Abstract. Persistent activation of signal transducer and activator of transcription 3 (STAT3) is associated with the progression of a range of tumors. In this report, we present the anticancer activity of 2-(1-(4-(2-cyanophenyl)1-benzyl-1H-indol-3-yl)-5-(4-methoxy-phenyl)-1-oxa-3-azaspiro(5,5)undecane (CIMO) against breast cancer cells. We observed that CIMO suppresses the proliferation of both estrogen receptor-negative (ER-) (BT-549, MDA-MB-231) and estrogen receptor-positive (ER+) (MCF-7, and BT-474) breast cancer (BC) cells with IC50 of 3.05, 3.41, 4.12 and 4.19 µM, respectively, and without significantly affecting the viability of normal cells. CIMO was observed to mediate its anti-proliferative effect in ER- BC cells by inhibiting the phosphorylation of JAK2 and STAT3 proteins. Quantitative PCR analysis demonstrated that CIMO decreases the relative mRNA expression of genes that are involved in cell cycle progression (CCND1) and cell survival (BCL2, BCL-xl, BAD, CASP 3/7/9, and TP53). In addition, CIMO was observed to arrest BC cells at G0/G1 phase and of the cell cycle. Furthermore, CIMO suppressed BC cell migration and invasion with concordant regulation of genes involved in epithelial to mesenchymal transition (CDH1, CDH2, OCLN and VIM). Thus, we report the utility of a synthetic azaspirane which targets the JAK-STAT pathway in ER- BC.

Introduction

Breast cancer (BC) is the leading cause of cancer-related death in females worldwide (1). Several studies have shown that the development of BC is associated with family history, age advancement, mutations in BRCA1, and BRCA2 genes, prolonged exposure to endogenous or exogenous estrogens and exposure to ionizing radiation (2-4). Diagnosis of BC at advanced stages may reduce the efficacy of therapeutic approaches such as surgery and chemotherapy. Approximately 65-70% of BCs are ER+ and BC patients with ER+ tumors respond positively to adjuvant anti-estrogen therapy, which has produced a significant improvement in survival and a reduction in disease relapse, especially in women with early BC and those with ER+ tumors, who may receive endocrine therapy (ET) alone or in combination with cytotoxic therapy. Approximately 10-20% of BCs are ER- and BC patients with ER- tumors do not respond to hormonal therapy or other targeted therapies (e.g., Herceptin). Therefore, improved prognostic outcomes for ER- BC depend on early detection and/or development of new therapeutics with higher efficacy for advanced stage cancer.

Signal transducer and activator of transcription 3 (STAT3) is a latent transcription factor residing in the cytoplasm (5). STAT3 is involved in relaying extracellular signals derived from multiple cytokines, hormones and growth factors to...
the nucleus in order to transcribe the genes involved in cell proliferation, apoptotic resistance, angiogenesis and immune evasion (6-8). Janus kinases and Src kinases are the upstream tyrosine kinases which phosphorylate STAT3 on Tyr-705. In turn, STAT3 undergoes dimerization to translocate into the nucleus to stimulate the transcription of genes involved in the aforementioned functions (9). STAT3 is reported to be constitutively activated in more than 20 types of cancer, including breast cancer, thereby contributing to cancer progression and poor prognosis (7,10). Hence, the critical role of STAT3 in breast cancer makes it an attractive therapeutic target for cancer treatment and potentially ER+ BC.

