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# STI571 sensitizes breast cancer cells to 5-fluorouracil, cisplatin and camptothecin in a cell type-specific manner

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ABSTRACT

Previously, we demonstrated that Abl kinases are highly active in invasive breast cancer cell lines, and contribute to survival in response to nutrient deprivation, invasion and proliferation. To determine whether an Abl kinase inhibitor, STI571 (Gleevec; imatinib mesylate) sensitizes breast cancer cells to chemotherapeutic agents, we treated three breast cancer cell lines (BT-549, MDA-MB-231, and MDA-MB-468) that have active Abl kinases, with STI571 in combination with several conventional chemotherapeutic drugs frequently used to treat breast cancer, and assessed the effect on cell viability, proliferation, and apoptosis. We found that STI571 had synergistic effects with cisplatin in BT-549 and to some extent in MDA-MB-468 cells; synergized with camptothecin using an alternate dosing regimen in MDA-MB-231 cells; and STI571 synergistically sensitized MDA-MB-468 cells to paclitaxel and to high doses of 5-fluorouracil. Significantly, STI571 increased the ability of cisplatin to inhibit constitutive activation of PI3K/Akt in BT-549 cells, synergized with camptothecin to increase the stability of IκB in MDA-MB-231 cells, and in MDA-MB-468 cells, camptothecin and 5-fluorouracil inhibited STI571-dependent activation of STAT3. In other cell line/drug combinations, STI571 had additive or antagonistic effects, indicating that the ability of STI571 to sensitize breast cancer cells to chemotherapeutic agents is cell type-dependent. Significantly, unlike cisplatin, paclitaxel, and camptothecin, mechloroethamine was strongly antagonistic to STI571, and the effect was not cell line-dependent. Taken together, these data indicate that the cellular milieu governs the response of breast cancer cells to STI571/chemotherapeutic combination regimens, which suggests that treatment with these combinations requires individualization.

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## 1. Introduction

The Abl family of non-receptor tyrosine kinases (Abl kinases) includes two proteins, c-Abl and Arg, which are encoded by *Abl1* and *Abl2* genes, respectively. The two kinases have homologous N-termini, which contain SH3, SH2 and kinase domains, but their C-terminal domains are more divergent as c-Abl contains nuclear localization signals and a DNA binding domain that are not present in Arg [1]. Both proteins have myristoylation signals that target the proteins to the plasma membrane. c-Abl and Arg are localized to the plasma membrane and cytoplasm, and c-Abl also is in the nucleus [1]. Subcellular localization of c-Abl is important for its function, as activation of nuclear c-Abl in fibroblasts by DNA damaging agents induces apoptosis, while activation of the cytoplasmic/membrane pool by growth factors and integrin engagement, promotes proliferation, survival, and migration [1,2].

Abl kinases are most known for their involvement in human leukemia. *Abl1* is translocated next to *BCR* forming a constitutively active BCR-Abl fusion protein, which drives the development of CML (chronic myelogenous leukemia) [3]. c-Abl and Arg are also translocated next to *Tel* in other forms of leukemia and myeloproliferative disease [3–5]. STI571 (Gleevec; imatinib mesylate) currently is first line therapy for patients with BCR-Abl$^+$ CML. Gleevec induces remission in patients with early stage disease, but patients in blast-crisis often relapse due to Gleevec resistance [6].

Breast cancer is the second leading cause of cancer-related deaths in U.S. women, and accounts for 25% of new cancer diagnoses each year [7]. Her-2-positive breast cancers are treated with Herceptin; however, many tumors develop resistance and others are Her-2-negative. Taxanes (e.g. paclitaxel; taxol), 5-

*Abbreviations:* 5-FU, 5-fluorouracil; CML, chronic myelogenous leukemia; ER, estrogen receptor; PR, progesterone receptor; (−), negative; (+), positive; topo I, topoisomerase I; PARP, poly(ADP-ribose) polymerase; TCA, trichloroacetic acid; CI, combination index; MMS, methyl methanesulfonate; Cis, cisplatin; PTX, paclitaxel; Cam, camptothecin; MCE, mechloroethamine; ERK, extracellular signal-regulated kinase; PDGF, platelet-derived-growth factor; FBS, fetal bovine serum.

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fluorouracil (5-FU), camptothecins, and cyclophosphamide, which metabolizes to mechloroethamine, are currently used for treatment of patients with $ER^-$ (estrogen receptor negative), $PR^-$ (progesterone receptor negative) tumors, and platinum (e.g. cisplatin) regimens are in preclinical trials [8–11]. Conventional chemotherapeutic approaches for treating patients with hormone-negative, Her-2-negative tumors are far from optimal, as the treatments often are not effective, resistance is common, and the drugs have significant side effects. Thus, the development of new combinations to treat this class of patients is critical. The use of targeted drugs together with conventional chemotherapeutic agents may increase the effectiveness of the agents, allowing doses to be decreased, thereby decreasing toxicity.

We showed that Abl kinases have dramatically increased activities in invasive breast cancer cells as compared to non-invasive MCF-7 breast cancer cells [12]. Interestingly, the mechanism of Abl kinase activation in breast cancer cells is unique from their activation in leukemia. In leukemia, chromosomal translocation activates the Abl kinases, whereas in $ER^-$, $PR^-$ breast cancer cells, Abl kinases are activated downstream of deregulated growth factor receptors such as EGFR, ErbB2/Her-2, and IGF-1R, as well as by activated Src family kinases [12,13]. Using STI571 and RNAi approaches, we showed that activation of Abl kinases in breast cancer cells has dramatic consequences as they promote proliferation, G1 → S transition in response to IGF-1, survival in response to nutrient deprivation, and invasion [12,13]. In this report, we demonstrate that STI571 synergizes with some conventional chemotherapeutic agents to inhibit proliferation and increase apoptosis of breast cancer cells in serum conditions. Interestingly, the effects of the drug combinations are cell type-specific, as STI571 synergizes with cisplatin in BT-549 cells, camptothecin in MDA-MB-231 cells, and 5-FU in MDA-MB-468 cells, whereas mechloroethamine has antagonistic effects with STI571 in all cell lines. Therefore, although all the cell lines are equally sensitive to the effects of STI571 alone [13], there are cell type-specific differences, which affect the ability of STI571 to synergize with conventional chemotherapeutic agents.

## 2. Materials and methods

### 2.1. Cell culture, reagents

Cell lines were purchased from the University of North Carolina Tissue Culture Facility. BT-549 cells were grown in RPMI/10% FBS (fetal bovine serum), and MDA-MB-231 and MDA-MB-468 cells were cultured in MEM/10% FBS/1 mM sodium pyruvate. STI571, kindly provided by Novartis (Basel, Switzerland), was dissolved in water at a concentration of 10 mM and stored at −80 °C. Antibodies to PARP (poly(ADP-ribose) polymerase; sc-8007), pan-ERK1/2 (sc-94), and IκB (sc-371) were obtained from Santa Cruz Biotechnologies (Santa Cruz Biotechnology; Santa Cruz, CA), GAPDH and STAT3 antibodies were obtained from BD Biosciences (Chicago, IL), phospho-Akt, Akt, and phospho-STAT3 (Y705) antibodies were from Cell Signaling Technology (Danvers, MA), and phospho-ERK1/2 antibody was from Promega (Madison, WI). Western blots were performed according to manufacturers' protocols.

### 2.2. Viability assays

Cells were plated in triplicate in 96-well dishes such that the vehicle-treated population was subconfluent at the end of the assay. The day after plating, the media was replaced with media containing drugs, and viability was assessed 72 h later using the Cell Titer-Glo Assay (Promega; Madison, WI). For 5-FU experiments, viability was assessed after 96 h. An equal volume of Cell Titer-Glo reagent (at room temperature) was added to each well, the plate was rocked, incubated at room temperature for 10 min, half of the mixture from each well was transferred to an opaque 96-well plate, and luminescence (all wavelengths of light emitted for 10 s) was measured on a Synergy 2 plate reader (Biotek; Winooski, VT).

### 2.3. Proliferation assays

Cells were plated in triplicate in 24-well dishes so that vehicle-treated cells were subconfluent on the day of harvest. The day after plating, the media was replaced with media containing drugs, and 72 h later (96 h for low dose 5-FU experiments) tritiated thymidine was added (2.5 μCi) for 2 h. Cells were harvested by washing with PBS, 10% trichloroacetic acid (TCA), incubating in 10% TCA for 45 min, solubilizing radioactivity in 0.2N NaOH, and reading on a scintillation counter.

### 2.4. Apoptosis assays

#### 2.4.1. PARP assay

Cells were plated in 60 mm dishes, the media was replaced with media containing drugs the next day, and 40 h later, detached and attached cells were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton-X 100, 0.1% SDS, 1% sodium deoxycholate, 1 mM PMSF, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 10 μg/ml leupeptin, aprotinin, pepstatin). Total protein was quantitated with a Lowry DC kit (BioRad; Hercules, CA), equal protein was loaded on SDS-PAGE gels, and blots were incubated with antibodies to PARP and GAPDH.

#### 2.4.2. Caspase-Glo-3/7 assay (Promega; Madison, WI)

Cells were plated as described above, lysed in caspase lysis buffer (50 mM HEPES pH 7.4, 5 mM CHAPS, 5 mM DTT), clarified by centrifugation, 15 μl of the supernatant or buffer (blank) and 35 μl of buffer were added to wells in an opaque 96-well plate, and an equal volume (50 μl) of room temperature Caspase-Glo substrate was added to each well. The plate was rocked, and luminescence (all wavelengths of light emitted) from each well was read 40–60 min after addition of Caspase-Glo using the Synergy 2 plate reader. Luminescent values were normalized to protein concentration using the Bradford protein assay (BioRad; Hercules, CA).

