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Inhibitors of PI3K/ERK1/2/p38 MAPK Show Preferential Activity Against Endocrine-Resistant Breast Cancer Cells

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Current mainstream pharmacological options for the treatment of endocrine-resistant breast cancer have limitations in terms of their side effect profile and lack of discrimination between normal and cancer cells. In the current study, we assessed the responses of normal breast epithelial cells MCF10A, estrogen receptor-positive (ER⁺) MCF-7, and ER-silenced pII breast cancer cells to inhibitors (either individually or in combination) of downstream signaling molecules. The expression/activity of ERK1/2, p38 MAPK, and Akt was determined by Western blotting. Cell proliferation, motility, and invasion were determined using MTT, wound healing, and Matrigel assays, respectively. Morphological changes in response to variation in external pH were assessed by light microscopy. Our results demonstrated that the inhibitors of ERK1/2 (PD0325901), p38 MAPK (SB203580), and PI3K (LY294002) preferentially reduce breast cancer cell proliferation. In pII cells, they also reduced motility, invasion, and bleb formation induced by alkaline conditions. Combination treatment with lower concentrations of inhibitors was significantly more effective than single agents and was more effective against the cancer cell lines than the normal MCF10A. In contrast, the commonly used cytotoxic agent paclitaxel did not sufficiently discriminate between the MCF10A and the cancer cells. We concluded that combination therapy using ERK1/2 inhibitor and either p38 MAPK or PI3K inhibitor may provide a greater therapeutic benefit in treating breast cancer by specifically targeting cancer cells with lower doses of each drug than needed individually, potentially reducing unwanted side effects.

Key words: Breast cancer; Endocrine resistance; pH; Invasion; ERK1/2; p38 MAPK; Akt

INTRODUCTION

Breast cancer is the major cause of tumor-associated mortality in women. Endocrine-based therapies remain the preferred option for patients with estrogen receptor-positive (ER⁺) status in terms of both length and quality of life¹. However, the occurrence of either de novo or acquired resistance to this form of therapy necessitates the use of general cytotoxic agents with many disadvantages in terms of safety profile, lack of specificity, and poor expectations for quality of life. Most mainstream drugs in current use aim principally to block cell proliferation by inhibiting DNA replication/transcription or cell division, but the doses needed inflict unacceptable collateral damage to normal tissues and lead to an increased risk for opportunistic infections as a result of immunological impairment². Monoclonal antibodies such as trastuzumab against epidermal growth factor receptor (EGFR) and bevacizumab against vascular endothelial growth factor receptor (VEGFR) provide improved but limited alternatives. Identification of alternative growth stimulatory

pathways in endocrine-resistant cancers has led to the development of a number of small-molecule inhibitors of EGFR/ERBB2 and platelet-derived growth factor receptor (PDGFR)-associated tyrosine kinase activity such as erlotinib and imatinib^{3,4}. Other drugs in preclinical development target the mitogen-activated protein kinase family (MAPK) of serine/threonine kinases, the extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), Big MAPK, and p38 MAPK⁵.

We have previously shown that endocrine resistance in breast cancer can be induced by functional loss of ER and a parallel cellular transition from epithelial-to-mesenchymal phenotype (EMT) with cell scattering and loss of polarity leading to increased aggressiveness⁶⁻⁸. We have also demonstrated a dramatic morphological transformation, specifically in ER-silenced breast cancer cells upon brief exposure to alkaline pH, with the formation of dynamic actin-rich blebs on the outer membrane and further enhancement in their invasive properties (in part due to enhanced MMP activity)⁹⁻¹¹.

In the present study, we determined the effect of inhibitors (either alone or in combination) of p38 MAPK, ERK1/2, and phosphatidylinositol 3-kinase (PI3K) on the proliferation of normal breast cells (MCF10A) compared with ER-expressing (MCF-7) and ER-silenced (pII) cancer cells. We also assessed their effect on pII cells with respect to motility, invasion, and behavior in an alkaline pH. The data presented indicate that inhibiting the function of one or more of these key signaling molecules has significant beneficial effects in reducing proliferation, motility, and invasion and is more discriminative between normal and cancer cells than paclitaxel, a commonly used anticancer drug. We have shown that these agents are particularly effective against endocrine-resistant cells that have increased sensitivity to EGF.

MATERIALS AND METHODS

Cell Lines

MCF10A normal breast epithelial cells cultivated in the laboratory of Dr. E. Saunderson of St. Bartholomews Hospital, London, were kindly made available by Dr. J. Gomm. MCF-7 (ER⁺) and MDA-MB-231 (ER⁻) breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The pII (ER⁻) cell line was established in this laboratory by transfection of MCF-7 with ER-directed shRNA plasmid as previously described^{6,8}. For routine cultures, the cancer cell lines were maintained as monolayers in advanced Dulbecco's minimum essential medium (DMEM) containing phenol red and supplemented with 5% fetal bovine serum (FBS), 600 µg/ml L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 6 ml/500 ml 100× non-essential amino acids (all from Invitrogen, Carlsbad, CA, USA), and grown at 37°C in an incubator gassed with an atmosphere of 5% CO₂ and maintained at 95% humidity. MCF10A were grown in DMEM F12 supplemented with 5% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, EGF (20 ng/ml), insulin (10 µg/ml), hydrocortisone (0.5 µg/ml), and cholera toxin (100 ng/ml).

Proliferation Assay

Approximately 10⁴ cells were seeded into triplicate wells of 12-well plates and allowed to attach overnight. Either vehicle only or various concentrations of PD0325901, SB203580, LY294002, or paclitaxel (10 nM–10 µM, either alone or in combination) were then added to the cells. Growth was assessed by MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay after 4 days of incubation. Briefly, after removal of medium, 1 ml of MTT reagent (0.5 mg/ml; Promega, Madison, WI, USA) was added to each well, and plates were incubated at 37°C for 30–60 min followed by the

addition of 1 ml of acidic isopropanol and vigorous resuspension of the converted blue crystals. Absorbance of the suspension was measured at 595 nm with background subtraction at 650 nm. The effect of the inhibitor used was compared to vehicle-treated cells (taken as 100%).

Motility Assay

Cells were grown in complete DMEM in six-well plates to 80–90% confluence. After addition of either vehicle or various concentrations of the inhibitors, a scratch was created in the cell monolayer using a sterile p1000 pipette tip. A photograph of the scratched area was taken immediately (0 h), and the plates were then placed in a 37°C, 5% CO₂ gassed incubator. After 24 h of incubation, another photograph was taken of the same scratched area. The width of the scratch at 24 h was calculated as a percentage of the width at 0 h.