Azaspirane derivatives are known for their tyrosine kinase inhibitory activity and many are in clinical trials for the treatment of various cancers (11,12). Midostaurin, atiprimod, lestaurtinib and K252a are some of the major azaspirane based multi-tyrosine kinase inhibitors (13). Midostaurin is an analogue of azaspirane and a derivative of staurosporine which have been tested in phase-II clinical trials for the treatment of acute myeloid leukemia and it potently inhibits protein kinase C, VEGFR2, PDGFR, KIT and FLT3 tyrosine kinases (14,15). Atiprimod proved to be a potent JAK2/ JAK3 inhibitor in preclinical studies (16). Lestaurtinib is an inhibitor of JAK2, FLT3 and TrkA and undergoing phase-III clinical trials in combination chemotherapy to treat acute lymphoblastic leukemia (17-19). K252a is a cell-permeable staurosporine based fungal alkaloid with inhibitory activity against protein kinase C and trk family kinases (20). K252a has also been reported to block leukemia inhibitory factor-induced STAT3 activation in olfactory receptor neurons (21). We have recently reported the synthesis and anticancer effect of various azaspirane derivatives and demonstrated their mechanism of action in several types of cancers (7,22,23). In our previous study, we reported the development of azaspirane based small molecule, 2-[(4-(2-cyanophenyl)-1-benzyl-1H-indol-3-yl)-5-(4-methoxy-phenyl)-1-oxa-3-azaspiro(5,5)undecane (CIMO) and demonstrated inhibition of the JAK-STAT pathway in hepatocellular carcinoma (7). In continuation of effort in demonstrating the pharmacological properties of various heterocyclic compounds (24-30), in this investigation, we evaluated the effect of CIMO in both ER+ and ER- BC cell lines.

Materials and methods

Cell culture and reagents. BC cell lines MCF-7, T47D, BT-474, MDA-MB-231, and BT-549 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and were cultured as per ATCC propagation instructions. MDA-MB-231 and BT549 cell lines were cultured in Dulbecco's modified Eagle's medium while MCF-7 and BT-474 cell lines were cultured in Roswell Park Memorial Institute. All growth media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Cell viability assay. The BC cell lines (MCF-7, T47D, BT-474, MDA-MB-231, BT-549) were seeded at 2.5x10⁴/ml in a 96-well plate. After an overnight incubation of cells, the medium was changed to the indicated concentration of CIMO ranging from 0.01 to 10 µM. Following 72 h incubation, alamarBlue® dye was added and incubated for 4 h in the dark, followed by measuring fluorescence activity at an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

ApoTox-Glo™ Triplex assay. Cells (2x10⁴) were seeded with complete medium in each well of a 96-well plate, 5 µM of CIMO and vehicle control (DMSO) was added to the respective well and incubated for 24 h. Thereafter, 20 µl of viability/cytotoxicity reagent containing both GF-AFC substrate and bis-AAF-R110 substrate was added to all the wells, and mixed by orbital shaking (300-500 rpm for ~30 sec) and incubated for 30 min at 37˚C. After which fluorescence measurement was obtained at the two wavelength sets: 400 Ex/505 Em (viability) 485 Em/520 Em (cytotoxicity). After measurement, 100 µl of Caspase-Glo® 3/7 reagent was added to all the wells, and mixed by orbital shaking (300-500 rpm for ~30 sec) and incubated for 30 min at room temperature followed by luminescence measurement with an integration time between 0.5-1 sec.

3D Matrigel proliferation assay. 3D Matrigel (100%) (BD BioCoat™ Matrigel™) was coated on 48-well plates and given time to solidify. A 2% Matrigel containing 5,000 MDA-MB-231 cells were cast above the 100% Matrigel layer and given time to solidify. Thereafter, cells were allowed to grow till they formed a 3D morphology before subjecting them to treatment with different concentration of CIMO at 5, 2.5 and 1.25 µM in 2% FBS + 1% P/S containing high glucose DMEM. The media in the wells were changed every 2 days, with microscopy images obtained every day to observe the drug-induced effects on cells present in a 3D culture.

Flow cytometric analysis. To determine the effect of CIMO on the cell cycle, cells were treated with CIMO at the indicated concentrations ≤5 µM. Thereafter, cells were washed, fixed with 70% ethanol, and incubated for 30 min at 37˚C with 0.1% RNase A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 µg/ml propidium iodide for 30 min at room temperature. Cell distribution across the cell cycle was examined with a Beckman Coulter flow cytometer.

