

Crizotinib, a MET inhibitor, inhibits growth, migration, and invasion of breast cancer cells in vitro and synergizes with chemotherapeutic agents

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Abstract: MET is a receptor tyrosine kinase known for its pleiotropic effects in tumorigenesis. Dysregulations of MET expression and/or signaling have been reported and determined to be associated with inferior outcomes in breast cancer patients rendering MET a versatile candidate for targeted therapeutic intervention. Crizotinib is a multi-targeted small-molecule kinase inhibitor for MET, ALK, and ROS1 kinases. This study evaluated the anti-proliferative, cytotoxic, anti-migratory, and anti-invasive effects of crizotinib in breast cancer cells in vitro. Cell viability was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay. In vitro wound-healing assay was used to examine the effect of crizotinib on breast cancer cell migration. The expressions of Ki-67, MET, and phospho-MET receptors were characterized using immunofluorescence staining. Results showed that crizotinib has significant anti-proliferative activity on all mammary tumor cells with IC_{50} values of 5.16, 1.5, and 3.85 μ M in MDA-MB-231, MCF-7, and SK-BR-3 cells, respectively. Crizotinib induced cytotoxic effects in all breast cancer cells examined. Combined treatment of small dose of crizotinib with paclitaxel or doxorubicin exhibited a highly synergistic inhibition of growth of MDA-MB-231 and MCF-7 cells with combination index values <1 while no significant effect was observed in SK-BR-3 cells compared with individual compounds. Treatment with crizotinib demonstrated a remarkable reduction in the expression of Ki-67 protein in all 3 tested cell lines. Crizotinib inhibited migration and invasion of MDA-MB-231 cells in a dose-dependent fashion. Crizotinib reduced MET receptor activation in MDA-MB-231 cells when treated at effective concentrations. In conclusion, crizotinib suppressed proliferation, migration, and invasion of breast cancer cells in vitro. The results of this study demonstrated that combined treatment of crizotinib with chemotherapeutic agents resulted in a synergistic growth inhibition of specific breast cancer cell lines.

Keywords: crizotinib, breast cancer, MET receptor, chemotherapy, migration, invasion

Introduction

Breast cancer is not a single disease but is highly heterogeneous at both the molecular and clinical level.¹ Comprehensive gene expression profiling of large sets of tumors has revealed 4 major molecular subtypes of breast cancer: luminal A, luminal B, HER2-enriched, and basal-like breast cancer.²⁻⁵ Both luminal A and luminal B breast cancers are hormone receptor-positive and have expression patterns reminiscent of the luminal epithelial component of the breast.⁶ HER2-enriched tumors are identified with overexpression/amplification of the *ErbB2/HER2* gene and are generally hormone

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receptor-negative.^{1,2} Basal-like tumors are predominantly triple-negative lacking expression of hormone receptors and HER2.² These subtypes have been associated with distinct pathological features and clinical outcomes in which patients with luminal A tumors have the best prognosis, while those with basal-like breast cancer have the worst prognosis.^{1,2} Despite advancements in targeted therapies, cytotoxic chemotherapy remains a cornerstone treatment of breast cancer.^{7,8}

Multiple receptor tyrosine kinases (RTKs) were identified for their oncogenic potential in breast cancer.^{9,10} Recently, strong evidence has supported the role of the hepatocyte growth factor (HGF) and its receptor, MET, in the development and progression of breast carcinoma.¹¹ Activation of MET induces receptor dimerization and tyrosine autophosphorylation within the catalytic site regulating kinase activity. The phosphorylated tyrosines create a multifunctional docking site for a wide spectrum of transducers and adaptors, including PI3K, viral oncogene homolog (Src), GRB2, Shc, PLC- γ , SHP2 phosphatase, and STAT.^{12,13} The involvement of such a diverse number of effectors allows the activation of different downstream pathways, including the Akt-NF κ B and the RAS-MAPK signaling pathways.¹⁴ Ultimately, activation of MET resulted in upregulation of diverse tumor cell functions, including cell proliferation, survival, motility, invasion, angiogenesis, and metastasis.^{15,16} Clinical studies showed that MET is overexpressed in 20%–30% of breast cancer cases and is a strong, independent predictor of decreased survival which correlated with poor patient outcome.^{17–20} Breast cancer cells have been shown to express MET and thus could be sensitive to MET inhibitors.^{21–23}

Because of its diverse roles in cellular processes important in oncogenesis and cancer progression, MET is considered to be an important target in anti-cancer therapy. Recently, it has been proposed that inhibition of MET may be a targeted therapy regardless of the type of cancer.²⁴ Several strategies have been developed to suppress MET activity, including monoclonal antibodies directed against MET, inhibitors of MET expression, and small-molecule tyrosine kinase inhibitors.^{25,26} In this regard, small molecule kinase inhibitors offer the most versatile approach by inhibiting HGF-dependent tumors as well as tumors driven by other MET-dependent mechanisms, such as receptor amplification and activating mutations.²⁷ Crizotinib is an oral small-molecule tyrosine kinase inhibitor of ALK, MET, and ROS1 kinases.²⁸ Crizotinib obtained European and USA Food and Drug Administration (FDA) approval for the treatment of non-small-cell lung cancer (NSCLC)

patients having ALK rearrangements.^{29,30} Crizotinib showed remarkable anti-proliferative activity, anti-angiogenic, and cytotoxic effects in multiple types of cancers.^{31–33} Despite the availability of this MET inhibitor, limited number of studies in literature had assessed the anti-cancer effects of crizotinib in breast cancer.^{24,34,35}

This study aimed to investigate in vitro activity of crizotinib in different molecular subtypes of breast cancer. In addition, the effect of combined crizotinib treatment with cornerstone chemotherapeutic agents available clinically for management of breast cancer has been examined in this study.

Methodology

Chemicals, reagents, and antibodies

Crizotinib, paclitaxel, and doxorubicin were purchased from Tocris Bioscience Company (Bristol, UK). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Sigma Aldrich (St Louis, MO, USA). Primary antibodies for Ki-67, MET, and phospho-MET as well as goat anti-rabbit Alexa Fluor[®]594 secondary antibody, and Fluoroshield mounting medium with DAPI were purchased from Abcam (Cambridge, MA, USA).

Cell lines and culture conditions

Human breast cancer cell lines MDA-MB-231, MCF-7, and SK-BR-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 breast cancer cell line represents basal-like subtype which is negative for hormone receptors and HER2 expression.³⁶ MCF-7 cells represent luminal A subtype, which are positive for hormone receptors and negative for HER2. SK-BR-3 cancer cells represent HER2-positive subtype, which are negative for hormone receptors and positive for overexpression/amplification of HER2.³⁶ Cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. All cells were maintained at 37°C in an environment of 95% air and 5% CO₂ in a humidified incubator. For subculturing, cells were washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) and incubated in 0.05% trypsin containing 0.02% EDTA in PBS for 5–15 min at 37°C.

Experimental treatments

Crizotinib was dissolved in dimethyl sulfoxide (DMSO) to provide a final 40 mM stock solution. Paclitaxel and doxorubicin were dissolved in DMSO to form a 10 mM stock solution of each. These stock solutions were used to prepare various

concentrations of treatment media. The final concentration of DMSO was maintained the same in all treatment groups within a given experiment and never exceeded 0.1%.

Measurement of viable cell number

Viable cell count was determined using the MTT colorimetric assay.³⁷ Briefly, treatment media were replaced with fresh media containing 0.42 mg/mL MTT and incubated for 3 h at 37°C in a humidified incubator. At the end of incubation period, media were removed and formazan crystals were dissolved in DMSO (100 µL/well for 96-well plates). Optical density was measured at 570 nm on a microplate reader (BioTech, Winooski, VT, USA). Number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined using a hemocytometer at the start of each experiment.