Invasion Assay

An invasion assay was performed using 24-well cell invasion chambers from Trevigen (Gaithersburg, MD, USA) according to the manufacturer's instructions. The chambers of the upper insert wells were coated with basement membrane extract solution (BME; 50 µl/well) and allowed to solidify overnight at 37°C in the 5% CO₂ incubator. Meanwhile, pII cells were grown to 80–90% confluence and then incubated in serum-free media containing various concentrations of the inhibitors (or vehicle) for 16 h. On the following day, cells were harvested by trypsinization and centrifugation and washed twice by resuspension in PBS (and centrifugation), then resuspended in PBS and loaded into the upper well (50,000 cells/well). Plates were left at 37°C in the 5% CO₂ incubator overnight to allow cells to invade through the BME into the lower well containing either 150 µl of PBS (control) or DMEM containing 10% FBS. On the next day, the lower chamber was gently rinsed with 1× washing buffer after removing the media, and 100 µl of calcein solution was added (12 µl of calcein AM solution mixed with 1 ml of 1× cell dissociation solution) and left at 37°C in the 5% CO₂ incubator for 30 min. Chambers were disassembled and the plates scanned in a Fluroskan Ascent plate reader (Thermo-Electron Corporation, Waltham, MA, USA) at 520-nm emission with 485-nm excitation. Fluorescence from intracellular dye was taken as an indirect measurement of comparative cell number.

Western Blotting

Cells were cultured in six-well plates to 80–90% confluence and treated for 30 min with vehicle (control) or EGF (10 and 50 ng/ml). After aspiration of media, cell monolayers were harvested by scraping and resuspension

in 300 μ l of lysis buffer containing 50 mM HEPES, 50 mM NaCl, 5 mM EDTA 1% Triton X, 100 μ g/ml PMSF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Protein concentration was determined by the Bradford assay using BSA as the standard, and 6 μ g of protein lysate was mixed with an equal volume of 2 \times SDS and heated at 90°C for 10 min. Samples were loaded onto a 10% SDS-polyacrylamide gel and electrophoresed at 150 V for 1 h. Proteins were transferred to a nitrocellulose membrane and blocked with 2% BSA for 1 h before being incubated overnight at 4°C with actin (control), ER, p-ERK1/2, p-p38 MAPK, p-Akt, or T-Akt antibodies (all from Cell Signaling, Danvers, MA, USA) prepared in 2% BSA at 1:1,000 dilution. The membrane was washed and incubated with anti-HRP-conjugated secondary antibody (1:500 dilution) for 1 h, developed with Super Signal ECL and visualized with Kodak X-ray film.

Morphological Changes Induced by Alkaline pH

Approximately 10^5 cells (treated with either vehicle or various concentrations of the inhibitors) were seeded into wells of a 12-well plate. After 24 h the culture plates were removed from the gassed incubator (i.e., from the 5% CO₂ atmosphere needed to maintain the buffering capacity of the DMEM). Several fields containing colonies were swiftly marked and photographed using an Olympus inverted microscope fitted with a camera and then immediately placed in an un-gassed incubator at 37°C. Exposure to normal atmospheric CO₂ conditions causes the medium to become alkaline (approximately pH 8.2–8.3) within a few minutes. Previously marked fields were rephotographed after 30 min, and resultant changes in cell size and shape were noted in each field and quantified in terms of the area occupied by cells using Adobe Photoshop CS4 Measuring Tool as previously described^{9,11}.

Statistical Analysis

Data were analyzed using GraphPad InStat (GraphPad Software, La Jolla, CA, USA). One-way ANOVA test followed by Bonferroni post hoc test was used to compare means of individual groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of EGF Treatment on the Expression/Phosphorylation of Signaling Molecules

EGF treatment (10–50 ng/ml, 30 min) enhanced the phosphorylated levels of ERK1/2 in both MCF-7 and pII cells. Akt and p38 MAPK phosphorylation was also enhanced, but only in pII cells, whereas total Akt remained unchanged (Fig. 1).

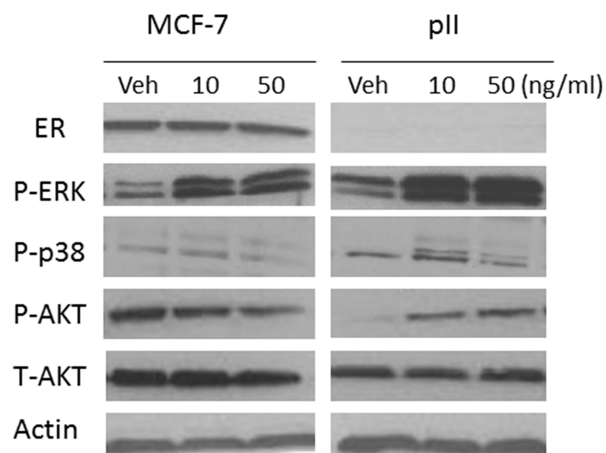


Figure 1. Effect of epidermal growth factor (EGF) on the expression/phosphorylation of downstream signaling molecules. Serum-starved (24 h) cells were treated with vehicle (Veh) or EGF (10 and 50 ng/ml, 30 min) and subsequently harvested by scraping directly into lysis buffer. Soluble lysate protein (6 μ g) was electrophoresed on 10% SDS polyacrylamide gel, blotted onto nitrocellulose membrane, and probed with antisera to estrogen receptor (ER), T-Akt, p-Akt, p-p38 mitogen-activated protein kinase (MAPK), p-extracellular signal-regulated kinase 1/2 (ERK1/2), and actin (control). This blot represents one of three separate experiments showing similar results.

Effect of PD0325901, SB203580, and LY294002 on Breast Cell Proliferation

MTT assay was performed for the normal breast epithelial cells (MCF10A) and the ER⁺ (MCF-7) and ER⁻ (MDA-MB-231 and pII) breast cancer cells after 4 days of treatment with inhibitors against ERK1/2 (Fig. 2A), p38 MAPK (Fig. 2B), and PI3K (Fig. 2C) signaling molecules. The growth of all three cancer cell lines was similarly inhibited by the ERK1/2 inhibitor from 10 nM upward, whereas MCF10A was not significantly affected until the inhibitor concentration was a thousandfold higher at 10 μ M (Fig. 2A). The p38 MAPK inhibitor effectively reduced growth in all cancer lines at 100 nM but did not show any dose dependence thereafter. In contrast, the growth of MCF10A cells was actually stimulated at all the tested concentrations (Fig. 2B). In the case of the PI3K inhibitor, only the endocrine-resistant pII cells were affected, with approximately 35% inhibition at 100 nM–1 μ M and 80% at 10 μ M (Fig. 2C). Neither the estrogen-responsive MCF-7 nor the normal MCF10A was significantly affected by LY294002 even up to 10 μ M.

