Pan-TAM Tyrosine Kinase Inhibitor BMS-777607 Enhances Anti–PD-1 mAb Efficacy in a Murine Model of Triple-Negative Breast Cancer

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Abstract

Tyro3, Axl, and Mertk (TAM) represent a family of homologous tyrosine kinase receptors known for their functional role in phosphatidylserine (PS)-dependent clearance of apoptotic cells and also for their immune modulatory functions in the resolution of inflammation. Previous studies in our laboratory have shown that Gas6–PS-mediated activation of TAM receptors on tumor cells leads to subsequent upregulation of PD-L1, defining a putative PS–TAM receptor–PD-L1 inhibitory signaling axis in the cancer microenvironment that may promote tolerance. In this study, we tested combinations of TAM inhibitors and PD-1 mAbs in a syngeneic orthotopic E0771 murine triple-negative breast cancer model, whereby tumor-bearing mice were treated with pan-TAM kinase inhibitor (BMS-777607) or anti–PD-1 alone or in combination. Tyro3, Axl, and Mertk were differentially expressed on multiple cell subtypes in the tumor microenvironment. Although monotherapeutic administration of either pan-TAM kinase inhibitor (BMS-777607) or anti–PD-1 mAb therapy showed partial antitumor activity, combined treatment of BMS-777607 with anti–PD-1 significantly decreased tumor growth and incidence of lung metastasis. Moreover, combined treatment with BMS-777607 and anti–PD-1 showed increased infiltration of immune stimulatory T cells versus either monotherapy treatment alone. RNA NanoString profiling showed enhanced infiltration of antitumor effector T cells and a skewed immunogenic immune profile. Proinflammatory cytokines increased with combinational treatment. Together, these studies indicate that pan-TAM inhibitor BMS-777607 cooperates with anti–PD-1 in a syngeneic mouse model for triple-negative breast cancer and highlights the clinical potential for this combined therapy.

Significance: These findings show that pan-inhibition of TAM receptors in combination with anti–PD-1 may have clinical value as cancer therapeutics to promote an immunostimulatory tumor microenvironment and improve host antitumor immunity.

Introduction

Tyro3, Axl, and Mertk (TAM) receptors comprise a family of homologous type I receptor tyrosine kinases (RTK) that have been implicated as oncogenic kinases overexpressed in human malignancies, and more recently as inhibitory or tolerogenic receptors expressed on hematopoietic-derived cells [natural killer (NK) cells, dendritic cells, and macrophages] that promote immunosuppression and resolution of inflammation (1–3). The activation of TAMs is mediated by homologous endogenous ligands (Gas6 and Protein S; refs. 4–7) that act as hetero-bifunctional molecules that bridge TAMs with externalized phosphatidylserine (PS) on apoptotic cells, stressed cells, exosomes, and shed microvesicles derived from membrane fragments (8). The activation of TAMs by Gas6 requires carboxyl-glutamic acid posttranslational modification of the Gas6 Gla domain and direct binding to PS for activation (8). Findings show that pan-inhibition of TAM receptors in combination with anti–PD-1 may have clinical value as cancer therapeutics to promote an immunostimulatory tumor microenvironment and improve host antitumor immunity.
as viral infected tissues and in the tumor microenvironment (11). Indeed, the stromal microenvironment of many solid tumors display constitutively elevated PS due the combined high apoptotic index of proliferating tumors (12), the occurrence of metabolically stressed tumor cells and vascular endothelial cells (13, 14), and the release of tumor-derived exosomes from transformed cells (15). We have hypothesized that the PS–TAM receptor axis is constitutively activated in the cancer microenvironment and represents an important target axis in immunoncology (9, 11).

The function of TAM receptors as inhibitory receptors that promote immune tolerance and resolution of inflammation is supported from both systemic genetic knockout studies in mouse models, by conditional knockout studies in tumor models (16–19), and most recently by pharmacologic inhibitor studies with small-molecule tyrosine kinase inhibitors to TAMs (20–22). In the former case, TAM knockout mice (either single KO of Mertk or triple KO of all three TAMs) develop age-dependent autoimmune disease due to the failure to clear apoptotic cells under homeostatic conditions (23, 24). However, in tumor models, conditional knockout of Mertk on bone marrow–derived monocytes improved tumor immunity in a syngeneic breast cancer model that correlates with increased inflammatory cytokines and tumor-infiltrating lymphocytes (TIL; ref. 25). Using a similar genetic strategy, additional studies show that Merk on tumor macrophages acts as a therapeutic target to prevent tumor recurrence following radiotherapy, whereby loss of Merk is sufficient to prevent recurrence after C57/B6 Merk (−/−) mice challenged with 20 Gy × 1 of focal radiation to the tumor (26).

Preclinical studies with pharmacologic agents also showed that small-molecule TAM tyrosine kinase inhibitors, including BGB324 (27, 28), RXDX106 (29), UNC-2025 (30, 31), and Sitratavinib (32) have antitumor activity. Collectively, the implications are that TAMs may act akin to checkpoint inhibitors, as so-called “myeloid checkpoint inhibitors”, to alter the cancer microenvironment, break tolerance, and improve host antitumor immunity.