Cell growth and cytotoxicity studies

To evaluate the effect of crizotinib, chemotherapy, and the combination of both compounds on viability of breast cancer cells, growth studies were performed. Breast cancer cells were initially seeded at 1×10^4 cells/well (6 replicates/group) in 96-well plates in RPMI-1640 media containing 10% FBS and allowed to attach overnight. Next day, cells were divided into different treatment groups and were exposed to respective control or experimental treatments in mitogen-free media for 48 h. In cytotoxicity studies, cells were plated as described earlier and allowed to grow to a confluency of 70%–80%. Afterward, cells were divided into different treatment groups and fed fresh treatment or control media for 24 h. Viable cell number was determined at the end of the experiments using the MTT assay. Each experiment was repeated at least 3 times.

Immunocytochemical fluorescent staining

Breast cancer cells were seeded on 8-chamber culture slides (Ibidi Company, Martinsried, Germany) at 5×10^4 cells/chamber (2 replicates/group) in 10% FBS RPMI-1640 media and allowed to attach overnight. Next day, cells were washed with PBS and incubated in serum-free media supplemented with 0.5% FBS containing desired concentrations of crizotinib for 24 h. At the end of treatment, media were removed and cells were rinsed 3 times with pre-cooled PBS. MDA-MB-231 cells were then fixed in methanol pre-cooled to -20°C for 2 min, while MCF-7 and SK-BR-3 cells were fixed with 1:1 vol of methanol:acetone pre-cooled to -20°C for 2 min and permeabilized with 0.2% triton X-100 in PBS for 2 min. Fixed cells were washed with PBS and blocked with 5% goat

serum in PBS at room temperature for 1.5 h. Afterward, cells were incubated in specific primary antibodies to Ki-67 (1:250), MET (1:250), and phospho-MET (1:100) overnight at 4°C in 5% goat serum in PBS. Cells were washed in pre-cooled PBS followed by incubation with goat anti-rabbit Alexa Fluor 594-conjugated secondary antibody (1:3,000) in 5% goat serum in PBS for 1 h at room temperature. After final washing, cells were embedded in Fluoroshield mounting medium with DAPI (Abcam). Fluorescent images were captured using Nikon's Eclipse E600 microscope (Nikon Instruments, Melville, NY, USA) at a magnification of $20\times$. Percentage of cells expressing Ki-67, MET, or phospho-MET was calculated by counting numbers of positive cell staining for each marker as a proportion of the total number of cells counted (stained with DAPI). Cells were counted manually in 5 photomicrographs captured randomly for every treatment group.

Migration assay

In vitro wound-healing assay was used to assess directional cell motility in 2 dimensions. MDA-MB-231 cells were plated in sterile flat-bottom 12-well plates (3 replicates/group) and incubated in 10% FBS RPMI-1640 media and then allowed to form a subconfluent cell monolayer per well, overnight. A scratch was drawn at the center of each cell monolayer using sterile 200 µL pipette tips. Cells were washed with $1\times$ PBS and incubated in 0.5% FBS RPMI-1640 culture media containing desired concentrations of crizotinib for 24 h. At the end of incubation period, medium was removed, and MDA-MB-231 cells were washed in pre-cooled PBS, fixed in methanol previously cooled to -20°C , and stained with crystal violet solution. Wound healing was visualized at 0 and 24 h by BEL INV-100 LED microscope coupled with BEL EUREKAM 5.0 camera (Biovera, Azienda, Rome, Italy) at a magnification of $4\times$. Digital images were taken using BEL capturing Software microscope (Biovera). Distance travelled by cells was determined by measuring wound width at 24 h and subtracting it from the wound width at the start of treatment (time zero, t_0). Values obtained were then expressed as percentage wound closure, setting the gap width at t_0 as 100%. Each experiment was performed in triplicate, and distance migrated was calculated in 5 or more randomly selected fields per treatment group.

Invasion assay

Anti-invasive effect of crizotinib treatment in MDA-MB-231 breast cancer cells was measured using Trevigen Cultrex® BME Cell Invasion Assay (Trevigen Inc., Gaithersburg,

MD, USA). About 50 μL of basement membrane extract (BME) coat was added per well of the top chamber. After an overnight incubation at 37°C in a 5% CO_2 atmosphere, 5×10^4 cells per 50 μL of RPMI-1640 medium supplemented with 0.5% FBS were added per well of the top chamber containing the final desired concentration of crizotinib treatment. Next, 150 μL of 10% FBS RPMI-1640 medium was added to the lower chamber in which FBS served as chemoattractant. Cells were then incubated at 37°C under 5% CO_2 which allowed for cell invasion from the top to the lower chamber. After 24 h of incubation, the top and bottom chambers were aspirated and washed with washing buffer supplied within the kit. About 100 μL of 1 \times cell dissociation solution/calcein-AM solution was added to the bottom chamber and incubated at 37°C under 5% CO_2 for 1 h. Fluorescence of the samples was determined at λ_{ex} 485 and λ_{em} 520 nm, with GloMax[®] Discover System (Promega Corporation, Fitchburg, WI, USA). The number of cells that invaded through the BME coat was calculated by a standard curve.

Statistical analysis

Data analysis was performed using IBM SPSS statistical package (IBM Corporation, Version 21.0., Armonk, NY, USA). The results are presented as mean \pm SEM. Differences between various treatments groups were determined by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's honest significant difference (HSD) test. All *P*-values were 2-sided and differences were considered to be statistically significant at $P < 0.05$.

IC_{50} values (concentration that induced 50% cell growth inhibition) were determined by applying non-linear regression curve fit analysis using GraphPad[®] Prism software version 6. Assessment of the effect of combined treatment of crizotinib and chemotherapeutic drugs was done by combination index (CI), dose reduction index (DRI), and isobologram analyses. CI is a quantitative representation of the pharmacological interaction between 2 compounds.^{38,39} A CI value of 1 represented additive interaction, while CI values < 1 or > 1 indicate synergistic and antagonistic effects, respectively. CI values for the combinations in this study were calculated by the following equation:^{38,39}

$$\text{CI} = [\text{Cc}/\text{C} + \text{Xc}/\text{X}]$$

where C and X represent the IC_{50} values of crizotinib and chemotherapeutic drug when used alone for cell growth studies, while Cc and Xc are the IC_{50} values of crizotinib and chemotherapeutic drug that also inhibited cell growth

by 50% when used in combination. DRI values represent fold decrease in the dose of individual drugs when used in combination, as compared with the dose of a single drug that is required to induce the same effect level.^{38,39} DRI values of > 1 are considered favorable allowing less toxicity while retaining the therapeutic efficacy of individual compounds. DRI values for each of the compounds used in this study were calculated by the following equation:^{38,39}

$$\text{DRI} = \text{X}/\text{Xc}$$

where X is the IC_{50} value of the compound when used alone for cell growth studies, and Xc is the IC_{50} value for the same compound when used in combination.^{38,39}

Isobologram analysis is a graphical method to evaluate the effect of equally effective concentration pairs for a single-effect level.⁴⁰ It is created on a coordinate system comprised of the individual drug concentrations and shows a straight line which represents additive effects for data points on the line. The straight line in each isobologram figure was constructed by plotting IC_{50} concentrations of crizotinib and chemotherapeutic drug on y- and x-axes, respectively. Data points located above the line indicate antagonistic interaction, while those falling below the line represent synergistic interaction between 2 compounds when given in combination.^{40,41}

Results

Growth and cytotoxic effects of crizotinib on breast cancer cells

Effects of various doses of crizotinib on in vitro growth of multiple breast cancer cell lines are indicated in Figure 1A. Crizotinib resulted in a dose-dependent reduction in viability of breast cancer cells after 48 h treatment in mitogen-free cell culture media. In MDA-MB-231 cells, treatment with 2–12 μM of crizotinib significantly inhibited growth of MDA-MB-231 cells compared to vehicle-treated controls. In MCF-7 and SK-BR-3 cells, 0.5–7.5 and 1–8 μM crizotinib significantly inhibited growth of both cell lines, respectively. The IC_{50} values for crizotinib treatment in mitogen-free culture media were 5.16, 1.5, and 3.85 μM in MDA-MB-231, MCF-7, and SK-BR-3 cells, respectively. The cytotoxic effects of acute 24 h treatment exposure to various doses of crizotinib on breast cancer cell viability are shown in Figure 1B. Acute treatment with 5–20 μM crizotinib significantly reduced MDA-MB-231 viable cell number as compared to vehicle-treated controls (Figure 1B). Similarly, acute 24 h treatment with 2.5–20 and 1–15 μM crizotinib resulted in significant cytotoxic effects in MCF-7 and SK-BR-3 cells, respectively.