### 2.5. Subcellular fractionation

Cells were plated in 60 mm dishes, and cytoplasmic/membrane and nuclear fractions were isolated using the NE-PER kit (Pierce; Rockford, IL) according to the manufacturer's protocol.

### 2.6. Statistics/determination of combination indices

Statistics were performed using SigmaStat for Windows (Systat Software, Inc.; San Jose, CA). Combination indices were determined using CalcuSyn software (Biosoft; Cambridge, UK) utilizing STI571 and chemotherapeutic drug dose–response curves.

## 3. Results

### 3.1. STI571 sensitizes MDA-MB-468 cells to paclitaxel

We previously showed that a variety of breast cancer cell lines containing highly active Abl kinases are sensitive to STI571, which inhibits cell proliferation in serum and serum-free conditions [13]. In this report, we utilized three of the cell lines that we previously characterized: BT-549, which has highly active c-Abl and Arg, MDA-MB-231, which has highly active c-Abl, and MDA-MB-468, which

**Table 1**
Combination indices for STI571/chemotherapeutic combinations.

| Drug | Cell line | Drug dose | STI571 dose | Viability CI | Proliferation CI |
|---|---|---|---|---|---|
| Paclitaxel | MDA-MB-231 | 5 nM, 10 nM | 10 μM | 1.1, 1.4 | |
| | BT-549 | 10 nM, 30 nM | 10 μM | 1.8, 2.4 | |
| | MDA-MB-468 | 10 nM, 50 nM | 10 μM | 0.99, 0.64 | |
| | MDA-MB-468 | 2.5 nM | 1, 5, 10 μM | | 1.0, 1.3, 1.1 |
| | MDA-MB-468 | 5 nM | 1, 5, 10 μM | | 1.2, 1.1, 1.1 |
| Cisplatin | MDA-MB-468 | 1.5 μM | 10 μM | 0.9, 0.72 | |
| | BT-549 | 10, 15 μM | 10 μM | 0.73, 0.47 | |
| | MDA-MB-231 | 25, 50 μM | 10 μM | 1.3, 1.3 | |
| | BT-549 | 0.5 μM | 2, 5, 10 μM | | 0.8, 0.7, 0.6 |
| | BT-549 | 1 μM | 2, 5, 10 μM | | 0.5, 0.4, 0.3 |
| | MDA-MB-468 | 0.125 μM | 2, 5, 10 μM | | 0.6, 0.9, 0.8 |
| | MDA-MB-468 | 0.5 μM | 2, 5, 10 μM | | 0.7, 0.8, 0.9 |
| Camptothecin | MDA-MB-468 | 0.5, 1 μM | 10 μM | 1.1, 1.1 | |
| | BT-549 | 0.01, 0.025 | 10 μM | 0.92, 1.2 | |
| | MDA-MB-231 | 0.5, 7.5 μM | 10 μM | 1.5, 1.2 | |
| | MDA-MB-231-Alt. dose | 0.5, 7.5 μM | 10 μM | 0.34, 0.64 | |
| | MDA-MB-231-Alt. dose | 0.0025 μM | 2, 5, 10 μM | | 1.1, 0.7, 0.9 |
| | MDA-MB-231-Alt. dose | 0.01 μM | 2, 5, 10 μM | | 1.2, 0.9, 0.9 |
| | MDA-MB-468 | 0.0125 μM | 2, 5, 10 μM | | 1.0, 1.3, 0.94 |
| | MDA-MB-468 | 0.035 μM | 2, 5, 10 μM | | 0.85, 0.95, 0.79 |
| 5-FU | MDA-MB-468 | 100, 500 μM | 10 μM | 0.44, 0.09 | |
| | BT-549 | 10, 500 μM | 10 μM | 1.6, 1.9 | |
| | MDA-MB-231 | 50, 500 μM | 10 μM | 2.0, 1.4 | |
| | MDA-MB-468 | 1.25 μM | 2, 5, 10 μM | | 2.1, 2.9, 1.8 |
| | MDA-MB-468 | 3.5 μM | 2, 5, 10 μM | | 1.5, 2.4, 1.5 |
| | MDA-MB-468 | 100 μM | 2, 5, 10 μM | | 0.9, 1.1, 1.1 |
| | MDA-MB-468 | 250 μM | 2, 5, 10 μM | | 1.0, 0.9, 0.5 |
| | MDA-MB-468 | 500 μM | 2, 5, 10 μM | | 0.7, 0.6, 0.4 |
| MCE | MDA-MB-468 | 1.5, 30 μM | 10 μM | 5.1, 2.9 | |
| | BT-549 | 1, 10 μM | 10 μM | 3.1, 3.4 | |
| | MDA-MB-231 | 20, 50 μM | 10 μM | 2.5, 3.6 | |

Combination indices (CI) were determined using CalcuSyn software and dose–response curves for STI571 and chemotherapeutic drugs (data not shown). CI values shown are representative of three independent experiments, and correspond to the combination of a single dose of chemotherapy with a single dose of STI571. Values greater than one indicate antagonism; values equal to one indicate additivity; and values less than one indicate synergism.

has highly active Arg and also expresses another STI571 target, c-Kit [12]. To determine whether STI571 sensitizes breast cancer cells to conventional chemotherapeutic agents, we treated the above cell lines in serum conditions with vehicle, STI571, chemotherapeutic drug, or STI571 + chemotherapeutic drug, and assessed the effect on viability, proliferation, and apoptosis. First, we tested the taxane, paclitaxel, a frequently used breast cancer drug, which promotes assembly of microtubules from tubulin dimers, leading to hyperstabilization and decreased depolymerization, thereby preventing microtubule reorganization, which is essential for interphase and mitotic cellular functions [8]. Breast cancer cells were treated with STI571 and paclitaxel alone or in combination, and viability was assessed using the Cell Titer-Glo assay, which quantitates ATP, a measure of metabolically active cells. A dose of 10 μM STI571 was used for these assays because we previously showed that 10 μM was required to effectively (65–75%) reduce phosphorylation/activities of active endogenous c-Abl and/or Arg [12]. Combination indices (CI), a measure of how well the drugs act together, were determined with CalcuSyn software, using STI571 and paclitaxel dose–response curves (Table 1; data not shown). CI values that are greater than one indicate drug antagonism, values equal to one indicate that the drugs act in an additive fashion, and values that are less than one indicate drug synergism.

In MDA-MB-231 and BT-549 cells, treatment with STI571 antagonized the effects of paclitaxel on viability (Fig. 1A and B; Table 1), whereas in MDA-MB-468 cells, STI571 slightly sensitized the cells to paclitaxel in an additive → synergistic manner (Fig. 1C; Table 1). To determine whether STI571 sensitized MDA-MB-468 cells to paclitaxel by inhibiting proliferation, we performed tritiated thymidine assays. Since tritiated thymidine assays are extremely sensitive, low doses of the drugs have a very dramatic effect, and thus low doses were utilized in order to accurately assess $IC_{50}$ values. We found a dose-dependent decrease in proliferation mediated by STI571 and paclitaxel alone in MDA-MB-468 cells, and the combination of the two drugs produced additive effects (Fig. 1D; Table 1). To determine whether apoptosis contributed to the additive → synergistic effects on viability, we measured two outcomes indicative of apoptosis: cleavage of PARP, a nuclear polymerase that is cleaved from 115 to 89 kDa during apoptosis [14]; and quantitation of caspase-3/7 activities using a luminescent caspase assay, Caspase-Glo (Promega; Madison, WI). Although PARP cleavage and caspase-3/7 assays are good indicators of apoptosis, Annexin V staining would be required to definitively confirm the presence of apoptotic cells. The caspase-3/7 proluminescent substrate contains the signature DEVD peptide sequence. Lysed cells release active caspase-3 or -7, which cleaves the DEVD substrate from aminoluciferin, and oxidation of luciferin by luciferase produces light. The amount of light produced is proportional to the amount of caspase activity. STI571 had no effect on the ability of paclitaxel to induce PARP cleavage or activate caspase-3/7 (Fig. 2A and B). Taken together, these data indicate that STI571 produces additive → synergistic effects with paclitaxel in MDA-MB-468 cells by decreasing proliferation, whereas STI571 antagonizes the effects of paclitaxel in BT-549 and MDA-MB-231 cells. To identify the mechanism by which STI571 reduces proliferation of MDA-MB-468 cells treated with paclitaxel, we blotted lysates with phospho-specific antibodies to Akt, ERK1/2 (Extracellular Signal-Regulated Kinase 1, 2), and

