UM-164: A Potent c-Src/p38 Kinase Inhibitor with In Vivo Activity against Triple-Negative Breast Cancer

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Abstract

Purpose: c-Src has been shown to play a pivotal role in breast cancer progression, metastasis, and angiogenesis. In the clinic, however, the limited efficacy and high toxicity of existing c-Src inhibitors have tempered the enthusiasm for targeting c-Src. We developed a novel c-Src inhibitor (UM-164) that specifically binds the DFG-out inactive conformation of its target kinases. We hypothesized that binding the inactive kinase conformation would lead to improved pharmacologic outcomes by altering the noncatalytic functions of the targeted kinases.

Experimental Design: We have analyzed the anti-triple-negative breast cancer (TNBC) activity of UM-164 in a comprehensive manner that includes in vitro cell proliferation, migration, and invasion assays (including a novel patient-derived xenograft cell line, VARI-068), along with in vivo TNBC xenografts.

Results: We demonstrate that UM-164 binds the inactive kinase conformation of c-Src. Kinome-wide profiling of UM-164 identified that Src and p38 kinase families were potently inhibited by UM-164. We further demonstrate that dual c-Src/p38 inhibition is superior to mono-inhibition of c-Src or p38 alone. We demonstrate that UM-164 alters the cell localization of c-Src in TNBC cells. In xenograft models of TNBC, UM-164 resulted in a significant decrease of tumor growth compared with controls, with limited in vivo toxicity.

Conclusions: In contrast with c-Src kinase inhibitors used in the clinic (1, 2), we demonstrate in vivo efficacy in xenograft models of TNBC. Our results suggest that the dual activity drug UM-164 is a promising lead compound for developing the first targeted therapeutic strategy against TNBC.

Introduction

Breast cancer is the most common potentially lethal cancer in women and the second leading cause of cancer deaths in the United States (3). Breast cancers have profound heterogeneity with respect to their genetic makeup, gene expression, and phenotype, leading to a current model of five subtypes (4). Among individual breast cancer subtypes, those classified as triple negative are especially lethal due to their high metastatic potential and propensity to early recurrence (5, 6). As a group, triple-negative breast cancers (TNBCs) lack expression of estrogen and progesterone receptors (ER and PR) and also lack overexpression of human epidermal growth factor receptor 2 (ErbB2/HER-2). Thus, therapies directed against these robust targets are not effective against TNBCs (7). There have been tremendous advances in the treatment of breast cancer over the last three decades, particularly for patients whose tumors overexpress ErbB2 and/or have expression of a hormone receptor, all of which have resulted in drastic improvements in survival (8). In contrast, TNBC is a large (and itself heterogeneous) subset of breast cancers for which there are no FDA-approved targeted therapies and cytotoxic agents remain the mainstay of therapy (7). Despite advances in chemotherapy schedules, the residual risk of recurrence in patients with nonmetastatic TNBC after aggressive treatment is close to 40%, substantially higher than patients with breast cancers expressing hormone receptors or HER-2 overexpression, where the risk of recurrence is less than 25% (9). Multiple factors result in poor clinical outcomes for patients with TNBC, with the lack of plausible predictive markers for response to any given targeted therapy playing a substantial role.

Next-generation sequencing of breast cancers and metastases has furthered our understanding of clonal evolution as a conduit for tumor heterogeneity, challenging the paradigm that all breast cancers can be treated with therapies targeting a single pathway (4, 10). Therefore, there is an increasing interest in inhibiting multiple pathways. Targeting parallel pathways, which may connect at one or more hubs, is of particular interest. Here, we present a multitarget kinase inhibitor with activity against c-Src and p38 kinases that shows promise in models of TNBC.
Together, these effects coalesce into producing UM-164 0 conformation alters the localization of c-Src in TNBC cells. We demonstrate that binding of UM-164 to c-Src in its inactive conformation dissociates tumor was strained using a 70-μm filter to achieve a single-cell suspension. The cells were then purified using the Miltenyi Biotec Mouse Cell Removal Kit (MB; 130-104-694) and LD Columns (MB; 130-042-401). Cells were then plated onto tissue culture–treated plates in RPMI 1640 medium, supplemented with 10% FBS and antibiotics. After several passages, tumor cells began to grow and were successfully passaged using standard methods. One additional round of mouse cell removal was performed to eliminate any remaining mouse cells from the culture. The resulting cell line, VARI-068, can be routinely split at 1:6 on a weekly basis in either RPMI 1640 or DMEM with L-glutamine, supplemented with 10% FBS and antibiotics. The resulting VARI-068 cell line was verified to maintain its TNBC status.

