows a representative of three independent experiments. (C) The same cells were untreated or treated with either metformin (10 mmol/L) or LY294002 (20 μmol/L) for 6 h. Cells were collected and subjected to western blot analyses with specific antibodies directed against P-Akt, Akt or β-actin. (D) The cells were plated onto 96-well plates and incubated for 24 h. The culture medium was then replaced with fresh medium containing 0.5% FBS or the same medium containing the indicated concentrations of LY294002 for another 72 h. The percentages of surviving cells from each cell line relative to control, defined as 100% survival, were determined by reduction of MTS. Bars, SD. Data shows a representative of three independent experiments.

were incubated with horseradish peroxidase-labeled secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), the signals were detected using enhanced chemiluminescence reagents (Amersham Life Science, Piscataway, NJ).

Statistical analyses. Statistical analyses of the experimental data were performed using a two-sided Student's t test. Significance was set at a p value of < 0.05. The EC_{50}s were calculated using GraphPad Prism version 4.01 (La Jolla, CA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are grateful to Dr. Francisco Esteva (MD Anderson Cancer Center) for providing SKBR3-pool2 cells; and to Ms. Lisa Litzenberger for her excellent assistance in arts preparation. This work was supported in part by a Susan G. Komen for the Cure Research Grant (BCTR0707449) to B.L.

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