Small-Molecule Inhibitor BMS-777607 Induces Breast Cancer Cell Polyploidy with Increased Resistance to Cytotoxic Chemotherapy Agents

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Abstract

The RON receptor tyrosine kinase is a therapeutic target for cancer treatment. Here, we report therapeutic effect and phenotypic change of breast cancer cells in response to BMS-777607, a RON tyrosine kinase inhibitor. Treatment of breast cancer cells with BMS-777607 at therapeutic doses inhibited cancerous clonogenic growth but had only minimal effect on cell apoptosis. Significantly, BMS-777607 induced extensive polyploidy with multiple sets of chromosomes in cancer cells. This effect is independent of RON expression. Knockdown of RON in T-47D and ZR-75-1 cells by specific siRNA did not prevent polyploid formation. Immunofluorescent analysis of α-tubulin and γ-tubulin expression in polyploid cells revealed that BMS-777607 disrupts bipolar spindle formation and causes multipolar-like microtubule assembly. Also, both metaphase equatorial alignment and chromosomal segregation were absent in polyploid cells. These results suggest that cellular mitosis arrests at prophase/pro-metaphase and fails to undergo cytokinesis. By analyzing kinase-inhibitory profiles, aurora kinase B was identified as the target molecule inhibited by BMS-777607. In BMS-777607–treated cells, aurora kinase B was inhibited followed by protein degradation. Moreover, BMS-777607 inhibited Ser10 phosphorylation of histone H3, a substrate of aurora kinase B. Chemosensitivity analysis indicated the resistance of polyploid cells toward chemotherapeutics. Treatment with doxorubicin, bleomycin, methotrexate, and paclitaxel significantly increased cellular IC50 values. These findings highlight the theory that BMS-777607 acts as a multikinase inhibitor at therapeutic doses and is capable of inducing polyploidy by inhibiting aurora kinase B. Increased resistance of polyploid cells to cytotoxic chemotherapeutics could have a negative impact on targeted cancer therapy using BMS-777607. Mol Cancer Ther; 12(5); 725–36. ©2013 AACR.

Introduction

The RON receptor tyrosine kinase belongs to the MET proto-oncogene family (1), which has been implicated in epithelial tumorigenesis and malignancy (2–4). Overexpression of RON, accompanied by generation of various constitutive RON variants, occurs in different types of tumors including colon, breast, and pancreatic cancers (5, 6). Moreover, activated RON transduces signals that regulate tumorigenic activities including cell growth, migration, survival, and invasion of extracellular matrixes (7). These activities facilitate malignant progression, which is characterized as epithelial-to-mesenchymal transition (EMT; ref. 8). Accumulated evidence also indicates that RON signaling is integrated into the cellular signaling machinery that is essential for cancer cell growth and survival (9–11). In colon and breast cancer cells, knockdown of RON expression by specific siRNA significantly attenuates tumor cell growth and increases apoptotic cell death (10–12). These observations provide the rationale for targeting RON as a potential cancer therapy (13, 14). At present, inhibition of RON by therapeutic monoclonal antibodies (mAb) and small-molecule inhibitors (SMI) has been studied in preclinical models (10, 15–17). Partial inhibition of tumor growth in animal xenograft models has been observed (10, 15–17). Further inhibition is achieved by RON-targeted mAb or SMI in combination with chemotherapeutic agents (15). In light of these findings, we propose that combinational therapy is the optimal strategy for RON-targeted cancer treatment to achieve maximal antitumor activity.

SMIs that specifically block RON are under intensive investigation (17, 18). Because of similarities with other tyrosine kinases such as MET, SMIs specific only to RON...
have not been reported. SMIs including PHA665752 (19), Compound-I (17), and BMS-777607 (18) have been found to inhibit RON-mediated tumorigenic activity using in vitro and in vivo models, although the major focus is on inhibition of MET signaling. BMS-777607 is a highly selective inhibitor of the MET kinase family (18). The primarily targeted tyrosine kinases as determined by in vitro enzymatic activities are RON (IC50: 1.8 nmol/L), MET (IC50: 3.9 nmol/L), Tyro-3 (IC50: 4.3 nmol/L), and Axl (1.1 nmol/L; ref. 18). Compared with other SMIs, BMS-777607 is the only one that inhibits RON kinase activity with IC50 at 1.8 nmol/L (17–19). However, BMS-777607 at high concentrations also inhibits other protein tyrosine kinases such as Mer (IC50: 14.0 nmol/L), Flt-3 (IC50: 16 nmol/L), Aurora B (IC50: 78 nmol/L), Lck (IC50: 120 nmol/L), and VEGFR2 (IC50: 180 nmol/L; ref. 18). Aberrant expressions of these proteins are known to regulate tumorigenic activities (20, 21).