Reagents and antibodies
Dasatinib (pharmacologic grade) was purchased from LC Laboratories. Primary antibodies [SRC-Y416 #2101, AKT, p-AKT (S473/S380), ERK1/2, p-SRC (Y419), SRC, p-EGFR, and EGFR] were obtained from Cell Signaling Technologies. Polyclonal antibodies to BAK and Bax-2 were obtained from Upstate Biotechnology and Santa Cruz Biotechnology, respectively.

Imaging of c-Src localization
MDA-MB 468 cells were trypsinized and allowed to adhere overnight to #1.5 cover glass in a 6-well plate. Cells were then treated with 5 μmol/L dasatinib, 5 μmol/L UM-164, or vehicle (DMSO) for 4 hours. Cells were then fixed with 4% paraformaldehyde for 15 minutes at room temperature followed by three washes with PBS. The fixed cells were treated with 1 μmol/L of an irreversible turn-on Src fluorophore (23) for 1 hour followed by three washes with PBS. Cells were then mounted on slides with UltraCruz Hard-set Mounting Medium w/ DAPI (Santa Cruz Biotechnology; sc-359850) and stored for 30 minutes at 4°C in the dark. Imaging was performed using an Olympus Fluoview 500 Laser Scanning Confocal Microscope with a 60X/1.4NA oil immersion objectives (Olympus). Samples were sequentially excited by Diode (405 nm) and Argon (488 nm) lasers for the DAPI and coumarin fluorophores, respectively. Emission signals were collected by barrier filters set to 430 nm for DAPI and 505 to 525 nm for the coumarin fluorophore. Differential Interference Contrast images were collected using the Argon laser channel. Images of 1,024 x 1,024 pixel dimensions were taken using the medium setting for scan speeds and Kalman filtering (averaging) of at least four scans. A zoom factor of $2 \times$ was used. The Z-resolution for each image was 0.5 μm.

Determination of dose-dependent toxicity
BALB/c mice were divided into three different groups: the control group and two treatment groups, which received vehicle, 40 mg/kg, or 140 mg/kg of UM-164 intraperitoneally for 5 consecutive days, respectively. The mice that were given 140 mg/kg...
showed signs of toxicity, whereas the 40 mg/kg dose showed no toxicity. Necropsy of all major organ systems, including small intestine, pancreas, spleen, lymphoid organs, thymus, kidney, liver, heart, lung, ovary, intestine, and bone marrow, was performed by the University of Michigan Veterinary Diagnostic Laboratory.

**Xenograft study**

NGR/nude mice, 6 weeks of age, were obtained from Taconic Biosciences and housed in pathogen-free conditions. Mice were anesthetized by injecting ketamine/xylazine combination at a concentration of 100 mg/kg:10 mg/kg. A total of 10,000 MDA-MB 231 cells were mixed with Matrigel (BD Biosciences) in a 1:1 ratio by volume and injected into both left and right fourth mammary gland fat pads. Mice were randomized into treatment groups once the tumors were palpable. UM-164 was dissolved in a mixture of DMSO/propylene glycol (1:9; Sigma-Aldrich). The volume of administration was 0.05 ml/mouse. The control group received 10 % DMSO/propylene glycol, and the treatment groups received 10 mg/kg, 15 mg/kg, or 20 mg/kg of drug. Mice were treated every other day by intraperitoneal injection, for up to 48 consecutive days. The tumors were monitored twice weekly, and body weight was measured once weekly. Tumor volume was calculated using the following formula: tumor volume = \( l \times b^2 \times 0.5 \), where \( l \) and \( b \) are the largest perpendicular diameters. The mean tumor volume and the tumor weight for each treatment were compared with the vehicle-treated group for statistical significance using a linear mixed effects model, generated for each cell line and tumor measurement (volume and weight) separately with an unstructured correlation matrix. Fixed covariates in the model include group, time, and the interaction of group and time. Quadratic and cubic terms were included for time and the interaction of time and group if needed. A random intercept allowed for baseline variation, and a random coefficient for the slope of time allowed for growth patterns to vary. Pairwise comparisons using contrasts were generated to test the growth difference between groups. All statistical models were performed using SAS 9.4 (SAS Institute). Statistical significance was determined at a threshold of 0.05 unless otherwise stated. Bonferroni multiple comparison corrections were made to adjust for multiple testing threshold of 0.05 unless otherwise stated. Bonferroni multiple comparisons using contrasts were generated to test the growth difference between groups once the tumors were palpable. UM-164 was dissolved in a mixture of DMSO/propylene glycol (1:9; Sigma-Aldrich).

