Abstract. Background: Triple-negative breast cancer (TNBC) is an aggressive sub-type of breast cancer. Dasatinib and bosutinib are FDA-approved Src/Abl kinase inhibitor drugs. Dasatinib potently inhibits the proliferation of many TNBC cell lines. Materials and Methods: The cell viability/proliferation for a panel of 4 TNBC cell lines was measured by detection of cellular ATP levels and cell numbers were directly determined by automated cell counting. Results: Bosutinib (≤1 μM) had little to no inhibitory activity on cell viability/proliferation, while dasatinib-alone generated potent IC50 values of <100 nM. Combination treatment of cells with both dasatinib and bosutinib resulted in reduced efficacy of dasatinib in all four cell lines, with two of them displaying a dramatic loss of efficacy. Direct cell counting confirmed that bosutinib enhanced cell proliferation in the presence of dasatinib. Conclusion: Bosutinib potently reduced the in vitro anti-proliferative efficacy of dasatinib in TNBC cell lines. We, hereby, report on a novel drug-induced loss in dasatinib sensitivity.

Approximately 15-20% of breast cancers are negative for the expression of estrogen receptor (ER) and progesterone (PR) expression and lack ErbB2 overexpression (1). Hence, this clinical sub-type of breast cancer is referred to as triple-negative breast cancer (TNBC). This clinical phenotype significantly overlaps with the basal-type breast cancer subtype established, based on whole-transcriptome microarray gene expression analysis of breast cancers (2). The diagnosis of TNBC correlates with aggressive cancer and there are limited treatment options, with no efficacious targeted-therapies.

TNBC has the general characteristic of aggressive relapses i.e. initially the cancer responds to chemotherapy, but then rapidly gains resistance and grows back (3). There are at least three general mechanisms for acquired resistance to targeted or traditional anticancer therapy. One of the most straightforward mechanisms is mutation of the drug target such as mutations in the kinase inhibitor targets EGFR, Abl and ALK (4-8). Another mechanism for acquired resistance is the activation of alternate pathways that bypass the original target which allow cells to proliferate and/or survive, as has been demonstrated for Met and ERBB2 (9, 10). Resistance to chemotherapy can also arise due to up-regulation of ABC transporters that actively pump drugs out of the cell, thereby reducing the intracellular drug concentration (11).

Dasatinib (BMS-354825) is a Food and Drug Administration (FDA)-approved cancer drug which has been approved for treatment of acute lymphoblastic leukemia (ALL) and chronic myelogenous leukemia (CML) (12). Dasatinib is an orally-active ATP-competitive small-molecule kinase inhibitor that potently inhibits Abl kinase, Src family kinases and other kinases (13). From in vitro biochemical kinase profiling data, this drug also potently (IC50≤1 nM) inhibits various other kinases including c-Kit, PDGFR and kinases in the EPH family. In addition to its activity against leukemia cells, dasatinib has also been shown to inhibit cell proliferation in numerous different types of tumor cell lines, including basal-type/TNBC (14), gastric (15), pancreatic (16), head and neck (17), ovarian (18), prostate (19) and lung cell lines (20, 21). The activity in solid tumors has been proposed to be due to the inhibition of Src family kinases. However, the DDR2 kinase was identified as a target of dasatinib in squamous cell lung cancer cells (22, 23). In mouse models of prostate and pancreatic cancer, dasatinib
inhibited tumor growth and metastasis (24, 25). Therefore, numerous clinical trials are on-going in which dasatinib is being assessed as monotherapy or in combination with other cancer drugs for efficacy against many cancer types.

Most basal-type and post-EMT cell lines have been discovered to be highly sensitive to cell growth inhibition by dasatinib in vitro with IC50 values <1 μM (14, 20, 26). In contrast, luminal-subtype breast cancer cell lines are generally resistant (IC50 >1 μM) (14). In general, the effect of dasatinib on dasatinib-sensitive breast cancer cell lines is predominately anti-proliferative with modest to no induction of apoptosis and cell death (27, 28). In the dasatinib sensitive TNBC cell line MDA-MB-231, dasatinib treatment induces G1 accumulation with little apoptosis, disrupts cell morphology and blocks in vitro migration and invasion (28). Despite this potent in vitro activity, dasatinib as a monotherapy failed to demonstrate significant efficacy against TNBC in a Phase 2 clinical trial (29). Other clinical trials are on-going using dasatinib in combination with other chemotherapies for TNBC.

Bosutinib (SKI-606) is also an FDA-approved drug for the treatment of Philadelphia chromosome positive (Ph+) CML in adult patients with resistance or intolerance to prior therapy (30). Like dasatinib, bosutinib is an ATP competitive Abl and Src inhibitor (31). However, chemical proteomics approaches have identified 40 to >45 target kinases for bosutinib and not an isomer (34).

We report that bosutinib potently inhibits the anti-proliferative effect of dasatinib on four TNBC cell lines. In a direct measure of cell number, the combination of dasatinib and bosutinib on MDA-MB-231 cells resulted in increased cell number compared to dasatinib alone.