In addition to the aforementioned suppressive functions of TAMs on myeloid expressing cells (NKs, DCs, Macrophages) that assist tumors to evade host antitumor immunity, we recently demonstrated that TAMs, when overexpressed on human breast cancer cells promote TAM-mediated epithelial efferocytosis (33) and, in doing so, activate a signaling cascade to upregulate PD-L1 (33, 34), an inhibitory checkpoint that binds to its receptor PD-L1–programmed death receptor-1 on T effector cells to induce T-cell anergy and tolerance (35). More recently, in an AML model, Mertk inhibition by either genetic manipulation or by Mtx-2843 tyrosine kinase inhibitor significantly decreased PD-L1 and PD-L2 in the tumor microenvironment (36). The inhibitory PD-L1/PD-1 checkpoint has gained much traction in recent years and motivated the current development of anti–PD-1/PD-L1 therapeutic strategies that have been clinically successful in a variety of indications, including melanoma, NSCLC, and more recently anti–PD-1 has shown some effect for breast cancer treatment (37, 38).

On the basis of our previous reports that TAMs, acting as PS sensing receptors, could induce epithelial effrocytosis and the subsequent upregulation of PD-L1, we propose that in vivo, combinations of PS targeting, TAM therapeutics, and anti–PD-L1/PD-1 may have additive or synergistic activities as combined therapeutics. Indeed, prior studies by Gray and colleagues have shown that combined PS targeting antibodies (upstream of TAM receptors) with anti–PD-1 function synergistically in a syngeneic breast cancer model (39, 40). The current study shows that Tyro3, Axl, and Mertk are differentially expressed on several cell subtypes that contribute to the tumor microenvironment, whereby Axl is preferentially expressed on E0771 tumor cells, while macrophages, including peritoneal macrophages, bone marrow–derived macrophages, and tumor-associated macrophages have higher Mertk/Axl ratios. Subsequently, we used a combination strategy with a pan-TAM inhibitor, BMS-777607 (41–43), and anti–PD-1 mAb to test the therapeutic potential in a preclinical model of triple-negative breast cancer. Our data demonstrated that combining of TAM kinase inhibitor and anti–PD-1 antibody significantly inhibited tumor growth compared with either single therapy regimen alone or control (vehicle drug) and decreased incidence of lung metastasis. Flow cytometry analysis of tumors revealed that combination treatment increased infiltrating T cells and dendritic cells; in contrast to myeloid derived suppressor cells (MDSC) whereby infiltration of MDSCs was less in the tumor microenvironment of combination therapy compared with vehicle control. Finally, immune-profiling analysis based on tumors RNAs demonstrated that combination of TAM kinase inhibitor (BMS-777607) and anti–PD-1 synergistically enhanced expression of proinflammatory cytokines and proimmune cells over control, and addition of BMS-777607 to anti–PD-1 treatment downregulated immunosuppressive cytokine expression in tumor microenvironment. Hence, these studies support the idea that combination therapies targeting TAMs and PD-1/PD-L1 may have potential to treat human breast cancer as immunotherapeutic modalities.

Materials and Methods

Cell culture

The murine triple-negative breast cancer cell line E0771 (CH3 BioSystems LLC) were maintained in RPMI1640 medium (Sigma-Aldrich) supplemented with 10% v/v heat-inactivated FBS (Sigma-Aldrich), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich). Cells were grown at 37°C in a humidified 5% CO2 incubator. After thawing, cells were used for up to 5 passages and their authenticities were checked by STR analysis according to the manufacturer’s protocol latest in October 2017 (GenePrint 10 System, Promega). Cells are routinely checked for Mycoplasma contamination.

Measuring TAM surface expression and receptor activation

Peritoneal macrophages were harvested from 80 μg/mL Concavanalin A (Sigma-Aldrich) injected C57Bl/6j mice (4 days after Concavanalin injection). Bone marrow–derived macrophages (BMDM) were isolated as described below. BMDCs were isolated from C57Bl/6j mice by culturing bone marrow cells in GM-CSF (20 ng/mL) and IL4 (20 ng/mL) for 7 days. Cells were ascertained by FACS to be >70% CD11b+ F4/80+ and >70% CD11c+ F4/80− for BMDMs and BMDCs, respectively. To determine the surface expression of TAMs by flow cytometry; E0771 cells, peritoneal macrophages, BMDMs and BMDCs were detached from the plates using accutase (Sigma-Aldrich) and then stained with anti-mouse Merk (R&D Systems #AF591), anti-mouse Axl (R&D Systems #FAB5841), and anti-mouse Tyro3 (R&D Systems #MAB759)
following the manufacturer’s protocol. In addition, TAM activation was analyzed in E0771 and BMDMs cells. Briefly, 1.0 × 10^6 E0771 cells or BMDMs were seeded into 35-mm tissue culture plates and then, cells were serum-starved for 6 hours. Later, cells were incubated with Gas6-induced conditioned medium (~250 nmol/L Gas6) and 5 × 10^5 apoptotic Jurkat cells (ATCC; prepared as described previously; ref. 33) at 37°C for 30 minutes. For inhibition of TAM activation, 300 nmol/L BMS-777607 (Selleckchem) was added and 293T (ATCC) conditioned medium was used as untreated control. After incubation, cells were washed twice with PBS and lysed using HTNT buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol) as described previously (33). Phosphorylated TAM proteins in the detergent lysates were analyzed by immunoblotting with primary antibodies: phospho-Merk (Aviva Systems Biology # OASG04503; Fabgennix #PMKT-140AP), phospho-Axl (Cell Signaling Technology #5724), total mouse Merk (Santa Cruz Biotecnology #sc-365499), total mouse Axl (Santa Cruz Biotecnology #sc-1096), and total Tyro3 (Cell Signaling Technology #5585).