Effect of Drug Combination on Breast Cancer Cell Proliferation

The effect of the ERK1/2 inhibitor (50 nM) with increasing concentrations (10 nM–10 μ M) of either the p38 MAPK or PI3K inhibitor on MCF-7 and pII cell

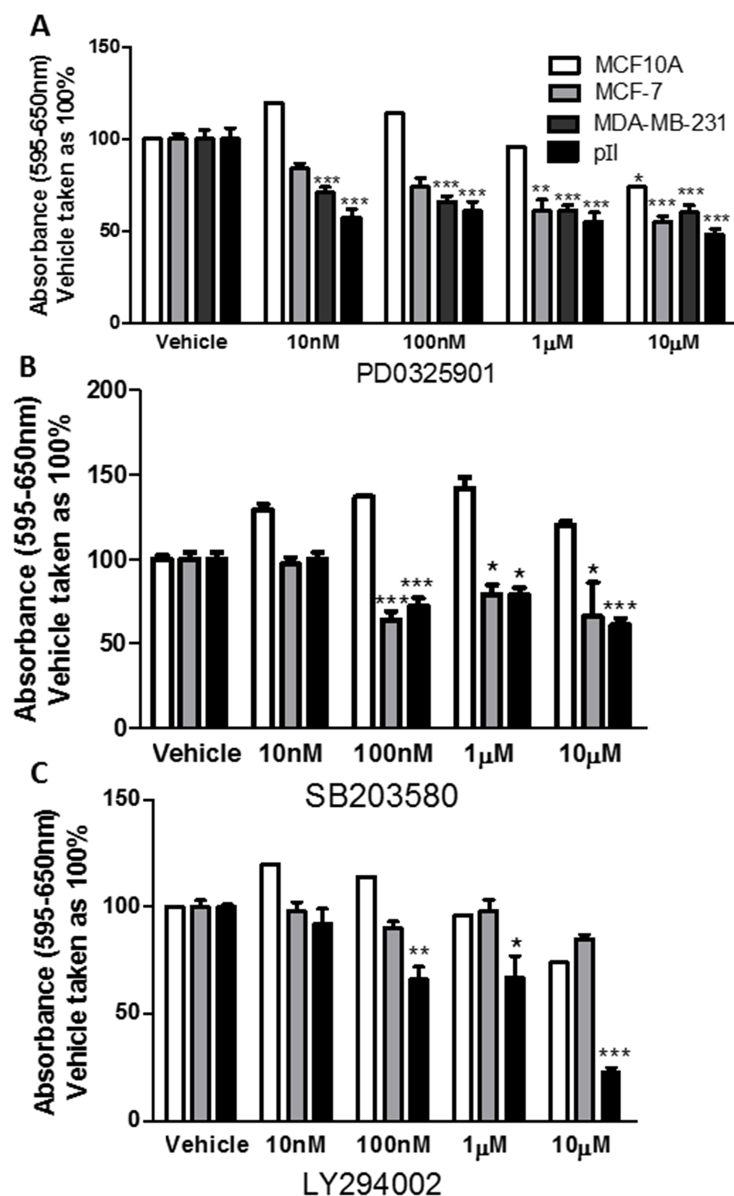


Figure 2. Effect of prolonged exposure to PD0325901/SB203580/LY294002 on cell proliferation. Approximately 10^4 normal breast epithelial cells (MCF10A, open bars), ER⁺ (MCF-7, gray bars), or ER⁻ breast cancer cells (MDA-MB-231 and pII, solid bars) were seeded into microwell plates and allowed to grow over 4 days in the presence of vehicle or various concentrations (10 nM–10 μ M) of the inhibitors as indicated. Cells were harvested, and growth was determined by the MTT assay. Histograms represent means \pm SEM of at least three independent determinations. Significant difference from vehicle control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

proliferation is shown in Figures 3 and 4. Whereas the ERK1/2 inhibitor at 50 nM did not affect MCF-7 cell proliferation, the p38 MAPK inhibitor significantly reduced proliferation (40%) at 100 nM–10 μ M. The combination of the two drugs did not further increase the effect of SB203580. Neither the ERK1/2 nor the P3IK inhibitor alone affected MCF-7 cell proliferation at the tested doses, but the combination significantly inhibited proliferation from the lowest dose of 10 nM LY294002 combined with the fixed dose of 50 nM PD0325901. In the

case of pII cells, both the ERK1/2 inhibitor at 50 nM and the p38MAPK inhibitor from 100 nM significantly inhibited proliferation. The combination produced an increased synergistic effect, which was also observed with the combination of PD0325901 with LY294002.

Effect of Paclitaxel on Cell Proliferation

Paclitaxel had a very potent inhibitory effect on both normal breast epithelial cells and the breast cancer cell lines (Fig. 5). At 10 nM, MCF10A and pII cell proliferation