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The current study focuses on phenotypic changes of breast cancer cells in vitro in response to increased therapeutic doses of BMS-777607. The role of RON in breast cancer pathogenesis and carcinogenesis has been studied extensively through genetic, biochemical, and biologic models (2, 3, 14, 22). Overexpression and constitutive activation of RON are implicated in breast cancer malignancy and drug resistance (2, 3, 14). Thus, the objective of this study is to determine whether targeted RON inhibition by BMS-777607 achieves a therapeutic effect on breast cancer cells. Specifically, we want to determine tumorigenic phenotypes of breast cancer cells in response to increasing concentrations of BMS-777607, which should inhibit not only RON/MET but also other protein tyrosine kinases. Consequently, knowledge of BMS-777607-mediated inhibition of multi-tyrosine kinases should help us not only to improve future therapeutic efficacy of the targeted RON/MET therapy but also to uncover survival or resistant mechanisms of breast cancer cells in response to multi-kinase inhibition.

Materials and Methods

Cell lines and reagents

Breast cancer T-47D, ZR-75-1, and MCF-7 cells were from American Type Culture Collection. Mouse mAb Zt/g4 and rabbit polyclonal IgG antibody R5029 specific to RON were used as previously described (5, 15, 23, 24). Mouse or rabbit IgG antibodies specific to MET, α-tubulin, γ-tubulin, and other signaling proteins were from Cell Signaling. Mouse or rabbit IgG antibodies specific to human aurora kinase B (AURK-B), histone H3 phosphoSer10 residue, and histone H3 were from BD Transduction Laboratories; and PHA665752, compound-I (CP-I), BMS-777607, and R0-3306 were from Selleck Chemicals. Doxorubicin, bleomycin, cisplatin, methotrexate, and paclitaxel were from Fisher Scientific.

Immunoprecipitation and Western blot analysis

These methods were conducted as previously described (23, 24). For immunoprecipitation, cellular proteins (250 μg per sample) were mixed with 1.5 μg/mL anti-RON mAb Zt/g4 coupled to protein G Sepharose beads. Proteins were separated in an 8% SDS-PAGE under reduced conditions. RON or other signaling proteins were detected by Western blotting using R5029 or corresponding antibodies and visualized using enhanced chemiluminescent reagents. The membranes also were reprobed with antibodies to β-actin to ensure equal sample loading.

Assays for chromosome spreading and counting

T-47D or ZT-75-1 cells were treated with 5 μmol/L BMS-777607 for 72 hours, suspended in hypotonic solutions, and then preserved with fixative solution (3:1 methanol:acetic acid) according to a previously described method (25). Cells were then dropped onto glass slides and stained with Hema-3 staining solution. Chromosomes were observed under an Olympus BKF71 microscope and photographed with a charge-coupled device (CCD) camera. The number of chromosomes from individual cells was manually counted from individual samples.

Immunofluorescent detection of cellular proteins

This method was conducted as previously described (15). Briefly, cells at 1 × 104 cells per well in a 24-well plate were cultured in RPMI-1640 with 10% FBS and treated with increasing amounts of BMS-777607 for various time periods. To detect AURK-B or histone H3, cells were fixed with cold acetone and incubated with individual specific antibodies followed by goat anti-mouse IgG coupled with fluorescein isothiocyanate (FITC). Normal mouse IgG was used as the control. Cellular immunofluorescence was observed under the Olympus BKF71 microscope equipped with DUS/fluorescent apparatus as previously described (15).

Methods for silencing RON mRNA expression

Synthetic siRNA specific to human RON were acquired from Dharmaco. T-47D or ZT-75-1 cells were cultured overnight and then transfected with 1 nmol/L of scrambled or RON-specific siRNA according to the manufacturer’s instructions (Dharmaco). After incubation for 24 or 48 hours, cells were washed and then processed for

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Western blot analysis or treated with BMS-777607 for induction of polyploidy.