IC_{50} determination

Advanced Cellular Dynamics tyrosine kinase cell-based assay (Carna Biosciences) was used for IC_{50} according to the manufacturer’s protocol. IL3-dependent Ba/F3 cells were transfected with human recombinant Tyro3, Axl, Merk, and VEGFR2 kinases and treated with different concentration of BMS-777607 followed by cell survival and proliferation analysis.

BMDMs and efferocytosis assay

BMDMs were generated from tibiae and femurs of male C57BL/6J mice (The Jackson Laboratory). The bone marrow cells were flushed with PBS, resuspended in DMEM supplemented with 10% heat-inactivated FBS, 20% L929-conditioned medium, and cultured for 7 days. For efferocytosis assay, apoptotic cells were labeled with pHrodo according to the manufacturer’s protocol (Thermo Fisher Scientific). Then, BMDMs were treated with pHrodo-labeled apoptotic cells and Gas6 with/out BMS-777607 for 30 minutes. Cells were washed 5 times with PBS for eliminating nonspecific binding of apoptotic cells and fluorescent intensity of pHrodo was evaluated by using BD FACSCalibur flow cytometry.

qRT-PCR analysis of TAM mRNA in E0771 cells, BMDCs, BMDMs, peritoneum, and tumor-associated macrophages

For tumor-associated macrophages, E0771 tumor-bearing mice were mechanically and enzymatically dissociated and assessed; and macrophages were gated and sorted according to CD11b “F4/80” for RNA isolation. Total RNAs from E0771 cells, BMDCs, BMDMs, peritoneum, and tumor-associated macrophages were isolated using the TRIzol reagent according to the manufacturer’s instructions. cDNA was obtained using the SuperScript III system (Thermo Fisher Scientific). qRT-PCR for mouse Tyro3, Axl, and Merk was performed using the FAST SYBR Green Master Mix (Thermo Fisher Scientific) with the following primers (IDT): Tyro3: Fwd – GCTTCCCCAATTGGCCGCTCA, Rev – CCAGACCGTGGTACATGAGATCA. Axl: Fwd – ATGGCGGACATTTGGCACGTTG, Rev – CCAGTATACCTCCCGTGTAG. Merk: Fwd – CAAGGCGCTTACCGAGGGA, Rev – TGTGTGCCTGGATGTATCTTC. Each sample was repeated in triplicate and normalized to the expression of housekeeping gene, β-actin.

Induced PD-L1 surface expression

To study IFNγ-induced PD-L1 surface expression, E0771 cells (1 × 10^6) were seeded in 6-well plates and incubated with 100 ng/ml recombinant mouse IFNγ (Biolegend) for 48 hours. Untreated and IFNγ-treated E0771 cells were harvested and stained with PE-conjugated anti-mouse PD-L1 (BioLegend) according to the manufacturer’s protocol and analyzed by flow cytometry. For Gas6 and apoptotic cell-mediated PD-L1 surface expression, E0771 cells (1 × 10^6) were seeded in 6-well plates, were serum-starved for 6 hours and incubated with Gas6-containing conditioned media (~250 nmol/L Gas6) or apoptotic cells opsonized with Gas6-conditioned medium (~250 nmol/L Gas6; ref. 33) with either vehicle (DMSO) or 300 nmol/L BMS-777607 (SelleckChem). 293T-conditioned medium not expressing Gas6 was used as untreated control medium. After 12 hours, apoptotic cells were washed away with RPMI1640 medium twice and E0771 cells were incubated in RPMI1640 medium supplemented with 0.5% FBS for an additional 12 hours. Subsequently, E0771 cells were collected and stained with PE-conjugated anti-mouse PD-L1 (BioLegend) and expression was measured by flow cytometry.

In vivo mouse experiments

E0771 cells (1 × 10^6) were suspended in 0.15 ml Matrigel (50 % v/v; Corning) in RPMI1640 medium and injected into the 9 of 10 mammary fat pads of 7-week-old female C57BL/6 mice (n = 8/group) from Charles River Laboratories. The chimeric anti-mouse PD-1 antibody (41H2; kindly gifted by Bristol-Myers Squibb) and the Pan-TAM kinase inhibitor BMS-777607 (SelleckChem) were used as treatment regimens. DMSO (100 μl/mice/day) and anti-mouse IgG1 isotype antibody control (5 mg/kg on day 10, 12, 14, and 16) as vehicle control, BMS-777607 (25 mg/kg/day) alone, anti-mouse PD-1 (100 μg/mouse on day 10, 12, 14, and 16) alone or their combination were administered via intraperitoneal injection after tumors became palpable (on day 10 following tumor implantation). Doses were selected though preliminary MTD studies (44, 45). Body weights and tumor growth was assessed every three days by caliper measurement of tumor diameter in the longest dimension (L) and at right angles to that axis (W). Tumor volumes were estimated using the formula, L × W × π/6. Toxicity and weight loss were not encountered in the studies. Mice were sacrificed with CO₂ on day 28; tumors, lungs, and spleens were collected for further analysis. When metastases were found, the organ was removed and fixed for quantification and histopathology analysis. Metastasis incidences were calculated by counting metastatic nodules in the lungs under magnification microscope. Mouse experiments were performed in accordance with the guidelines and under the approval from Institutional Animal Care and Use Committee at the Rutgers University, New Jersey Medical.

