Synergistic Activities of MET/RON Inhibitor BMS-777607 and mTOR inhibitor AZD8055 to Polyploid Cells Derived from Pancreatic Cancer and Cancer Stem Cells

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Running Title: Synergism of BMS-777607 and AZD8055 on Polyploid PDAC Cells

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ABSTRACT

Tyrosine kinase inhibitor BMS-777067 is an inhibitor of RON/MET receptor tyrosine kinases currently under clinical trials. Here, we report the synergistic activity of BMS-777607 in combination with mTOR inhibitor AZD8055 in killing chemoresistant pancreatic cancer and cancer stem cells. Treatment of pancreatic cancer L3.6pl cells with BMS-777067 alone inhibited clonogenic growth and moderately induced apoptotic death. However, BMS-777607 caused extensive polyploidy in L3.6pl cells through inhibition of aurora kinase B activity, independent of RON expression. In contrast, L3.6pl-derived cancer stem cells were highly resistant to BMS-777607-induced growth inhibition and apoptosis. The effect of BMS-777607 on induction of cancer stem cell polyploidy also was weak. BMS-777607-induced polyploidy features a predominant cell population with 8N chromosome content in both L3.6pl and cancer stem cells. These cells also showed decreased sensitivity towards chemotherapeutics by increased survival of IC50 values in response to doxorubicin, cisplatin, methotrexate, 5-fluorouracil, and gemcitabine. Among a panel of chemical inhibitors that target different signaling proteins, we found that BMS-777607 in combination with mTOR inhibitor AZD8055 exerted synergistic effects on L3.6pl and cancer stem cells. More than 70% of L3.6pl and cancer stem cells lost their viability when both inhibitors were used. Specifically, BMS-777607 in combination with inhibition of mTORC2 but not mTORC1 was responsible for the observed synergism. Our findings demonstrate that BMS-777607 at therapeutic doses exerts inhibitory activities on pancreatic cancer cells but also induces polyploidy insensitive to chemotherapeutics. Combination of BMS-777607 with AZD8055 achieves the maximal cytotoxic effect on pancreatic cancer and cancer stem cells.
INTRODUCTION

Overexpression of the recepteur d’origine nantais (RON) receptor tyrosine kinase in cancer cells has been implicated in tumorigenic activities and malignant progression (1-4). Aberrant RON expression also has been molecularly targeted in mouse xenograph models of pancreatic ductal adenocarcinoma (PDAC) (5-7). PDAC is a highly malignant disease with limited treatment options (8). Studies using immunohistochemical staining have confirmed that RON is overexpressed in more than 30% of primary PDAC cases (1,5,6). Aberrant RON expression also regulates PDAC cell migration, survival, and invasion through multiple signaling pathways (4-6,9,10). In certain PDAC cell lines, knockdown of RON expression by specific siRNA causes PDAC cell apoptosis and increase their sensitivity in response to chemotherapeutics (4). These preclinical studies indicate that aberrant RON signaling is critical for PDAC cell growth and survival. Moreover, aberrant RON signaling serves as a mechanism for acquired chemoresistance. However, RON expression in correlation with clinico-pathophysiological parameters has not been fully established (11). Thus, further investigation is required to demonstrate the pathogenic role of RON in PDAC malignancy.

Targeted inhibition of RON to treat PDAC is currently under investigation (6,7,12,13). Both tyrosine kinase inhibitors (TKIs) and therapeutic monoclonal antibodies have been tested in preclinical models (7,14). Moreover, anti-RON antibodies have been used as a drug delivery method to improve chemotherapeutic efficacy (13). Currently, several TKIs including PHA665752, Compound-I, and BMS-777607 that target RON signaling have been characterized in preclinical model (4-6,14-16). BMS-777607 is a MET superfamily inhibitor with a high specificity to RON at the enzymatic IC$_{50}$ of 1.8 nM (16). This is the highest inhibitory IC$_{50}$ value observed among various TKIs showing RON-inhibitory activity (14-16). The other targets of BMS-777607 include MET (IC$_{50}$: 3.9 nM), Tyro-3 (IC$_{50}$: 4.3), and Mer (IC$_{50}$: 14.0 nM) (16). Studies from tumor xenograft models have shown that BMS-777607 at 6.25 mg/kg (C$_{max}$ = 4.5...
μM) has a therapeutic effect, which significantly inhibits tumor growth mediated by gastric GTL-16 cancer cells that overexpress MET (16). We have recently observed that BMS-777607 inhibits clonogenic growth and induces apoptosis in breast cancer cells exclusively expressing RON (17). Moreover, BMS-777607 induces extensive polyploidy in breast cancer cells at therapeutic doses by inhibition of auroras kinase B (AUKB) (17). In this sense, BMS-777607 can be considered as a multi-tyrosine kinase inhibitor (16,17). Currently, BMS-777607 is under clinical phase I trials for patients with advanced cancers (http://clinicaltrials.gov; trial identification number: NCT01721148).

The study described here was to determine phenotypic changes of PDAC cells in response to BMS-777607 and to find a strategy to overcome BMS-777607-induced chemoresistance. PDAC L3.6pl cells and their derived cancer stem cells were used as the model. Currently, the effect of BMS-777607 on PDAC and its derived stem cells are unknown. It also is equally unknown how PDAC cells and PDAC stem cells change their tumorigenic behaviors in response to RON-targeted BMS-777607. L3.6pl cells are derived from a metastatic PDAC patient with tumorigenic features resembling to PDAC in vivo (18). Moreover, PDAC stem cells can be isolated from L3.6pl cells (13,19). RON, but not MET, is highly expressed in L3.6pl cells and sustained in L3.6pl-derived cancer stem cells (13). These properties make L3.6pl cells and their derived cancer stem cells a suitable in vitro model for studying the effect of BMS-777607. Knowledge gained from current studies should help to understand survival and resistance mechanisms of PDAC cells in response to molecularly-targeted therapeutics and to find a strategy to improve future RON/MET-targeted therapy.