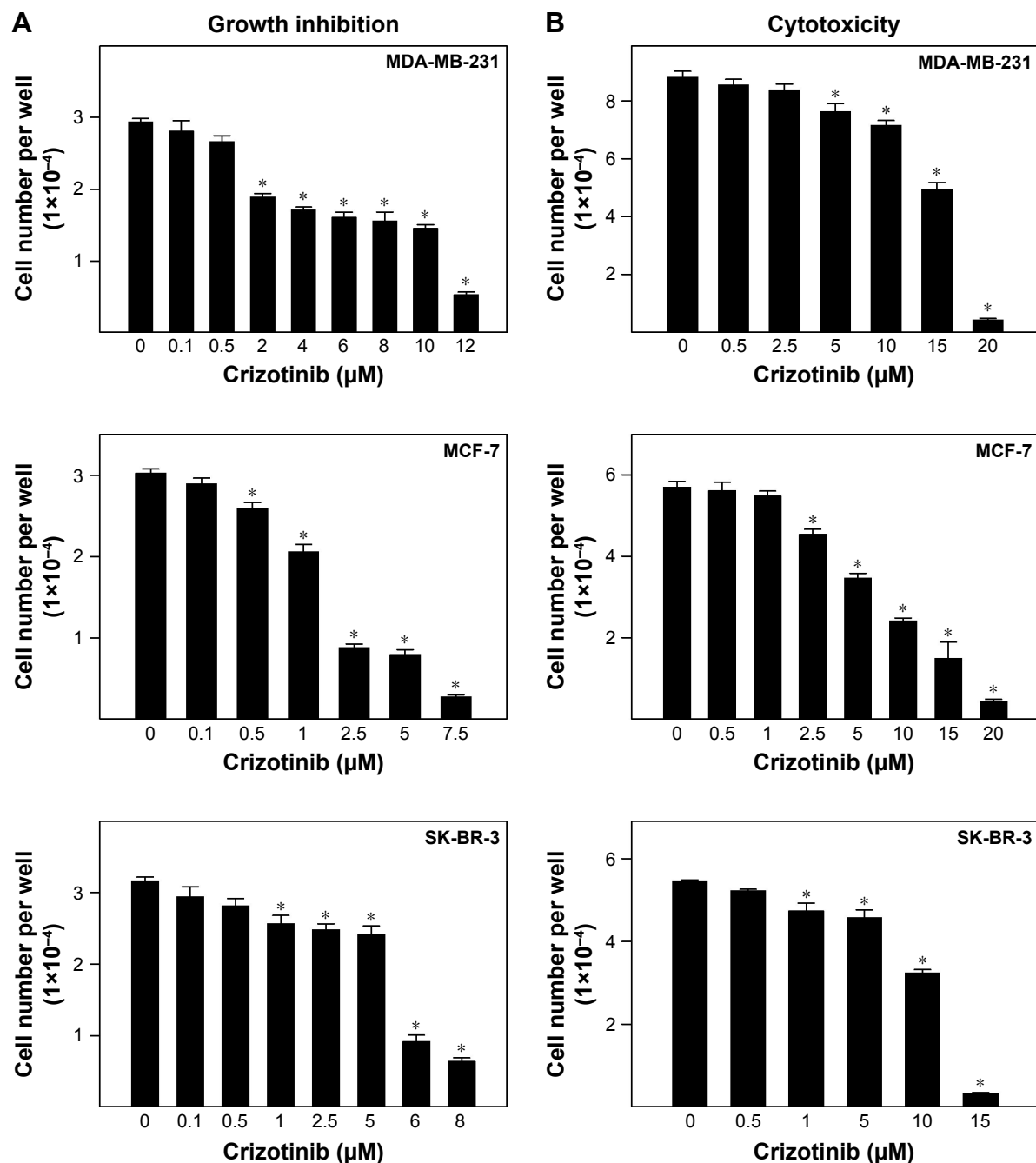


Figure 1 Growth and cytotoxic effects of crizotinib on breast cancer cells.

Notes: (A) Antiproliferative effects of crizotinib on breast cancer cells in vitro. (B) Cytotoxic effects of crizotinib on breast cancer cells in vitro. Vertical bars indicate the mean cell count \pm SEM in each treatment group. * $P < 0.05$ as compared with vehicle-treated control group.

Effects of chemotherapeutic agents on growth of breast cancer cells

The effects of chemotherapeutic agents on the viability of breast cancer cells are shown in Figure 2. Exposure to increasing concentrations of the taxane drug, paclitaxel, resulted in a dose-dependent suppression of breast cancer cell growth in vitro (Figure 2A). Paclitaxel treatment with 0.05–10, 0.05–10, and 0.01–10 μ M significantly reduced viability of MDA-MB-231, MCF-7, and SK-BR-3 cells,

respectively, as compared to vehicle-treated control cells (Figure 2A). The IC_{50} values for paclitaxel were 1.326, 0.04, and 3.682 μ M for MDA-MB-231, MCF-7, and SK-BR-3 cells, respectively. Similarly, the anthracycline drug doxorubicin inhibited growth of breast cancer cells after 48 h treatment in a dose-dependent manner (Figure 2B). Doxorubicin significantly suppressed growth of MDA-MB-231 cells in a concentration range of 0.01–10 μ M with IC_{50} value of 1.3 μ M (Figure 2B). Doxorubicin treatment also resulted