Flow cytometric analysis

Half of each tumor excised from the mice was physically dissociated and digested in Hank’s balanced salt solution buffer supplemented with 1 mg/ml collagenase (Sigma-Aldrich), 0.1 mg/ml hyaluronidase (Sigma-Aldrich), and 200 U/mL DNase type IV (Sigma-Aldrich) for 50 minutes at 37°C and passed through a 70-μm filter (Falcon). Then, cells were washed with PBS and treated with red blood cell lysis buffer (Roche).
for 10 minutes at room temperature. Cells were washed twice with PBS and stained with antibodies according to the manufacturer's protocols. Antibodies were used in flow cytometry analysis: PerCP-Cy5.5-conjugated CD45 (BioLegend), APC-conjugated CD4 (eBioscience), FITC-conjugated CD3 (BioLegend), PerCP-Cy5.5-conjugated CD25 (BioLegend), PerCP-Cy5.5–conjugated CD11b (BioLegend), PE-conjugated CD8 (eBioscience), APC-conjugated Ly-6C (BioLegend), FITC-conjugated Ly-6G (eBioscience), and FITC-conjugated CD11c (BioLegend).

NanoString immune-profiling analysis
RNAs were isolated from 3 E0771 tumors from each treatment group (vehicle, BMS77607 alone, anti–PD-1 alone, and BMS77607 + anti–PD-1 combination) by using Direct-zol RNA MiniPrep Plus Kit (Zymo Research) by using manufacturer's protocol. All the RNA samples have passed quality control (assessed by OD 260/280) and were subjected to analysis by nCounter murine PanCancer Immune Profiling Panel according to manufacturer's protocol at NYU Genomic Center (NanoString Technologies). Normalization of raw data was performed using the nSolver 3.0 analysis software (NanoString Technologies). The mean of each gene expression (represented in log2) for each group was calculated and data were imported to Graphpad Prism software for statistical analysis and graphics. Further advanced immune-profiling analysis was performed using nSolver 3.0 analysis software with nCounter advanced analysis package (NanoString Technologies) with identified immune cell types (classified by Newman and colleagues; ref. 46). Probes used to classify cell type were as follows: for T cells (Cd2, Cd3d, Cd3e, Cd3g, Cd6, Lck, Cd96, Sh2d1a); for regulatory T cells (Treg) Foxp3; for CD8+ T cells (Prf1, Cd8a, Gzmm, Cd8b, Fli3g); for type 1 T helper cells [T1; C1a4, Lta, Ifng, Cd38, Ccl4]; for DCs (Cd1e, Cd1b, Ccl17, Ccl22, Cd1a); for macrophage (Cd84, Cybb, Cd163, Cd68); for neutrophils (C1r, Col3a1), and for NK cells (Spn, Xcd2, Nr1c1).

Statistical analysis
Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc.). In the figures, data represent one of the triplicate experiments result. Differences between groups were tested by the two-tailed Student t test or Tukey test or Tukey follow-up or Newman and colleagues (1, 47). Here, employing an E0771 orthotopic triple negative breast cancer mouse model, we observe that TAMs are expressed on both tumor cells as well as myeloid-derived cells that contribute to the tumor microenvironment (Fig. 1). When peritoneal macrophages were elicited by Concanavalin A, or cells from the bone marrow were differentiated with L929 conditioned media (containing M-CSF) to become BMDCs, CD11b/F4/80 positive (positive) or with GM-CSF and IL-4 to become BMDCs (CD11c+/F4/80 negative), TAMs exhibit differential expression in these subsets, as determined by either qPCR (Fig. 1A) or flow cytometry using TAM-specific antibodies to detect surface receptor expression (Fig. 1B). Using a similar approach to detect TAM expression on E0771 cells, E0771 cells preferentially express Axl at both the mRNA and protein level, with lesser but detectable Merk and Tyro3 (Fig. 1C). Finally, in the tumor-associated macrophages isolated from the E0771-orthotopically proximal transplanted tumors (in this study), Merk was also preferentially expressed relative to Axl (Fig. 1D), similar to results obtained with BMDMs and peritoneal macrophage expression, which showed high Merk/Axl ratio's in both subsets (Fig. 1A). These data suggest that TAMs are coexpressed on both tumor cells, as well as on macrophages and DCs that comprise the tumor microenvironment.

The aforementioned coexpression of Tyro3, Axl, and Merk on both tumor and tumor microenvironment (immune cells) implies that a pan-TAM inhibitor might be expected to directly target tumor cells as well as indirectly targeting the tumor microenvironment via the TAM expression on macrophages and DCs. To employ a pan-TAM inhibitor, we tested TAM tyrosine kinase inhibitors, BMS-777607 and BGB324, using a Ba/F3 tyrosine kinase cell-based IC50 assay, which measures viability of IL3 dependency to promote cell survival such that IC50 of tyrosine kinase inhibitors can be measured by a decrease in survival (shown as percent receptor inhibition; Fig. 2A; ref. 48). Using this assay, we observe BMS-777607 as a broad-based pan-TAM inhibitor with similar IC50 values (low to mid nM Kds) towards Tyro3, Axl, and Merk, while BGB324 showed selectivity towards Axl. In parallel, employing a CHO-based assay whereby each intracellular tyrosine kinase domain of TAMs was cloned as an EGFR-TAM chimeric receptor (to normalize postreceptor signaling and ligand-dependent kinase activity of each TAM), BMS-777607 also showed efficacy as a pan-TAM inhibitor using phosphorylated-TAMs as a readout in cells stimulated with EGF to activate TAM postreceptor signaling (Fig. 2B; ref. 41).