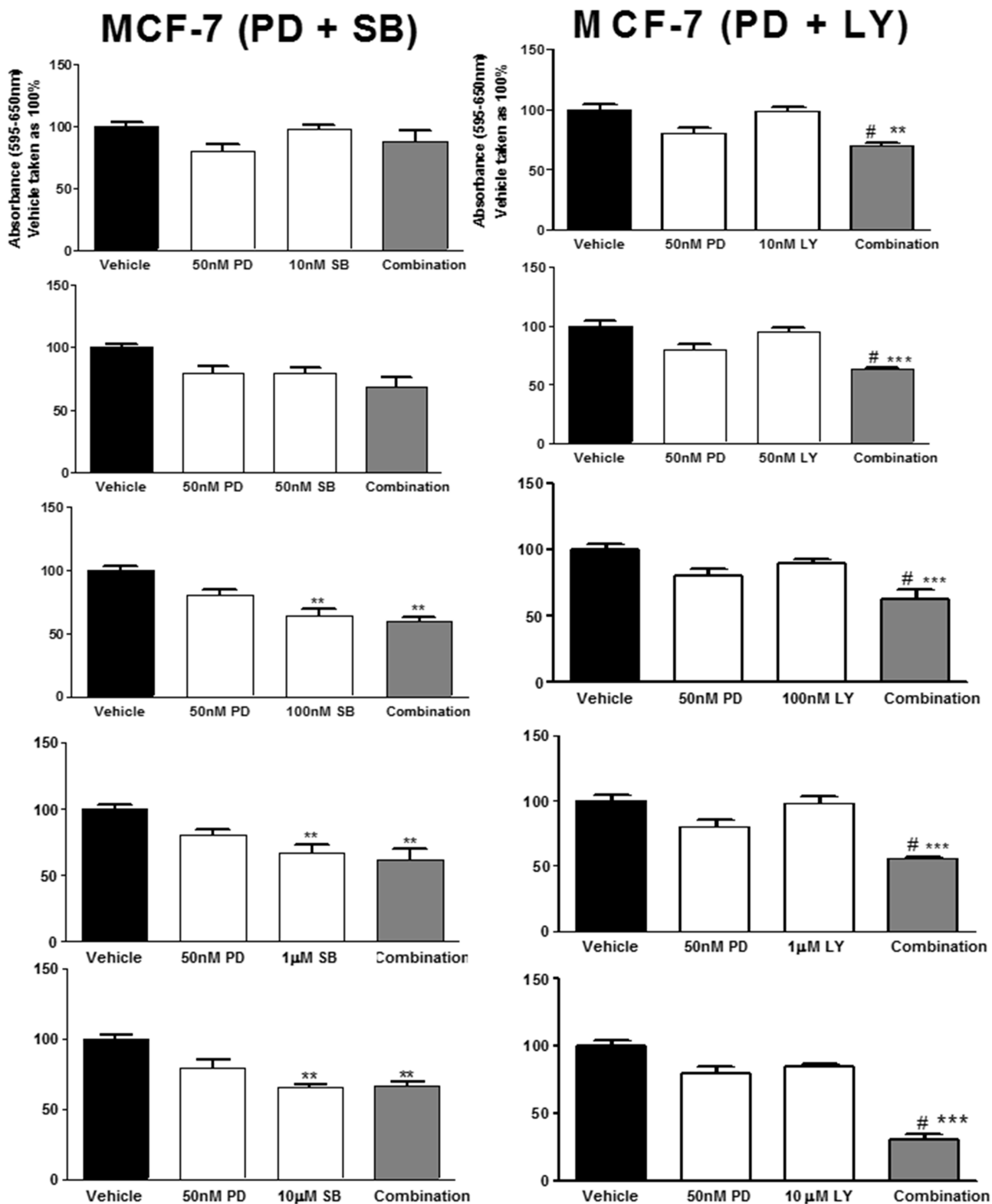


Figure 3. Effect of drug combination on MCF-7 cell proliferation. Approximately 10^4 MCF-7 cells were seeded into microwell plates and allowed to grow over 4 days in the presence of vehicle (solid bars), PD0325901 (50 nM, open bars), increasing concentrations of SB203580 or LY294002 (10 nM–10 μ M, open bars), or a combination (gray bars). Cells were harvested and growth was determined by the MTT assay. Histograms represent means \pm SEM of at least three independent determinations. Significant difference from vehicle control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. #Difference between single agent and combination.

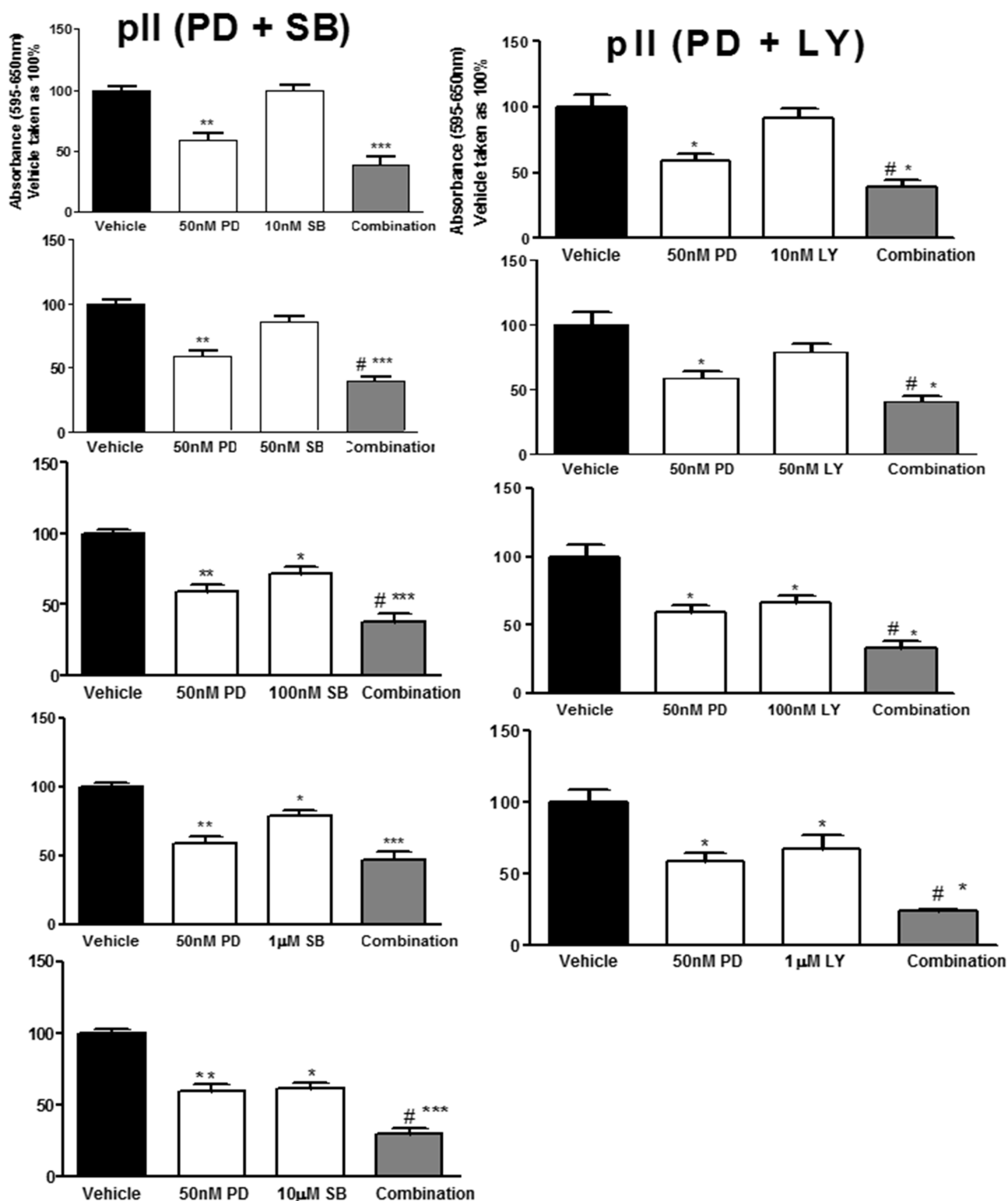


Figure 4. Effect of drug combination on pII cell proliferation. Approximately 10^4 of pII cells were seeded into microwell plates and allowed to grow over 4 days in the presence of vehicle (solid bars), PD0325901 (50 nM, open bars), increasing concentrations of SB203580 or LY294002 (10 nM–10 μ M, open bars), or a combination (gray bars). Cells were harvested, and growth was determined by the MTT assay. Histograms represent means \pm SEM of at least three independent determinations. Significant difference from vehicle control: * p <0.05, ** p <0.01, *** p <0.001. #Difference between single agent and combination.