Western blotting. Western blot analysis was performed as previously described (31,32). Briefly, CIMO treated MDA-MB-231 whole-cell extracts were lysed in lysis buffer (20 mM Tris, pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaVO₃. Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and protein concentration was quantified. Thereafter, proteins were resolved on SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and probed with various antibodies overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally examined by chemiluminescence (ECL; GE Healthcare).

Real-time PCR. Quantitative analysis of mRNA expression by real-time PCR was performed using ABI 7700 real-time PCR system (Applied Biosystems) as previously described
Briefly, total cDNA (5 ng) from each stable cell line was added to a 20 µl reaction containing SYBR GreenER qPCR SuperMix and forward and reverse primer mix. All reactions were performed in triplicate in a 384-well plate using a two-step amplification program with 24 initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 20 sec and 6°C for 30 sec. Relative mRNA expression between cDNA samples was calculated using comparative Ct method and normalized against a panel of housekeeping genes including β-actin, HPRT, and GAPDH. Relative expression was computed as: Fold expression = 2^{-∆Ct} where ∆Ct = Ct difference of sample relative to control (∆Ctsample-control). Positive and negative relative expression indicates increase and decrease in mRNA levels, respectively. A P-value <0.05 was considered as statistically significant.

Wound healing assay. The migration of cells was investigated using a wound healing assay. MDA-MB-231 cells were seeded in a 6-cm culture dish with complete medium and allowed to grow until ~80% confluent. A wound was created using a pipette tip and rinsed with PBS to remove detached cells before the treatment with varying concentration of CIMO. The microscopic observation of the cells was recorded as described previously (34).

Invasion assay. The invasion assay was performed with slight modifications in a method described previously (35). A BD Biocoat Matrigel™ invasion chamber with 8-µm pores in the light-tight polyethylene terephthalate membrane and was coated with a reconstituted basement membrane gel (BD Biosciences). MDA-MB-231 cells (1x10^4) were suspended in serum-free DMEM and seeded into the Matrigel Transwell chambers. The cells were incubated with different concentrations of CIMO (1.25, 2.5 and 5 µM). After 24-h incubation, the wells were gently removed with cotton swabs. The Transwell insert was fixed in 4% PFA for 15 min at 4°C. Thereafter, the insert was washed twice in PBS and stained with Hoechst. The invading cells were then counted in randomly selected areas under microscopic observation.

Data analysis. All data analysis was done using the GraphPad Prism (V.60f) software. The data given in this study are...
CIMO suppresses proliferation of ER+ and ER- BC cells. Initially, we evaluated the effect of CIMO on a panel of five BC cell lines (ER+: MCF-7, T47D, and BT-474 and ER-: MDA-MB-231 and BT-549) using an alamarBlue cell viability assay. The dose-response curve indicated that CIMO was able to produce a substantial decrease in cell viability in all cell lines with BT-549 exhibiting relatively the lowest IC50 value of 3.05 µM followed by MDA-MB-231, MCF-7 and BT-474 with the IC50 values 3.41, 4.12 and 4.19 µM, respectively (Fig. 1A). The ApoTox-Glo™ triplex assay results indicated a decreased cellular viability with higher apoptotic activity in both MDA-MB-231 cells than BT-549 cells (Fig. 1B-D). Nevertheless, across all BC cell lines, significantly higher apoptotic levels were detected with a corresponding decrease in cellular viability. CIMO exhibited no substantial cytotoxicity against normal immortalized mammary epithelial cells and/or against BC cell lines.

CIMO decreases proliferation of MDA-MB-231 cells in 3D culture. Tumor cells are more resistant to anticancer agents in three-dimensional multicellular spheroidal conformation compared to monolayer culture (36). Therefore, we analyzed the effect of CIMO on 3D culture of MDA-MB-231 cells. BC cells were cultured in Matrigel, treated with CIMO at indicated doses and cellular viability was measured with alamarBlue on day 10. Treatment with CIMO decreased the
cell viability by >50% compared to vehicle control in 3D culture (Fig. 2A).