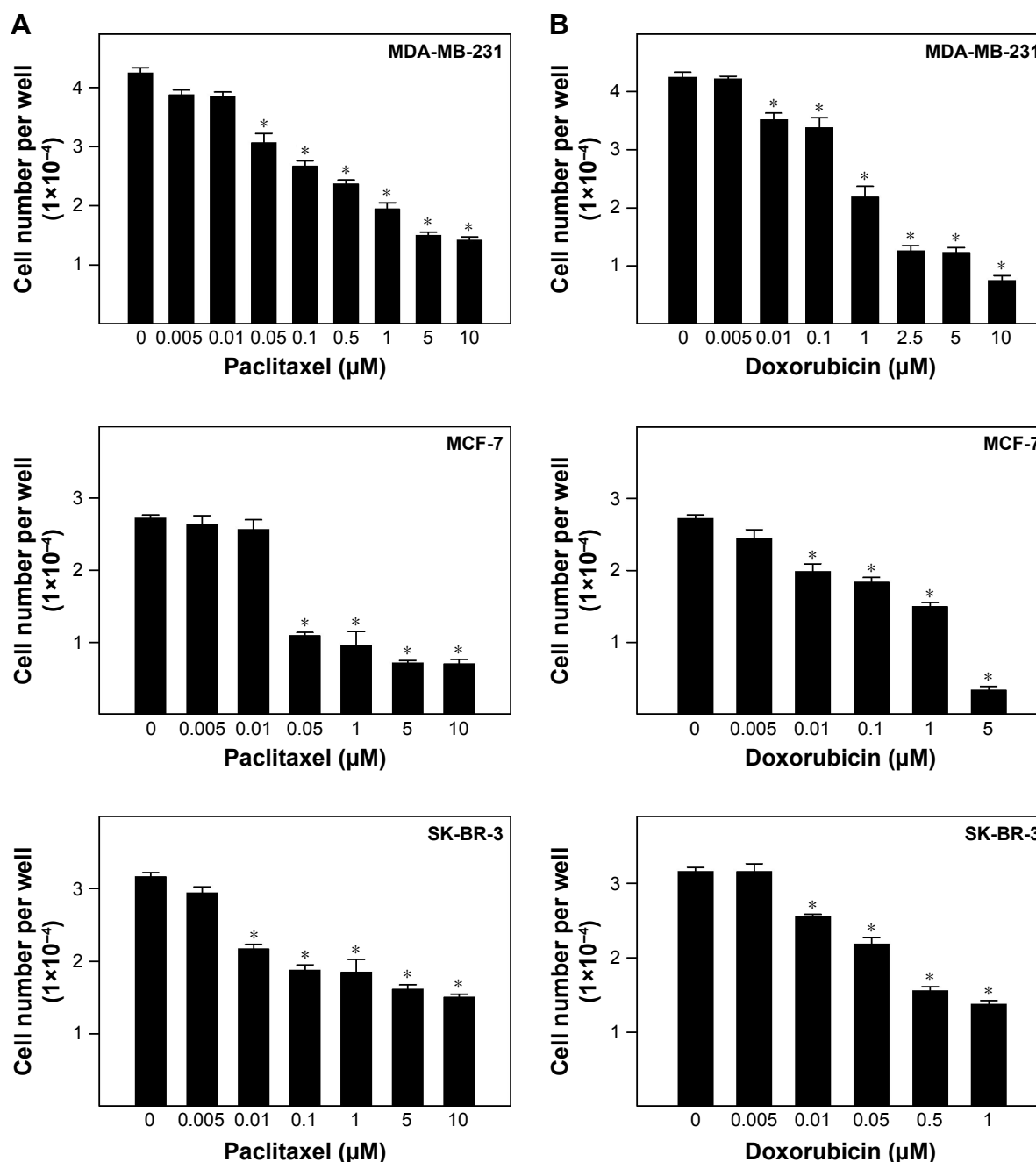


Figure 2 Effects of chemotherapeutic agents on growth of breast cancer cells.

Notes: (A) Antiproliferative effects of paclitaxel on breast cancer cells in vitro. (B) Antiproliferative effects of doxorubicin on breast cancer cells in vitro. Vertical bars indicate the mean cell count \pm SEM in each treatment group. * $P < 0.05$ as compared with vehicle-treated control group.

in a dose-dependent inhibition of MCF-7 and SK-BR-3 cell growth in mitogen-free media with IC_{50} values of 0.915 and 0.352 μ M, respectively (Figure 2B).

Effects of combined treatment of crizotinib and chemotherapeutic agents on growth of breast cancer cells

The combination of a single concentration of crizotinib to a dose range of paclitaxel resulted in suppression of breast

cancer cell growth in a dose-dependent manner after 48 h of treatment in culture (Figure 3A). Combined treatment of 2 μ M crizotinib with 0.005–0.05 μ M paclitaxel significantly inhibited MDA-MB-231 cell growth compared to both vehicle-treated control cells and the respective paclitaxel-treated cells (Figure 3A). Similarly, combination of 0.5 μ M crizotinib with the taxane significantly suppressed MCF-7 cell growth over a dose range of 0.005–10 μ M of paclitaxel compared to respective treatment groups with the taxane alone.

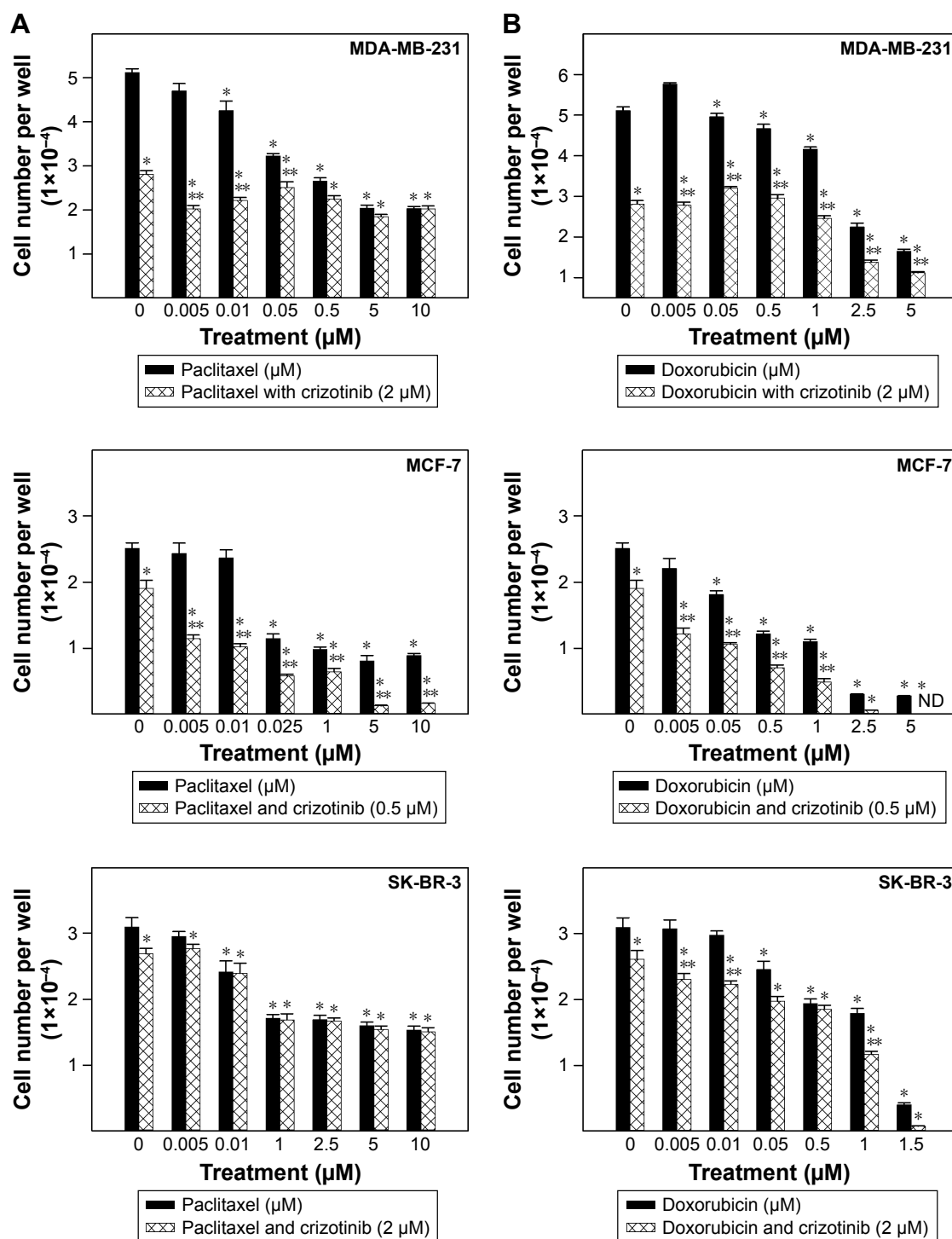


Figure 3 Effects of combined treatment of crizotinib and chemotherapeutic agents on growth of breast cancer cells.

Notes: (A) Effects of combined crizotinib and paclitaxel treatment on growth of breast cancer cells after 48 h of treatment duration. (B) Effects of combined crizotinib and doxorubicin treatment on growth of breast cancer cells after 48 h of treatment duration. Vertical bars represent the mean cell count \pm SEM in each treatment group. * $P < 0.05$ compared with respective vehicle-treated control group, and ** $P < 0.05$ compared to respective group with individual chemotherapy treatment.