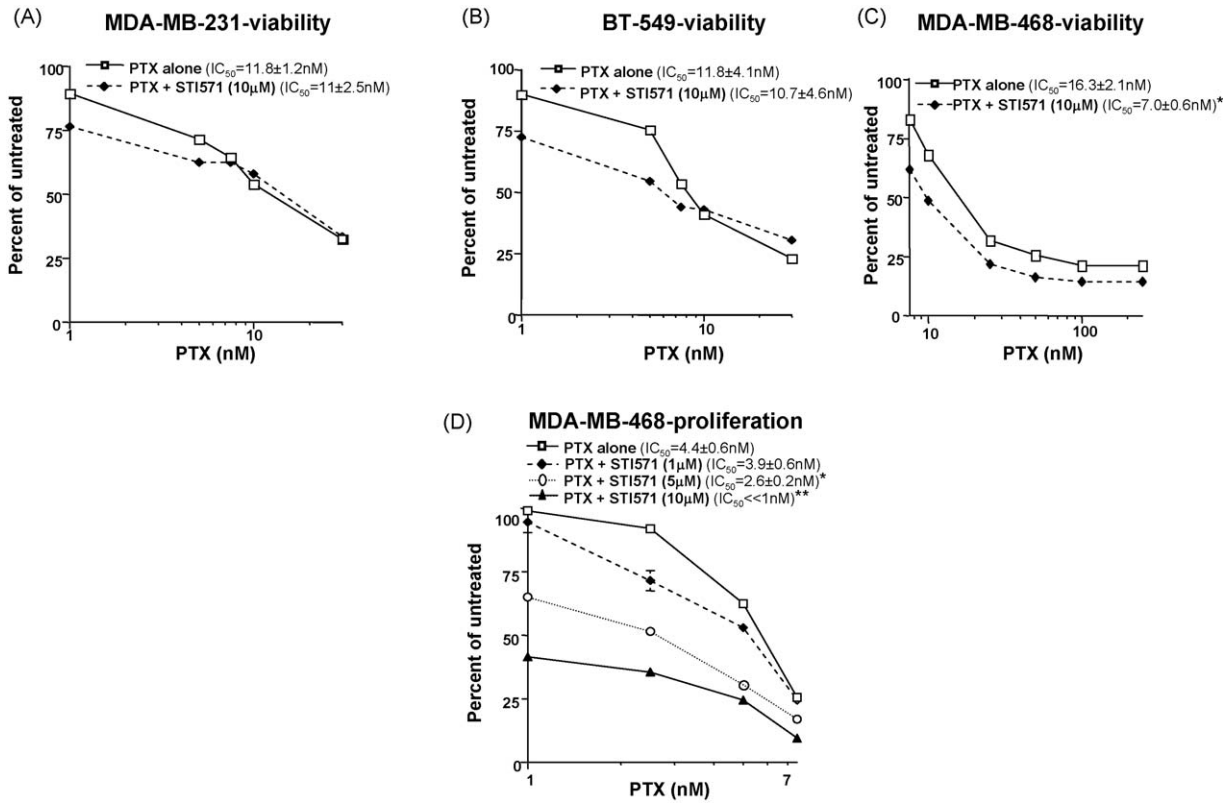


**Fig. 1.** Paclitaxel has additive or antagonistic effects with STI571 in breast cancer cells. (A–C) Cells were plated in triplicate in 96-well dishes, the media was replaced the next day with media containing drugs, and three days later viability was assessed by Cell Titer-Glo Assay. Experiments were performed three times in triplicate, and representative experiments are shown. Data is expressed as a percentage of values obtained from vehicle-treated cells. Mean ± S.E.M. Some error bars are too small to be visualized. $IC_{50}$ values are mean ± S.E.M. for three independent experiments. $^{*}p < 0.05$ using a Student's *t*-test. (D) MDA-MB-468 cells were plated in 24-well dishes in triplicate, the media was replaced with media containing drugs the next day, and proliferation was assessed 72 h later by tritiated thymidine assay. Experiments were performed in triplicate three times, and a representative experiment is shown. Mean ± S.E.M. Some error bars are too small to be visualized. $IC_{50}$ values are mean ± S.E.M. for three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.005$ using Student's *t*-tests.

STAT3, and with antibody to IκB, in order to assess whether activation of PI3K/Akt, Ras/ERK, STAT3, or NF-κB signaling pathways, which are involved in breast cancer cell proliferation and survival [15–19], are altered in the presence of STI571. IκB stability was not altered by STI571 and/or paclitaxel treatment (Fig. 2B). Moreover, Akt phosphorylation was increased by paclitaxel treatment, which may be a mechanism by which cells resist the effects of paclitaxel; however, STI571 had no effect on this upregulation (Fig. 2B). Interestingly, STI571 induced phosphorylation of ERK1/2 and STAT3 (Fig. 2B), which may be mechanisms by which cells resist the effects of STI571. Interestingly, treatment of cells with paclitaxel reduced the STI571-dependent induction of STAT3 and ERK1/2 phosphorylation (Fig. 2B), indicating that paclitaxel may sensitize cells to STI571 by inhibiting STI571-dependent activation of STAT3 and ERK1/2.

### *3.2. STI571 acts synergistically with cisplatin in BT-549 and MDA-MB-468 breast cancer cells to inhibit viability, proliferation, and survival*

Cisplatin is not commonly used to treat breast cancer; however, there are a number of preclinical and clinical studies underway to test whether cisplatin may be efficacious [11]. Cisplatin, a DNA damaging agent, crosslinks guanine bases in DNA strands, which inhibits the ability of the strands to uncoil and separate, thereby blocking cell division [11]. DNA damaging agents such MMS (methyl methanesulfonate), mitomycin C, and cisplatin induce apoptosis of non-transformed fibroblasts by activating the nuclear pool of c-Abl [20]. Since cisplatin induces apoptosis by activating nuclear c-Abl in fibroblasts, we hypothesized that STI571 treatment will inhibit cisplatin-induced activation of nuclear c-Abl, thereby antagonizing the effects of cisplatin. To our surprise, STI571 treatment of MDA-MB-468 cells reduced the cisplatin viability $IC_{50}$ nearly 3-fold, and STI571 demonstrated synergism with cisplatin at multiple doses (Fig. 3A; Table 1; data not shown). In BT-549 cells, the effect of STI571 on the cisplatin $IC_{50}$ was not as great (1.6-fold) as that observed in MDA-MB-468 cells; however, at certain cisplatin doses (10 μM, 15 μM), strong synergy was observed (Fig. 3B; Table 1). In contrast to BT-549 and MDA-MB-468 cells, STI571 slightly antagonized the effects of cisplatin in MDA-MB-231 cells (Fig. 3C; Table 1).

To determine the mechanism by which STI571 sensitizes BT-549 and MDA-MB-468 cells to cisplatin, we assessed the effect of combination treatment on proliferation and apoptosis. In both BT-549 and MDA-MB-468 cells, STI571 synergized with cisplatin to inhibit proliferation (Fig. 3D and E). In BT-549 cells, STI571 also dramatically sensitized cisplatin-treated cells to apoptosis, as evidenced by increased PARP cleavage and caspase-3/7 activity (Fig. 4A and B), whereas in MDA-MB-468 cells, STI571 treatment did not increase PARP cleavage or caspase-3/7 activity (Fig. 4C and D). Taken together, these data indicate that although cisplatin induces apoptosis by activating nuclear c-Abl in non-transformed cells, inhibition of Abl kinases using STI571 in BT-549 and MDA-MB-468 breast cancer cells does not prevent cisplatin from inhibiting viability, but rather sensitizes cells to cisplatin. In BT-549 cells, STI571 synergizes with cisplatin to inhibit cell viability by affecting both proliferation and apoptosis, whereas in MDA-

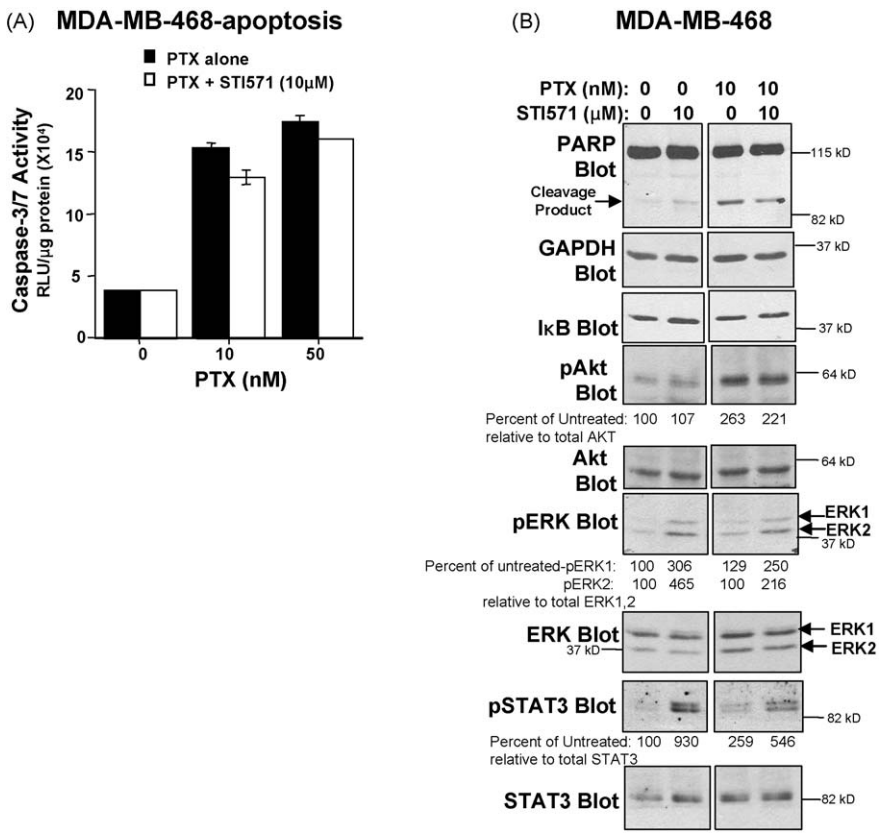


**Fig. 2.** Paclitaxel inhibits STI571-induced phosphorylation of STAT3 and ERK1/2 in MDA-MB-468 cells. MDA-MB-468 cells were plated in 60 mm dishes, treated with STI571 and/or paclitaxel the next day, and 40 h later, detached and attached cells were lysed and blotted with PARP and GAPDH antibodies (B) or caspase-3/7 activity was assessed by Caspase-Glo assay from duplicate samples (A). Mean ± S.E.M. from three independent experiments. Some error bars are too small to be visualized (A). PARP and GAPDH blots were stripped and probed with other antibodies (B).

MB-468 cells, the synergistic effect of STI571 on viability is due to an effect on proliferation. In contrast, in MDA-MB-231 cells, STI571 antagonized the effects of cisplatin (Fig. 3C; Table 1), which suggests that there is something intrinsically different about the MDA-MB-231 cell line, which prevents STI571 from sensitizing the cells to cisplatin.