CIMO arrests MDA-MB-231 cells at G0/G1 phase. In order to evaluate the effect of CIMO on the distribution of the cell cycle in MDA-MB-231 cells, we performed flow cytometric analysis. MDA-MB-231 cells were treated with CIMO at different time intervals up to 48 h and stained with propidium iodide to analyze the cell cycle distribution. We observed that CIMO increased the accumulation of cells in G0/G1 phase of the cell cycle (Fig. 2B). The treatment of BC cells with 5 µM of CIMO for 48 h resulted in an increased G0/G1 population of 77.1% compared to 60.82% in vehicle control.

CIMO suppresses the basal activation of STAT3 in ER+ and ER- cells. Azaspiranes have been reported to possess inhibitory activity against the JAK-STAT pathway. Therefore, we further evaluated the inhibitory potential of CIMO towards the activity of JAK2 and STAT3 in ER+ (MDA-MB-231) and STAT3 in ER- cells by western blotting via antibodies recognizing phospho-JAK2 (Tyr-1007/1008) and phospho-STAT3 (Tyr-705). We observed that, CIMO significantly inhibited the phosphorylation of JAK2 and STAT3 in a dose-dependent manner, with a maximum inhibition identified at 5 µM and 6 h. At the same time, the expression of total JAK2 and STAT3 proteins remained unaltered (Fig. 2C and D).

CIMO downregulates the expression of STAT3 targeted genes in MDA-MB-231 cells. Activated STAT3 has been reported to modulate the expression of antiapoptotic proteins (37,38). Therefore, we evaluated whether CIMO modulates the expression of various STAT3-regulated genes. Real-time PCR analysis demonstrated that exposure of MDA-MB-231 cells to CIMO decreased mRNA levels of CCND1, CCNE1, CDK2 and CDK4 required for cell cycle progression (39). In addition, the mRNA levels of CDKN2A an inhibitor of CDK4 was increased in MDA-MB-231 cells treated with CIMO relative to vehicle exposed cells (40). CIMO treated MDA-MB-231 cells exhibited decreased mRNA levels of the pro-survival gene, BCL-xL. Concordantly, the mRNA levels of genes encoding proapoptotic MDM2, S100A4, BAX and CDKN1B were increased after CIMO exposure in MDA-MB-231 cells (Fig. 3A and B). Furthermore, western blot analysis demonstrated that protein levels of CCND1 and BCL2 and BCL-xL were decreased in MDA-MB-231 cells after treatment with CIMO in a dose-dependent manner (Fig. 3C).
CIMO promotes apoptosis via the mitochondrial pathway in MDA-MB-231 cells. Cleavage of pro-caspase 9 serves as a marker of cells undergoing apoptosis via the mitochondrial pathway with subsequent activation of the executioner caspase 3 and 7 (41). We therefore investigated whether CIMO promoted apoptosis through the intrinsic pathway in MDA-MB-231 cells. We observed that, CIMO treatment produced a decrease in the level of pro-caspase 9 and increased levels of cleaved caspase 3 and 7 as direct evidence of mitochondrial mediated apoptosis (Fig. 4). Dephosphorylation of BAD protein at Ser-136 results in dimerization with BCL2 and BCL-xL to induce the release of cytochrome c to promote apoptosis via the intrinsic pathway (42). Treatment with CIMO decreased BAD phosphorylation in a dose-dependent manner. In addition, we also observed an increased expression of TP53 (Fig. 4).