Materials and Methods

Materials. All common reagents such as dimethyl sulfoxide (DMSO) were reagent-grade quality and obtained from Thermo Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich (St. Louis, MO, USA). Tissue culture media, fetal bovine serum (FBS) and supplements were purchased from Thermo Fisher Scientific. The CellTiter-Glo™ viability assay kit (cat# G7573) was obtained from Promega (Madison, WI, USA). Dasatinib and bosutinib were obtained from LC Laboratories (Woburn, MA, USA) and Selleck Chemicals (Houston, TX, USA), respectively. The manufacturer asserted that this was genuine bosutinib and, in addition, we subjected our lot of bosutinib to NMR analysis and confirmed the structure of the bosutinib used in this report as being genuine bosutinib and not an isomer (34).

Cell culture. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C in an atmosphere of 5% CO2 and 95% humidity in their respective media. MDA-MB-231 and Hs578T cells were grown in DMEM-high glucose media supplemented with 10% FBS, 200 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 15 mM HEPES. HCC1143 and HCC1806 were grown in RPMI-1640 supplemented in the same way. Cells used for experiments did not exceed passage 15.

Cell viability assay. Cells were harvested from tissue culture flasks for assay with trypsin/EDTA solution (Versene, Fisher). 80 μl of cells in normal growth media were added to a 96-well plate such that 2,500 cells/well were plated. After an overnight incubation (16-24 h) in the incubator, cells were treated with compounds. Compound stock solutions were made in 100% DMSO. Serial dilutions of compounds for IC50 determinations were initially performed in 100% DMSO, followed by appropriate dilutions in growth culture media, producing a constant 0.1% DMSO in all wells. Subsequently, 10 μl of each diluted compound (or 20 μl of DMSO for controls or 10 μl diluted DMSO for single-compound treatments) were added to the wells for a final volume of 100 μL and 0.1% DMSO. After a 72-h exposure to compounds, cell viability was assessed using the CellTiter Glo kit (Promega) per manufacturer’s protocol. Luminescence was measured using a BMG Pherastar plate reader. Relative luminescence units (RLU), were normalized to DMSO (100% activity) and “no cells” (0% activity) controls as maximum and minimum signals, respectively. Compound concentration response curves were generated using data points that represent the average of three determinations per concentration. All IC50/EC50 values provided are averages of at least three independent determinations. The IC50/EC50 values were calculated from concentration-response data using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) employing either a four-parameter or a three-parameter (fixed bottom or top) curve fit.

Cell counting assay. MDA-MB-231 cells were seeded in 6-well plates at 120,000 cells per well and incubated at 37°C and 5% CO2 for 16-24 h. Cells were treated with the compound as described above. At the time of harvesting, treatment media were removed (and saved); cells were washed briefly in phosphate buffered saline (PBS), trypsinized and complete growth media added. In order to ensure the collection of all cells (living and dead), supernatants, washes, trypsinized cells, and recovery media were pooled and cell count/viability determined using trypan blue and a Vi-CELL XR cell viability analyzer (Beckman Coulter, Brea, CA, USA) following manufacturer’s guidelines.

Results

We tested a panel of kinase inhibitor drugs to distinguish the ones that could induce cell death in the presence of the anti-proliferative drug dasatinib, using the TNBC cell line MDA-MB-231. Surprisingly, we discovered that one drug, bosutinib, appeared to inhibit the anti-proliferative activity of dasatinib. In the present report, we have used a panel of 4 dasatinib-sensitive TNBC cell lines and cell viability/proliferation assays to confirm this initial observation. Within the time frame of our 96-well cell viability assay (72 h), these cell lines approximately doubled in number and hence complete inhibition of proliferation resulted in approximately half the signal, representing the originally plated cells. We performed bosutinib concentration response determinations in the
presence or absence of 100 nM dasatinib for the TNBC cell lines MDA-MB-231, HCC1143, HCC1806 and Hs578T (Figure 1). Bosutinib (up to 1 μM)-alone had little to no effect on the cell viability assay for all the cell lines tested (Figure 1). These data also indicated that bosutinib does not affect the assay detection method itself (e.g. enhancing luciferase activity). At 100 nM, dasatinib almost completely blocked cell proliferation in these cell lines (Figure 2) and is similar to the 90 nM serum concentration achieved in human clinical trials (35). In the presence of a constant 100-nM dasatinib, bosutinib demonstrated a dose-dependent increase in assay signal for all four cell lines (Figure 1). The MDA-MB-231 and HCC1143 breast cancer cell lines showed the most dramatic response to bosutinib, with maximal response at 1 μM bosutinib resulting in signals greater than 90% of control values. Since these are partial curves, we fixed the top of the bosutinib/dasatinib curve at 100% of controls to obtain relative effective concentration of bosutinib required for a 50% reversal of dasatinib-mediated inhibition (EC50). The calculated average bosutinib EC50 values (n=3) for MDA-MB-231, HCC1143, HCC1806 and Hs578T were 159±83 nM, 407±287 nM, 823±303 nM and >300 nM (highest bosutinib concentration tested). Thus, bosutinib potently reduced the dasatinib-mediated inhibition in this cell viability/proliferation assay.