**Results**

**UM-164 inhibits c-Src in an inactive conformation**

All clinically used c-Src inhibitors (e.g., dasatinib, bosutinib, saracatinib) act by binding the active conformation of the kinase. We hypothesized that inhibiting c-Src in a specific inactive conformation (termed DFG-out) would have improved efficacy against TNBCs. Preclinical target validation of c-Src has largely been performed using genetic techniques that ablate the entire c-Src gene, whereas pharmacologic intervention with small molecules (such as dasatinib) inhibits only the kinase catalytic activity. Meanwhile, inhibiting a kinase in the DFG-out inactive conformation can have dramatic effects on the noncatalytic functions of the kinase (24). To test our hypothesis that DFG-out conformation inhibitors of c-Src will be superior to FDA-approved c-Src inhibitors, we synthesized an analogue of dasatinib (termed UM-164; Fig. 1) that binds c-Src and forces a DFG-out inactive conformation (25–27). Crystallographic studies confirmed that UM-164 binds c-Src in the DFG-out inactive conformation (PDB code: 4YBJ; ref. 28).

In biochemical assays, UM-164 is a highly potent inhibitor of c-Src with a binding constant comparable with dasatinib (Supplementary Fig. S1: UM-164 \( K_d = 2.7 \) nmol/L, dasatinib \( K_d = 0.7 \) nmol/L). To confirm that UM-164 is capable of inhibiting the activation of c-Src in vitro, we examined the effect of UM-164 on the c-Src autophosphorylation in two TNBC cell lines (MDA-MB 231 and SUM 149). Inhibition of c-Src autophosphorylation was detected in a concentration- and time-dependent manner (Fig. 1B and C). At 120 minutes, complete abrogation of c-Src autophosphorylation was observed at 50 nmol/L, demonstrating that UM-164 is a potent c-Src inhibitor in vitro.

**UM-164 has potent anti-TNBC activity in vitro**

TNBCs are highly diverse and heterogeneous (29, 30). To approximate the diversity of TNBC, we selected a panel of five TNBC cell lines and determined the expression of activated c-Src by probing the total cell lysate with an antibody that specifically recognizes the activated forms of c-Src (pY419), p-EGFR, and p-p38MAPK (31). Three of the selected cell lines (MDA-MB 231, HCC1937, and MDA-MB 468) have large amounts of activated c-Src, and two (SUM 149 and Hs578T) have relatively lower amounts. Each TNBC cell line in our panel has a large amount of P-p38MAPK (Supplementary Fig. S2A). Notably, our panel includes cell lines previously reported to be both sensitive and resistant to FDA-approved c-Src inhibitors (dasatinib resistant: MDA-MB 468, HCC 1937; dasatinib sensitive: Hs578T, MDA-MB 231, and SUM 149; refs. 11, 29).

We next compared the effect of UM-164 and dasatinib on cellular proliferation using our TNBC cell line panel. UM-164 has potent antiproliferative activity (average GI\(_{50} = 160 \) nmol/L) in all TNBC cell lines tested (Table 1A). Notably, UM-164 is significantly more potent than dasatinib (average GI\(_{50} = 2,300 \) nmol/L). UM-164 has consistent antiproliferative activity across heterogeneous TNBC cell lines, whereas dasatinib only inhibits a subset of this group of cell lines. Moreover, UM-164 is active against a primary, low-passage, patient-derived TNBC cell line VARI-068 (Supplementary Fig. S2B). VARI-068 was derived from a TNBC patient lumpectomy and, while sensitive to UM-164, is relatively resistant to dasatinib. Together, these data are consistent with the poor responses observed in clinical trials for TNBC tumors treated with dasatinib (20, 21).