To determine the mechanism by which STI571 synergistically inhibits proliferation and induces apoptosis in cisplatin-treated BT-549 cells, we analyzed PI3K/Akt, ERK1/2, STAT3, and NF-κB signaling pathways. Interestingly, STI571 and cisplatin increased the stability of IκB (Fig. 4A), which negatively regulates NF-κB signaling. However, STI571/cisplatin combination treatment did not result in increased IκB stabilization (Fig. 4A), which suggests that inhibition of NF-κB signaling is not a likely mechanism by which STI571 sensitizes BT-549 cells to cisplatin. Similarly, cisplatin treatment resulted in a significant reduction in STAT3 phosphorylation; however, STI571 treatment had no additional effect (Fig. 4A). Significantly, STI571 increased the ability of cisplatin to inhibit constitutive Akt phosphorylation (Fig. 4A), which indicates that STI571 is likely to increase apoptosis by inhibiting constitutive activation of the PI3K/Akt pathway. STI571 treatment also increased phosphorylation of ERK2, and cisplatin treatment further increased ERK2 phosphorylation (Fig. 4A). Activation of ERK2 by STI571 and cisplatin could be a mechanism by which BT-549 cells resist the effects of STI571 and cisplatin. However, it is also possible that ERK2 upregulation is a mechanism by which both drugs inhibit proliferation and induce apoptosis, since STI571 treatment of BCR-Abl$^+$ CML cells *increases* ERK1/2 activity, and treatment with a MEK1/2 inhibitor increases suppression of CML progenitors, and sensitizes cells to STI571-dependent apoptosis [21,22].

Interestingly, in MDA-MB-468 cells, STI571 and cisplatin had no effect on IκB stability or Akt phosphorylation, and similar to BT-549 cells, STI571 increased ERK2 activation in a synergistic manner with cisplatin (Fig. 4C). In addition, STI571 increased STAT3 phosphorylation, and cisplatin treatment decreased the STI571-dependent effect (Fig. 4C). Therefore, cisplatin may synergize with STI571 in MDA-MB-468 cells by inhibiting the STI571-dependent increase in STAT3 phosphorylation and/or by increasing ERK2 phosphorylation.

### 3.3. STI571 chemosensitizes MDA-MB-231 cells to camptothecin

Camptothecin binds topisomerase I (topo I), and prevents it from religating DNA, which results in single strand breaks in DNA, and camptothecin also stabilizes the topoisomerase I/DNA complex so that single strand nicks result in double strand breaks [9]. STI571 sensitized MDA-MB-468 and BT-549 cells to camptothecin in an additive fashion, and there was a 2-fold reduction in the $IC_{50}$ in the presence of STI571 (Fig. 5A and B; Table 1). In MDA-MB-231 cells, STI571 had an additive → antagonistic effect with camptothecin, when camptothecin was added at the same time as STI571 (Fig. 5C; Table 1). However, when MDA-MB-231 cells were treated with camptothecin 24 h prior to addition of STI571 (alternate dosing), STI571 synergistically sensitized cells to camptothecin, reducing the camptothecin $IC_{50}$ 5-fold (Fig. 5D; Table 1). An alternate dosing regimen also was tested with STI571 and other chemotherapeutic agents in MDA-MB-231 cells, and a

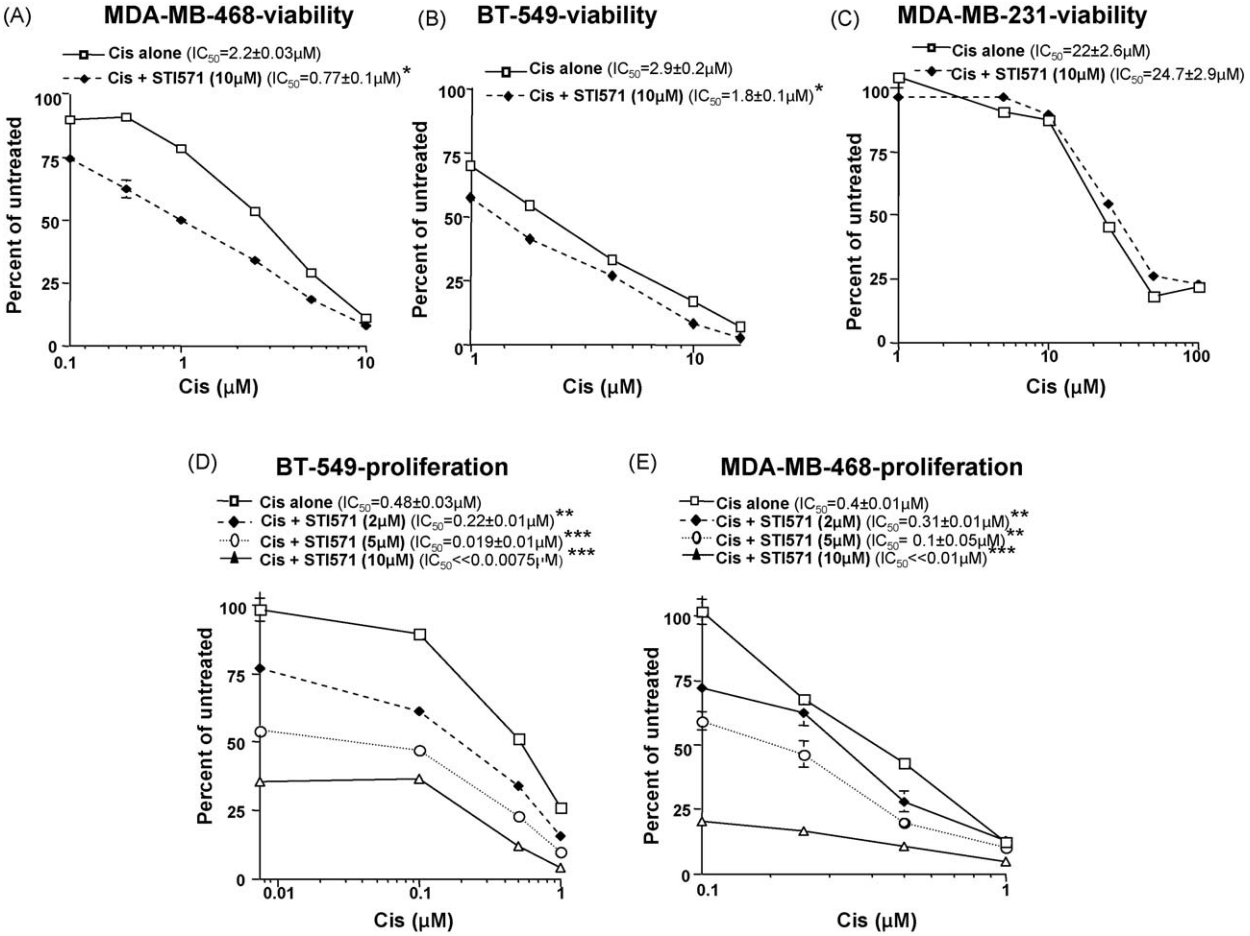


**Fig. 3.** STI571 synergistically sensitizes MDA-MB-468 and BT-549 breast cancer cells to cisplatin. (A–C) Cells were plated, treated, and viability assessed as in Fig. 1A–C. Experiments were performed in triplicate three times, and representative experiments are shown. Mean ± S.E.M. $IC_{50}$ values are mean ± S.E.M. for three independent experiments. $^{*}p < 0.05$, using a Student's *t*-test. (D and E) Proliferation was assessed by tritiated thymidine assay as in Fig. 1D. Graphs shown are representative of three experiments. Mean ± S.E.M. $IC_{50}$ values are mean ± S.E.M. for three independent experiments. $^{**}p < 0.005$, $^{***}p < 0.001$ using Student's *t*-tests.

similar response was not noted (data not shown), indicating that the effect of alternate dosing is specific for the STI571 + camptothecin combination. Similarly, synergistic effects of STI571 and camptothecin were observed on the proliferation of MDA-MB-231 cells, using an alternate dosing regimen (Fig. 5E; Table 1). In MDA-MB-468 cells, STI571 also sensitized cells to the anti-proliferative effects of camptothecin, and synergized with camptothecin at higher doses (0.035 μM) (Fig. 5F; Table 1). To determine whether STI571 increases the ability of camptothecin to induce apoptosis, PARP and caspase-3/7 assays were performed. In MDA-MB-231 cells, STI571 had no effect on the ability of camptothecin to induce apoptosis (Fig. 6A and B), whereas in MDA-MB-468 cells, STI571 antagonized the apoptotic effects of camptothecin (Fig. 6C and D). Thus, in MDA-MB-468 cells, STI571 synergistically increases the ability of camptothecin to inhibit proliferation, while it only has additive effects on viability. This is likely due to the fact that STI571 antagonizes the ability of camptothecin to induce apoptosis, resulting in a decreased overall effect on viability. In MDA-MB-231 cells, synergistic effects of STI571 and camptothecin were observed both in proliferation and viability assays, whereas STI571 had no effect on apoptosis, indicating that camptothecin inhibits the viability of MDA-MB-231 cells solely by affecting cell proliferation.