**MATERIALS AND METHODS**

**Cell lines, antibodies, inhibitors, and chemoagents:** Human pancreatic cancer cell line L3.6pl was provided by Dr. G.E. Gallick (University of Texas M.D. Anderson Cancer Center,
Houston, TX) (18) and authenticated in 2011 by the University of Virginia with DNA profiling, cytogenesis, flow cytometry, and immunocytochemistry. L3.6pl-derived CD24⁺CD44⁺ESA⁺ triple-positive cancer stem cells (designated as CSCs⁺24/44/ESA⁺) were generated by us from L3.6pl spheroids by sequential magnetic cell sorting methods as previously described (13). Human MSP, mouse monoclonal antibody (mAb) Zt/g4, and rabbit polyclonal IgG antibody R5029 to RON were used as previously described (20,21). Mouse or rabbit IgG antibodies specific to phospho-tyrosine (clone PY-100), p85/70S6K, phospho-p85/70S6K (Thr⁴²¹/Ser⁴²⁴), AKT, and phospho-AKT (Ser⁴⁷³) were from Cell Signaling (Danvers, MA). Mouse or rabbit IgG antibodies to AUKB were from BD Transduction Laboratories (Lexington, KY). Chemical inhibitors including BMS-777607, AZD8055, AZD1152, XAV939, GDC-0449, SB216763, wortmannin, PD98059, RAD001, and PP242 were from Selleck Chemicals (Houston, TX). Chemical structures of BMS-7776-7 and AZD8055 have been previously published (16,22). Doxorubicin, cisplatin, gemcitabine, methotrexate, and 5-fluorouracil were from Fisher Scientific (Hanover Park, IL).

Protein phosphorylation, immunoprecipitation, and Western blot analysis: These methods were performed as previously described (21). For inducting RON phosphorylation, cells (3 x 10⁶ cells) were stimulated at 37°C with 1 nM MSP for 15 min. Cellular proteins (250 µg per sample) were mixed with 1.5 µg/ml Zt/g4 coupled to protein G Sepharose beads. Proteins were separated in an 8% of SDS-PAGE under reduced conditions. Phosphorylated RON and other signaling proteins were detected by Western blotting using PY-100, R5029, and other antibodies and visualized using enhanced chemiluminescent reagents (21). Membranes also were reprobed with rabbit IgG antibody to β-actin to ensure equal sample loading.

Methods for silencing RON mRNA expression: Synthetic siRNA specific to human RON was from Dharmacon (Chicago, IL). L3.6pl cells and CSCs⁺24/44/ESA⁺ were cultured overnight and then transfected with 1 nM scrambled or RON-specific siRNA according to the manufacturer's
instructions (Dharmacon, Chicago, IL). After incubation for 48h, cells were processed for
immunoprecipitation followed by Western blot analysis. Transfected cells also were treated with
BMS-777607 for induction of polyploidy followed by drug sensitivity analysis.

**Assays for chromosome spreading and counting:** L3.6pl cells and CSCs^{+24/44/ESA} were
treated with 5 μM BMS-777607 or 1 μM AZD1152 for 72h, suspended in hypotonic solutions,
and then preserved with fixative solution (3:1 methanol: acetic acid) according to a previously
described method (23). Cells were then dropped onto glass slides and stained with Hema-3
staining solution. Chromosomes were observed under an Olympus BK71 microscope and
photographed with a CCD camera. The number of chromosomes from individual cells was
counted from individual samples.

**Immunofluorescent detection of cellular proteins:** Cells at 1x 10^4 cells per well in a 24-well
plate were cultured in DMEM with 10% FBS and treated with various amount of BMS-777607
for various periods. To detect AUKB expression, cells were fixed with cold acetone and
incubated with a specific antibody followed by FITC coupled anti-mouse IgG. Normal mouse IgG
was used as the control. Cellular immunofluorescence was observed under the Olympus BK81
microscope equipped with DUS/fluorescent apparatus as previously described (13).

**Assays for cell growth, viability, apoptotic death, and DNA content:** Cell growth and
survival was determined by the clonogenic assay (24). L3.6pl cells in the presence or absence
of BMS-777607 were cultured for 12 days to allow clonogenic growth as previously described
(24). CSCs^{+24/44/ESA} in stem cell culture media were cultured in an ultra-low adhesion plate
coated with 0.2% agarose to facilitate cell anchoring. After incubation for 18 days, clonogenic
growth was determined. The effect of BMS-777607 or other chemotherapeutic agents on cell
viability was determined by the MTS assay as previously described (13). Cells were treated with
various drugs in a 96-well culture plate in triplicate for 72h followed by the MTS assay (13). The effect of BMS-777607 on apoptotic death of L3.6pl cells and CSCs\(^{+24/44/ESA}\) was determined by treating cells with or without 2.5 \(\mu\)M and 5 \(\mu\)M BMS-777607 for 72 h followed by the Annexin V-propidium iodide-labeling assay (25). For measuring cellular DNA content, L3.6pl and CSCs\(^{+24/44/ESA}\) were treated with or without 5 \(\mu\)M and 20 \(\mu\)M BMS-777607 or 1 \(\mu\)M and 5 \(\mu\)M AZD1152 for 72h, labeled with propidium iodide, and analyzed by flow cytometer as previously described (23).