Assays for cell growth, viability, apoptotic death, and DNA content
Cell growth and survival was determined by the clonogenic assay. T-47D or ZR-75-1 cells in the presence or absence of BMS777607 were cultured for 10 days to allow clonogenic growth as previously described (26). The effect of BMS-777607 or other chemotherapeutic agents on cell viability was determined by the MTS assay as previously described (27). The effect of BMS-777607 on apoptotic death of T-47D or ZR-75-1 cells was measured using the Annexin V/propidium iodide labeling method as previously described (28). For measuring cellular DNA contents, cells were treated with BMS-777607 for 3 days, labeled with propidium iodide, and analyzed by flow cytometer as previously described (25).

Statistical analysis
Graphpad Prism 5 software was used for statistical analysis. Results are shown as mean ± SD. The data between control and experimental groups were compared using Student t test. Statistical differences at P < 0.05 were considered significant.

Results
Inhibitory effect of BMS-777607 on breast cancer cells
T-47D, ZR-751, and MCF-7 were selected as the model cell lines due to their variable levels of MET and RON expression as determined by Western blot analysis (Supplementary Fig. S1). We used the clonogenic assay to determine the effect of BMS-777607 on survival and proliferation of breast cancer cells. BMS-777607 inhibited clonogenic growth of T-47D and ZR-75-1 cells in a dose-dependent manner (Fig. 1A and B). In MCF-7 cells that barely express RON and MET, BMS-777607 at 5 μmol/L only slightly affected clonogenic growth (Fig. 1A and B). The apoptotic effect of BMS-777607 on T-47D and ZR-75-1 cells was detected by the Annexin V/propidium iodide labeling method (28). BMS-777607 at 1.0 to 2.5 μmol/L had no cytotoxic effect on 3 of the cell lines tested (data not shown). However, a slight increase in the rate of apoptotic cell death in T-47D (10.3%) and ZR-75-1 cells (9.7%) compared with control MCF-7 cells (2.2%) was observed when 5 μmol/L BMS-777607 was used (Fig. 1C). We further conducted time-lapse photography to observe the effect of BMS-777607 on breast cancer cells. Representative images from T-47D cells treated with 5 μmol/L BMS-777607 are shown in Supplementary Fig. S2. Untreated cells undergoing proliferation served as the control. After BMS-777607 treatment, cell growth inhibition and apoptotic death were observed in a time-dependent manner. These results, together with those in Fig. 1, show that BMS-777607 treatment ranging from 1 to 5 μmol/L has an inhibitory effect on survival and proliferation of breast cancer T-47D and ZR-75-1 cells, which express RON or MET. BMS-777607 also caused apoptotic death in T-47D and ZR-75-1 cells, although this effect was relatively weak.

Induction of polyploidy by BMS-777607 in breast cancer cells
An interesting finding after BMS-777607 treatment was the appearance of polyploid cells (Fig. 2A and B). Polyploidy results from subsequent cycles of DNA replication in the absence of cytokinesis and is associated with mitotic checkpoint defects (29). The minimal amount of BMS-777607 required to induce polyploidy is at a 1 μmol/L for both T-47D and ZR-75-1 cells (Fig. 2A). At this concentration, 14% of T-47D and 11% of ZR-75-1 cells underwent polyploidy. The percentages were significantly higher when BMS-777607 was used at 5.0 μmol/L (78% for T-47D and 82% for ZR-75-1) to 10.0 μmol/L (86% for T-47D and 88% ZR-75-1). The effect of BMS-777607 on polyploidy of MCF-7 cells was relatively weak. Only 36% to 59% of MCF-7 cells were affected when BMS-777607 was used at 5.0 to 10.0 μmol/L, respectively. Time-dependent polyploidy is shown in Fig. 2B. Polyploidy was seen as early as 24 hours after 5.0 μmol/L BMS-777607 treatment. At 72 hours, more than 70% of cells underwent polyploidy in T-47D and ZR-75-1 cells, respectively. Again, the effect of BMS-777607 on MCF-7 cells was relatively weak. Polyploid cells were observed only 48 hours after BMS-777607 administration and prolonged treatment only slightly increased the rate of polyploid cells. Thus, BMS-777607 can induce polyploidy in breast cancer cells in both dose- and time-dependent manners and the polyploid cells most likely undergo into a premature senescence phenotype.