CIMO suppresses cell invasion and migration in MDA-MB231 cells. STAT3 regulated gene products are also reported to be associated with migration and invasion of cancer cells (6,7). To evaluate whether CIMO repressed the motility of cancer cells, we performed a wound healing assay. CIMO significantly inhibited cell migration at 5 µM (Fig. 5A). Further investigation using Transwell invasion chamber demonstrated that CIMO inhibited the invasion of BC cells (Fig. 5B). Loss of CDH1 and OCLN promotes invasiveness, and increased expression of CDH2 and VIM is correlated with metastasis and poor prognosis in human cancers (43-46). Given the anti-invasive property of CIMO, we further analyzed the expression of epithelial-mesenchymal transition proteins including CDH1, CDH2, OCLN and VIM. Fig. 5C demonstrates the upregulation of CDH1 and OCLN and downregulation of CDH2 and VIM in a dose-dependent manner.

We next evaluated the effect of siRNA-mediated deletion of STAT3 transcripts on cell invasion. Transient transfection of STAT3-directed siRNA in MDA-MB-231 cells showed decreased levels of phospho-STAT3 and STAT3 protein compared with their respective controls which was confirmed using western blot analysis. We also observed the decreased phosphorylation of STAT3 on treatment with CIMO without altering the levels of total STAT3 (Fig. 6). Cells transfected and/or exposure to CIMO exhibited reduced invasion and migratory properties when compared to DMSO treated cells indicating that deletion/inhibition of STAT3 plays a critical role in motility of cancer cells.

Discussion

STAT3, upon phosphorylation, dimerizes and translocates to the nucleus where it relays its oncogenic signals via regulating genes involved in cell growth, survival, angiogenesis, and cell migration (22,39,40,42). Hence, it is no surprise that a mutation in this gene alone can support oncogenic activity and give rise to uncontrolled cell proliferation (31). JAK2, a non-receptor tyrosine kinase promoting STAT3 activation was observed to be constitutively activated in >50-60% of primary breast tumors and tumor-derived cell lines with drug resistance (32). Hence, inhibition of JAK2/STAT3 signaling is an attractive approach in disrupting aggressive subtypes of breast cancer including ER BC. The aim of this study was to further investigate the effects of the oxazine-based compound CIMO, that has been recently reported to disrupt the JAK-STAT pathway in hepatocellular carcinoma. In ER+ and ER- BC, CIMO inhibits the kinase activation of JAK2 and hence subsequently reduces the JAK2 mediated activation of STAT3 specifically at Y705. As previous studies have demonstrated that CIMO has no effect on S727 phosphorylation of STAT3 in hepatocellular carcinoma cells, the phosphorylation activity at S727 of STAT3 was not investigated.

Constitutive activation of STAT3 by receptor tyrosine kinases EGFR, HER2, fibroblast growth factor receptor (FGFR), IGFR, HGF and platelet-derived growth factor receptor (PDGFR), growth hormone, prolactin, receptor-associated kinases (JAK) and non-receptor kinases (Src and ABL) through phosphorylation has been documented in BC cells (47-49). This constitutive STAT3 activation leads to increased expression of proteins such as MMP-2, MMP1, MEK5, c-Fos and VEGF and promote invasion (25). As such, the ability of CIMO to suppress invasion was evident and due to its ability to disrupt the JAK2/STAT3 pathway. Constitutive STAT3 activation is able to promote EMT via STAT3 promoted SNAIL1 expression including increased