To confirm that the antiproliferative activity of UM-164 is not due to general cytotoxicity, we examined its effect on the growth of primary human mammary epithelial cells (HMEC) and an immortalized, nonmalignant cell line (MCF-10A). Both HMEC and MCF-10A cells are relatively resistant to the antiproliferative activity of both UM-164 (HMEC GI\(_{50} = 2.8 \) \( \mu \)mol/L; MCF-10A GI\(_{50} = 4.6 \) \( \mu \)mol/L) and dasatinib.

Relative to two-dimensional (2D) substrates (such as polystyrene), three-dimensional (3D) cell growth in extracellular matrix is generally accepted as more closely resembling the natural growth environment for tumor cells. Indeed, 3D cell culture has been proposed as an early predictor for in vitro activity (32–35). Thus, we measured the antiproliferative activities of UM-164 and dasatinib on MDA-MB 231, SUM 149, and VARI-068 cells grown in a 3D matrix of basement membrane extract. We found the
activity of UM-164 was preserved in 3D cell culture (average GI_{50} = 0.3 \text{ mol/L}). In contrast, dasatinib has a striking loss of activity in 3D cell culture (average GI_{50} = 14 \text{ mol/L}; Table 1B). The lack of activity for dasatinib in 3D cell culture with TNBC cell lines has previously been reported (18, 36) and again correlates with the poor outcomes of patients with TNBC treated with dasatinib in clinical trials (20–22).

Comparing the antiproliferative activities against TNBC cell lines grown in 3D, cell culture of primary human cells (e.g., HME cells) can provide an approximate cellular TI (GI_{50} for HMEC/average GI_{50} for TNBC in 3D). The cellular TI can be used to estimate compound efficacy and prioritize compounds for in vivo assessment. UM-164 has a promising TI (8.4), whereas dasatinib's TI is very low (0.14). These data demonstrate that UM-164 has antiproliferative activity that specifically inhibits the growth of TNBC cells.

Table 1. Cellular growth inhibition of UM-164 and dasatinib in TNBC. A, IC_{50} for UM-164 and dasatinib in noncancer and TNBC cell lines grown in 2D culture on polystyrene. B, IC_{50} for UM-164 and dasatinib in selected TNBC cell lines grown in 3D culture on basement membrane extract.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>UM-164 IC_{50} (nmol/L)</th>
<th>Dasatinib IC_{50} (nmol/L)</th>
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<tr>
<td>A</td>
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<td></td>
<td>MCF-10A</td>
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<td></td>
<td>HCC1937</td>
<td>120</td>
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<tr>
<td></td>
<td>Hi578t</td>
<td>9.1</td>
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<tr>
<td></td>
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<td></td>
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<td></td>
<td>VARI-068</td>
<td>800</td>
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Figure 1. Chemical structures of dasatinib and UM-164. A, UM-164 is a dasatinib analogue with an appended trifluoromethyl amide group (colored red) that causes binding to the inactive conformation of c-Src. B and C, MDA-MB 231 and SUM 149 cells were treated with UM-164 for 15, 30, 60, and 120 minutes at the indicated concentrations, and the whole-cell lysate was probed for P-Src/Tyr-419. The blots were stripped and probed for total c-Src and β-actin to control for protein loading.
Kinome-wide profiling of UM-164

We performed chemical genetic profiling of both UM-164 and dasatinib in MDA-MB 231 lysate to identify the kinase targets for both inhibitors. We found that UM-164 and dasatinib have nearly identical target profiles, with the exception of the p38 kinases (Supplementary Fig. S3A). UM-164 is a potent inhibitor of p38α and p38β, whereas no FDA-approved c-Src inhibitor (e.g., dasatinib, bosutinib) potently inhibits both p38α and p38β. The kinome profiling result suggesting that UM-164 is a potent p38 inhibitor was validated in biochemical assays (Supplementary Fig. S1). Consistent with UM-164 being a potent p38 inhibitor, p38MAPK phosphorylation was completely absent in SUM 149 cells treated with 50 nmol/L of UM-164 (Fig. 2).

