Characterization of a Dual Rac/Cdc42 Inhibitor MBQ-167 in Metastatic Cancer

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Abstract

The Rho GTPases Rac (Ras-related C3 botulinum toxin substrate) and Cdc42 (cell division control protein 42 homolog) regulate cell functions governing cancer malignancy, including cell polarity, migration, and cell-cycle progression. Accordingly, our recently developed Rac inhibitor EHop-016 (IC50, 1,100 nmol/L) inhibits cancer cell migration and viability and reduces tumor growth, metastasis, and angiogenesis in vivo. Herein, we describe MBQ-167, which inhibits Rac and Cdc42 with IC50 values of 103 and 78 nmol/L, respectively, in metastatic breast cancer cells. Consequently, MBQ-167 significantly decreases Rac and Cdc42 downstream effector p21-activated kinase (PAK) signaling and the activity of STAT3, without affecting Rho, MAPK, or Akt activities. MBQ-167 also inhibits breast cancer cell migration, viability, and mammosphere formation. Moreover, MBQ-167 affects cancer cells that have undergone epithelial-to-mesenchymal transition by a loss of cell polarity and inhibition of cell surface actin-based extensions to ultimately result in detachment from the substratum. Prolonged incubation (120 hours) in MBQ-167 decreases metastatic cancer cell viability with a GI50 of approximately 130 nmol/L, without affecting noncancer mammary epithelial cells. The loss in cancer cell viability is due to MBQ-167–mediated G2–M cell-cycle arrest and subsequent apoptosis, especially of the detached cells. In vivo, MBQ-167 inhibits mammary tumor growth and metastasis in immunocompromised mice by approximately 90%. In conclusion, MBQ-167 is 10× more potent than other currently available Rac/Cdc42 inhibitors and has the potential to be developed as an anticancer drug, as well as a dual inhibitory probe for the study of Rac and Cdc42.

Introduction

Rho GTPases regulate migration and invasion, cytoskeletal organization, transcriptional regulation, cell-cycle progression, apoptosis, vesicle trafficking, and cell-to-cell and cell-to-extracellular matrix adhesions. The Rho GTPases Rac and Cdc42 are potent inducers of actin polymerization and extension of actin structures at the leading edge of motile cells. In addition, Cdc42 plays a critical role in cell polarity and thus promotes directed and persistent migration (1).

Studies have implicated hyperactive Rac and Cdc42 with increased cancer cell survival, proliferation, and invasion as well as in Ras and other oncogene-mediated transformation (2, 3). Furthermore, oncogenic cell surface receptors, such as tyrosine kinase, cytokine, and G protein–coupled receptors, activate Rac and Cdc42 via regulation of their upstream effectors guanine nucleotide exchange factors (GEF; refs. 4, 5). Accordingly, Rac and Cdc42 proteins are generally not mutated in cancer but rather overexpressed or hyperactivated (6, 7). Even though approximately 9% of melanomas contain an activating Rac (P29S) mutation (8), and the hyperactive splice variant Rac1b is overexpressed in some cancers (7), a majority of the Rac and Cdc42 in human cancer are activated because of upregulated GEFs (9). Therefore, targeting the binding of GEFs to Rac and Cdc42 is a rational strategy to inhibit their activation.

Of the direct downstream effectors of Rac and Cdc42, p21–activated kinases (PAK) are overexpressed in a number of cancers and contribute to cancer transformation and progression by regulating key cellular functions, including cytoskeletal organization, cell migration, adhesion, growth, and development (10, 11). Therefore, a number of PAK inhibitors have been developed as anticancer therapeutics. However, these have been limited by specificity, bioavailability, and toxicity and have yet to successfully complete clinical trials (12).

Both Rac and Cdc42 have also been used as drug targets, although to our knowledge, none of the available inhibitors have entered clinical studies. NSC23766 was the first Rac inhibitor shown to block the interaction of Rac with the GEFs Trio and Tiam1; however, its high effective concentrations...
(IC50 > 75 μmol/L) limits its therapeutic use (13). Therefore, we developed a panel of putative Rac and Cdc42 inhibitors (14), which led to the identification of EHop-016 (15). EHop-016 blocks the interaction of the GEF Vav2 with Rac and inhibits Rac activity at an IC50 of approximately 1.1 μmol/L, which makes it approximately 100× more potent than NSC23766. EHop-016 also inhibits Cdc42 activity at concentrations of ≥10 μmol/L, without affecting Rho activity (16).