Abbreviation: ND, not detectable.

In SK-BR-3 cells, addition of crizotinib to a dose range of paclitaxel did not result in a significant suppression of SK-BR-3 cell growth compared to individual compound treatment of the taxane (Figure 3A). The same pattern for the

combined treatment was found with the addition of a single concentration of crizotinib to a dose range of doxorubicin in MDA-MB-231, MCF-7, and SK-BR-3 cells (Figure 3B). In both MDA-MB-231 and MCF-7 cells, addition of 2 and

0.5 μM crizotinib to a dose range of doxorubicin significantly reduced viability of MDA-MB-231 and MCF-7 cells compared to their respective individual anthracycline-treated cells (Figure 3B). However, in SK-BR-3 cells, combined treatment of crizotinib and doxorubicin did not result in a significant inhibition of SK-BR-3 cell growth for most of the dose range applied.

Isobologram analysis for the effect of combination treatment of crizotinib and chemotherapeutic drugs is shown in Figure 4. Results showed that the growth inhibitory effect of combined treatment of crizotinib with paclitaxel was synergistic in both MDA-MB-231 and MCF-7 cells as indicated by data point in each isobologram located well below the line defining an additive effect (Figure 4A). However,

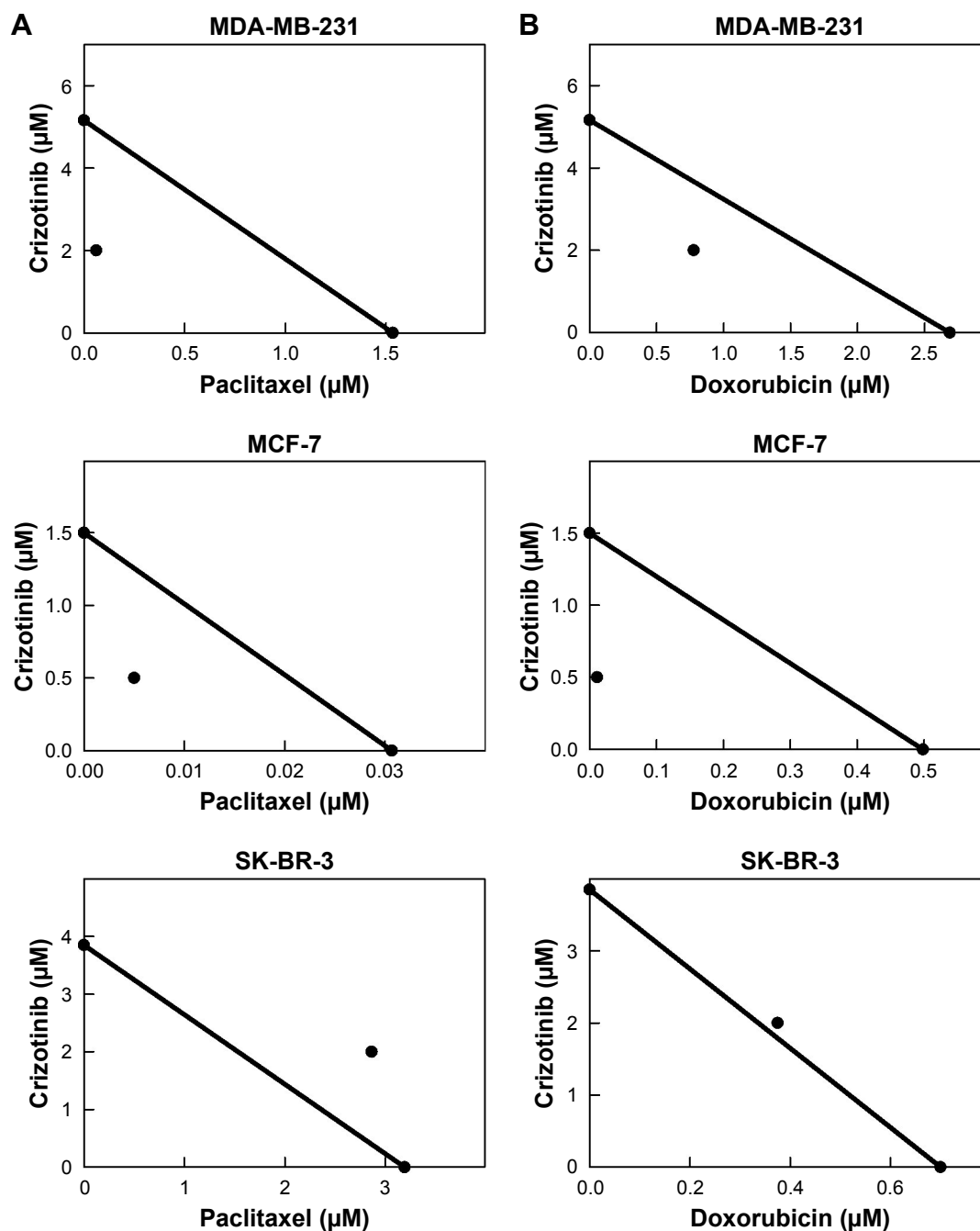


Figure 4 Isobolograms for the anti-proliferative effects of combined treatment of crizotinib and chemotherapeutic drugs in breast cancer cells.

Notes: (A) Isobolograms of crizotinib and paclitaxel anti-proliferative effects on breast cancer cells. (B) Isobolograms of crizotinib and doxorubicin anti-proliferative effects on breast cancer cells. The data point on each isobologram represents the actual concentrations of crizotinib and chemotherapeutic drug which induced 50% inhibition of cell growth when used in combination.

isobologram for the combined treatment of crizotinib and paclitaxel in SK-BR-3 cells indicated antagonism as shown by data point located above the line of additivity (Figure 4A). Isobologram analysis for the effect of combination treatment of crizotinib and doxorubicin showed similar pattern suggestive of synergistic interaction between both compounds for growth inhibition of MDA-MB-231 and MCF-7 cells and antagonistic interaction in SK-BR-3 cells (Figure 4B). The IC_{50} values calculated for paclitaxel treatment in combination with crizotinib were 0.061 μ M for MDA-MB-231, 0.006 μ M for MCF-7, and 2.866 μ M for SK-BR-3 cells. For doxorubicin, IC_{50} values calculated for the anthracycline in combination with crizotinib were 0.777, 0.011, and 0.375 μ M for each of MDA-MB-231, MCF-7, and SK-BR-3 cells, respectively. CI calculated for growth suppressive effects of combined crizotinib and chemotherapy treatment indicated high level of synergism with values <1 for MDA-MB-231 and MCF-7 breast cancer cells (Table 1). In addition, DRI values calculated for combined crizotinib and chemotherapy treatments showed multiple-fold reductions for both compounds when used in combination among MDA-MB-231 and MCF-7 cell lines (Table 1). However, antagonism was shown as indicated by CI values of >1 when both therapies were combined in treatment of SK-BR-3 cells (Table 1).

Effect of crizotinib treatment on Ki-67 labeling in breast cancer cells

Positive expression of the nuclear protein Ki-67 is a known marker of cell proliferation.⁴² In vehicle-treated control cells, Ki-67 expression was observed in 93.3%, 91.8%, and 36.4% of MDA-MB-231, MCF-7, and SK-BR-3 cancer cells, respectively (Figure 5). In MDA-MB-231 cells, treatment with 2.5–7.5 μ M crizotinib resulted in a dose-dependent reduction of Ki-67 expression in treatment groups as compared to control cells after 24 h duration in cell culture (Figure 5A). Similarly, the number of Ki-67 positive cells was significantly reduced in MCF-7 cells treated with 0.1–3 μ M crizotinib compared to vehicle-treated control group (Figure 5B).

In SK-BR-3 cells, crizotinib treatment resulted in reduced Ki-67 expression at concentrations of 5–7.5 μ M compared to control cells (Figure 5C).