Dual inhibition of c-Src and p38 kinases

p38MAPK kinase activity is reported to have an important role in the metastasis of TNBC tumors (37). Thus, we hypothesized that UM-164’s ability to potently inhibit the p38 kinases is beneficial with regard to the observed anti-TNBC activities. To test this hypothesis, we examined the effect of combining dasatinib with BIRB-796, a potent and selective inhibitor of the p38 kinase family, on TNBC cell proliferation, migration, and invasion assays. Dasatinib + BIRB-796 accurately constitutes the targets of UM-164 (Supplementary Fig. S3A; ref. 38). We found that the dasatinib + BIRB-796 combination has improved antiproliferative efficacy over either compound dosed alone in 2D, 3D, and spheroid cultures (Supplementary Fig. S3B and S3C; Supplementary Fig. S11).

UM-164 abrogates key TNBC signaling pathways

c-Src regulates several key signaling transduction pathways involved in cell survival and cell proliferation (13–16). To probe the ability of UM-164 to inhibit c-Src-mediated signaling pathways, SUM 149 and MDA-MB 231 cells were treated with UM-164 or dasatinib, and the phospho- and total protein levels of c-Src, p38MAPK, EGFR AKT, and p44/42MAPK (ERK1/2) were examined by Western blot (Fig. 2). As expected for a potent c-Src inhibitor, both UM-164 and dasatinib are efficient at reducing...
the level of activated c-Src (P-Src). At low concentration, we found that UM-164, but not dasatinib, was a potent inhibitor of p38MAPK activation. Both UM-164 and dasatinib robustly reduce phosphorylation of EGFR/Tyr-1068, a known site of EGFR activation by c-Src. Intriguingly, UM-164 reduced phosphorylation of EGFR/Tyr-1068, an autophosphorylation site of EGFR known to be important in TNBC oncogenesis (39). Given that neither UM-164 nor dasatinib directly inhibits EGFR, this reduction in EGFR/Tyr-1068 phosphorylation is likely due to an indirect mechanism. Both dasatinib and UM-164 reduced ERK1/2 signaling, however, UM-164 did so at significantly lower concentrations.

The effect of UM-164 on c-Src-mediated signaling was also examined using the patient-derived VARI-068 cell line. UM-164 efficiently reduced the phosphorylation of these same key signaling pathways in a concentration-dependent manner (Fig. 2C) across diverse TNBC cell lines.

To determine whether the observed signaling differences were due to changes in compound potency or to distinct changes in the signaling pathways, we did a Western blot analysis in SUM 149 cells using IC_{50} values of each inhibitor (Supplementary Fig. S4). At their IC_{50} concentrations, UM-164 decreases activation of EGFR/Tyr-1068, whereas dasatinib has no effect. Thus, we conclude that at least part of UM-164’s excellent anti-TNBC effects are due to the ability for UM-164 to inhibit diverse signaling pathways, including EGFR/Tyr-1068 activation, which is known to be important in TNBC progression (40, 41).

To assess whether the observed signaling pathway changes result from the polypharmacology of UM-164, we examined the impact on TNBC signaling when dasatinib was dosed in combination with p38MAPK inhibitors. SUM 149 cells were treated with dasatinib (Supplementary Fig. S5), UM164, BIRB-796 (a pan-p38 inhibitor), VX-745 (a dual p38α/β inhibitor), Skepinone (a selective p38α inhibitor), or the combination of each p38 inhibitor with dasatinib (Supplementary Fig. S6). The combination of BIRB-796 with dasatinib inhibits p38MAPK phosphorylation comparable with UM-164; however, the combination does not have the same inhibitory effect on the phosphorylation of EGFR/Tyr-1068. These results suggest that the signaling changes manifested by UM-164 are due to factors other than its increased polypharmacology relative to dasatinib.