We further reported that at 25 mg/kg body weight (BW), EHOp-016 reduces mammary tumor growth, metastasis, and angiogenesis without apparent toxicity in nude mice. The pharmacokinetic analysis of EHop-016, after oral and intraperitoneal (i.p.) administration, demonstrated a bioavailability of approximately 30% with an average half-life of approximately 4.5 hours, indicating its potential as a cancer therapeutic in breast cancer (4, 14–18) and subsequently in other types of cancer (19–21).

Although other small-molecule inhibitors, such as the NSC23766 derivative Aza-1 (inhibits both Rac and Cdc42) and CID2950007/MIL141 (selective for Cdc42), are currently available, they are effective in the micromolar range (22–24). Our goal to develop a Rac/Cdc42 inhibitor with improved activities led to the identification of MBQ-167. Compared with EHOp-016, MBQ-167 is a 10× more potent inhibitor of Rac and a 100× more potent inhibitor of Cdc42, which resulted in an enhanced inhibition of cancer malignancy.

Materials and Methods

Synthesis of MBQ-167

All reagents were purchased from Sigma-Aldrich Chemical Company. The synthesis of 3-azido-9-ethyl-9H-carbazole 3 is described (Fig. 1).

Step 1. To a solution of 2.10 g (10.0 mmol) 9-ethyl-9H-carbazol-3-yl-amine 1 in 20 mL water, 2.0 mL (40.0 mmol) of concentrated H2SO4 was added. When all the amine was converted to the sulfate (green precipitate), additional 10 mL water was added and the suspension cooled to 0°C to 5°C in an ice water bath. A solution of 0.828 g (12.0 mmol) sodium nitrite (NaNO2) in 5 mL of water was added drop-wise and the mixture was stirred for 1 hour. Next, a solution of 0.780 g (12.0 mmol) of sodium azide (NaN3) in 5 mL of water was added drop-wise and stirred continuously for 2 to 8 hours (Caution!: This step should be carried out in a well-ventilated hood due to the formation of extremely toxic and potentially explosive hydrazoic acid.). After completion of the reaction, the reaction mixture was warmed to 25°C, 30 mL of ethyl acetate and 20 mL of distilled water were added; and after vigorous mixing, the layers were separated. The organic layer was extracted with 10 mL brine, separated, dried on sodium sulfate, filtered, and concentrated on a rotary evaporator. After silica gel chromatography using 3:1 hexanes/ethyl acetate as the eluent, 1H-NMR (CDCl3, 400 MHz) δ 1.47 (t, J = 7.22 Hz, 3H), 3.48 (q, J = 7.22 Hz, 2H), 7.26–7.33 (m, 6H), 7.36 (dd, J = 1.76, 8.60 Hz, 1H), 7.41 (d, J = 8.84 Hz, 1H), 7.46 (d, J = 8.32 Hz, 1H), 7.53 (t, J = 7.32 Hz, 1H), 7.93 (s, 1H), 8.20 (d, J = 7.84 Hz, 1H), 8.15 (d, J = 1.80 Hz, 1H); 13C (CDCl3, 100 MHz) δ 13.8, 37.9, 108.7, 108.9, 117.9, 119.5, 120.8, 122.5, 123.0, 123.2, 126.6, 127.1, 128.3, 128.5, 128.8, 129.0, 131.3, 138.0, 139.8, 140.7. LRGC-MS (rel%): [M]+ 338 (37), [M-C6H5]+ 310 (53), [M-C4H9N3]+ 295 (100), [M-C6H5N4]+ 179 (34).