To determine whether RON expression is required for BMS-777607-induced polyploidy, we compared the effect of compared BMS-777607 with CP-I and PHA665752 on polyploidy induction. PHA665752 and CP-I both inhibit MET/RON with enzymatic IC50 values at 9/68 and 4/9 nmol/L, respectively (17, 19). After treatment, polyploidy was observed only in BMS-777607-treated cells (Supplementary Fig. S3). Both PHA665752 and CP-I had no effect on induction of polyploidy. We further used specific siRNA to knockdown RON expression in T-47D and ZR-75-1 cells (Fig. 2C). Knockdown of RON expression had no preventive effect on T-47D cell polyploid formation after BMS-777607 treatment (Fig. 2D). The rates of polyploidy T-47D cells (79.4%) were comparable with those from control (83.5%) or scramble RNA–treated (81.1%) cells. Similar results were observed when ZR-75-1 cells were used (data not shown). These results indicate that RON expression is not required for BMS-777607–induced breast cancer cell polyploidy.

To study the fate of polyploid cells, polyploid T-47D cells induced by 5 μmol/L BMS-777607 for 72 hours were collected, cultured in RPMI-1640 with 5%FBS, and observed for up to 10 days (Fig. 2E). These cells retained their polyploid appearance and phenotype without BMS-777607. Moreover, polyploid cells did not undergo obvious proliferation or death during this period. Similar
results were also obtained with polyploid ZR-75-1 cells. These results indicate that polyploid cells induced by BMS-777607 are alive and their polyploid phenotypes are irreversible during the 10-day period of culture. Thus, BMS-777607–induced polyploidy has a long-lasting effect on breast cancer cells.

**Chromosome features of BMS-777607–induced polyploidy in breast cancer cells**

We first used the flow cytometric method to analyze DNA contents in BMS-777607-treated breast cancer cells. Results in Fig. 3A show the DNA contents at different time intervals in MCF-7, T-47D, and ZR-75-1 cells treated with 5 μmol/L BMS-777607. The percentages of polyploid cells with 8N and 16N chromosome contents are shown in Supplementary Table S1. Treatment of T-47D and ZR-75-1 cells with BMS-777607 for 24 hours produced a population dominated by cells with 8N chromosome contents (26.0% for T-47D cells and 14.0% for ZR-75-1 cells). A small population of cells with 16N chromosome contents also emerged in both cell lines. After treatment for 48 hours, the population featured by 16N was significantly
increased in T-47D (from 10.0% to 18.5%) and ZR-75-1 cells (from 5.0% to 32.2%). The percentages of cells with 8N chromosome contents were relatively stable from 48 to 72 hours (T-47D: from 23.0% to 21.0%; ZR-75-1: from 20.0% to 20.0%). The response of MCF-7 cells to BMS-777607 was different. Treatment of cells for 24 hours only caused a small fraction of polyploid cells with 8N chromosome contents (14.0%). However, the percentage of cells with 8N chromosome contents increased to 25.5% after incubation for 48 hours. This population of cells increased slightly (29%) 72 hours after stimulation. Interestingly, BMS-777607 failed to induce a cell population with 16N chromosome contents (14.0%). The decrease of cells with 8N chromosome contents increased to 25.5% after incubation for 48 hours. This population of cells increased slightly (29%) 72 hours after stimulation. Interestingly, BMS-777607 failed to induce a cell population with 16N chromosome contents in MCF-7 cells. Currently, we do not know the different responses of MCF-7 and T-47D/ZR-75-1 cells to BMS-777607. Chromosome count in metaphase spreads confirmed the presence and percentages of T-47D or ZR-75-1 cells with 8N and 16N chromosome numbers (Fig. 3B). However, MCF-7 cells with 16N chromosome numbers were not observed. Thus, results in Fig. 3 show that BMS-777607 at a relatively high concentration induces a dominant population of breast cancer polyploid cells with 8N or 16N chromosome contents.