**Data analysis and statistical significance:** Results of individual drug IC\(_{50}\) values, minimum inhibitory concentrations (MICs), combination indexes, and statistical analysis were calculated using Graphpad Prism 6 software (GraphPad Software, Inc. La Jolla, CA). The MICs for cell viability are defined as the lowest concentration of a drug that reduces cell viability with a statistical difference from the viability of control cells in the absence of drug. Results are shown as mean \(\pm\) SD. The data between control and experimental groups were compared using paired t test. Statistical differences at \(p < 0.05\) were considered significant.

**RESULTS**

**Growth inhibitory and apoptotic effects of BMS-777607 on PDAC L3.6pl cells and CSCs\(^{+24/44/ESA}\):** L3.6pl and CSCs\(^{+24/44/ESA}\) were selected as the model due to their sustained RON but not MET expression (13). We first determined the effect of BMS-777607 on MSP-induced RON phosphorylation, which was inhibited in a dose-dependent manner (Fig. 1A). We then determined the effect of BMS-777607 on cell growth/survival using the clonogenic assay (24). BMS-777607 inhibited L3.6pl clonogenic growth in a dose-dependent manner (Fig. 1B). More than 70% reduction in clonogenic growth was observed when BMS-777607 was used at 5 \(\mu\)M (Fig. 1C). In contrast, CSCs\(^{+24/44/ESA}\) were resistant to the inhibitory effect of 5 \(\mu\)M of BMS-777607. Only a slight reduction (~20%) was observed.
The apoptotic effect of BMS-777607 on L3.6pl and CSCs\textsuperscript{+24/44/ESA} was detected by the Annexin V-propidium iodide-labeling method (23). BMS-777607 at 1 \( \mu \)M had no cytotoxic effect on L3.6pl or CSCs\textsuperscript{+24/44/ESA} (data not shown). However, we observed an increase of apoptosis in L3.6pl cells from 13.7\% to 24.3\% compared to control L3.6pl cells (less than 1\%) when BMS-777607 was increased from 5 \( \mu \)M to 10 \( \mu \)M, respectively (Fig. 1D). However, a statistical significance was only observed in L3.6pl cells treated with 10 \( \mu \)M BMS-777607. CSCs\textsuperscript{+24/44/ESA} were resistant to BMS-777607-induced apoptotic cell death. Only a slight increase in the rate of apoptotic cells (4\% at 5 \( \mu \)M and 8.5\% at 10 \( \mu \)M) compared to control cells (2.4\%) was observed when the concentration of BMS-777607 was increased from 5 \( \mu \)M to 10 \( \mu \)M. The differences were not statistically significant. Thus, results in Fig. 1 demonstrate that BMS-777607 inhibited RON phosphorylation in a dose-dependent manner. L3.6pl cells are relatively sensitive to BMS-777607-induced inhibition of clonogenic growth and apoptosis. However, L3.6pl cell-derived CSCs\textsuperscript{+24/44/ESA} were resistant to BMS-777607.

**Induction of polyploidy by BMS-777607 in L3.6pl cells and CSCs\textsuperscript{+24/44/ESA}**: An interesting observation upon BMS-777607 treatment was the appearance of polyploid cells from L3.6pl and CSCs\textsuperscript{+24/44/ESA} (Fig. 2). L3.6pl cell-derived polyploid cells were observed as early as 24h after addition of BMS-777607. However, polyploidy from CSCs\textsuperscript{+24/44/ESA} were not found until 48h after BMS-777607 treatment (Fig. 2A). For L3.6pl cells, the minimal amount of BMS-777607 required to induce polyploidy was about 1 \( \mu \)M (Fig. 2B and 2C). At this concentration, 23\% of cells were shown having polyploidy. The percentage rose when BMS-777607 was used at 5.0 \( \mu \)M (84\%). Further increases in BMS-777607 concentrations did not significantly increase the percentages of polyploid cells (10 \( \mu \)M: 91\% and 20 \( \mu \)M: 86.7\%). Thus, the maximal polyploidy-inducing concentration is at about 5 \( \mu \)M. Similar results also were observed when PDAC Panc-1 and BxPC-3 cell lines were used (supplementary Fig. 1A). The polyploid effect of BMS-777607 on
CSCs^+24/44/ESA was relatively weak. Only 17.4% of CSCs^+24/44/ESA showed polyploidy when BMS-777607 was used at 5.0 μM. The significant increase in the rate of polyploidy was observed only when CSCs^+24/44/ESA were treated with 10 and 20 μM BMS-777607 (polyploidy at 42.4% and 49%, respectively).

We used a specific AUKB inhibitor AZD1152 (IC₅₀: 0.37 nM) (26) as the control for BMS-777607-induced polyploidy (Fig. 2B, lower panel). L3.6pl polyploid cells were readily observed 72h after treatment with 0.05 μM AZD1152 (38.3%). More than 90% of cells underwent polyploidy when L3.6pl cells were treated with 0.5 μM AZD1152. In contrast, polyploidy was observed only when CSCs^+24/44/ESA were treated with 0.5 μM AZD1152. Further increase in AZD1152 concentrations resulted in significant numbers of cell death instead of polyploidy. To verify these results, we performed immunofluorescent analysis to determine expression and localization of AUKB in BMS-777607-treated L3.6pl cells (Fig. 2D). AUKB was expressed and localized with condensed chromosomes in a bi-polar fashion in metaphase of control L3.6pl cells. However, in AZD1152-induced L3.6pl polyploid cells, AUKB expression/localization were disorganized, showing a multi-polar-like expression pattern, which was associated with disorganized and condensed DNA. This expression/localization pattern also was observed in L3.6pl polyploid cells induced by BMS-777607. Thus, results in Fig. 2 demonstrate that BMS-777607 induces PDAC cell polyploidy in a dose-dependent manner. Pancreatic CSCs^+24/44/ESA were insensitive to the polyploidy-inducing effect of BMS-777607. The induction of polyploidy by BMS-777607 is mediated by inhibition of AUKB.