**UM-164 alters the cellular localization of c-Src**

Inhibiting kinases via an inactive conformation can alter non-catalytic functions of the protein and/or its cellular localization (15, 42). UM-164 is an inhibitor of the DFG-out conformation of c-Src (24), whereas all c-Src inhibitors used clinically bind the active conformation of c-Src. Thus, we hypothesized that some of UM-164’s impressive anti-TNBC activities could be a result of UM-164 forcing c-Src into an inactive conformation. To explore this hypothesis, we performed imaging of c-Src in MDA-MB 468 cells treated with dasatinib or UM-164 [23]. We chose the MDA-MB 468 TNBC cell line because in this cell line, c-Src alone inhibition by dasatinib did not result in growth inhibition. Importantly, while MDA-MB 468 is growth resistant to dasatinib, dasatinib is a highly efficacious inhibitor of c-Src in this cell line, as demonstrated in Supplementary Fig. S10, which shows that c-Src phosphorylation is nearly absent when MDA-MB 468 cells are treated with 50 μmol/L dasatinib. As in most TNBC cell lines (43, 44), c-Src is membrane associated in MDA-MB 468 cells. We observed a profound change in c-Src localization upon treatment with UM-164, but no change in localization upon treatment with dasatinib (Fig. 3). Based on these data, we propose that binding the inactive conformation of c-Src causes its dissociation from the membrane. This plausible mechanism is consistent with our finding that UM-164 (and not dasatinib) reduces EGFR/Tyr-1068 phosphorylation, given that c-Src and EGFR cannot interact when c-Src is not membrane associated.

**Effect of UM-164 on cell apoptosis and cell-cycle progression in TNBC cells**

To further elucidate the antiproliferative mechanisms of UM-164, cell apoptosis markers were examined and a cell-cycle analysis was performed. UM-164 exhibits a dose-dependent decrease in cell proliferation in all of the tested TNBC lines; however, no significant change in the proapoptotic proteins Bax and Bak proteins (45, 46) was observed (Supplementary Fig. S7B). Consistent with this finding, flow cytometry experiments demonstrate that UM-164 treatment of MDA-MB 231 and SUM 149 increased the proportion of G0–G1 cells by 25% and 28%, respectively, and concurrently decreased the fraction of S cells by 16% and 19%, respectively. The fraction of apoptotic cells (sub-G_1) was very low in both treated and untreated MDA-MB 231 and SUM 149 samples, indicating that the decrease in cellular proliferation is not due to apoptosis but to cell-cycle arrest at G_1–S (Supplementary Fig. S7A). Finally, we performed a FACS analysis using Annexin V–FITC and PI staining and observed no apoptotic cells upon treatment with UM-164 (Supplementary Fig. S7C). These findings are consistent with the mechanism of action for UM-164 being more prominently cytostatic rather than cytotoxic.

**UM-164 is an inhibitor of TNBC cell motility and invasion**

c-Src is an important mediator of cell migration signaling pathways through its role in modulating the dynamics of focal adhesions (47, 48). Thus, we examined the effect of UM-164 on
cell motility and invasion using the MDA-MB 231 and SUM 149 cell lines, the latter being characterized by prominent motility (49). We found that UM-164 can suppress both cell motility and invasion with an IC_{50} = 50 nmol/L (Fig. 4A and B). To confirm that the observed effect is not due to cell death, the MDA-MB 231 and SUM 149 cell lines were pretreated with UM-164 for 24 hours. After pretreatment, the viable cells were plated for both motility and invasion. In this control, a similar reduction of migration and invasion was observed, confirming that the observed effect is due to inhibition in the invasive properties of the cells rather than to cell death. Enhanced migratory activity is linked to increased cross activation of c-Src and FAK activity (50, 51). As shown in Fig. 4C and D, FAK phosphorylation is inhibited by UM-164 in SUM 149 cells. Furthermore, phosphorylation of paxillin, which serves as an adaptor protein in cell adhesion and is a substrate for the FAK–Src complex (52, 53), is likewise inhibited by UM-164 in SUM 149 cells.