**Figure 2.** Induction by BMS-777607 of polyploidy in breast cancer cells. A, BMS-777607 induces polyploidy in breast cancer cells in a dose-dependent manner. Cells (5,000 cells per well) were incubated at 37°C in RPMI-1640 with 5% FBS and treated with different amounts of BMS-777607 for 72 hours. Polyploid cells were photographed as described in Fig. 1A. B, time-dependent induction of polyploidy by BMS-777607. Cells were treated with 5 μmol/L BMS-777607 for various time intervals. Stained cells were photographed. C, knockdown of RON expression in breast cancer cells by specific siRNA. Cells (2 × 10⁵ cells in a 60-mm diameter culture dish) were transiently transfected with 1 μmol/L scramble or RON-specific siRNA for 48 hours. Cell lysates (50 μg per sample) were subjected to Western blot analysis using rabbit anti-RON IgG antibody. D, effect of RON expression on BMS-777607-induced polyploidy. T-47D cells were transiently transfected with scramble or RON-specific siRNA for 48 hours as described in C and then treated with 5 μmol/L BMS-777607 for 72 hours. Polyploidy was monitored and photographed. E, fate of polyploid cells in culture. Polyploid T-47D cells were isolated, washed, and then cultured in BMS-777607-free Dulbecco’s Modified Eagle Media with 10% FBS for 10 days. Cells were observed and photographed at various times. Results shown here are from 1 of 2 experiments with similar results.

**Disruptive effect of BMS-777607 on cellular mitotic spindle assembly and function**

Results from above studies indicate a delayed progression of cellular mitosis in the presence of BMS-777607, which strongly suggests that microtubule dynamics are affected. We therefore conducted immunofluorescent staining of T-47D cells to find any abnormalities of α-tubulin and γ-tubulin in spindle assembly (Fig. 4A). The untreated control cells exhibited well-formed mitotic spindles with chromosomes aligned at the equatorial plate. Both α-tubulin and γ-tubulin were shown in a bipolar spindle pattern associated with condensed DNA. However, in T-47D cells treated with BMS-777607, the mitoses were severely disorganized, which is marked by
an absence of functional bipolar spindle assembly, highly condensed/irregular-shaped chromosomes, and a complete lack of both metaphase equatorial alignment and chromosomal segregation. This effect was observed as early as 24 hours after BMS-777607 treatment. Moreover, we observed the formation of multipolar spindles, which were maintained up to 72 hours after BMS-777607 treatment. These effects caused cell-cycle arrest in a pro-metaphase-like state with dysfunctional mitotic spindle. Under such conditions, cells underwent polyploidy and failed to advance into endoreduplication cycle. Similar results also were observed in ZR-75-1 and MCF-7 cells (data not shown). Thus, BMS-777607 treatment has a disruptive effect on microtubule dynamics and impairs the functional mitotic spindle assembly.

Next, we determined whether removal of BMS-777607 relieves and facilitates cell cycle progress. T-47D cells were treated with 5 μmol/L BMS-777607 for 24 hours to induce a pro-metaphase arrest. Cells were then allowed to enter cell cycle by removing the inhibitor. The assembly of mitotic spindles was monitored by immunofluorescent staining of α-tubulin and γ-tubulin. As shown in Fig. 4B, T-47D cells entered into cell cycle rapidly after removal of R0-3306. Within 60 minutes after removal of the inhibitor, the T-47D cells began to form bipolar mitotic spindles and division was completed after 120 minutes. However, the removal of BMS-777607 failed to relieve and to facilitate T-47D cells entering into telophase and cytokinesis (Fig. 4C). These cells were arrested at metaphase with disorganized multipolar mitotic spindles. Observation for up to 3 days revealed the persistent presence of multipolar mitotic spindles in these polyploid cells (data not shown). These results are consistent with those described in Fig. 2E, showing that BMS-777607–treated cells maintained the polyploid phenotype for up to 10 days. Thus, BMS-777607 has a disruptive effect on the assembly of mitotic spindles, displays a long-lasting disruptive effect on cell cycles, and causes cells to arrest at the metaphase-like state.