To determine if RON is involved in polyploidy, we used specific siRNA to knockdown RON expression followed by BMS-777607 treatment. Results in Supplementary Fig. 2A show the effectiveness of specific siRNA in silencing RON expression. However, by checking polyploidy, knockdown of RON expression had no effect on the formation of L3.6pl polyploid cells after
BMS-777607 treatment (Supplementary Fig. 2B, top panel). The percentage of polyploid L3.6pl cells (80%) was comparable to that of control cells (82%) or scramble RNA-treated cells (84%). We observed similar results when CSCs^{+24/44/ESA} were used (Supplementary Fig. 2B, bottom panel). In this case, polyploid cells were formed after 10 μM BMS-777607 treatment and knockdown of RON expression had no effect on development of polyploidy by CSCs^{+24/44/ESA}. These results demonstrate that RON expression is not required for BMS-777607-induced polyploidy by L3.6pl cells and CSCs^{+24/44/ESA}.

We used the flow-cytometric method to analyze cell cycle and DNA content in BMS-777607-treated L3.6pl cells and CSCs^{+24/44/ESA}. Results in supplementary Fig. 3A indicate a dramatic change in cell cycle, showing a decrease in G0/G1 phase, a relatively stable and/or increased G2/M phase, and appearance of 8N DNA content in L3.6pl cells and CSCs^{+24/44/ESA} treated with 5 and 10 μM BMS-777607 or 1 and 5 μM AZD1152. Treatment of L3.6pl cells with BMS-777607 and AZD1152 produced a population dominated by cells with 8N chromosome content. Chromosome count in metaphase spreads confirmed the presence of L3.6pl polyploid cells with 8N chromosome numbers (Supplementary Fig. 3B). The percentage changes in DNA content from 2N, 4N, and 8N are shown in supplementary Table 1. After treatment with 5 μM BMS-777607, cells with 8N chromosome content (67.3%) appeared as the predominant population. The response of CSCs^{+24/44/ESA} to BMS-777607 was different. Treatment of CSCs^{+24/44/ESA} with 5 μM BMS-777607 caused only about 10% of 8N chromosome content (Supplementary Table 1). However, the percentage increased up to 22.3% when CSCs^{+24/44/ESA} were treated with 20 μM BMS-777607.

The polyploid effect of AZD1152 on L3.6pl cells was strong. AZD1152 at 1 μM was sufficient to induce 64% of 8N chromosome content. Chromosome count also confirmed the presence of 8N chromosome numbers (Supplementary Fig. 3B). However, CSCs^{+24/44/ESA} were
relatively resistant to AZD1152. Treatment of 1 μM AZD1152 induced only 19.6% of 8N chromosome content, which is significantly lower than that observed by the same treatment in L3.6pl cells. Nevertheless, an increase of AZD1152 up to 5 μM further increased the percentage of 8N chromosome content in CSCs^{24/44/ESA}. Thus, results in Supplementary Fig. 3 and Supplementary Table 1 demonstrate that BMS-777607 at relatively high concentrations induces a population of L3.6pl polyploid cells with 8N chromosome content. CSCs^{24/44/ESA} were resistant to such effect.

Decreased sensitivity of BMS-777607-induced L3.6pl- and CSCs^{24/44/ESA} polyploid cells in response to chemotherapeutics: The appearance of polyploid cells after BMS-777607 treatment prompted us to determine their sensitivity in response to cytotoxic chemotherapeutics. Polyploid cells were isolated from BMS-777607-treated L3.6pl cells and CSCs^{24/44/ESA} by flow cytometric methods and cultured for 72h in the presence or absence of various amounts of gemcitabine, cisplatin, methotrexate, doxorubicin, and 5-fluorouracil. Analysis of cell viability revealed that in comparison with parental cells, L3.6pl polyploid cells acquired reduced sensitivity in response to cytotoxic chemotherapeutics (Fig. 3). Individual IC_{50} values of polyploid cells to all five chemoagents increased significantly compared to those of control L3.6pl cells (Supplementary Table 2). The IC_{50} ratios derived from L3.6pl polyploid cells to parent cells were increased at variable levels from 1.60 to 61.67 (Supplementary Table 2). In the case of gemcitabine, the IC_{50} from polyploid cells increased up to nine fold (1.7 ± 0.3 μM) compared to parental L3.6pl cells (0.19 ± 0.04 μM). We also observed similar results when polyploid cells from CSCs^{24/44/ESA} were used. CSCs^{24/44/ESA} were highly resistant to chemoagents and induction of polyploidy rendered these cells even more resistant to cytotoxic therapeutics (Fig. 3B and supplementary Table 2). Results in Supplementary Table 3 compare the MICs for BMS-777607 and individual chemoagents between L3.6pl cells and CSCs^{24/44/ESA}. MICs were increased at variable levels in polyploid L3.6pl and CSCs^{24/44/ESA}.
compared to control L3.6pl and CSCs^{24/44/ESA}, Thus, BMS-777607-induced polyploid cells developed resistance in response to chemotherapeutics.