Effect of crizotinib treatment on migration and invasion of breast cancer cells

Effect of various concentrations of crizotinib on migration and invasion of MDA-MB-231 cancer cells is shown in Figure 6. Almost complete closure of inflicted wounds in MDA-MB-231 cells was observed after 24 h of incubation in media supplemented with 0.5% FBS. Treatment with crizotinib resulted in a dose-dependent inhibition of cell motility after 24 h in culture (Figure 6B). Exposure to crizotinib treatment at the concentration range of 0.5–5 μ M significantly suppressed migration of MDA-MB-231 cells as compared to vehicle-treated control group (Figure 6B). Crizotinib treatment in concentration of 7.5–30 μ M significantly suppressed invasiveness of MDA-MB-231 cells compared to vehicle-treated control group (Figure 6C).

Effect of crizotinib treatment on total levels of MET and phospho-MET in breast cancer cells

Immunofluorescent staining of MET indicated abundant expression of the receptor in MDA-MB-231 cells with cytoplasmic and nuclear localization (Figure 7A). Crizotinib treatment with a dose-range of 1–7.5 μ M did not result in a reduction in total levels of MET as compared to vehicle-treated control group (Figure 7A). Immunocytochemical fluorescent staining of phosphorylated MET receptors (phospho-MET) in MDA-MB-231 cells is shown in Figure 7B. The phosphorylated MET corresponds to the phosphorylation of tyrosine moieties Y1230/Y1234/Y1235 of kinase domain corresponding to receptor activation. Phospho-MET receptors are found located both on cell membranes and in cytoplasm of the cells (Figure 7B). Levels of phospho-MET receptors were significantly reduced when

Table 1 CI and DRI values for combined treatment of crizotinib and chemotherapeutic drugs resulting in 50% reduction in growth of multiple breast cancer cell lines

Breast cancer cell line	Classification	Receptor status	CI		DRI		
			Crizotinib and paclitaxel	Crizotinib and doxorubicin	Crizotinib	Paclitaxel	Doxorubicin
MDA-MB-231	Triple-negative	ER–, PR–, HER2–	0.427	0.68	2.5	25	3.5
MCF-7	Luminal A	ER+, PR±, HER2–	0.518	0.355	3	5.4	45.3
SK-BR-3	HER2-enriched	ER–, PR–, HER2+	1.423	1.06	ND	ND	ND

Abbreviations: CI, combination index; DRI, dose reduction index; ND, not determined.

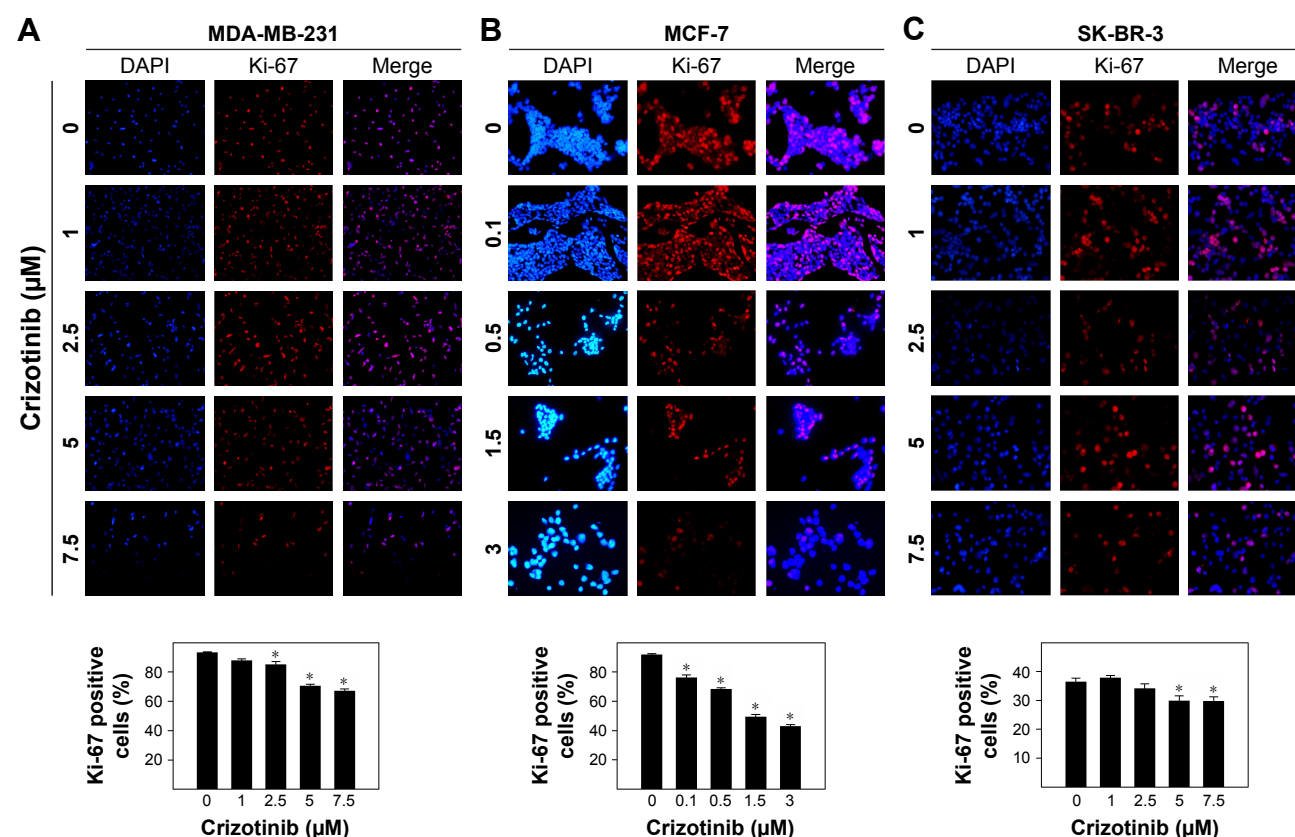


Figure 5 Effect of crizotinib treatment on Ki-67 labeling in breast cancer cells.

Notes: Effect of crizotinib treatment on Ki-67 expression in (A) MDA-MB-231 cells, (B) MCF-7 cells, and (C) SK-BR-3 cells. Upper panel: immunofluorescent staining for Ki-67 in breast cancer cells. Red color indicates positive fluorescence staining for Ki-67, while blue color represents counterstaining of cell nuclei with DAPI. Magnification of each image is 20×. Bottom panel: percentage of positive Ki-67 cancer cells in proportion to the total number of cells. Vertical bars represent percentage of cells with positive Ki-67 staining \pm SEM in each treatment group. * $P < 0.05$ compared to vehicle-treated control group.

cells were treated with 7.5 μ M crizotinib compared to respective control group after 24 h treatment (Figure 7B).