**Inhibitory effects of BMS-777607 on aurora kinase B and histone H3 phosphorylation**

Specific inhibitors of tubulins only cause prolonged metaphase arrest and rarely induce polyploidy (29). The
induction of prominent polyploidy in breast cancer cells indicates that BMS-777607 inhibits additional signaling proteins essential for polyploid formation. To this end, we conducted immunofluorescent staining to determine AURK-B expression and localization in BMS-777607–treated T-47D cells according to the kinase profiling of BMS-777607 (ref. 18; Fig. 5A). We also studied the Ser10 phosphorylation of histone H3 (Fig. 5B), which is known to be a substrate of AURK-B (30, 31). In control T-47D cells, AURK-B was localized with chromosomes in the prophase and metaphase stages and also was observed in cytokinesis between the 2 dividing cells (Fig. 5A, top). In contrast, AURK-B expression and localization in BMS-777607–treated cells were disorganized, showing a multipolar-like expression pattern. AURK-B was associated only with condensed DNA in such expression pattern, which was detected as early as 24 hours following treatment with BMS-777607 (Fig. 5A). The disorganized AURK-B also was observed 48 and 72 hours after BMS-777607 treatment. However, the amount of AURK-B in BMS-777607–treated cells detected by immunofluorescent analysis was progressively reduced in a time-dependent manner. Another interesting finding was that AURK-B was not detected in prominent polyploid cells. Analysis of more than 100 polyploid cells without condensed DNA found no positive staining of AURK-B. Consistent with these observations, results from Western blot analysis (Fig. 5C) show that BMS-777607 treatment results in reduced AURK-B expression in a time-dependent manner in T-47D and ZR-75-1 cells. However, this effect was not observed in MCF-7 cells. Ser10 phosphorylation of histone H3 was detected by immunofluorescence staining in mitotic condensed chromosomes in control T-47D cells (Fig. 5B). At early stages of BMS-777607 treatment (24 hours), we detected Ser10 phosphorylation in histone H3, which was associated with condensed DNAs but not with chromosomes in polyploid cells. However,
after incubation of cells for 48 to 72 hours, Ser10 phosphorylation was progressively reduced in BMS-777607–treated cells (Fig. 5B). This observation was further confirmed by Western blot analysis showing that Ser10 phosphorylation in histone H3 was progressively diminished in a time-dependent manner (Fig. 5C). Nevertheless, the reduction was not associated with histone H3 protein expression. We used proteasome inhibitor lactacystin to determine whether BMS-777607–induced reduction of AURK-B is mediated by intracellular protein degradation. As shown in Fig. 5D, lactacystin effectively blocked BMS-777607–induced reduction of AURK-B in both T-47D and ZR-75-1 cells, which indicates that the decrease of AURK-B after BMS-777607 treatment is caused by proteasome-mediated protein degradation. Taken together, results in Fig. 5 show that BMS-777607 inhibits AURK-B function and induces its protein degradation. BMS-777607 also suppressed AURK-B–mediated Ser10 phosphorylation of histone H3.

Increased resistance of BMS-777607–induced polyploid cells in response to cytotoxic chemotherapeutics

To determine sensitivity of polyploid cells to cytotoxic chemotherapeutics, polyploid cells were collected from BMS-777607–treated T-47D and ZR-75-1 cells and then treated for 72 hours with various amounts of chemotherapeutic agents including doxorubicin, bleomycin, cisplatin, paclitaxel, and methotrexate. Analysis of cell viability indicated that polyploid T-47D and ZR-75-1 cells developed a reduced sensitivity in response to cytotoxic chemotherapeutics compared with those of control cells (Fig. 6). The IC50 values of polyploid T-47D cells and ZR-75-1 cells significantly increased in response to doxorubicin, bleomycin, paclitaxel, and methotrexate when compared with those of control T-47D or Z-75-1 cells (Supplementary Table S2). The only chemotherapy agent to which polyploid cells did not show a significant increase in IC50 values is cisplatin. Thus, BMS-777607–induced polyploid cells developed cellular resistance to chemoagent-induced cellular cytotoxicity.
Doxorubicin conc (nmol/L) Bleomycin conc (nmol/L) Cisplatin conc (nmol/L) MTX conc (nmol/L) Paclitaxel (nmol/L)