In vivo efficacy of UM-164 in xenograft models of TNBC

On the basis of the promising in vitro anti-TNBC activity of UM-164, we proceeded to study the efficacy of UM-164 in orthotopic xenograft models of TNBC. Prior to the xenograft study, we assessed the toxicity of UM-164 at two doses: 140 and 40 mg/kg, dosed for 5 consecutive days. Mice dosed with 140 mg/kg showed signs of toxicity. The significant alterations in affected animals were pancreatic degeneration and inflammation, villus blunting, epithelial degeneration, marked lymphoid depletion/atrophy, and erythroid depletion in the bone marrow (Supplementary Fig. S8). However, the treatment group that was injected with the 40 mg/kg dose of UM-164 for 5 consecutive days showed no signs of toxicity in any organ.

We next performed a xenograft study using NCr/nude mice implanted with MDA-MB 231 and SUM 149 cell lines. Once the tumors became palpable, the mice were randomized into control and treatment groups. Mice were injected intraperitoneally with either drug (10 and 20 mg/kg in both xenograft studies; a 15 mg/kg dose was added to the SUM 149 xenograft studies) or vehicle every other day (n = 5 for each group). At the selected doses of UM-164, there was no significant weight loss or gross abnormalities observed in the treated animals, even after 52 days of treatment (Fig. 5C and D). However, tumor growth was significantly inhibited in both the 10 mg/kg and 20 mg/kg dose groups compared with the vehicle-treated group (P < 0.026 and P < 0.004, respectively; Fig. 5A and B). We performed an additional MDA-MB 231 xenograft study comparing 20 mg/kg dasatinib with 20 mg/kg UM-164 (Supplementary Fig. S9). We found

![Figure 4](image-url)

**Figure 4.**

UM-164 treatment inhibits cell motility and invasion through c-Src-mediated FAK activation. A, MDA-MB 231 and SUM 149 cells were seeded in serum-free medium into the upper part of the Matrigel invasion chambers. The lower chambers were filled with medium containing 1% serum to promote invasion through the Matrigel. Cells were treated immediately with DMSO vehicle or UM-164 as indicated. Twenty-four hours later, noninvading cells were removed and the invaded cells were fixed and stained. Cells in all the wells were counted. The average numbers of invading cells from three independent experiments are shown in the graph. Each experiment was performed in triplicate. The image shown represents one entire well from a single experiment. B, MDA-MB231 and SUM 149 cells were seeded at a density of 500 to 600 cells/well on a lawn of blue microscopic fluorescent beads. Cell motility is indicated by the dark track left by the cell movement. The average track area covered by the cells within a 24-hour time period is graphed (*P < 0.0001 control vs. treatment group). Error bars represent SD of triplicate experiments. C and D, UM-164 alters FAK and paxillin activation. MDA-MB 231 (C) and SUM 149 (D) cells were treated with increasing concentrations of UM-164 for 24 hours. The whole-cell lysates were then analyzed to determine the amount of P-FAK (Y576/577) and P-Paxillin (Y118), relative to total FAK and paxillin.
that UM-164 was highly efficacious in this model, whereas dasatinib was ineffective. These data are consistent with previous work reporting that dasatinib is ineffective in xenograft models of TNBC (1, 2).

To explore the molecular basis for the antitumor effects of UM-164, total protein from tumor lysates of the control and treated groups was analyzed for protein levels of P-p38MAPK, P-EGFR/Tyr-1068, and P-Src. A significant decrease in the phosphorylation of P-Src, P-p38MAPK, and EGFR/Tyr-1068 was observed at 20 mg/kg treatment group in MDA-MB 231 tumor samples (Fig. 5E and F; Supplementary Fig. S12), showing the same mechanisms as previously observed in our in vitro experiments.

**Discussion**

In preclinical studies, c-Src has been shown to play a pivotal role in breast cancer progression, metastasis, and angiogenesis. In the clinic, however, limited efficacy of the existing c-Src inhibitors has tempered the enthusiasm for c-Src inhibition as a strategy in TNBC (20, 21). Motivated by the biologic potential of c-Src inhibition, we developed a novel c-Src inhibitor (UM-164) that specifically binds the DFG-out inactive conformation of target kinases. We hypothesized that binding the inactive kinase conformation could improve pharmacologic outcomes for our c-Src inhibitor (24, 54). In this study, we demonstrate that UM-164 is highly active against diverse TNBC cell lines in vitro and in vivo and furthermore, we describe a conserved signaling modulation both in vivo and in vitro.