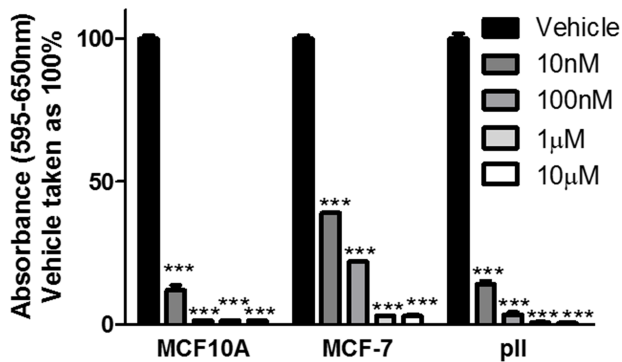


Figure 5. Effect of paclitaxel treatment on cell proliferation. Approximately 10^4 MCF10A, MCF-7, or pII cells were seeded into microwell plates and allowed to grow over 4 days in the presence of vehicle (solid bars) or various concentrations (10 nM–10 μ M) of paclitaxel as indicated. Cells were harvested and growth was determined by the MTT assay. Histograms represent means \pm SEM of at least three independent determinations. Significant difference from vehicle control: *** $p < 0.001$.

was inhibited by 90%, while MCF-7 was inhibited by 60%. At higher concentrations from 100 nM–10 μ M, the degree of inhibition was similar in all cell lines (90%).

Effect of 24 h of Treatment With PD0325901, SB203580, and LY294002 on pII Cell Proliferation

We also evaluated the effect of shorter-term (24 h) treatment with the inhibitors on pII cell proliferation (Fig. 6) to ensure that any effect observed with the functional assays was not due to inhibition of cell proliferation. This was confirmed for all concentrations.

Effect of PD0325901, SB203580, and LY294002 on pII Cell Motility

Neither MCF10A nor MCF-7 showed any movement in the scratch assay (data not shown), while pII cells completely closed the gap after 24 h. In the presence of the ERK1/2 inhibitor, at doses from 100 nM to 10 μ M, the gap closure was significantly reduced by approximately 50%. Neither the PI3K nor the p38 MAPK inhibitor affected cell motility at any of the tested concentrations, with the gap being completely closed (Fig. 7).

Effect of PD0325901, SB203580, and LY294002 on pH-Induced Morphological Changes in pII Cells

Brief exposure (30 min) of pII cells to extracellular alkaline environment (effected by exposure to normal atmospheric conditions) induced dramatic morphological changes with cell rounding and shrinkage as illustrated in the uppermost panel of Figure 8. There was also extensive blebbing at the cell surface (not shown but observable at higher magnification). These effects were prevented by pretreatment (30 min) with each of the inhibitors.

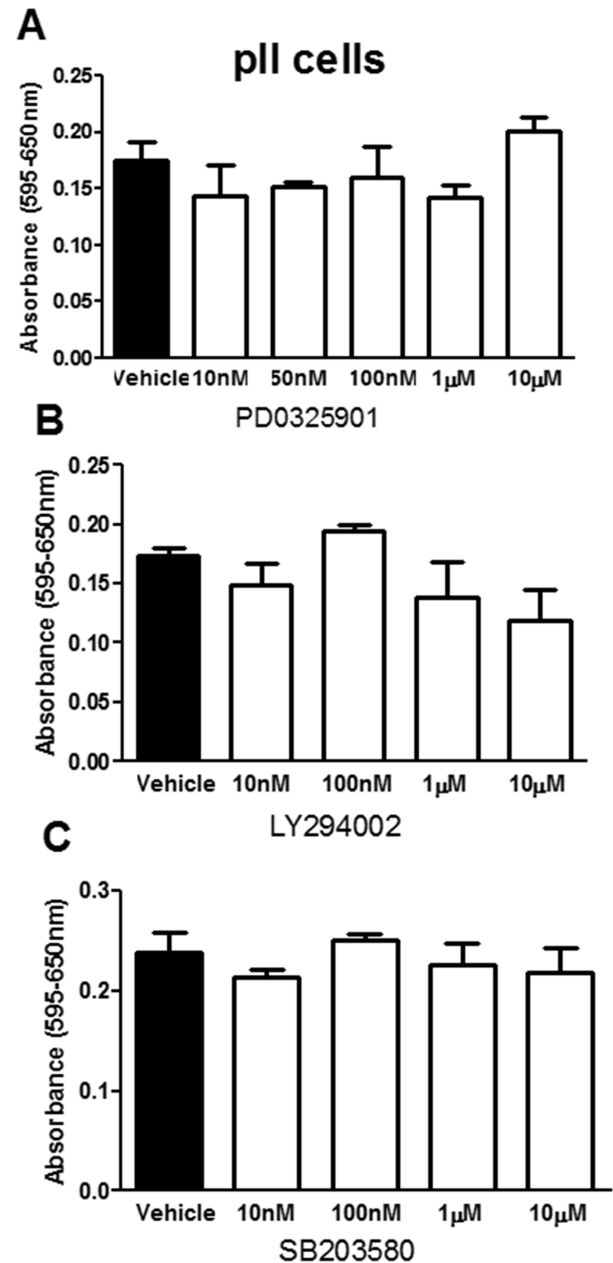


Figure 6. Effect of short-term PD0325901/SB203580/LY294002 on pII cell proliferation. Approximately 10^4 of pII cells were seeded in microwell plates and allowed to grow over 1 day in the presence of vehicle (solid bars) or various concentrations (10 nM–10 μ M, open bars) of the inhibitors as indicated. Cells were harvested, and growth was determined by the MTT assay. Histograms represent means \pm SEM of at least three independent determinations.