To directly assess the effect of bosutinib on the anti-proliferative activity of dasatinib, we determined viable cell numbers using the trypan blue exclusion and an automated cell counter. We chose the MDA-MB-231 cell line for this assay where we exposed cells for 72 h to DMSO (control), 100 nM dasatinib, 500 nM bosutinib or the combination of dasatinib/bosutinib at these same concentrations (Figure 2). As expected, treatment with dasatinib resulted in approximately...

Figure 1. Bosutinib concentration response in cell viability assays with and without dasatinib. Bosutinib concentration response data were generated in the presence and absence of 100 nM dasatinib using the ATP detection cell viability assay. The assay was performed with the cell lines MDA-MB-231 (A), HCC1143 (B), HCC1806 (C) and Hs578T (D). Data were normalized to cells treated with compound vehicle (DMSO) and wells without cells and expressed as a percentage of these controls. Data points represent the average of three determinations per variable and error bars represent standard deviations. Data are representative of three independent experiments.
half the number of viable cells compared to cells treated with DMSO (vehicle control). While bosutinib-alone had no significant effect on cell number, bosutinib partially reversed dasatinib-mediated inhibition of cell proliferation.

In order to further characterize the apparent antagonistic effect of bosutinib on dasatinib activity, we performed dasatinib IC50 determinations with and without a fixed concentration of bosutinib (Figure 3). Depending on the cell line, bosutinib was fixed at 0.3-1 μM. The average relative IC50 values for all four cell lines ranged from 32-57 nM. In the presence of bosutinib, the relative potency of dasatinib in MDA-MB-231, HCC1806 and Hs578T did not change significantly – less than two-fold (range: 40-90 nM IC50 values). However, the maximal efficacy (inhibition) was significantly reduced in these three cell lines, especially dramatic for MDA-MB-231 (see Figure 3). Bosutinib appeared to reduce the maximal efficacy achieved in all three cell lines. For HCC1143, the IC50 for dasatinib shifted from an average relative IC50 of 32 nM to >3,000 nM in the presence of 1 μM bosutinib. Consistent with results from Figure 1, MDA-MB-231 and HCC1143 demonstrated the most dramatic reversal of dasatinib’s activity by bosutinib.

**Discussion**

We used a cell viability/proliferation assay to characterize the effect of bosutinib on the anti-proliferative activity of dasatinib using a panel of 4 TNBC cell lines. We have discovered a surprising effect of the anti cancer drug bosutinib. By itself, bosutinib at ≤1 μM had little to no inhibitory activity on cell viability/proliferation in these cell lines, while dasatinib alone generated potent IC50s. Treatment of all cell lines with bosutinib showed a dose-dependent increase in signal in the presence of a fixed concentration of dasatinib. We confirmed that bosutinib was inducing cell proliferation in the presence of dasatinib by performing direct cell counting. This partial reversal of the effect of dasatinib was reflected in IC50 determinations where the presence of bosutinib shifted the IC50 and/or efficacy of dasatinib in all 4 TNBC cell lines. Thus, in these cell lines, bosutinib appeared to antagonize the effect of dasatinib.

There were significant differences in the magnitude of activity that bosutinib had in the cell lines. The MDA-MB-231 and HCC1143 cell lines showed dramatic responses to bosutinib in the presence of dasatinib, especially of HCC1143 which almost lost responsiveness to dasatinib. For HCC1806 and Hs578T, bosutinib reduced the inhibitory activity (efficacy), but to a lesser extent than the other 2 lines. Therefore, the magnitude of inhibiting the effect of dasatinib on cells is clearly cell line dependent.

The mechanism for this bosutinib-induced resistance to dasatinib is unknown. Both dasatinib and bosutinib are frequently referred to as Src or Src family inhibitors. However, both compounds have multiple potential cellular kinase targets as indicated by selectivity studies (32). Kinome re-programming is a term for the activity of certain kinase inhibitors that have been discovered to paradoxically activate cell signaling pathways. For example, MEK inhibitors have been shown to indirectly induce receptor tyrosine kinase (RTK) activation in cells (36). To date, bosutinib has not been reported to trigger kinome re-programming. We postulate that bosutinib induces kinase re-programming of the cell signaling cascades in TNBC cell lines. Bosutinib may activate or enhance alternate cell signaling pathways that circumvent the blockade of cell proliferation caused by dasatinib.

There are several significant aspects to these results. This is the first report to show that bosutinib induces resistance to another drug. Whether this effect is specific to dasatinib or not will require further studies with other anti-proliferative drugs. These data also suggest that TNBC cells may be able to readily-mutate or over-express a pathway component – the same one postulated to be activated by bosutinib – and gain resistance to dasatinib chemotherapy. Of note, use of dasatinib-alone to treat TNBC patients has failed to show efficacy in clinical trials (29). This may be due to rapid selection of cancer cells that activate other pathways to circumvent the dasatinib blockade. Finally, the clinical use...
of the combination of dasatinib and bosutinib for treatment of TNBC patients, and perhaps other cancers, should be avoided without further studies.

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References