**Effect of BMS-777607 in combination with different small molecule inhibitors on L3.6pl cell growth and polyploidy:** Decreased chemosensitivity of L3.6pl polyploid cells prompted us to find a pharmaceutical way to increase BMS-777607 efficacy in L3.6pl cells. We first tested BMS-777607 in combination with a panel of chemical inhibitor on the growth of L3.6pl cells. L3.6pl cells were treated with 5 μM BMS-777607 and individual inhibitors alone or BMS-777607 in combination with each inhibitor. Individual inhibitors were used at the concentration that specifically targets their intracellular signaling molecules. Preliminary experiments using 5 μM BMS-777607 with different concentrations of individual inhibitors have found that the selected dose for the combination experiments in **Fig. 4A** achieves the maximal synergistic effect. When used alone, none of the six inhibitors, XAV939 (Wnt inhibitor), GDC-0449 (Hedgehog inhibitor), SB216763 (GSK-3α/β inhibitor), AZD8055 (mTOR inhibitor), wortmannin (PI-3K inhibitor), and PD98059 (MEK inhibitor) significantly decreased the growth of L3.6pl cells as measured by viable cells (**Fig. 4A**). However, BMS-777607 in combination with AZD8055 or PD98059 displayed a synergistic effect on reduction of L3.6pl cell growth. The combination index is 2.0. Viable L3.6pl cells, was also reduced to 33% after BMS-777607 plus PD98059 treatment. The combination index is 1.6. Interestingly, analysis of cell apoptosis showed that BMS-777607 in combination with AZD8055 does not increase the rate of cell death (**Supplementary Fig. 4**). Thus, the viability reduction in cells treated with BMS-777607 plus AZD8055 is caused by synergistic effect that inhibits cell growth. Taken together, results in **Fig. 4A** suggest that BMS-777607 in combination with AZD8055 or PD98059 effectively inhibits L3.6pl cell proliferation.

We further determined the effect of combinational treatment on formation of L3.6pl polyploid cells (**Fig. 4B**). Among viable cells treated with BMS-777607 plus AZD8055, only 4%
showed polyploidy. BMS-777607 in combination with PD98059 also significantly reduced the percentage of polyploid cells. The effect of other inhibitors in combination with BMS-777607 on L3.6pl cell polyploidy was not statistically significant. We also used aurora kinase-B inhibitor AZD1152 in combination with these inhibitors to determine the growth inhibitory and polyploid effect. Consistent with results from BMS-777608 in combination with AZD8055, AZD1152 in combination with AZD8055 caused a significant reduction in L3.6pl cell viability. Moreover, the percentage of polyploid cells in remaining viable cells also was significantly reduced compared to cells treated with AZD1152 alone. We also studies cyclin B and myeloid leukemia cell differentiation protein 1 (MCL1) expression in L3.6pl cells treated with BMS-777607 and AZD8055 (Supplementary Fig. 5). Cyclin B expression was dramatically decreased after 5 μM BMS-777607 treatment and reduced further in the presence of AZD8055 (Supplementary Fig. 5). In contrast, MCL1 expression was no affected by BMS-777607 but induced by AZD8055 (. These results suggest that changes in cyclin B and MCL1 expression occur in BMS-777607 and ZD8055 treated cells. Taken together, results in Fig. 4 demonstrate that BMS-777607 in combination with AZD8055 or PD98059 has a synergistic effect on decreased cell viability and on reduced polyploid cells.

**Synergistic effect of BMS-777607 and mTOR-2 inhibitor on reduction of L3.6pl cell viability:** The findings from Fig. 4 prompted us to determine which mTOR complex (mTORC1 or mTORC2) is involved in the synergistic effect. We first determined the inhibitory effect of AZD8055 (mTOR inhibitor) (27), RAD001 (mTORC1 inhibitor) (28), and PP242 (dual mTORC1/mTORC2 inhibitor) (29) on phosphorylation of their corresponding substrates (Fig. 5A). BMS-777607 treatment had no effect on the protein expression of mTORC1 substrate p85/70S6K or mTORC2 substrate AKT. BMS-777607 also had no effect on phosphorylation of p85/79S6 kinase Thr^{421}/Ser^{424} and AKT Ser^{473}. In contrast, AZD8055, RAD001, and PP242 reduced p85/70S6K protein expression and inhibited phosphorylation of their corresponding substrates.
Upon confirming these results, we tested the synergistic effect of AZD8055, RAD001, and PP242 with BMS-777607 on L3.6pl cell viability (Fig. 5B). BMS-777607 was used at 1 μM, which has a slight effect on reduction of L3.6pl cell viability (<10%). Consistent with the results shown in Fig. 4, AZD8055 in combination with BMS-777607 reduced L3.6pl cell viability in a dose-dependent manner. ADZ8055 at 0.5 μM is sufficient to synergize with BMS-777607 to achieve a 50% reduction in cell viability. In contrast, RAD001 in combination with BMS-777607 did not exert the synergistic effect on L3.6pl cell viability. However, PP242 in combination with BMS-777607 showed a significant synergism in reducing L3.6pl cell viability. Importantly, PP242 at 0.5 μM is sufficient to synergize with BMS-777607 to cause a 50% reduction in L3.6pl cell viability. We also examined the polyploidy in cells treated with different combinations. Percentages of polyploid cells from 1 μM BMS-777607 plus 5 μM AZD8055 or 1 μM PP242 were less than 5% in viable cells. However, the percentage of polyploid cells from BMS-777607 plus 1 μM RAD001 was about 24% in viable cells.