Effect of PD0325901, SB203580, and LY294002 on pII Cell Invasion

The Cultrex assay was used to assess the invasion of pII cells through a layer of basement membrane components. These were detected in the lower chamber by

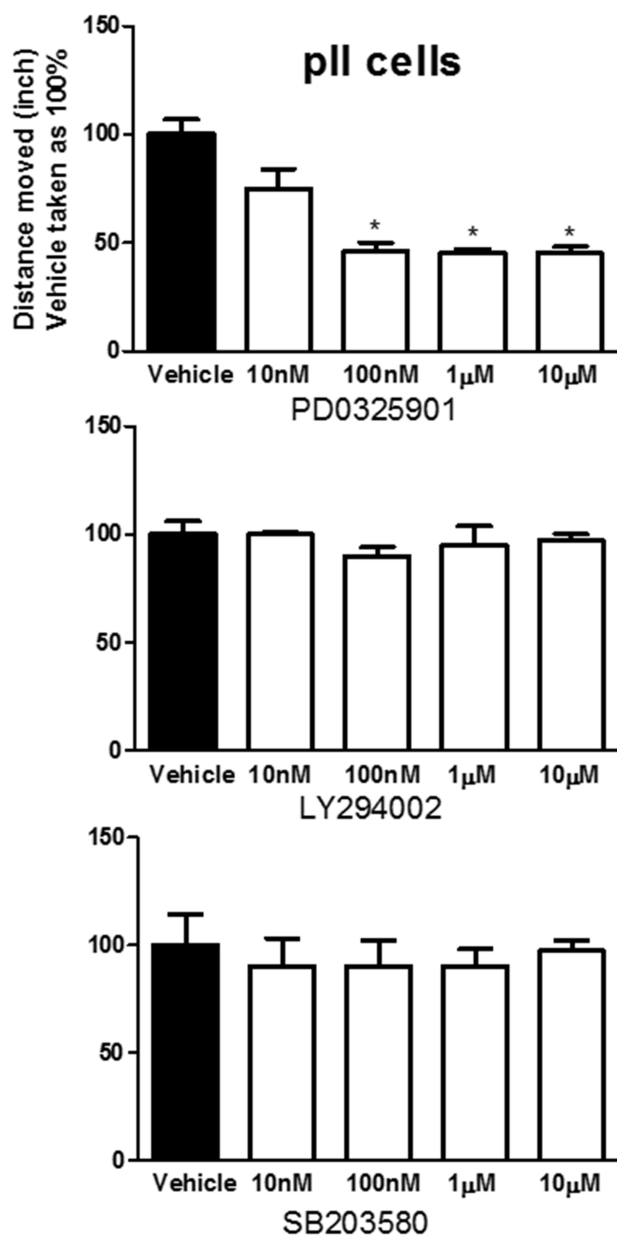


Figure 7. Effect of PD0325901/SB203580/LY294002 on pII cell motility. The mean distance moved after 24 h as an indication of motility of pII cells pretreated with vehicle (solid bars, taken as 100%) or various concentrations (10 nM–10 µM, open bars) of inhibitors was determined as described in Methods. Histograms represent means \pm SEM of at least three independent determinations. Significant difference from vehicle control: * $p < 0.05$.

uptake of calcein. Addition of the PI3K inhibitor to the cell suspension had no effect on cell invasion (Fig. 9). With the ERK1/2 inhibitor, we observed a marginal (10%) but statistically significant inhibition of invasion at 1 µM. However, the p38 MAPK inhibitor did show a marked inhibitory effect (approximately 30% at 10 nM).

A schematic synopsis of the above-described study findings is depicted in Figure 10.

DISCUSSION

In our previous studies using ER-silenced cell lines^{6,8}, we demonstrated that EGF plays a significant role in breast cancer cell proliferation, motility, and invasion and is likely to be a major factor in the progression of endocrine-resistant cancer cells. In this study, we show the effect of targeting three key downstream mediators that are known to be regulated through EGF-induced receptor phosphorylation, namely, ERK1/2, PI3K (Akt), and p38 MAPK in a normal breast line MCF10A, the ER⁺ MCF-7 line, the ER⁻ MDA-MB-231 line, and the ER-silenced pII cell line. While EGF-induced ERK1/2 activity was common to both MCF-7 and pII, only the latter showed increased phosphorylation of p38 and Akt, suggesting a greater involvement of these pathways in estrogen-desensitized cells. Agents that are known to selectively target these molecules displayed selective killing of breast cancer cells compared with the normal epithelial cells, conferring a significant advantage over classic anticancer agents such as paclitaxel, which affected both normal and cancer cells without sufficiently discriminating between them. Combining the ERK1/2 inhibitor with either the p38 MAPK or PI3K inhibitor produced a synergistic effect in inhibiting the growth of pII cells and was also achieved with lesser doses of each drug than needed separately. The pH-induced morphological changes characterizing pII cells were prevented by all three inhibitors. Only the ERK1/2 inhibitor blocked motility, while the p38 MAPK inhibitor was most effective in reducing invasion.

The MAPK signaling transducer pathway is activated through various cytokines, growth factor receptors, and G protein-coupled receptors (GPCRs), which ultimately leads to the activation of several downstream molecules and transcription factors such as AIB1, ER coactivators, heat-shock proteins (hsp) 25 and 27, ATF2, MEF-2, Elk-1, and CREB^{12–19}. The Ras/MAPK pathway is a key regulator of proliferation, differentiation, survival, and invasion, as well as epithelial-to-mesenchymal transition (EMT), which is also linked to endocrine resistance in breast cancer^{20–22}. Genetic mutations in proteins involved in this pathway have been noted in 2–10% of breast cancer patients, suggesting their involvement in the disease pathogenesis^{23,24}. Positive correlations between enhanced phosphorylation of p38 MAPK and lymph node metastasis have been demonstrated in breast cancer patients²⁵, and some preclinical studies have indicated the usefulness of MEK inhibitors in combination with either PI3K or Src inhibitors^{26,27}. Multiple effects of TGF- β in the induction of EMT, proliferation, and invasion through both Smad-dependent and Smad-independent mechanisms involve activation of p38 MAPK^{28–30}. Furthermore, p38 MAPK induces the