In MDA-MB-231 cells, STI571 and camptothecin had little effect on STAT3 phosphorylation, Akt was not constitutively phosphorylated in this cell type, as a band the correct size was not detected, and ERK1/2 phosphorylation was decreased by camptothecin treatment, but this inhibition was not enhanced by STI571 (Fig. 6A). Conversely, the stability of IκB was enhanced in MDA-MB-231 cells treated with STI571 or camptothecin, and the combination regimen further increased IκB stability (Fig. 6A). These data indicate that inhibition of NF-κB signaling is a likely mechanism by which STI571 sensitizes MDA-MB-231 cells to the anti-proliferative effects of camptothecin. In MDA-MB-468 cells, STI571 induced a modest decrease in Akt phosphorylation; however, this inhibition was not reproducible and camptothecin had no additional effect on Akt phosphorylation (Fig. 6C). Interestingly, STAT3 phosphorylation was induced by STI571, and treatment with camptothecin prevented this upregulation (Fig. 6C), similar to what was observed in MDA-MB-468 cells treated with paclitaxel (Fig. 2B). These data indicate that camptothecin may synergize with STI571 to inhibit proliferation in MDA-MB-468 cells by preventing STI571-dependent upregulation of STAT3. ERK1/2 also was upregulated by STI571 treatment and addition of camptothecin increased this upregulation (Fig. 6C), similar to what was observed in BT-549 cells treated with STI571 and cisplatin (Fig. 4A).

### 3.4. STI571 synergistically sensitizes MDA-MB-468 cells to 5-fluorouracil

5-Fluorouracil (5-FU) is an anti-metabolite; FUTP incorporates into RNA, FdUTP incorporates into DNA, and FdUMP forms a complex with thymidylate synthase [10]. 5-FU is incorporated in the place of

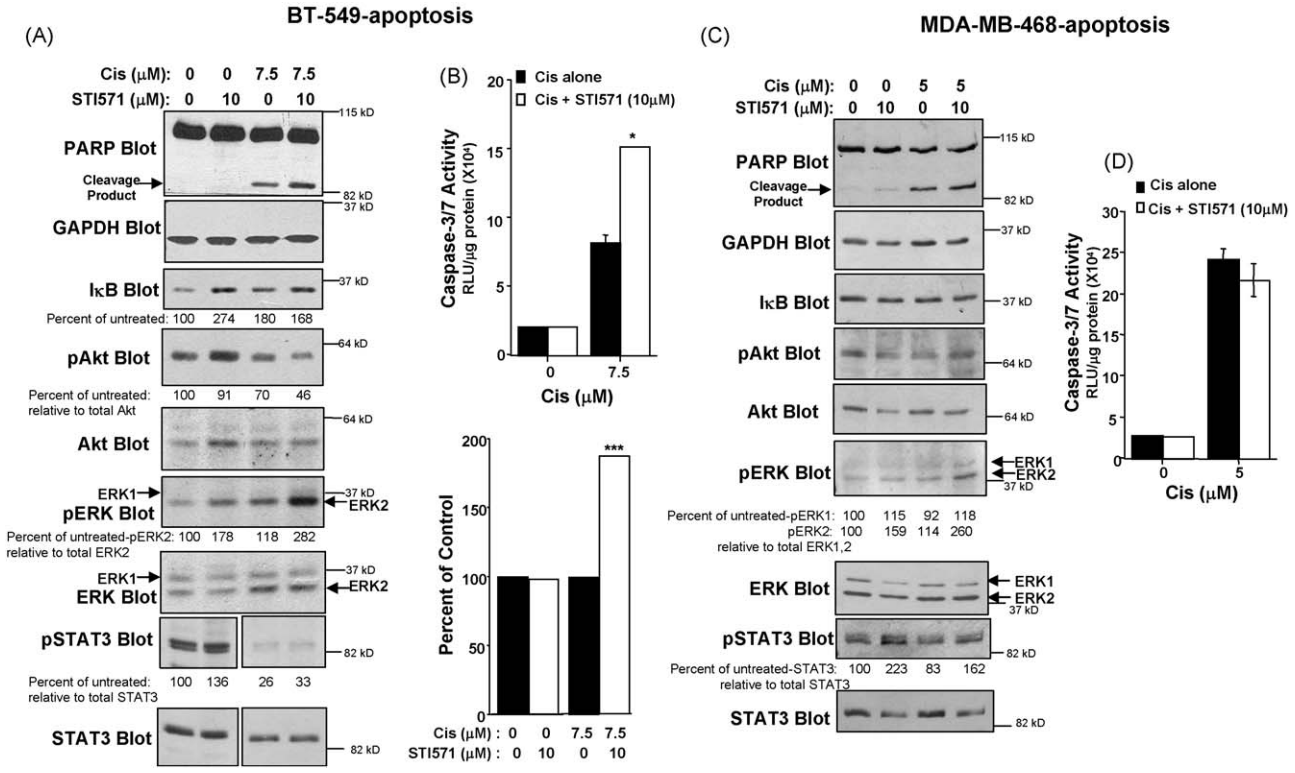


**Fig. 4.** Cisplatin and STI571 synergize to induce apoptosis in BT-549 cells. BT-549 and MDA-MB-468 cells were plated in 60 mm dishes, treated with STI571 and/or cisplatin the next day, and 40 h later, detached and attached cells were lysed and blotted with antibodies to PARP and GAPDH (A and C) or caspase-3/7 activity was assessed by Caspase-Glo assay from duplicate samples (B and D). PARP and GAPDH blots were stripped and reprobed with other antibodies (A and C). Experiments shown are representative of three independent experiments. Mean ± S.E.M. (B-top, D). $^{*}p = 0.007$ using a Student's *t*-test. Mean ± S.E.M. of three independent experiments where STI571 alone is normalized to untreated, and Cis + STI571 is normalized to cisplatin alone (B-bottom). Some error bars are too small to be seen (B). $^{***}p < 0.001$ using a Student's *t*-test.

purines or pyrimidines, and prevents purine/pyrimidine incorporation into DNA during S-phase, which inhibits cell division [10]. 5-FU also inhibits thymidine incorporation into DNA, which inhibits DNA/RNA synthesis [10]. STI571 sensitized MDA-MB-468 cells to 5-FU in a synergistic manner only at higher doses of 5-FU (100–500 μM), but did not significantly alter the $IC_{50}$ due to additive/antagonistic effects at lower doses (Fig. 7A; Table 1). These data indicate that STI571 may kill or prevent the proliferation of a small population of MDA-MB-468 cells that are intrinsically resistant to 5-FU and survive high doses of the drug. In contrast, in BT-549 and MDA-MB-231 cells, STI571 antagonized the effects of 5-FU on cell viability (Fig. 7B and C; Table 1). Interestingly, although STI571 reduced the viability of 5-FU-treated MDA-MB-468 cells in a synergistic manner, synergism was not observed in proliferation assays, and in fact STI571 actually antagonized the effect of 5-FU on proliferation at several 5-FU doses (e.g. 1.25, 3.5 μM; Fig. 7D; Table 1). This may be due to the fact that lower doses of 5-FU were utilized in proliferation assays. To test this hypothesis, we repeated the proliferation assays using high doses of 5-FU, and indeed, STI571 synergized with high doses of 5-FU to inhibit proliferation (Fig. 7E; Table 1). These data confirm that STI571 induces synergism by preventing the proliferation of cells that survive high doses of 5-FU. We performed PARP and caspase-3/7 assays to determine whether STI571 also reduces the viability of MDA-MB-468 cells by increasing apoptosis. Significantly, STI571 dramatically sensitized MDA-MB-468 cells to apoptotic death induced by high doses of 5-FU (Fig. 8A and B). Thus, STI571 sensitizes MDA-MB-468 cells to 5-FU by increasing its ability to induce apoptosis as well as by inhibiting proliferation, and STI571 may specifically target residual cells that are resistant to 5-FU.

STI571 did not increase IκB stability in MDA-MB-468 cells treated with 5-FU (Fig. 8A) nor did STI571 alter Akt phosphorylation, indicating that STI571 does not increase apoptosis or reduce proliferation by inhibiting NF-κB or PI3K/Akt signaling. However, as was observed in MDA-MB-468 cells treated with cisplatin (Fig. 4C), STI571 increased STAT3 phosphorylation (Fig. 8A), and addition of 5-FU prevented STI571-dependent STAT3 upregulation (Fig. 8A), indicating that this may be a mechanism by which the combination treatment inhibits proliferation. In addition, ERK1/2 phosphorylation was dramatically increased in the combination treatment regimen, which indicates that Ras/ERK signaling may also be involved (Fig. 8A).

### 3.5. STI571 strongly antagonizes the effects of mechloroethamine in all breast cancer cell lines

Mechlorethamine, a metabolite of cyclophosphamide, is an alkylating agent that: (1) attaches alkyl groups to DNA bases, which results in DNA fragmentation; (2) induces DNA damage by forming crosslinks that prevent DNA strands from separating; and (3) induces mispairing of nucleotides, which leads to mutations [23]. Interestingly, STI571 strongly antagonized the effects of mechloroethamine on viability in all breast cancer cell lines examined (Fig. 9; Table 1), which indicates that Abl kinases or another STI571 target is likely to be required for the action of mechloroethamine in breast cancer cells.