Discussion

Historically, RTKs have been shown to correlate with the development and progression of most forms of human malignancies.⁴³ Therefore, targeting RTKs has become an attractive therapeutic option in cancer therapy.^{43–45} MET is a RTK known to play a significant role in neoplastic transformation, tumor growth, survival, angiogenesis, migration, invasion, metastasis, and resistance to targeted therapies.^{46,47} Highly expressed levels of MET have been detected in human breast cancer and are associated with high tumor grade and poor prognosis.^{17,18,48} In addition, multiple clinical studies indicated that overexpression of MET is associated with decreased survival and poor outcomes in terms of increased risk of recurrence and death among breast cancer patients.^{19,20} In line with this, expression data and tissue microarrays revealed that high MET expression correlates mainly with ER/HER2-negative and the basal subtypes of human breast

tumors.^{13,17,19,20,45} In animal models, studies had shown that activated MET induced a high incidence of mammary tumors with diverse histopathological phenotypes including tumors with basal and luminal characteristics.^{19,20}

The potential implication of HGF/MET axis in the progression of breast carcinomas as well as other cancers has led to a great interest in applying anti-MET therapies clinically.⁴⁴ Small-molecule tyrosine kinase inhibitors of MET have been developed and evaluated as monotherapy or in combination with other targeted therapies.²⁵ Crizotinib is a multi-targeted RTK inhibitor of MET, ALK, and ROS1.^{49,50} In this study, crizotinib induced dose-dependent inhibition of growth of multiple breast cancer cell lines in vitro. In addition, acute exposure to crizotinib resulted in cytotoxic activity in the different cell lines investigated. Mammary cancer cell lines provided in these studies represent in vitro models for major molecular subtypes of breast cancer commonly encountered in clinical settings. Earlier findings in literature showed that the human breast cancer cell lines MDA-MB-231, MCF-7, and SK-BR-3 express MET which is also activated in response

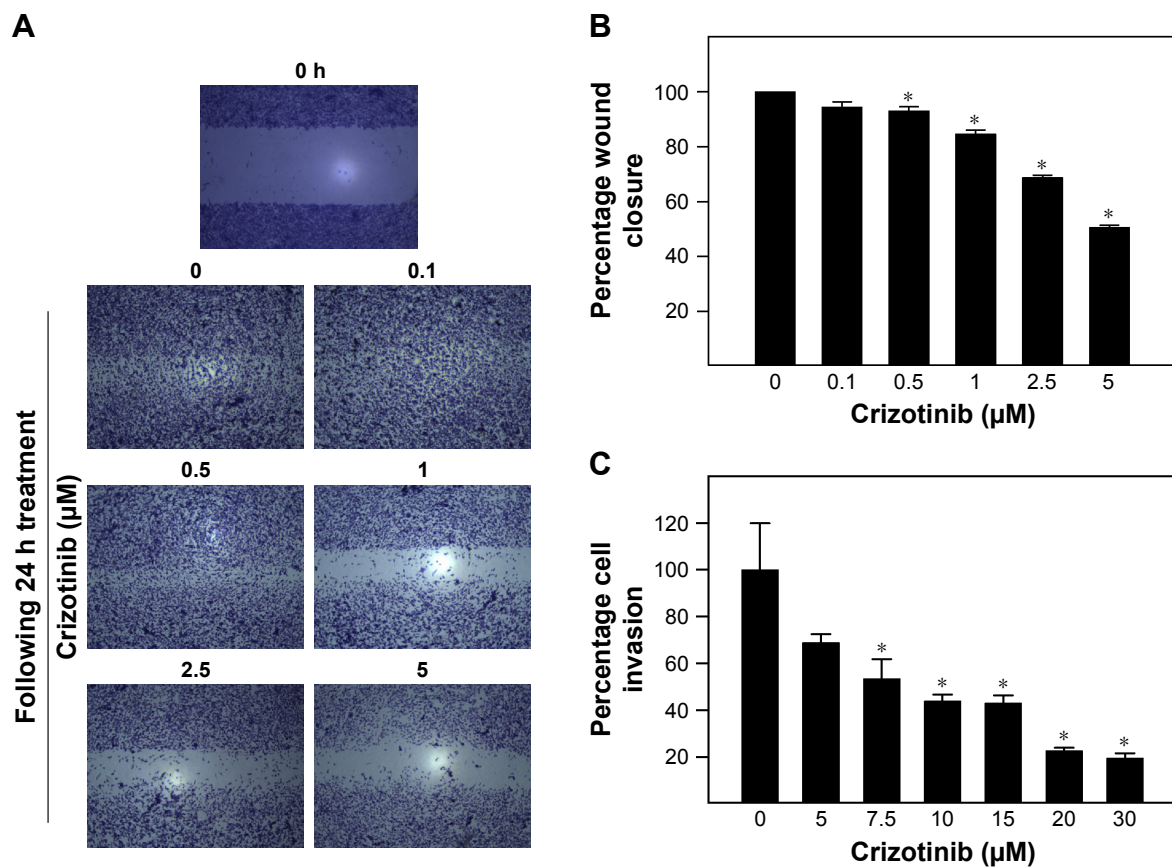


Figure 6 Effect of crizotinib treatment on migration and invasion of breast cancer cells.

Notes: (A) Photomicrographs of crizotinib treatment on migration of MDA-MB-231 cancer cells using the in vitro wound-healing assay. Photomicrographs (4× magnification) were taken at the beginning and end of the treatment period after cell fixation. (B) Quantitative analysis of wound closure in each experimental group. Vertical bars represent percent migration ± SEM. * $P < 0.05$ compared to vehicle-treated control group. (C) Anti-invasive effect of crizotinib on MDA-MB-231 cells using Trevigen Cultrex BME cell invasion assay. Bars represent average percentage ± SEM of invading cells out of 4 replicates for each treatment group. * $P < 0.05$ compared to vehicle-treated control group.

Abbreviation: BME, basement membrane extract.

to ligand exposure.^{21–23} In this study, the luminal MCF-7 cells were most sensitive to crizotinib anti-proliferative effects, followed by HER2-enriched SK-BR-3 cells and the triple-negative MDA-MB-231 cells. Inhibition of MDA-MB-231 and SK-BR-3 cells was observed at a higher range of micromolar concentrations compared to MCF-7 cells. It is noteworthy to mention that the breast cancer cell lines used in this study have, in addition to different molecular profiles, different doubling times, expression of other growth factor receptors, and the potential cross-talk between different growth factor signaling pathways. Altogether, it is not unexpected that different breast cancer cells would respond to different concentrations of crizotinib. Results in this study revealed that crizotinib treatment reduced the expression of the proliferation marker Ki-67 at concentrations that were effective to inhibit the growth of breast cancer cells. Ki-67 is a nuclear protein detectable in nuclei of proliferating cells in all phases of cell cycle and correlated with growth fraction of any given cell population.^{51,52} Crizotinib reduced the percentage

of Ki-67-positive cells most notably in MCF-7 treated cells compared to both MDA-MB-231 and SK-BR-3 cells. This could explain, in-part, the greater sensitivity of MCF-7 cells to growth inhibition of crizotinib. However, reduced viability of breast cancer cells could also be mediated by the ability of crizotinib to modulate other growth signaling pathways that were not investigated in this study. Megiorni et al⁵³ indicated that crizotinib inhibition of rhabdomyosarcoma cell proliferation and survival was mediated by MET- and ALK-independent mechanisms involving induction of autophagy and accumulation of reactive oxygen radicals.

Chemotherapy clearly remains a cornerstone of breast cancer management in different stages of the disease.⁸ Among chemotherapeutic agents available for the treatment of breast cancer, anthracycline- and taxane-based regimens proved to be the most powerful systemic treatment.^{54,55} However, administration of such treatments could be limited by drug toxicity and unfavorable adverse effects. Recently, combination treatment of chemotherapy with novel targeted therapy