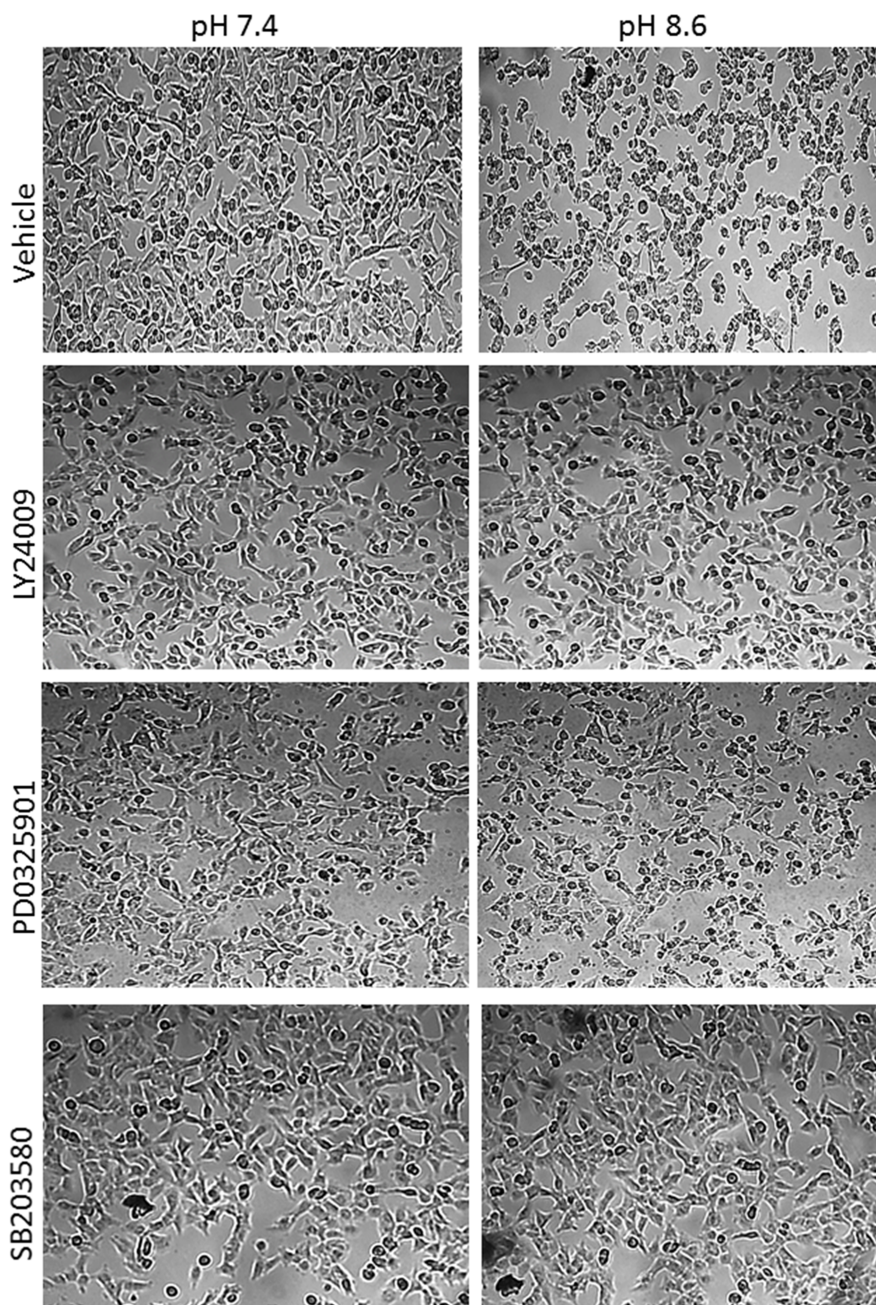


Figure 8. Effect of PD0325901/SB203580/LY294002 on pH-induced morphological changes in pII cells. pII cells, grown in complete DMEM in a 12-well plate, pretreated with vehicle or inhibitors (10 μ M, 30 min) were photographed immediately after removal from the CO₂ gassed incubator (pH 7.4) and after exposure to atmospheric conditions for 30 min (which increased the pH to 8.3). Magnification: \times 20.

activity of various MMPs^{31–33}, uPA^{34,35}, and COX-2³⁶ as well as mediating the effects of H-Ras in the induction of MMP-2 activity and cell migration³², and of hsp-27 on cell migration and invasion³⁷. SB203580 has been observed to inhibit the invasion of BT549 and MDA-MB-231 breast cancer cells through Matrigel, in part by reducing the uPA/uPAR expression^{34,38}. Another report

suggested that WAVE3 (a member of the WASP/WAVE family of proteins) regulates actin cytoskeleton polymerization and motility in MDA-MB-231 breast cancer cells through the activation of p38 MAPK and production of MMPs 1, 3, and 9³⁹.

The PI3K signaling pathway is activated downstream of several growth factor receptors (EGFR, ERBB2),

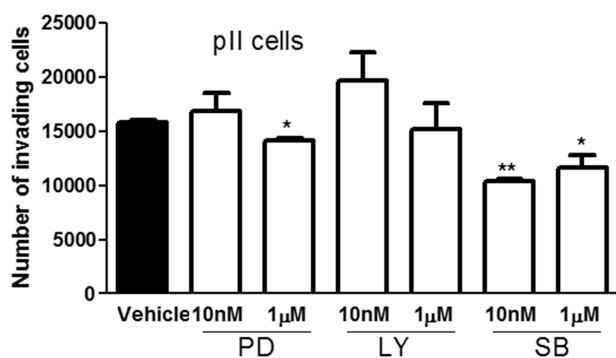


Figure 9. Effect of PD0325901/SB203580/LY294002 on pII cell invasion. Histobars indicate the number of pII cells invading through the Matrigel toward serum components present in the lower chamber for cells either pretreated with vehicle (solid bar) or various concentrations (10 nM and 1 μ M, open bars) of the indicated inhibitors. Each histobar represents mean \pm SEM of at least three independent determinations. Significant difference from vehicle control: * p <0.05.

insulin-like growth factor 1 receptor (IGF1-R), and GPCRs to phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP₂) and produce phosphatidylinositol 3,4,5-triphosphate (PIP₃). This in turn recruits and activates several adaptor proteins such as phosphatidylinositol-dependent kinase-1 (PDK1) and protein kinase-B (Akt) to regulate processes such as cell proliferation, differentiation, metabolism, survival, and angiogenesis, and/or further activate other downstream pathways such as the mammalian target of rapamycin (mTOR)/Raptor complex-1 (mTORC1), GSK-3 β , and tau^{40,41}. The phosphatase and tensin homolog (PTEN) and the inositol polyphosphate-4-phosphatase type II (INPP2B) work as negative regulators of this pathway by dephosphorylating PIP₃ to produce PIP₂. It has been demonstrated that activating mutations of the PI3K catalytic subunit (p110), Akt, and upstream regulators (ERBB2), or loss-of-function mutations in PTEN/INPP4B occur in breast cancer⁴²⁻⁴⁴. Somatic mutations have been identified in PIK3CA (36% encode for the catalytic subunit p110), PIK3R1 (3% encode for the regulatory subunit p85), Akt1 (2%), and PTEN (3%) genes in breast cancer patients²⁴.