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Figure 6. Cytotoxic effects of different chemotherapy agents on BMS-777607–induced breast cancer polyploid cells. T-47D and ZR-75-1 cells (3 × 10^5 cells in 100-mm diameter culture dishes) were treated with 5 μmol/L BMS-777607 for 72 hours. Polyploid cells were collected, washed, and cultured at 5,000 cells per well in a 96-well culture plate. Regular T-47D and ZR-75-1 cells (5,000 cells/well) without BMS-777607 treatment were used as the control. Cells were treated for 72 hours with different amounts of doxorubicin, bleomycin, cisplatin, paclitaxel, or methotrexate in triplicate followed by the MTS assay to determine cell viability (27). The IC_{50} values from individual drugs were calculated as previously described (27). The calculated IC_{50} values of bleomycin for treated for 72 hours with different amounts of doxorubicin, bleomycin, cisplatin, paclitaxel, or methotrexate in triplicate followed by the MTS assay to
cells per well in a 96-well culture plate. Regular T-47D and ZR-75-1 cells (5,000 cells/well) without BMS-777607 treatment were used as the control. Cells were

Discussion
The major finding in this study is the induction of polyploidy by BMS-777607 in breast cancer cells, which results in increased resistance to cytotoxic chemotherapeutics. Preclinical studies have shown that RON is overexpressed and activated in breast cancer cells, which facilitates malignant tumor progression (13). Here, we determined the therapeutic effect of BMS-777607 on RON-mediated signaling in breast cancer cells. BMS-777607 inhibited growth and survival of T-47D and ZT-75-1 cells in a dose-dependent manner. It also caused apoptosis, although the effect was relatively weak. These activities contribute directly to BMS-777607–mediated inhibition of RON/MET signaling, as BMS-777607 had marginal effect on growth, survival, and apoptosis of MCF-7 cells that barely express RON or MET. To our surprise, BMS-777607 at therapeutic doses caused extensive polyploidy in breast cancer cells. This effect was not related to RON expression because knockdown of RON expression by specific siRNA did not prevent polyploidy formation. In contrast, this effect was directly related to BMS-777607–mediated inhibition of AURK-B, a vital regulator for mitosis (29). Furthermore, we showed that polyploid cells induced by BMS-777607 display a chemoresistant phenotype, which renders tumor cells less sensitive to cytotoxic activities of various chemotherapeutics. Thus, BMS-777607–directed growth inhibition of breast cancer cells is associated with generation of polyploid cells that are resistant to certain chemotherapeutic agents. Such activities could have an impact on therapeutic efficacy of BMS-777607 against tumors in vivo.

Targeted inhibition of RON for potential cancer treatment has achieved significant success in preclinical studies (15–18). We and others have shown that therapeutic antibodies specific to RON, administered repeatedly as a single agent, inhibited tumor growth mediated by colon, breast, and pancreatic cancer cells in mouse tumor xenograft models (15, 16). Similar results were also observed in studies using specific SMIs (10, 17). BMS-777607 is a tyrosine kinase inhibitor targeting RON with the enzymatic IC_{50} of 1.8 nmol/L (18). Studies using BMS-777607 to target MET has shown the effectiveness in MET-mediated tumor cell migration, matrix invasion, and distance metastasis (32, 33). However, the effect of BMS-777607 on RON-mediated tumorigenesis is unknown. We found that BMS-777607 has a growth-inhibitory effect on breast cancer cells expressing RON. However, the ability of BMS-777607 to induce apoptosis is relatively weak. Thus, BMS-777607 has the ability to target breast cells expressing RON for potential therapeutic application.

BMS-777607-induced polyploidy is featured by accumulation of 4N, 8N, and 16N DNA contents with...
corresponding numbers of chromosome in T-47D, ZR-75-1, and MCF-7 cells. The increase of 4N DNA content cells is likely caused by defect in cytokinesis. However, the appearance of 8N and 16N DNA is most likely manifested by other mechanisms. Increased endoreduplication without proper mitosis and/or cytokinesis is a potential cause, which leads to formation of 8N and 16N DNA contents. Endoreduplication, also known as endoreduplication, is a form of cell cycle in which mitosis is skipped and cells repeatedly replicate their DNA, resulting in cell polyploidy with 8N and 16N DNA contents (29). It appears that BMS-777607 has the ability to increase endoreduplication in breast cancer cells.