We also confirmed the therapeutic effect of BMS-777607 in combination with mTOR inhibitors on viability of CSCs^24/44/ESA (Fig. 6). In this case, BMS-777607 at 5 μM is required in combination with 1 μM AZD8055 or PP242 to achieve the significant reduction of CSCs^24/44/ESA viability. Again, we found that BMS-777607 in combination with AZD8055 or PP242 decreased the percentages of CSCs^24/44/ESA-derived polyploid cells. Thus, results in Fig. 5 and 6 demonstrate that BMS-777607 in combination with AZD8055 and PP242 is effective in reducing viabilities of L3.6pl cells and CSCs^24/44/ESA. Inhibition of mTORC2 but not mTORC1 is primarily responsible for the observed synergistic effect on cell viability.

**DISCUSSION**

Previous studies have shown that RON is overexpressed and activated in primary PDAC samples and established cell lines (1-6). Here, we determined the therapeutic effect of BMS-
BMS-777607 on RON signaling in L3.6pl cells and CSCs^{24/44/ESA}. Our findings demonstrate that BMS-777607 inhibits growth and survival of L3.6pl cells in a dose-dependent manner. It also causes moderate apoptotic cell death. In contrast, CSCs^{24/44/ESA} were resistant to BMS-777607-induced growth inhibition. CSCs^{24/44/ESA} also are insensitive to BMS-777607-induced apoptosis. An interesting observation is that BMS-777607 at therapeutic doses causes extensive polyploidy in L3.6pl cells. CSCs^{24/44/ESA} also undergo polyploidy, although at a moderate level. The polyploid effect was not related to RON expression. Knockdown of RON expression by specific siRNA did not prevent polyploidy. Instead, this effect was directly related to BMS-777607 inhibition of AUKB, a vital regulator of cellular mitosis (30). We further demonstrate that polyploid cells display a chemoresistant phenotype, which renders PDAC cells less sensitive to various chemotherapeutics. However, BMS-777607 in combination with mTOR inhibitors AZD8055 and PP242 achieve a synergistic effect on reduction of L3.6pl cell viability. Thus, BMS-777607-directed growth inhibition of PDAC cells is associated with generation of polyploid cells, which could have a negative impact on efficacy of BMS-777607. Combinational treatment should achieve the maximal therapeutic activity in MET/RON-targeted therapy against PDAC and their CSCs.

Inhibition of RON has implicated its signaling for PDAC cell growth and survival in preclinical studies [2-6.10]. Various studies have shown that therapeutic antibodies and TKIs specific to RON inhibit tumor growth mediated by pancreatic, colon, and breast cancer cells in mouse tumor xenograft models (7,12,13,31). Studies using BMS-777607 to target MET have shown the effectiveness in inhibiting MET-mediated tumor cell migration, matrix invasion, and distance metastasis (32,33). However, the effect of BMS-777607 on RON-mediated PDAC tumorigenesis is unknown. Using L3.6pl cells and CSCs^{24/44/ESA} as the models, we demonstrate that BMS-777607 exerts a growth inhibitory effect on L3.6pl cells expressing RON. More than 70% of clonogenic growth was inhibited by 5 μM BMS-777607. This dose also causes moderate...
levels of L3.6pl cell apoptosis. These observations are consistent with a previous in vivo study showing that 5 μM BMS-777607 is the minimal dose required to show therapeutic activity against tumor growth (16). We reason that the effect of BMS-777607 is due mainly to moderate dependence of L3.6pl cells on RON signaling for growth and survival. In contrast, the role of RON signaling in CSCs^24/44/ESA growth and survival is minimal, although RON is highly expressed. As shown in Fig. 1, the ability of BMS-777607 to inhibit clonogenic growth and to induce apoptosis in CSCs^24/44/ESA is marginal. Less than 15% of clonogenic growth was inhibited by 5 μM BMS-777607. Thus, BMS-777607 has a therapeutic effect on regular PDAC cells expressing RON, even though its effect on PDAC CSCs is negligible.

An interesting discovery in this study is the BMS-777607 induction of chemoresistant polyploid L3.6pl cells and CSCs^24/44/ESA. In L3.6pl cells, polyploidy was observed as early as 24h after BS-777607 treatment. BMS-777607 at 1 μM is sufficient to induce more than 20% of viable cells undergoing polyploidy. More than 80% of viable cells showed polyploidy after a 72h treatment, suggesting that the polyploid effect of BMS-777607 on L3.6pl cells is significant. In contrast, CSCs^24/44/ESA are insensitive to BMS-777607-induced polyploidy, which developed slowly and required high concentrations of BMS-777607. The polyploid effect is not associated with RON expression but is manifested by BMS-777607-targeted inhibition of AUKB. Knockdown of RON expression by specific siRNA did not prevent BMS-777607-induced polyploidy.

AUKB is a protein that functions in the attachment of the mitotic spindle to the centromere during cellular mitosis (34). Inhibition of AUKB is featured by cellular polyploidy (35). Analysis of BMS-777607-targeted kinase profiles confirms that BMS-777607 impairs AUKB activity with the enzymatic IC_{50} value at 78 nM (16). We observed by immunofluorescent analysis that the dynamic association of AUKB with centromeres had disappeared in polyploid cells after BMS-
treatment. Instead, AUKB is associated with condensed and disorganized chromosomes in prophase/pro-metaphase leading to the multi-polarized expression pattern. The fact that BMS-777607 is highly effective in induction of polyploidy in L3.6pl cells suggests that PDAC cells are highly sensitive to BMS-777607-targeted inhibition of AUKB. Supporting this notion, we also observed extensive polyploidy in BMS-777607-treated PDAC cell lines Pan-1 (RON negative) and BxPC-3 (RON positive) cells. In this sense, BMS-777607 can be considered as a multiple kinase inhibitor affecting not only RON and MET, but also AUKB activity. Clearly, the induction of polyploidy constitutes a mechanism of action by which BMS-777607 exerts its effect on phenotypes of PDAC cells.