To translate aforementioned in vitro results into a more functional outcome, we treated naive peritoneal macrophages that express Merk and Axl (Fig. 1A) with either BMS-777607 or BGB324 followed by activation by Gas6 (a pan-TAM ligand; Fig. 2C). Notably, 300 nmol/L BMS7706 effectively blocked Gas6-inducible pAkt activation (~85%), while Axl-specific tyrosine kinase inhibitor BGB324 only modestly inhibited pAkt (~16%; Fig. 2C). These data suggest that BMS-777607 is more effective than BGB324 to inhibit (pan)-TAM receptor activation on macrophages. Notably, of other potential BMS-777607 targets, including Tyro3 and Axl, and non-TAM tyrosine kinases FLT, RON, MET, VEGFR2, these receptors are less abundant on peritoneal macrophages relative to Merk (shown are uncorrected qPCR values; Fig. 2D). However, the role of off-target tyrosine kinases affected by BMS-777607 in vivo (below) cannot be ruled out. Further, to examine effects of BMS-777607 on E0771 tumor cells, 300 nmol/L BMS-777607 blocked both Gas6-mediated, and Gas6-opsonized AC-mediated activation of Axl on E0771 cells, as

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measured by immunoblotting with a pAxl or pAkt Abs (Fig. 2E). Together, these data suggest that pan-TAM BMS-777607 concomitantly suppresses TAM activation on both tumor cells and macrophages.

BMS-777607 blocks macrophage efferocytosis and Gas6-PS–opsonized apoptotic cells; TAM mediated upregulation of PD-L1 on E0771 mouse breast cancer cell lines

To extend TAM kinase activation studies, we pretreated M-CSF–elicited BMDMs or E0771 cells with BMS-777607 to assay efferocytosis (a macrophage outcome; Fig. 2F) or Gas6/AC-induced PD-L1 upregulation (a tumor cell outcome), respectively (Fig. 2G and H). Notably, under these conditions, BMS-777607 suppressed macrophage efferocytosis in a cell-based engulfment assay using pHRodo-labeled apoptotic cells (23% inhibition at 1 μmol/L; 77% inhibition at 10 μmol/L; Fig. 2F). Previously, using a series of human breast cancer cell lines, we showed that TAM-expressing tumor cell lines, when stimulated with Gas6–opsonized apoptotic cells, promoted epithelial efferocytosis and the upregulation of the checkpoint inhibitor ligand PD-L1 (33). Consistent with these previous findings with human cell lines, when E0771 cells were cocultured with Gas6 (Fig. 2H) or Gas6–opsonized ACs (5:1 ratio; Fig. 2G, middle panel) for 12 hours, followed by washout and incubation for an additional 12 hours, surface PD-L1 was upregulated as detected by flow cytometry using a mouse-specific anti–PD-L1 mAb, and this effect was potently blocked by BMS-777607 (Fig. 2G, right). Treatment of cells with IFNγ was used as a positive control for PD-L1 expression. Hence, in the E0771 cells, BMS-777607 inhibited both the early acute tyrosine phosphorylation of TAMs (Fig. 2E) as well as the later upregulation in PD-L1 (Fig. 2G and H). These data suggest that TAMs are activated by Gas6/AC in a kinase dependent-manner in E0771 cells, predict a functional AC−TAM−PD-L1 axis on E0771 cells, and support the rationale to combine the pan-TAM inhibitors with the checkpoint PD-1/PD-L1 inhibitors using in vivo models.

Synergistic antitumor/metastatic effects of pan-TAM BMS-777607 and anti–PD-1 mAbs in the E0771 xenograft model

To assess functional relevance in an in vivo model of tumor progression and immune subversion, E0771 cells were implanted into mammary fat pad of C57BL/6 female mice in a longitudinal study. Previous studies by Gray and colleagues showed
Figure 2.
BMS-777607 is a pan-TAM inhibitor and blocks Axl- and Merk-dependent signaling in E0771 tumor cells and macrophages. A, Inhibition of TAMs by BMS77706 (left) and BGB324 (right) and assessment of IC50 activities using a Ba/F3 cell-based assay. Following IL3 withdrawal of TAM-expressing Ba/F3 cells, tyrosine kinase inhibitors were titrated to derive IC50s (% receptor inhibition). VEGFR was used as a non-TAM tyrosine kinase control. B, Schematic illustration of EGFR-TAM chimeric receptors (left). TAM receptors phosphorylation levels were evaluated by Western blotting after 30 minutes EGF treatment with or without BMS-777607 (300 nmol/L; more than 10-fold higher than IC50 value) in EGFR-TAM chimeric cell lines (right). (Continued on the following page.)
antitumor activity in the E0771 model is enhanced by anti–PD-1 therapeutics, implying the tumor microenvironment in the C57 BL/6 background provides an immune competent milieu to test checkpoint inhibitors (40). In this study, after 10 days, when tumors reached volumes approximately 80 to 100 mm³, mice were treated with intraperitoneal injections of either vehicle/isotype antibodies alone, with BMS-777607 at a concentration of 25 mg/kg/day, with anti–PD-1 (5 mg/kg, 4 doses every 2 days), or combined BMS-777607 and anti–PD-1 combination as described in Materials and Methods (Fig. 3). Measurements of tumor volume and tumor (wet) weight showed that single regimes of either BMS-77707 or anti–PD-1 mAb partially inhibited the tumor growth compared with vehicle or isotype control treatment in the E0771 syngeneic model. Notably, however, combinatorial BMS-777607 and anti–PD-1 mAb treatment showed enhanced antitumor effects (volumes and wet weights) compared with monotherapy (P < 0.0001; Fig. 3B and C), as well as reduced lung metastatic nodules (Fig. 3D; P < 0.01). Despite efficient suppression of tumor growth, no evidence of weight loss or overt toxicity was observed in any treatment group, consistent with previous reports that TAM TKIs (± PD-1) are tolerable in vivo (44, 45).