### 3.6. MDA-MB-468 and MDA-MB-231 cells have similar levels of nuclear and cytoplasmic c-Abl

In MDA-MB-231 cells, STI571 had antagonistic effects with all chemotherapeutic agents examined except for camptothecin. In contrast, in MDA-MB-468 cells, STI571 had either additive or

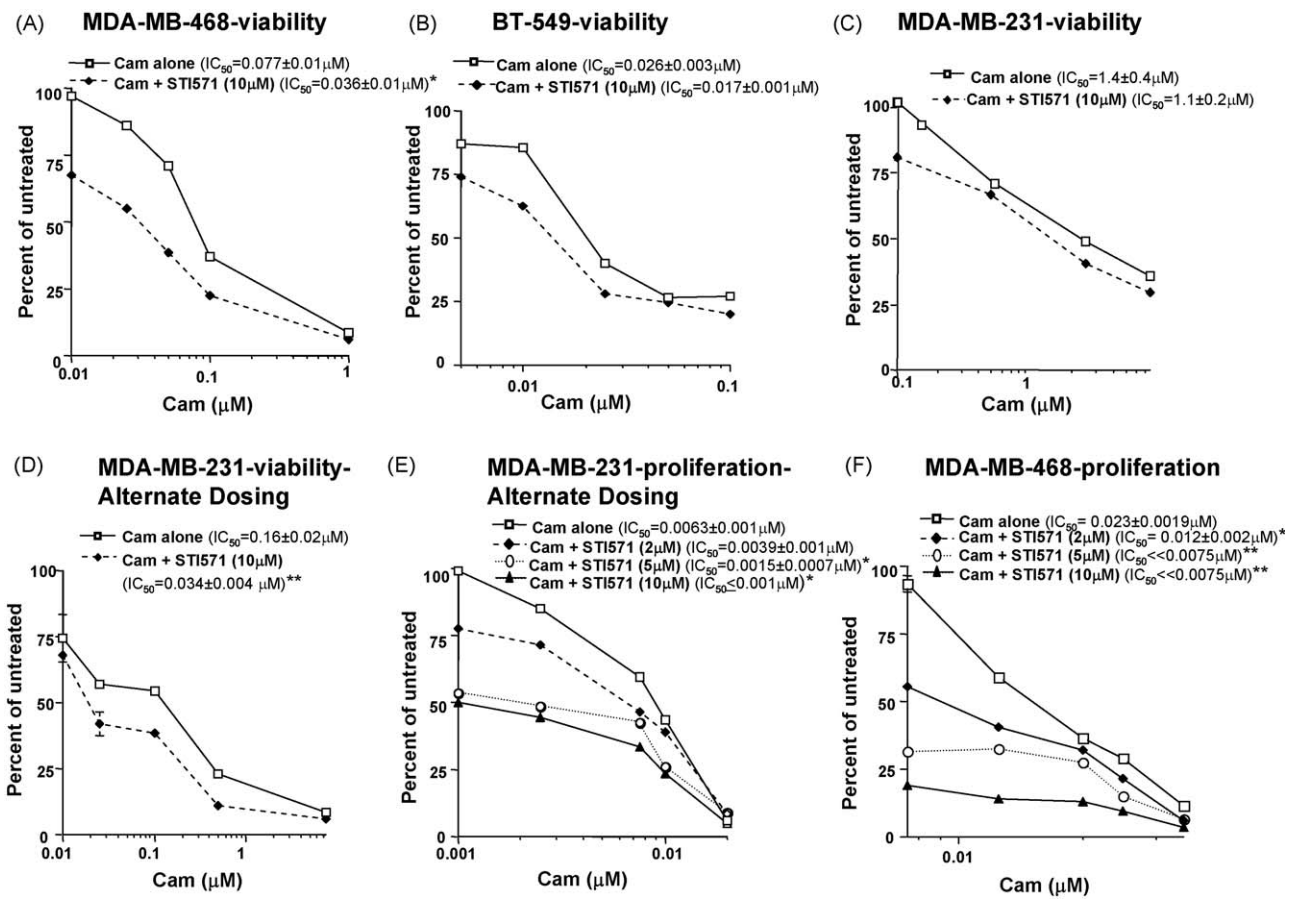


**Fig. 5.** STI571 and camptothecin synergize to inhibit the viability and proliferation of MDA-MB-231 cells when utilizing an alternate dosing schedule. (A–D) MDA-MB-468, BT-549, and MDA-MB-231 cells were plated, treated as described in Fig. 1A–C, and viability assessed. For alternate dosing experiments, MDA-MB-231 cells were plated in triplicate in 96-well dishes, camptothecin was added the next day, and the following day, the media was changed and camptothecin and STI571 were added. Cells were harvested for the Cell Titer-Glo assay 72 h later (D). Experiments were repeated three times, and representative experiments are shown. Mean ± S.E.M. $IC_{50}$ values are mean ± S.E.M. for three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.005$ using Student's *t*-tests. (E) MDA-MB-231 cells were plated in 24-well dishes, and treated for 24 h with camptothecin alone, followed by 72 h of STI571 + camptothecin, and tritiated thymidine incorporation was measured. $^{*}p < 0.05$ using a Student's *t*-test. (F) MDA-MB-468 cells were set-up and treated as described in Fig. 1D, and tritiated thymidine incorporation assessed. Experiments were repeated three times, and representative experiments are shown. Mean ± S.E.M. $IC_{50}$ values are mean ± S.E.M. for three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.005$ using Student's *t*-tests.

synergistic effects with all the chemotherapeutic agents examined, except for mechloroethamine. DNA damaging agents induce apoptosis in non-transformed fibroblasts by activating the nuclear pool of c-Abl [20]. Thus, STI571, which is likely to inhibit nuclear c-Abl, could antagonize the effects of chemotherapeutic agents if there is a large pool of c-Abl localized in the nucleus. To determine whether MDA-MB-231 cells have higher levels of nuclear c-Abl than MDA-MB-468 cells, we performed subcellular fractionation. Both cell lines contained high levels of c-Abl in the cytoplasm, and lower levels of c-Abl in the nucleus (Fig. 10). These data indicate that increased expression of nuclear c-Abl is not likely to be the reason that MDA-MB-231 cells are more resistant to the effects of STI571 in combination with chemotherapeutic agents than MDA-MB-468 cells (Fig. 10).

## 4. Discussion

In this report, we demonstrate for the first time that STI571 sensitizes breast cancer cells containing highly active Abl kinases to several conventional chemotherapeutic agents. Interestingly, sensitization is cell type-specific in that STI571 synergizes with paclitaxel, cisplatin, and 5-FU in MDA-MB-468 cells, while STI571 only synergizes with cisplatin in BT-549 cells, and with camptothecin in MDA-MB-231 cells. These data indicate that there are intrinsic differences between the three cell lines, which govern their susceptibility to STI571/conventional chemotherapy combination regimens. Interestingly, the three cell lines all have fairly equal sensitivities to the effect of STI571 alone on viability (data not shown). When tritiated thymidine incorporation and growth kinetic assays are used to measure the sensitivity of the cell lines to STI571, BT-549 and MDA-MB-468 cells are equally sensitive, while STI571 takes more time to inhibit the growth of MDA-MB-231 cells [13]. However, the overall effect after 4–5 days of STI571 treatment is similar between the three cell lines [13]. Activation of particular signaling pathways or increased expression of drug transporters may explain the differential sensitivity of the cell lines to different STI571/chemotherapeutic combination regimens. However, regardless of the mechanism, our data are significant because they indicate that STI571 may be effective in certain combination regimens.

Previously, we showed that the effects of STI571 on proliferation, survival, anchorage-independent growth, and invasion of breast cancer cells containing highly active Abl kinases were Abl kinase-dependent as RNAi produced similar results [12,13]. In addition, except for MDA-MB-468 cells, cell lines containing high c-Abl/Arg activities were sensitive to STI571 even though they did not express other STI571 targets (c-Kit or PDGF (platelet-derived-growth factor) receptors) [12]. To determine whether STI571

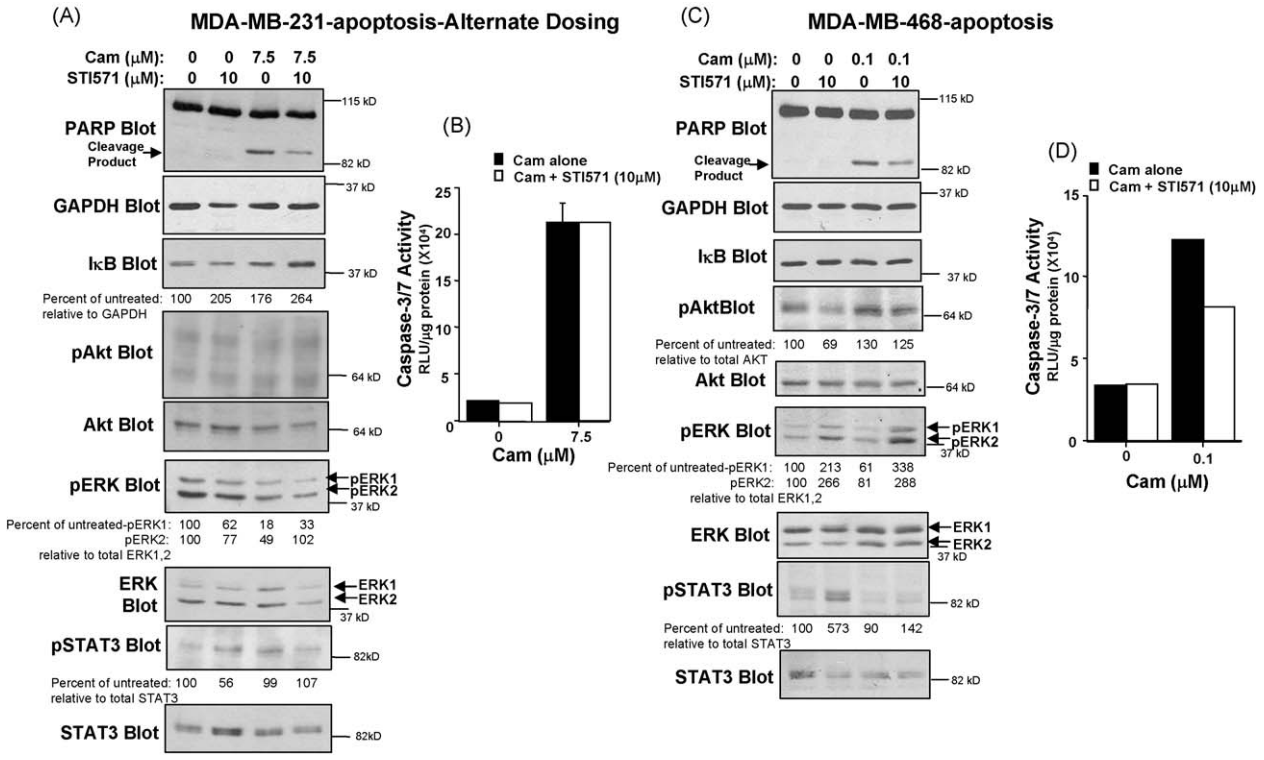


**Fig. 6.** STI571 synergizes with camptothecin to increase the stability of IκB in MDA-MB-231 cells, but STI571 has no effect on the ability of camptothecin to induce apoptosis. Cells were plated in 60 mm dishes, treated with STI571 and/or camptothecin the next day, and 40 h later, detached and attached cells were lysed and blotted with antibodies to PARP and GAPDH (A and C) or caspase-3/7 activity was assessed by Caspase-Glo assay (B and D). Mean ± S.E.M. for three independent experiments.