Resistance of BMS-777607-induced polyploid cells to chemotherapeutics indicates the existence of a protective and survival mechanism in PDAC cells. Preclinical studies have found that chemotherapy-induced resistance, characterized by senescence and prolonged cell cycle arrest, is responsible for the survival of cancer cells (36,37). Results from this study indicate that BMS-777607-induced PDAC polyploid cells could utilize a similar mechanism against chemotherapeutics. As shown in Fig. 3, L3.6pl polyploid cells displayed a significant increase in IC₅₀ values against doxorubicin, 5-fluorouracil, cisplatin, methotrexate, and gemcitabine when compared to control cells. Since the mechanisms of action differ among these five chemoagents, BMS-77767-induced polyploid cells appear to develop a common resistance mechanism, which reduces cellular sensitivity in response to cytotoxic activities of different chemotherapeutics. Such mechanism could protect cancer cells from cytotoxic death and facilitate their survival in a hostile environment.

Development of chemoresistant polyploid cells prompted us to find a therapeutic means to increase efficacy of BMS-777607. Among a panel of chemical inhibitors screened, we found that BMS-777607 in combination with mTOR inhibitors AZD8055 and PP242 significantly reduced
cell viability and polyploidy by L3.6pl cells. The mTOR pathway regulates various cellular activities, such as survival, motility, and metabolism by integrating signaling from upstream molecules including growth factors and receptor tyrosine kinases (38-40). Currently, two mTOR complexes, namely mTORC1 and mTORC2, have been identified (38-40). mTORC1 functions as a nutrient/energy/redox sensor and controls protein synthesis and mTORC2 acts primarily as a regulator of cell cycle and motility (38-40). By analyzing cell viability and polyploidy, we demonstrate that inhibition of mTOR signaling alone by AZD8055 at 1 μM is not sufficient to significantly reduce L3.6pl cell viability. However, when used in combination, BMS-777607 and AZD8055 showed a synergistic effect not only on decreased cell viability but also on reduced polyploidy. The action of AZD8055 in combination with BMS-777607 is likely to be mediated by its inhibitory effect on both mTOR1 and mTOR2. Using RAD001 and PP242 that differentially inhibit mTORC1 and mTORC2, respectively, we demonstrate that inhibition of mTORC2 but not mTORC1 has synergistic activity with BMS-777607. These results provide a mechanistic explanation of why mTOR1 inhibitor RAD001 is less effective than AZD8055 in combination with BMS-777607 to achieve the synergistic activity.

AUTHORS’ CONTRIBUTIONS:


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REFERENCES


FIGURE LEGENDS

Figure 1: inhibitory and apoptotic effects of BMS-777607 on PDAC L3.6pl and L3.6pl-derived CSCs$^{+24/44/ESA}$. (A) Dose-dependent inhibition of RON phosphorylation by BMS-777607 was performed by stimulation of L3.6pl cells or CSCs$^{+24/44/ESA}$ (3 x10$^6$ cells/sample in 1 ml PBS) with 1 nM MSP in the presence or absence of different amounts of BMS-777607 for 20 min. RON was immunoprecipitated from cell lysates (250 μg/ml) using anti-RON mAb Zt/g4 followed by Western blot analysis using TY-100 specific to phospho-tyrosine as previously described [13].

(B) The inhibitory effect of BMS-777607 on survival and proliferation of L3.6pl and CSCs$^{+24/44/ESA}$ was determined by the clonogenic assay (23). Briefly, L3.6pl cells (6000 cells per well) in MEM...
with 5% FBS were cultured in duplicate in a 24-well plate and then treated with different amounts of BMS-777607 for 12 days. CSCs*24/44/ESA in stem cell culture media were incubated for 18 days in the ultra-low attachment culture plate coated with a thin layer of 0.2% of agarose to facilitate cell anchored growth. Clonogenic cells were stained with Hema-3 staining solution (Fisher Scientific), photographed using an Olympus BK71 microscope equipped with CCD camera, and counted. The number of clonogenic growth from individual groups is presented. (C) Numbers of clonogenic cells from L3.6pl cells and CSCs*24/44/ESA in duplicate were counted. Clonogenic growth from the cell control was set as 100%. (D) Apoptotic cell death was measured by the Annexin V-propidium iodide-labeling method (24). Cells were treated with 5 μM and 10 μM of BMS-777607 for three days, labeled, and then analyzed by flow cytometer as previously described [24]. Results are from one of three experiments with similar results.