Combined TAM TKI and anti–PD-1 mAb display increased tumor-infiltrating lymphocytes

To test whether the aforementioned in vivo antitumor responses observed with combinatorial BMS-777607 and anti–PD-1 mAb showed antitumor immunity, we dissociated cells from total tumor mass from each treatment group and accessed the frequency of immune cells subsets, including TILs, by flow cytometry analysis. As indicated in Fig. 4, when we assessed CD45+ in tumor-bearing mice treated with single-agent BMS-777607 or single-agent anti–PD-1 mAb, only the latter showed increased immune infiltration, suggesting that in the E0771 model, pan-TAM TKI inhibitor alone was insufficient to increase immune cell infiltration into tumors. However, in combinatorially treated mice, the addition of BMS-777607 and anti–PD-1 mAb therapy significantly increased CD45+ levels to 56.3 % (P < 0.001 to vehicle, P < 0.05 to anti–PD-1 single treatment; Fig. 4A). Furthermore, levels of CD4+, CD8+, and CD8+ cells, discrete subpopulations of CD45+ cells, showed a similar trend over vehicle treatment [P < 0.01 (CD4+), P < 0.001 (CD8+), P < 0.0001 (CD8+)]. Moreover, combination of BMS-777607 and anti–PD-1 mAb therapy significantly enhanced more CD3+, CD4+, and CD8+ subpopulations over single anti–PD-1 therapy, while again, BMS-777607 single treatment did not show significant increase in TILs (Fig. 4B–D). This suggests that general pan-TAM inhibition, together with anti–PD-1, has a synergistic effect to increase immune infiltration into tumors. To better understand the molecular mechanisms by which TAM inhibitors and anti–PD-1 mAb alter the tumor microenvironment when used in combination, we profiled RNA expressions of genes in specific immune cells based on classifications described by Newman and colleagues (Fig. 4E–G; ref. 46). Consistent with the results of the mechanical dissociation/analysis of cell type frequency in the combination treatment of tumor-bearing mice with BMS-777607 and anti–PD-1 mAb, the expression of genes associated with TILs (Cd2, Cd3d, Cd3e, Cd3g, Cd6, Lck, Cd96, Sh2d1a) was also substantially increased in the tumors compared to anti–PD-1 or BMS-777607 treatment alone (Fig. 4F; P < 0.01 for T cells, anti–PD-1 vs. vehicle; P < 0.001 for T cells, combination vs. vehicle). Similar enhancement effects were observed in the expression of CD8+ effector T cells (Prf1, Cd8a, Gzmm, Cd8b, Flt3lg; Fig. 4G) and Treg cells (Fig. 4H; P < 0.01 for CD8+ T-cell combination versus vehicle; P < 0.05 for CD8+ T cells, combination vs. anti–PD-1).

Moreover, when total RNA was isolated from the aforementioned dissected tumors (Fig. 3) and analyzed globally using NanoString PanCancer Immune Profiling Panel arrays, we also observed the tumors from the combinatorial treatment groups (BMS-777607 + anti–PD-1) showed an enhanced immunogenic profile pattern. This profile included enhanced expression of TNFα (Fig. 5A), IL12 (Fig. 5B), and IFNγ (Fig. 5C; P < 0.05 for IL12 and P < 0.01 for IFNγ; combination versus vehicle) and suppressed expression of immunosuppressive cytokines IL10 (Fig. 5D), IL4 (Fig. 5E), and IL13 (Fig. 5F; P < 0.05 for IL10, P < 0.01 for IL4, P < 0.01 for IL13). Interestingly, however, we also observed that PD-L1 expression was increased by combination therapy (Fig. 5G), potentially explained by concomitant increased IFNγ expression seen in combination therapy (Fig. 5C) as it is well established that IFNγ can induce PD-L1 expression in cancer cells (49). In addition to PD-L1, its receptor PD-1 expression was significantly increased by combination therapy (Fig. 5H), which again may be explained by increased total CD45+ cell infiltration. Taken together, combination treatment of BMS-777607 and anti–PD-1 mAb decreased tumor growth by decreasing expression of some tumor-promoting (immune suppressor) cytokines and increasing tumor suppressor (immune activating) cytokines in the tumor microenvironment compared to mono treatment.

Although the above-mentioned profiling of TILs and accompanying cytokines suggested combined BMS-777607 and anti–PD-1 combinatorial treatments induced a T-cell mediated anti-tumor response, we also wanted to expand such profiling to analyze MDSCs (defined by CD11b, Ly-6C, Ly-6G markers) and DCs (by CD11b, CD11c, and CD8 markers). Compared with
control treatment, MDSCs’ mean value (22.2%), anti–PD-1 alone and combination treatment showed significant decreased level of MDSC infiltration to E0771 tumors (14.26%, P < 0.01 for anti–PD-1 treatment; 11.63%, P < 0.001 for combination treatment; Fig. 6A). Similar to CD45<sup>+</sup> and subpopulation of CD45<sup>+</sup> cells result, infiltrated DCs levels (percentage) were significantly increased with combination treatment compared with vehicle (P < 0.0001; Fig. 6B). Addition of BMS-777607 to anti–PD-1 further demonstrated significant enhancement in DCs levels (RNA expression based; probes: Cd1e, Cd1b, Cd17, Cd22, Cd1a) compared with vehicle and anti–PD-1 mAb alone treatment (P < 0.01 combination vs. vehicle; P < 0.05 combination vs. anti–PD-1; Fig. 6C). Taken together, our results in tumor-bearing E0771 mice model showed that anti–PD-1 single treatment was capable of increasing TILs and combining anti–PD-1 treatment with BMS-777607 agents significantly increases infiltrating antigen-presenting cells (DCs) further in tumor microenvironment. MDSCs, implicated as immune-suppressors in tumor microenvironment, were decreased in anti–PD-1 and anti–PD-1/BMS-777607 combination treated E0771 mice model. These data suggest that combination treatment (anti–PD-1 and BMS-777607) enhance antitumor response by promoting immune activation in tumor microenvironment.