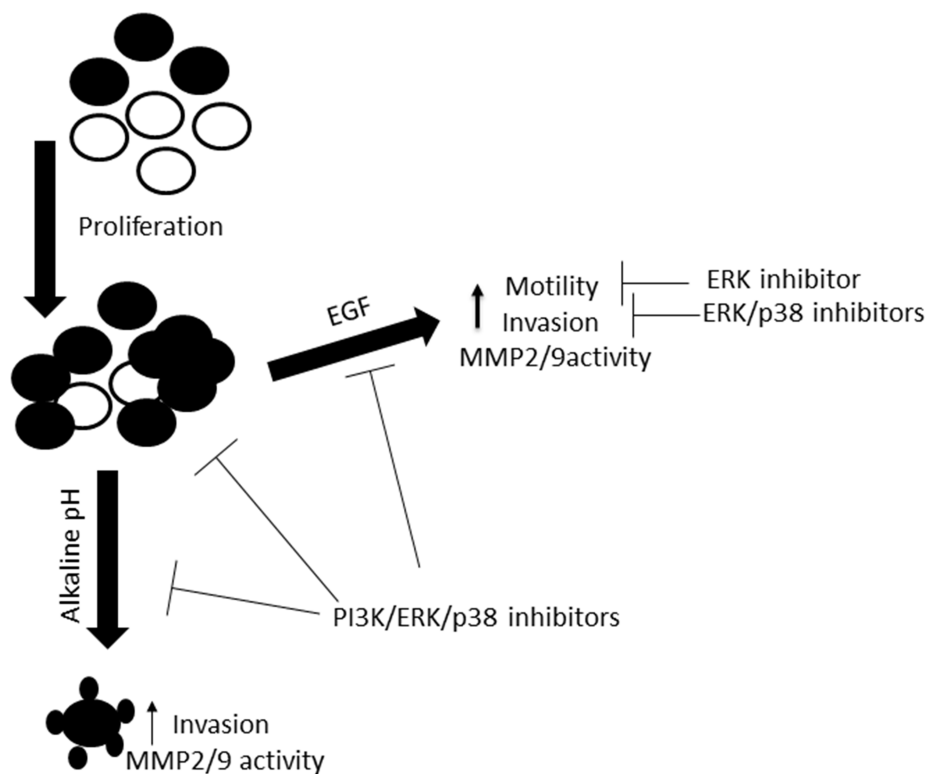


Figure 10. Schematic presentation of the effect of the inhibitors investigated in this study on endocrine-resistant pII cells. PI3K/ERK1/2/p38 MAPK inhibitors *specifically* reduce the proliferation of pII cells (solid circles) while preserving the normal epithelial cells (open circles), which presents a significant advantage over the classic anticancer drugs such as paclitaxel. Either EGF stimulation or short-term exposure to extracellular alkaline pH conditions leads to enhanced cell motility and invasion, which is also prevented by the indicated inhibitors.

Furthermore, enhancement of this pathway through the activation of Akt and mTOR substrates, or knockdown of PTEN, has been associated with de novo and acquired endocrine resistance in breast cancer^{45,46}. The PI3K/Akt/mTOR pathway also plays a significant role in breast cancer. The mTOR inhibitors rapamycin and everolimus have been shown to restore sensitivity to hydroxytamoxifen and fulvestrant in previously resistant MCF-7 cells. This was mediated in part through increased ER protein expression and a change in the ratio of phosphoser167 ER to total ER in the resistant cells⁴⁷. Combined treatment with the mTOR inhibitor RAD001 and tamoxifen had an additive antiproliferative effect on MCF-7 cells through modulation of the apoptotic machinery⁴⁸. These findings were also confirmed with temsirolimus and the ER antagonist ERA-923⁴⁹. Several preclinical studies have been conducted using the inhibitors of PI3K/mTOR in endocrine-resistant breast cancer patients, with promising outcomes⁵⁰⁻⁵². Various anticancer agents act by modulating Akt activity to inhibit breast cancer cell growth. These include paclitaxel, cisplatin, topotecan, docetaxel, lapatinib, and letrozole. In this study, the PI3K inhibitor was the most effective of the three agents tested in inhibiting cell proliferation (either alone or in combination).

We previously reported that brief exposure of specifically ER-silenced breast cancer cells to extracellular alkaline pH induced a dramatic morphological transformation accompanied by the formation of dynamic actin-rich bleb-like structures on the cell surface⁹⁻¹¹. These blebs promote ameboidal-like movement through an extracellular matrix, in part by increasing MMP 2/9 activity. This was prevented by all three inhibitors, suggesting that multiple signaling pathways are necessary for cytoplasmic streaming. Our current data show that the previously documented effects of EGF on breast cancer cell proliferation, invasion, motility, and EMT⁷ are mediated, at least in part, through enhancement of the phosphorylation (activity) of MAPK/Akt. This serves to highlight the potential benefit of inhibiting these signaling molecules as an effective treatment in breast cancer that has distinct advantages over nonspecific cytotoxic agents, particularly for endocrine-resistant cancers. A combination of the ERK1/2 inhibitor with either p38 MAPK or PI3K inhibitor may be useful in targeting multiple effector functions with lesser doses of each drug.

In summary, while we recognize that our data need to be replicated with a wider selection of endocrine-resistant cancer cell lines and other "normal" breast lines, our study provides promising preclinical evidence for the utility of inhibitors targeting downstream signaling molecules for breast cancer treatment. The most significant advantage that we have highlighted is the use of these

agents in combination with commonly used general cytotoxic drugs such as paclitaxel. A therapeutic effect may be achieved with significantly lower doses of the latter, lessening the occurrence of undesirable side effects on normal tissues, which is the major drawback of current chemotherapy. An additional advantage in adding another agent with an entirely independent mode of action may further reduce cellular drug resistance that is frequently encountered with chemotherapy alone⁶. Of course, as with all in vitro observations, it remains to be determined whether these can be effectively translated into the clinical setting. There will also be a need for additional preclinical and clinical confirmatory data on the safety and efficacy of the experimental treatments described in this study.

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