Kinome profiling of UM-164 identified p38α and p38β as being potently inhibited by UM-164, and we observed dose-dependent inhibition of p38MAPK kinases both in vitro and in vivo. p38α activity has been demonstrated to promote TNBC oncogenesis via increased invasion, inflammation, and angiogenesis (55). The role of p38α in breast cancers has been studied in depth and very recently proposed as a therapeutic target in TNBC (56, 57). ER-negative and mutant-p53 cell lines (which comprise the majority of TNBCs) were observed to be more sensitive to small-molecule inhibition of p38α than ER-positive and wt-p53 cell lines (58). The exact contribution of p38β to MAPK signaling has not been fully elucidated; however, there is growing evidence...
that p38δ activity correlates with increased proliferation of TNBC cells (57, 59, 60). Thus, we posited that adding p38 kinase inhibition would improve the anti-TNBC effects of c-Src kinase inhibitors. We observed that dasatinib had increased anti-TNBC activity when dosed in combination with BIRB-796, a pan-p38 kinase inhibitor. Taken together, we propose that one advantage of UM-164 over dasatinib is the potent inhibition of p38 kinases by UM-164. Importantly, UM-164 represents the only kinase inhibitor that potently inhibits c-Src, p38δ, and p38β while not potently inhibiting the majority of the kinase.

We found that UM-164 inhibits diverse signaling pathways previously reported to be important in TNBC oncogenesis. In addition to preventing activation of UM-164’s direct targets (c-Src and p38 kinases), UM-164 also efficiently reduces activation of EGFR (at both Tyr-845 and Tyr-1068). AKT, and ERK1/2, all of which are not direct targets of UM-164. Other c-Src inhibitors (e.g., dasatinib) are effective at reducing activation of EGFR-845, which is a known site of EGFR activation by c-Src. Tyr-1068, however, is a site of autophosphorylation of EGFR, and activation of EGFR-1068 has been reported to be important in TNBC oncogenesis.

Upon binding UM-164, the target kinase is forced into a DFG-out inactive conformation. Adopting inactive conformations can lead to changes in regulatory domain accessibility as well as changes in cell localization of a kinase. A recent study demonstrated a differential localization of c-Src in TNBCs versus non-TNBCs (17). Membrane-bound c-Src was detected in 78% of TNBCs versus 38% of non-TNBCs. These data suggest that the spatial positioning of c-Src at the membrane likely plays a role in the development and/or progression of aggressive breast cancers (17). We found that TNBC cells treated with UM-164 have altered localization of c-Src. Specifically, upon treatment with UM-164, c-Src is not found on the membrane, but in cytosolic puncta. In contrast, TNBC cells treated with dasatinib showed no change in c-Src localization. We propose that the altered localization of c-Src is consistent with c-Src adopting an inactive conformation and no longer being able to associate with the membrane (membrane association requires an active, open conformation of c-Src).

UM-164 displays cytostatic and noncytotoxic antiproliferation activity on TNBC cells. In addition, we found that UM-164 can suppress cell migration and invasion in TNBC cells. The antimigration and anti-invasion properties of UM-164 provide the initial basis for further research on the antimitastatic activity of this drug. In in vivo models, UM-164 displayed good efficacy with limited toxicity. At a very high dose (140 mg/kg), mild toxicity was observed, but no toxicity was observed at lower doses over long time periods (40 mg/kg and 20 mg/kg dosed over 52 days). The promising in vivo TI is consistent with the excellent cellular TI observed with diverse TNBC cell lines. The significant decrease in in vivo tumor growth correlated with a decrease in p-P38MAPK, p-EGFR-1068, and p-Src in the recovered tumors. Given that UM-164 is active in vivo with excellent anti-TNBC activity and limited toxicity as a single agent, UM-164 has potential for relevance in the clinic. Together, our results suggest that in inactive conformation binding, c-Src/p38 dual kinase inhibitors are a promising strategy for developing the first targeted therapeutic strategy against TNBC.

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

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