![Figure 4. Western blot. CIMO promotes apoptosis via the mitochondrial pathway in MDA-MB-231 cells. MDA-MB-231 cells (1x10^6 cells/ml) were treated with the indicated concentrations of CIMO for 24 h, after which whole-cell extract was prepared and resolved on SDS-PAGE gel, electrotransferred onto PVDF membrane, and probed for caspase 3, 7 and 9 along with CCND1, TP53, phospho-BAD and BAD. β-actin was used as input control.](image-url)
Figure 5. (A) Wound healing assay. CIMO inhibits cell migration. MDA-MB-231 cells (1x10^6) were plated on a 6-cm culture dish with complete medium. After 24-h incubation and ensuring cells were 100% confluent, media was removed and a scratch were performed on the plate using a P200 pipette tip. The cells were then rinsed twice with PBS before CIMO treatment. Images to monitor the migration of cells were taken at different time-points of 0, 24 and 48 h using microscopy under x5 and x10 magnifications. (B) Transwell assay. CIMO inhibits cell invasion. MDA-MB-231 cells suspended in serum-free DMEM were seeded in the top chamber of a 5% Matrigel (BD BioCoat Matrigel) gel layer in a 24-well Transwell insert (Greiner bio-one ThinCert 24-well culture insert; 8.0 µM). After which, CIMO or vehicle DMSO in serum-free media were added followed by the chemoattractant, DMEM containing 10% FBS was added to the 24-well plates. Following 24-h incubation, Transwell inserts were fixed in 4% PFA for 15 min at 4°C and washed with PBS before staining with Hoechst (1% Triton X-100 + 4 µg/ml Hoechst dye 33258). (C) Western blot. Whole cell lysate obtained after 24 h CIMO treatment and vehicle control DMSO were run on SDS-page gel and transferred to PVDF membrane which were probed for CDH1, CDH2, OCLN and VIM. β-actin was used as input control.

Figure 6. Comparative study of cell invasion between STAT3-siRNA-transfected and CIMO-treated MDA-MB-231 cells. Correspondingly, western blot analysis was used to assess the levels of phospho-STAT3 and STAT3 in MDA-MB-231 cells with siRNA-mediated depletion of STAT3 expression and upon exposure to CIMO. Whole-cell extract was prepared and resolved on SDS-polyacrylamide gel, electrotransferred onto nitrocellulose membrane, and probed for phospho-STAT3, and the same blot was stripped and reprobed with STAT3 antibody and β-actin was used as input control.
expression of mesenchymal proteins VIM and CDH2 (26,27). In particular, MDA-MB-231 cells exhibiting higher STAT3 activity have shown to have a higher VIM expression (50). Hence, CIMO was able to decrease the expression of EMT proteins via the inactivation of STAT3. This demonstrates the effect of CIMO on suppressing STAT3 mediated migration and invasion in ER-BC cells.

Inhibition of JAK2 and STAT3 activity by CIMO directly correlated with decreased expression of STAT3-regulated proteins BCL2, BCL-xL and CCND1. In association with other proteins, BCL2 and BCL-xL protein maintain the integrity of outer membrane of mitochondria in the cells. Herein, we demonstrated that CIMO exposure to ER-BC cells increased cleavage of caspase 9 by subsequently increased cleaved caspase 3 and 7, which signify the induction of late-phase intrinsic apoptosis. Concordantly, CIMO exposure to ER-BC cells also increased expression of BAD protein that indicated the induction of mitochondrial outer membrane permeabilization (MOMP) that leads to intrinsic apoptosis, as interaction between dephosphorylated-BAD and BCL2/BCL-xL protein consequent to permeabilization of the mitochondrial outer membrane (51). Moreover, CIMO exposure to BC cells also decreases expression of CCND1 and stimulates a proliferative arrest in G0/G1 phase that indicated the disruption of the cell cycle progression. Together, CIMO exposure to ER-BC cells inhibits cell cycle progression and survival.

In conclusion, the design of therapeutic agents against ER-BC remains as a prime challenge in clinical management of BC. Herein, we report that the azaspirane based small molecule, CIMO as an inhibitor of the JAK2-STAT3 pathway in ER-BC cells with no or very low cytotoxicity towards normal cells. CIMO promotes apoptosis through the repression of STAT3 activity on target genes. In addition, CIMO suppressed cellular migration and invasion mediated via STAT3 regulated EMT related proteins. Therefore, CIMO emerges as a potential inhibitor targeting the ER-BC cells whose growth is dependent on the constitutive activation of the JAK2/STAT3 signaling pathway (52).

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