**Effect of TAM kinase and PD-1 inhibition on RNA immunoprofile of tumor microenvironment**

We examined the expression of macrophage (genes analyzed: Cd84, Cybb, Cd163, Cd68; Fig. 6D), neutrophils (genes analyzed:
Effect of combinatorial therapy with TAM kinase inhibitor and anti–PD-1 antibody on TILs. E0771 tumor-bearing mice were treated with single agents or combination of BMS-777607 plus anti–PD-1 antibody. At the end of the experiment, three tumors were collected from each treatment group, and single-cell suspensions were prepared and then stained with specific antibodies against immune cell surface markers. Average percentage for positive surface marker was calculated by flow cytometry for each group, and data are presented for CD45⁺ cells (A) and subpopulation of CD45⁺ cells: CD3⁺ (B), CD4⁺ (C), CD8⁺ (D). RNAs were isolated from the E0771 tumors from treated mice and subjected to further analysis by utilizing NanoString PanCancer Immune Profiling Panel and nSolver advanced immune-profiling analysis software. Expression profile of CD45 surface markers (E) and profiling tumor-associated immune cell type markers: T cells (Cd2, Cd3d, Cd3e, Cd6, Lck, Cd96, Sh2d1a; F), CD8⁺ T cells (Prf1, CD8a, GzmM, CD8b, Flt3lg; G), Treg (FoxP3) cells (H). RNA expression values are presented in log₂ and are graphically represented by GraphPad Prism. Dots, mean (n = 3/group); bars, SD. Statistically significant differences between groups were defined by Student two-tailed t test; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 versus vehicle group. n.s., nonsignificant.
Figure 5.
Effect of combinatorial therapy with TAM on cytokine expression. RNAs isolated from E0771 tumors from different treated mice were subjected to NanoString PanCancer Immune Profiling Panel analysis and nSolver software. Expression profiles of immune activating and immune suppressor cytokines are shown for each treatment group (A–F). RNA expression values are presented in log₂ and are graphically represented by GraphPad Prism. Dots, mean (n = 3/group); bars, SD. Intratumor RNA expression analysis of PD-L1 (G), PD1 (H), and tumor microenvironment cytokines was performed for each treatment group. Statistically significant differences between groups were defined by Student two tailed t-test; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 versus vehicle group. n.s., nonsignificant.
Figure 6.
Effect of combinatorial therapy with TAM on other immune cell types. Average percentage of infiltrating MDSCs surface marker positive (CD11b, Ly-6C, and Ly-6G; A) and DCs surface marker positive (CD11c, CD11b, CD8 for flow cytometry analysis; C, D, etc. for expression analysis; B and C) cells are shown for each treatment group. RNA expressions were analyzed for macrophages (Cd84, Cybb, Cd163, Cd68; D), neutrophils (C1r, Col3a1; E), NK cells (Spn, Xcl1, Ncr1; F), Th1 cells (Cilia4, Lta, Ifng, Cd38, Ccl4; G). RNA expression values are presented in log2 and are graphically represented by GraphPad Prism. Dots, mean (n = 3/group); bars, SD. Statistically significant differences between groups were defined by Student two tailed t-test; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 versus vehicle. n.s., nonsignificant.
Combination of TAM kinase inhibitor and anti-PD-1 modulates tumor growth by altering tumor microenvironment. A, Representative heatmap for cell type abundance in each treatment; nCounter advanced analysis software was used for drawing heatmap. B, The model demonstrates the mechanism of action of TAM kinase inhibitor and anti-PD-1 cancer cells, macrophages, and T cells found in tumor microenvironment.

C1r, Col3a1; Fig. 6E), and NK (genes analyzed: Spn, Xcl2, Ncr1; Fig. 6F) cell markers, combination treatment significantly augmented expressions of these three immune cell markers over vehicle treatment. Similar to increased expression of Tregs (Fig. 4H) in combination treatment (gene analyzed: Foxp3) over vehicle ($P < 0.05$ for combination vs. vehicle), we observed
similar enhancements in type 1 T helper (Th1) cells (Fig. 6G) expressions (genes analyzed: Cila4, Lta, Ifng, Cd38, Cd4; P < 0.05 vs. vehicle group). Representative heatmap for cell type abundance was analyzed by nCounter advanced analysis software that RNA expression for immune cells was used for one sample from each group to obtain heatmap analysis (Fig. 7A). Heatmap data demonstrate that combination of treatment induced the abundance of infiltrating immune cells compared with vehicle treatment (Fig. 7A). According to the tumor microenvironment NanoString RNA expression data, these increased immune cells demonstrate pro-inflammatory functions by increasing proinflammatory cytokine and chemokine expression in dual treatment group. These data suggest that combination treatment of TAM kinase inhibitor and anti–PD-1 mAb enhanced infiltration of immune cells into tumor microenvironment compared with vehicle treatment and pan-TAM tyrosine kinase inhibitors are predicted to have pleiotropic effects in the tumor microenvironment (Fig. 7B cartoon).