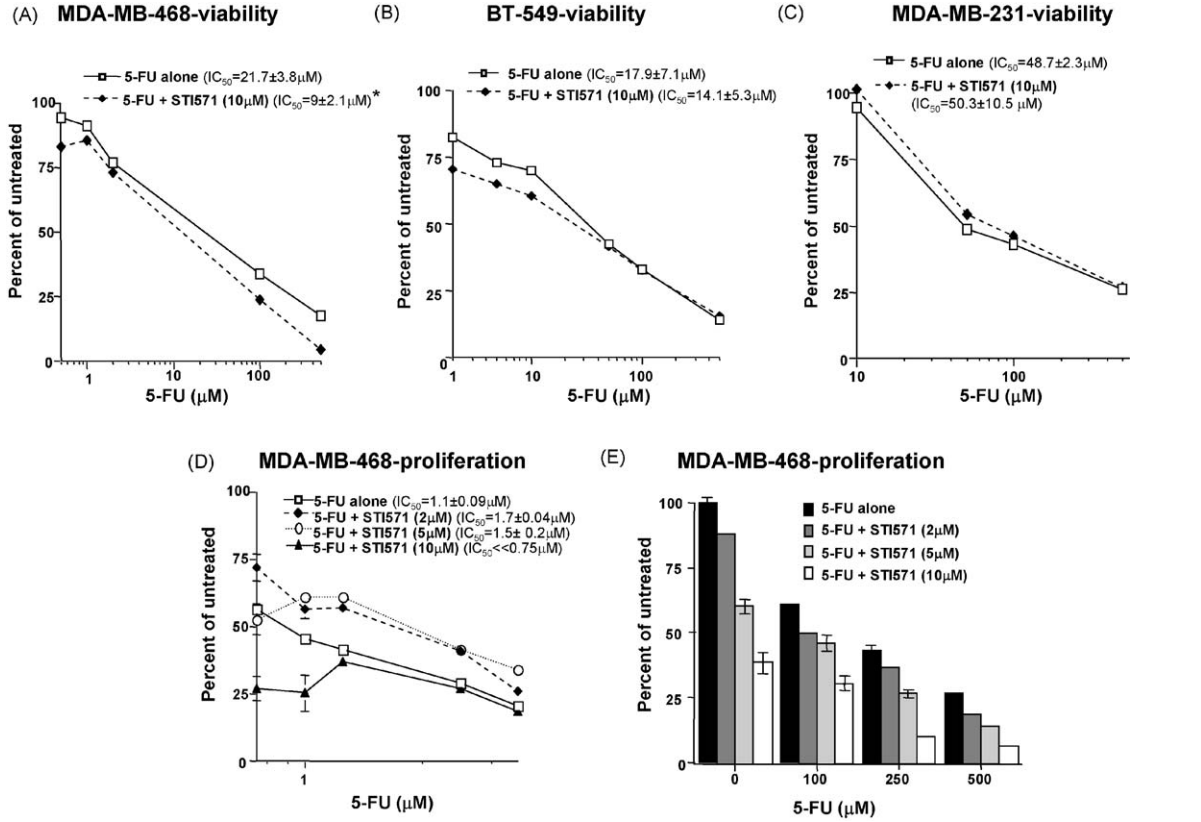


**Fig. 7.** STI571 synergizes with high doses of 5-fluorouracil in MDA-MB-468 cells, while STI571 antagonizes the effects of 5-fluorouracil in BT-549 and MDA-MB-231 cells. (A–C) Cells were plated, treated with STI571 and/or 5-fluorouracil for 96 h, and viability assessed as in Fig. 1A. Experiments were repeated three times, and representative experiments are shown. Mean ± S.E.M. $IC_{50}$ values are mean ± S.E.M. for three independent experiments. $^{*}p < 0.05$ using a Student's *t*-test. (D and E) MDA-MB-468 cells were set-up and treated with STI571 and/or 5-FU for 72 (E) or 96 h (D), and tritiated thymidine incorporation assessed. Experiments were repeated three times, and representative experiments are shown. Mean ± S.E.M. $IC_{50}$ values are mean ± S.E.M. for three independent experiments.

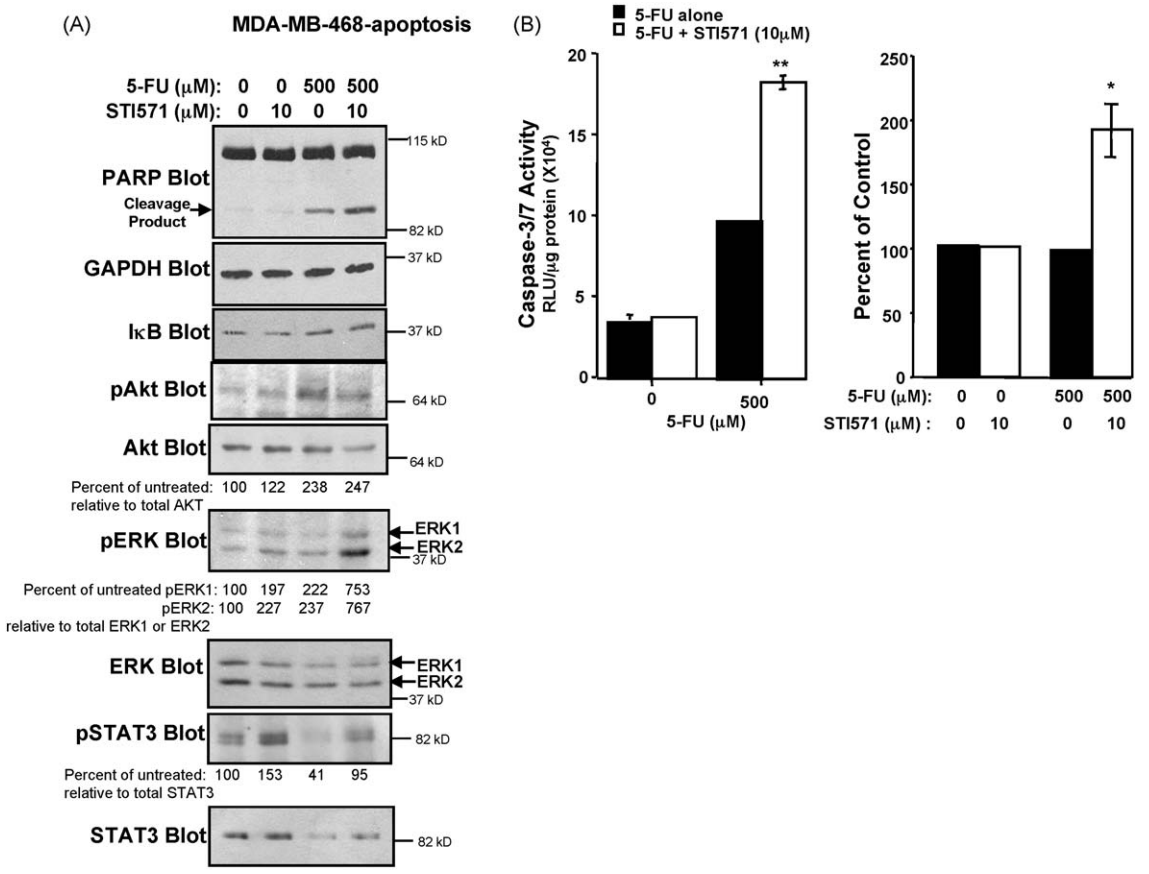


**Fig. 8.** STI571 increases the ability of 5-FU to induce apoptosis. (A) PARP cleavage, Western blots, and (B) caspase-3/7 activity were assessed as described in Fig. 2. Mean ± S.E.M. for three independent experiments. (B-left). $^{**}p = 0.003$ using a Student's *t*-test. Mean of three independent experiments where STI571 alone is normalized to untreated, and 5-FU + STI571 is normalized to 5-FU alone (B-right). Some error bars are too small to be seen. $^{*}p = 0.011$ using a Student's *t*-test.