Induction of breast cancer cell polyploidy by BMS-777607 is manifested by BMS-777607–targeted inhibition of AURK-B. We have shown that PHA665752 and compound-I, both MET / RON-specific inhibitors (17, 19), have no effect on induction of polyploidy. Moreover, knock-down of RON expression by specific siRNA did not prevent BMS-777607–induced polyploidy, which suggests that RON is not involved in BMS-777607–induced polyploidy. We also found that BMS-777607 induces polyploidy in MCF-7 cells, although this activity is relatively weak compared with its effect on T-47D and ZR-75-1 cells. Analysis of BMS-777607–targeted kinase profiles has directly confirmed that BMS-777607 impairs AURK-B activity with IC50 value at 78 nmol/L (18). The fact that BMS-777607 is highly effective in induction of polyploidy in breast cancer T-47D and ZR-75-1 cells suggests that RON/MET-expressing breast cancer cells may be highly sensitive to BMS-777607–targeted inhibition of AURK-B. In this sense, BMS-777607 can be considered as a multiple kinase inhibitor affecting not only RON and MET but also AURK-B activity. Such inhibitory effects constitute a mechanism of action by which BMS-777607 exerts its effect on phenotypes of breast cancer cells.

The evidence that BMS-777607 affects cell cycle and mitosis comes from our analysis of mitotic spindle assembly and function. Using immunofluorescence to analyze α-tubulin and γ-tubulin expression and localization, we discovered that BMS-777607 treatment results in disorganized mitotic spindle assembly in polyploid cells. The bipolar spindle assembly was completely disrupted and replaced with multipolarized mitotic spindles with condensed and disorganized multiple chromosomes. This effect was observed as early as 24 hours and lasted up to 72 hours after BMS-777607 treatment. Another interesting observation is that after removal of BMS-777607, the multipolarized spindles continued to appear in polyploid cells 3 days after BMS-777607 treatment. These results suggest that BMS-777607 affects microtubule dynamics. The effect on polyploidy is long-lasting and difficult to reverse.

We also analyzed the inhibitory effect of BMS-777607 on AURK-B and its substrate histone H3. AURK-B associates with spindle microtubules and is essential during chromosome segregation (31). In addition, AURK-B–mediated phosphorylation of histone H3 at Ser10 residue is crucial for chromosome condensation and cell-cycle progression during mitosis (30). We discovered that after BMS-777607 treatment, the dynamic association of AURK-B with centromeres had disappeared in polyploid cells. Instead, AURK-B is associated with condensed and disorganized chromosomes in prophase/pro-metaphase leading to the multipolarized expression pattern. Such irregular appearance highly resembled to that of mitotic spindle microtubules observed in analysis of α-tubulin and γ-tubulin. Again, the disorganized multipolar patterns of AURK-B were detected as early as 24 hours and then progressively diminished after prolonged BMS-777607 treatment. Consistent with these findings, Western blot analysis showed that BMS-777607 treatment results in decreased AURK-B expression in T-47D and ZR-75-1 cells, which is mediated through an intracellular proteomic degradation mechanism. This conclusion is based on our studies showing that inhibition of proteomic degradation activity by lactacystin effectively prevents BMS-777607–mediated AURK-B reduction. We reason that the interaction of BMS-777607 with AURK-B triggers the intracellular proteomic degradation mechanism, leading to degradation of AURK-B.

Resistance of BMS-777607–induced polyploid cells to various chemotherapeutics indicates the existence of a protective survival mechanism in breast cancer cells. Previous studies have shown that chemotherapy-induced resistance, characterized by senescence and prolonged cell-cycle arrest, is responsible for the survival of cancer cells (34, 35). Results from this study indicate that BMS-777607–induced polyploid cells could use a similar survival mechanism against the cytotoxic effect of chemotherapy agents. As shown in Fig. 6, polyploid T-47D and ZR-75-1 cells both displayed a significant increase in IC50 values against 4 chemotherapeutic agents including doxorubicin, bleomycin, paclitaxel, and methotrexate, when compared with control cells. Because the mechanisms of action differ among these 3 chemoagents, BMS-77767–induced polyploid cells seem to develop a common resistance mechanism, which reduces cellular sensitivity in response to cytotoxic activities of different chemotherapy agents. Such mechanism could protect cancer cells from cytotoxic death and facilitate their survival in a hostile environment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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