Discussion

Previously, using TAM-expressing cancer cell lines and a cell culture system, we showed that Gas6-opsonized apoptotic cells (externalizing PS) activated TAM receptors, induced epithelial efflorescence (34, 50), and induced up-regulation of the T cell checkpoint ligand PD-L1 (33). Consequently, these studies predict that PS-positive dying cells and tumors with high apoptotic indexes, in vivo, may have an unanticipated consequence to skew immune responses that impinge on the PD1/PD-L1 axis. In the present study, we extend the previous in vitro studies and show that combined in vivo administration of anti-PD-1 mAb with a pan-tyrosine kinase inhibitor (BMS-777607) enhances tumor-infiltrating lymphocytes and T cell mediated immunity with improved anti-tumor/anti-metastatic activity compared to single mono-therapeutics alone. Our study, combined with recent reports by Gray and colleagues showing augmentation in T cell-mediated immune responses by PS-targeting antibodies plus anti-PD-1 therapy in breast cancer (40), reports by Guo and colleagues that Axl-specific inhibitor R428 synergizes with PD1 therapeutics in colon cancer models (51), and most recent reports by Du and colleagues that sitravinib potentiates immune checkpoint blockade in refractory cancer models (32) support the further exploitation of the putative PS–PSR (i.e. TAM receptors)–PD-L1 axis as an immune checkpoint target in cancer for future preclinical and future human clinical trials.

Although the present study provides proof-of-concept and supports the idea that pan-TAM inhibitors, combined with anti-PD-1 or other checkpoint inhibitors, will have therapeutic benefit as combinatorial regimes in cancer, further mechanistic studies will be required to identify the repertoire of molecular targets of BMS-777607 in vivo. For example, in the E0771 triple negative murine model used in this study, E0771 cells mainly express Axl, although express other TAMs at lower levels. It is possible that MerTK and Tyro3 can individually impact tumor growth and survival, as well as induce PD-L1 up-regulation when activated by PS-positive apoptotic cells in other tumors that express these TAMs. Likewise, in recent years, it has been widely reported that TAMs are differentially and dynamically expressed on a variety of tumor associated myeloid cells, including M2 macrophages, DCs, NK cells, and MDSCs, although by commonality they all act as inhibitory receptors that suppress immune responses (1). Henceforth, pan-TAM inhibitors likely exert complex mechanisms of action on distinct target cell types in the tumor microenvironment, including macrophages, DCs, and NK cells. Adding additional complexity, recent studies also demonstrate that TAMs (as well as Pros1) can be expressed on activated memory T cells, and act in a feedback mechanism to limit antigen specific memory T cell responses (52, 53). Based on the broad and dynamic expression patterns of TAMs and their ligands on multiple cell types that comprise the tumor microenvironment, in future studies it will be important to investigate effects of specific TAM antagonists, for example, how Tyro3, Axl, and MerTK specific mAbs, or small molecule tyrosine kinase inhibitors, act in different combinations and in different cancer types.

Despite the board range of potential target cell types for BMS-777607 in the tumor microenvironment, both single therapy BMS-777607 and combined BMS-777607 therapy with anti-PD-1 showed tumor growth inhibition and concomitant enhanced infiltration of tumor-associated lymphocytes, the latter associated in many cancers, including breast cancer, with better overall survival. Indeed, compared with anti–PD-1 treatment alone, anti–PD-1 and BMS-777607 increased both TILs and intratumoral DCs (tumor antigen presenting cells), and substantially shifted the cytokine and chemokine profiles to that typically observed in "hot tumors". This includes increased intratumoral IL12 and IFNγ cytokines, while decreased immunosuppressive cytokines (IL10, IL4, IL13, and IL17). Interestingly, we also observed significantly increased intratumoral expression of PD1L, which may be counterintuitive given the putative PS–TAM–PD-L1 activation axis that we have proposed. However, it is also known that IFNγ has both tumor suppressive and tumor promoting activity, the former through the upregulation of MHC class I (54) and the latter via the upregulation of PD-L1 (55). It is possible that the increased levels of PD-L1 observed reflect the increased TILs that produce IFNγ, which in turn provide an inductive signal for PD-L1. This might act fortuitously in this model, whereby PD-1 mAbs are concomitantly administered. Collectively, in combination with anti–PD-1, pan-TAM inhibitors enhance proinflammatory/antitumor immune cells and cytokines in the tumor microenvironment and decrease immunosuppressive/tumor promoting immune cells and cytokines.

In recent years, the field onco-immunology has gained much traction as a therapeutic modality mainly with clinical observations that targeting PD1, and its ligand PD-L1, has produced significant clinical benefit in variety of cancers included melanoma, NSCLC, and renal cell carcinoma. However, it is also clear that PD-1 blockage is not sufficient to antagonize all resistance mechanisms in the cancer TME. Here, we support proof-of-concept that pan-TAM inhibitors, likely acting as PS receptors, can have synergistic activity with conventional anti–PD-1 therapeutics and should be further explored in preclinical models and future human clinical trials.

Disclosure of Potential Conflicts of Interest

M. Quigley has ownership interest (including stock, patents, etc.) in Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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**References**


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