Figure 2: Induction by BMS-777607 of polyploidy in L3.6pl cells and CSCs*24/44/ESA. (A) BMS-777607 induces polyploidy in L3.6pl cells and CSCs*24/44/ESA in a time-dependent manner. L3.6pl cells (6000 cells per well in a 96-well plate in MEM with 5% FBS) and CSCs*24/44/ESA (5000 cells per well) in stem cell culture media in an ultra-low adhesion plate were treated with 5 μM BMS-777607. Cells at different time intervals were fixed and stained with Hema-3 staining solution. Polyploid cells were identified and photographed. (B) BMS-777607 induces polyploidy in a dose-dependent manner. Cells were incubated as described in (A) and treated with different amounts of BMS-777607 for 72h. Polyploid cells were photographed as described in (A). AZD1152, an AURK-B inhibitor known to induce polyploidy, was used at different concentrations and served as the control. (C) Percentages of polyploid cells in BMS-777607-treated L3.6pl cells and CSCs*24/44/ESA. Cells from (B) were used to determine the percentages of polyploidy. Three hundred cells were counted from three independent areas. The percentages of polyploid cells were calculated by numbers of polyploid cells divided by total cell numbers. Results from BMS-777607-treated and control cells were compared using the paired t test with $P < 0.05$ as
the significance (*). **(D)** Effect of BMS-777607 on AURK-B expression in L3.6pl cells was analyzed by immunofluorescence using an AURK-B specific antibody. Cells (5000 cells per culture chamber) were treated at 37°C with 5 μM BMS-777607 and 1 μM AZD1152 for 72h, fixed with acetone, and then examined to detect AURK-B expression. Cells stained with DAPI for DNAs were used as the control. Immunofluorescence was observed using the Olympus BK71 microscope equipped with DSU/fluorescence apparatus. Results are from one of three experiments with similar results.

**Figure 3. Sensitivity of L3.6pl- and CSCs+24/44/ESA-derived polyploid cells in response to different cytotoxic chemotherapeutics.** L3.6pl cells (3 x10^6 cells in 100 mm diameter culture dishes) and CSCs+24/44/ESA (2 x10^6 cells with stem cell culture media in 100 mm diameter ultra-low adhesion dishes) were treated with or without 5 μM BMS-777607 for 72h. Polyploid cells were collected, washed, and cultured at 5000 cells per well in a 96-well culture plate. L3.6pl cells and CSCs+24/44/ESA (5000 cells/well, respectively) without BMS-777607 treatment were used as the control. Cells were treated for 72h in triplicate with different amounts of doxorubicin, cisplatin, 5-fluorouracil, methotrexate, and gemcitabine, followed by the MTS assay to determine cell viability (13). (A) Parental L3.6pl and L3.6pl-derived polyploid cells. (B) Parental CSC+24/44/ESA and CSC+24/44/ESA-derived polyploid cells. The IC_{50} values from individual drugs (shown in Supplementary Table 2) were calculated as previously described (13). Results are from one of two experiments with similar results.

**Figure 4. Effect of individual small molecule inhibitors on viability and polyploidy of L3.6pl cells treated with BMS-777607.** L3.6pl cells at 6000 cells/well were incubated in MEM with 5% FBS in triplicate in a 96-well culture plate and then treated alone with 5 μM BMS-777607, 1 μM XAV939, 1 μM AZD8055, 20 nM GDC-0449, 2 μM SB216763, 10 μM wortmannin, and 50 μM PD98059, or with BMS-777607 in combination with individual inhibitors. The dose of
individual compounds used in the figure was selected after testing various concentrations, which gives the maximal synergistic effect with BMS-777607. (A) Cell viability was determined by the MTS assay after incubation of cells for 72h. (B) Polyploidy was examined under BK71 Olympus microscope and photographed 72 h after treatment. Images from cells treated with XAV939, AZD8055, GDC-0449 SB216763, wortmannin, and PD98059 alone were similar to those from control cells and not included. The percentages of polyploid cells were calculated as described in Fig. 2. Results are from one of three experiments with similar results. Statistical significance marked as “*” was determined using the paired t test with \( P < 0.05 \) as the significance.

Figure 5. Synergistic effect of BMS-777607 with mTOR inhibitors in reduction of L3.6pl cell viability. (A) Phosphorylation of mTOR1 substrate p85/70S6K and mTOR2 substrate AKT was determined by Western blot analysis using specific antibodies. Cells (3 x \( 10^6 \) cells per dish) were treated for 120 min at 37°C with 5 \( \mu \)M BMS-777607, 1 \( \mu \)M AZD8055, 1 \( \mu \)M RAD001, and 1 \( \mu \)M PP242, or their different combinations. Cellular proteins (50 \( \mu \)g per sample) were separated in a 12% SDS-PAGE under reduced conditions followed by Western blot analysis. Same membranes were also reprobed with anti-p85/70S6K or AKT antibodies for loading control. (B) L3.6pl cells at 6000 cells/well were incubated in MEM with 5% FBS in triplicate in a 96-well culture plate and treated with different amounts of AZD8055, RAD001, and PP242 alone, or in combination with 1 \( \mu \)M BMS-777607. Cells were cultured for 72h followed by the MTS assay. Cell viability from control cells cultured in MEM alone was set at 100%. The combination index for BMS-77607+5 \( \mu \)M AZD8055, BMS-777607+1 \( \mu \)M RAD001, and BMS-777607+PP242 is at 2.1, 1.1, and 1.6, respectively.

Figure 6. Synergistic effect of BMS-777607 with mTOR inhibitors in reduction of CSCs*24/44/ESA viability. CSCs*24/44/ESA at 5000 cells/well with stem cell culture media in triplicate in an ultra-low adhesion plate were treated with 5 \( \mu \)M BMS-777607, 1 \( \mu \)M AZD8055, 1 \( \mu \)M
RAD001, and 1 μM PP242 alone, or in their different combinations. Cells were cultured for 72h. Cell viability was determined by the MTS assay (A). Cell viability from control cells cultured in MEM alone was set at 100%. Cells were also stained with Hema-3 solution to determine polyploid cells (B). Percentages of polyploid cells were determined by counting 300 cells from two different regions. Results shown here were from one of two experiments with similar results.
Synergistic Activities of MET/RON Inhibitor BMS-777607 and mTOR inhibitor AZD8055 to Polyploid Cells Derived from Pancreatic Cancer and Cancer Stem Cells

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