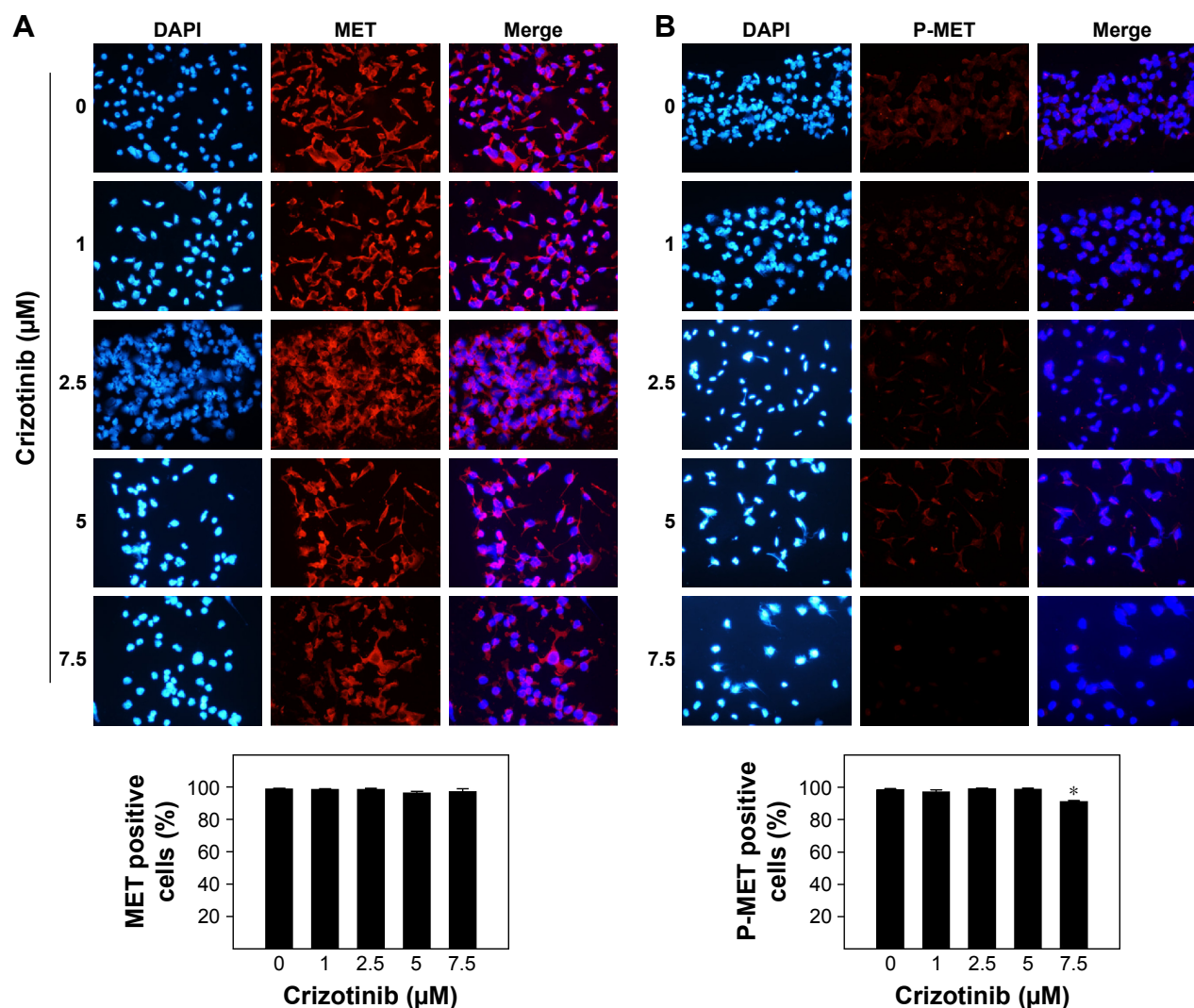


Figure 7 Effect of crizotinib treatment on total levels of MET and phospho-MET (P-MET) in breast cancer cells.

Notes: Effect of crizotinib treatment on levels of (A) MET and (B) P-MET in MDA-MB-231 cells. Upper panel: immunofluorescent staining for (A) MET and (B) P-MET in MDA-MB-231 cells. Red color in the photomicrographs indicates positive fluorescence staining for (A) MET and (B) P-MET, while blue color represents counterstaining of cell nuclei with DAPI. Magnification of each image is 20×. Bottom panel: percentage of cancer cells with positive staining for (A) MET and (B) P-MET in proportion to the total number of cells. Vertical bars represent percentage of cells with positive staining \pm SEM in each treatment group. * $P < 0.05$ compared to vehicle-treated control group.

became a common practice to overcome the limitation of conventional combination regimens in breast cancer.⁵⁶ Findings from this study indicated that the combination of crizotinib and the taxane paclitaxel or the anthracycline doxorubicin resulted in a synergistic growth inhibition in each of MDA-MB-231 and MCF-7 cells. Interestingly, combined treatment of crizotinib and chemotherapy in SK-BR-3 cells demonstrated only marginal additive effect and was antagonistic for the combination with paclitaxel. Accordingly, these findings suggest that the effect of combined treatment of crizotinib and chemotherapy could be cell-type specific. Despite the cytotoxic effects observed for crizotinib in breast cancer cells, it is possible that more specific molecular mechanisms are mediating the synergism observed in both MDA-MB-231

and MCF-7 cells. Recently, Krytska et al³² showed that crizotinib synergizes with topotecan and cyclophosphamide in human neuroblastoma-derived cell lines with varying ALK statuses. The synergism was explained in terms of higher caspase-dependent apoptosis in combined treatments compared to chemotherapy alone. Interestingly, a recent study investigating the effect of crizotinib in combination with cisplatin in 4 NSCLC cell lines showed remarkable antagonism among the 4 cell lines discouraging crizotinib and cisplatin combination in NSCLC.⁵⁷ Collectively, the effect of combined crizotinib and chemotherapy in cancer is variable and the level of interaction could be affected by the type of chemotherapeutic agents, type of cancer cells, and possible other factors.

Recent evidence indicated that overexpression of MET is being increasingly related to reducing cancer sensitivity to chemotherapeutic as well as biological treatments and emergence of resistance among different types of tumors.^{24,58–60} In this regard, the combination between chemotherapy and crizotinib could be appealing in terms of improving sensitivity and reducing resistance to chemotherapeutic drug therapy. Zhou et al³⁵ found crizotinib to reverse resistance and enhance cytotoxicity of chemotherapeutic drugs in multidrug-resistant (MDR) breast cancer cells in vitro and in vivo. These effects were attributed to inhibition of P-gp function in cancer cells, particularly ABCB1.³⁵ Taking into consideration the fact that paclitaxel and doxorubicin are substrates for multiple ABCB1/P-gp transporters potentially expressed by MDA-MB-231 and MCF-7 cells,^{61–64} it is possible that the synergistic effect for combined treatment is mediated, at least in-part, by inhibiting ABCB1 transporters allowing efficient accumulation of chemotherapy within cancer cells. Moreover, the toxicity profile of MET inhibitors is completely different from that of standard chemotherapy further encouraging evaluation of combined crizotinib and chemotherapeutic treatments.²⁵ Overall, crizotinib had a tolerable adverse effect profile, and most events were mild to moderate in severity in clinical studies.^{29,65}

Activation of MET is known to promote cancer cell scattering, migration, and invasion.⁶⁶ The basal-like MDA-MB-231 breast cancer cells are known to demonstrate invasive phenotype in vitro.³⁶ Findings from this study showed that crizotinib treatment significantly inhibited migration and invasion of MDA-MB-231 cells in a dose-dependent manner. Immunofluorescent staining for active MET (phospho-MET) was suppressed in MDA-MB-231 cells treated with crizotinib compared to vehicle-control group. Reduced MET activation may explain the anti-proliferative as well as the anti-migratory and anti-invasive effects of crizotinib in triple-negative breast cancer subtype.

To the best of our knowledge, this is the first study to characterize the anti-cancer effects of crizotinib in breast cancer cells. Crizotinib inhibited growth of hormone-dependent, HER2-enriched, and triple-negative breast cancer cells in vitro. The anti-proliferative effects could be explained in terms of reduced expression of Ki-67 in breast cancer cells. Combined treatment of crizotinib and chemotherapy resulted in synergistic growth inhibition in luminal and triple-negative breast cancer subtypes. In triple-negative breast cancer, crizotinib treatment reduced MET receptor activation resulting in inhibition of cell migration and invasion. Collectively, these findings provide insights into the potential activity of

crizotinib in tumors not known to have the classical ALK rearrangement. This study encourages further investigations for alternative combination treatments of crizotinib with other chemotherapeutic or targeted therapy in breast cancer analyzing potential molecular targets.

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Disclosure

The authors report no conflicts of interest in this work.

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