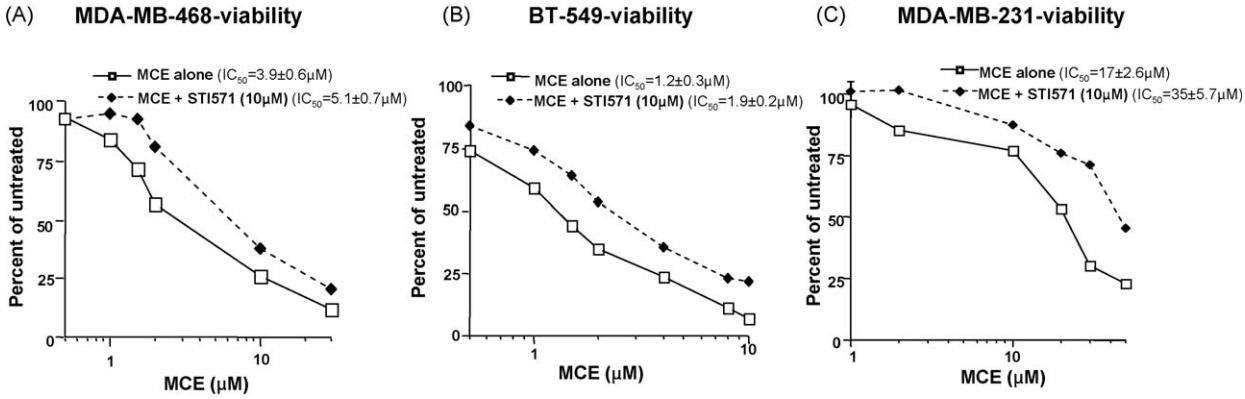


**Fig. 9.** Mechloroethamine strongly antagonizes STI571 in all breast cancer cell lines. Cells were plated, treated, and viability assessed as in Fig. 1A. Experiments were repeated three times, and representative experiments are shown. Mean ± S.E.M. $IC_{50}$ values are mean ± S.E.M. for three independent experiments.

synergizes with chemotherapeutic agents specifically by inhibiting Abl kinases, we attempted to test whether silencing Abl kinases sensitizes cells to the chemotherapeutic drugs. Since cells must be transfected with siRNAs or shRNAs prior to being treated with chemotherapeutic agents, these experiments were unsuccessful, as silencing Abl kinases significantly slowed cell growth, thereby decreasing the effectiveness of the chemotherapeutic drugs, which act on highly proliferating cells (data not shown). These data highlight the importance of inhibiting Abl kinases at the same time or after chemotherapeutic drug administration. In order to inhibit Abl kinases during this time frame, cell lines expressing inducible shRNAs must be obtained; a laborious process which often results in inefficient silencing. Regardless of whether or not STI571 targets Abl kinases to sensitize breast cancer cells to chemotherapeutic agents, the data we obtained are still significant, as they indicate that use of STI571 with some chemotherapeutic agents may be a viable treatment option for some STI571-sensitive breast cancers. Clearly, the treatment combinations will require individualization since some cells are responsive to certain combinations and others are not. STI571 also has been shown to synergize with camptothecin in small cell lung cancer cells [24], cisplatin in head and neck cancer [25] and lung cancer cells [26], carboplatin in ovarian [27], paclitaxel in ovarian [27], and 5-FU in colon cancer cells [28]. The target of STI571 in these cell lines also has not been

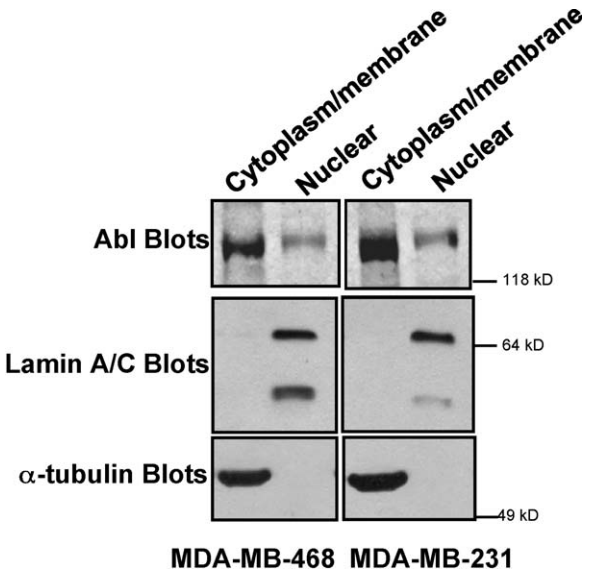


**Fig. 10.** MDA-MB-231 and MDA-MB-468 cells have similar levels of cytoplasmic and nuclear c-Abl. Cells were fractionated using NE-PER (Pierce; Rockford, IL) into cytoplasmic/membrane and nuclear fractions. Equal percentages of the fractions were loaded on an SDS-PAGE gel, and blotted with c-Abl antibody. Lysates also were blotted with antibodies to α-tubulin to assess the purity of the cytoplasmic fractions, and lamin A/C to assess nuclear fraction purity.

proven most likely due to the difficulty in using siRNA/shRNA approaches.

Interestingly, although STI571 synergizes with both cisplatin and 5-FU in MDA-MB-468 cells, it does so by different mechanisms. STI571 sensitizes MDA-MB-468 cells to cisplatin by increasing its ability to inhibit proliferation. In contrast, STI571 sensitizes MDA-MB-468 cells to high doses of 5-FU by dramatically increasing its ability to induce apoptosis, and by targeting cells that are resistant to high doses of 5-FU. These data demonstrate that STI571 acts not only as a cytostatic agent but also as a cytotoxic agent in breast cancer cells. The different mechanisms of action of the chemotherapeutic drugs are likely to affect the ability of STI571 to synergize with the chemotherapeutic agents. Previously, we showed that STI571 slows serum-induced G1 → S progression of breast cancer cells containing highly active Abl kinases [12,13]. Thus, by slowing S-phase progression, STI571 may prevent incorporation of 5-FU, which is incorporated into DNA in S-phase, resulting in drug antagonism at low 5-FU doses. Such an effect may not be observed at high doses, as STI571 may specifically target cells that are resistant to high doses of 5-FU, perhaps by inhibiting a drug transporter which effluxes 5-FU. Indeed, STI571 inhibits and/or is a substrate for ABCB1 and ABCG2/BCRP [29–34], and 5-FU is a substrate of ABCG2 [35] and ABCC5 [36].

Significantly, STI571 synergizes with cisplatin in both MDA-MB-468 and BT-549 cells. In MDA-MB-468 cells, STI571 increases the ability of cisplatin to inhibit proliferation, whereas in BT-549 cells, STI571 enhances both the anti-proliferative and pro-apoptotic effects of cisplatin. These data are interesting because previous data indicated that cisplatin induces apoptosis of non-transformed fibroblasts by activating the nuclear pool of c-Abl [20]. Clearly, cisplatin does not induce apoptosis of BT-549 breast cancer cells via this mechanism, as STI571 would be predicted to prevent cisplatin-induced apoptosis. However, unlike fibroblasts, breast cancer cells have highly active Abl kinases. Preliminary data in another solid tumor cell line, MDA-MB-435s, which also contains highly active Abl kinases [12,13], shows that the pool of c-Abl that is highly active is primarily the cytoplasmic/membrane pool and not the nuclear pool (data not shown). These data are not particularly surprising given that activation of nuclear c-Abl is associated with apoptosis rather than proliferation, and that c-Abl is activated by cytoplasmic/membrane receptor tyrosine kinases and Src kinases in breast cancer cells [12,13]. Thus, STI571 treatment of breast cancer cells may preferentially inhibit the highly active cytoplasmic/membrane c-Abl pool as opposed to the smaller, less-active nuclear pool. In addition, Arg is not located in the nucleus; Arg is the only Abl kinase that is highly active in MDA-MB-468 cells; and in BT-549 cells, although both c-Abl and Arg have elevated activities, Arg activity is much higher than c-Abl [12,13]. Therefore, it is possible that STI571 synergizes with cisplatin in these two cell lines by targeting Arg. In contrast, cisplatin has less of an effect in MDA-MB-231 cells, which have more highly active c-Abl than Arg. MDA-MB-231 cells, in general, are more resistant to STI571/chemotherapeutic combination regimens, and this could be due to the fact that c-Abl rather than Arg, is the main Abl kinase activated in these cells [12,13].

Although STI571 has cytotoxic effects in combination with cisplatin in BT-549 cells and 5-FU in MDA-MB-468 cells, the molecular mechanisms are different. In BT-549 cells, STI571 increases the ability of cisplatin to inhibit constitutive Akt phosphorylation, while in MDA-MB-468 cells treated with 5-FU, STI571 does not alter Akt phosphorylation. As mentioned earlier, STI571 may inhibit a 5-FU transporter in MDA-MB-468 cells, thereby sensitizing resistant cells to 5-FU. Future experiments will address this possibility. In MDA-MB-231 cells, STI571 sensitizes cells to camptothecin most likely by increasing the stability of IκB, thereby inhibiting NF-κB signaling which promotes proliferation. In MDA-MB-468 cells, STI571 treatment causes increased phosphorylation of STAT3, and treatment with paclitaxel, cisplatin, camptothecin or 5-FU inhibits the STI571-dependent STAT3 activation. Since STAT3 induces proliferation of breast cancer cells, MDA-MB-468 cells may resist the effects of STI571 by activating STAT3, and treatment with conventional chemotherapeutic agents, which prevents this upregulation, may prevent resistance. Thus, inhibition of STAT3 activation may be a common mechanism by which STI571 synergizes with chemotherapeutic agents to inhibit the proliferation of MDA-MB-468 cells. Taken together, these data indicate that the mechanisms by which STI571 potentiates the effects of chemotherapeutic agents are not only cell type-specific but also are specific for particular STI571/chemotherapeutic combinations.

In summary, our data demonstrate that STI571 cooperates with several conventional chemotherapeutic agents to inhibit viability by decreasing proliferation and/or increasing apoptosis. These data indicate that STI571 may be an effective agent in combination chemotherapy for treating breast cancer patients with $ER^-$, $PR^-$ tumors that have active Abl kinases. However, our data indicate that the cellular milieu dictates which cells will be sensitive to particular therapeutic combinations, which emphasizes the need to determine empirically, which patients will respond to particular STI571/chemotherapeutic combinations.

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