Step 2. Synthesis of 1-(9-ethyl-9H-carbazol-3-yl)-5-phenyl-1H-1,2,3-triazole 6 (MBQ-167): In a 25-mL three-neck round-bottom flask containing phenylacetylene 0.11 g (1.1 mmol) under a nitrogen atmosphere, a solution of ethylmagnesium bromide in THF (1.1 mmol) was added drop-wise at 25°C. After the Grignard reagent was added, the mixture was heated at 50°C for 15 minutes and cooled to 25°C. A solution of 0.24 g (1.0 mmol) of azide 3 in THF (1.0 mol/L) was added drop-wise and heated to 50°C for 1 hour. After quenching with 10% ammonium chloride, the products were extracted with ethyl acetate (3×). The organic layer was washed with 10 mL of brine, separated and dried on sodium sulfate, filtered and concentrated on a rotary evaporator to obtain crude material (0.33 g). The crude oil was purified via silica gel chromatography to obtain 0.29 g (0.86 mmol = 86%) of 1-(9-ethyl-9H-carbazol-3-yl)-5-phenyl-1H-1,2,3-triazole MBQ-167 as a white solid. Purity (>98%) was verified by TLC, NMR spectroscopy, and gas chromatography/mass spectrometry (GC/MS): Rf = 0.26 (3:1, hexane/ethyl acetate); 1H-NMR (CDCl3, 400 MHz) δ 1.47 (t, J = 7.22 Hz, 3H), 3.48 (q, J = 7.22 Hz, 2H), 7.26–7.33 (m, 6H), 7.36 (dd, J = 1.76, 8.60 Hz, 1H), 7.41 (d, J = 8.84 Hz, 1H), 7.46 (d, J = 8.32 Hz, 1H), 7.53 (t, J = 7.32 Hz, 1H), 7.93 (s, 1H), 8.20 (d, J = 7.84 Hz, 1H), 8.15 (d, J = 1.80 Hz, 1H); 13C (CDCl3, 100 MHz) δ 13.8, 37.9, 108.7, 108.9, 117.9, 119.5, 120.8, 122.5, 123.0, 123.2, 126.6, 127.1, 128.3, 128.5, 128.8, 129.0, 131.3, 138.0, 139.8, 140.7. LRGC-MS m/z (rel%): [M]+ 338 (37), [M-C6H5]+ 310 (53), [M-C4H9N3]+ 295 (100), [M-C6H5N4]+ 179 (34).

Cell culture

MDA-MB-231, MCF7 (ATCC), GFP-tagged bone metastatic variant of MDA-MB-435 (GFP-HER2-BM, characterized in (25), from Dr. Danny Welch, The University of Kansas Cancer Center, Kansas City, KS), and MCF10A mammary epithelial cells (ATCC) were cultured and maintained as previously described (16). MDA-MB-231 and MCF7 cell lines were obtained in 2000, the MCF10A cell line was purchased in 2013, and the GFP-HER2-BM cell line was a gift from Dr. Danny Welch in 2008. The cell lines were authenticated by ATCC in 2015.

Rac and Cdc42 activation assays

For the IC50 curves: Rac1/2/3 and Cdc42 activation was determined as described (16), using a G-LISA kit (Cytoskeleton, Inc.). MDA-MB-231 cell lysates were prepared from 24-hour MBQ-167 treatment by combining attached and detached cell populations (n = 3). Four-parameter dose–response IC50 curves were fitted using the nonlinear regression function of GraphPad Prism.

In addition, Rac, Cdc42, or Rac activation was determined by pull-downs using the P21-binding domain (PBD) of PAK or Rho-binding domain of Rhotekin as described (2, 16). The GFP-bound active Rac, Cdc42, or Rho was detected by Western blotting (n = 3).

Western blot analysis

Total cell lysates or pull-downs were Western blotted using routine procedures. The primary antibodies used were: Rac (Rac1,2,3), Cdc42, Bcl-xl, Bcl-2, Mcl-1, PAK1, PAK2, p-PAK1 (T423)/PAK2 (T402), p-PAK1 (S199/204)/PAK2 (S192/197), p-PAK1 (S144/204)/PAK2 (S141), LIM kinase (LIMK1), p-LIMK1/2 (Tyr507/Thr508), cofilin, p-cofilin (S3), STAT3, p-STAT3 (Y705), p-P-38 MAPK (T180/Y182), p-ERK (T202/204)
MDA-MB-231 cells were treated with vehicle or MBQ-167 at 250 or 500 nmol/L for 24 hours. Cells were fixed, permeabilized, and stained with rhodamine phalloidin to visualize F-actin and with p-tyrosine or vinculin to visualize focal adhesions, as described (2). Fluorescence micrographs were acquired at 600× in an Olympus BX40 fluorescence microscope using a Spot digital camera.

Wound-healing scratch assay. MDA-MB-231 cells plated on 6-well plates at equal cell density were incubated in 10% FBS until confluent. The media were changed to 2% FBS, and a single scratch was made in the center of the monolayer culture with a pipet tip. MBQ-167 was added at 0, 250, or 500 nmol/L immediately following wounding. Images were digitally acquired from an Olympus microscope (4× magnification) at 0, 8, 12, and 24 hours after staining

Mammosphere formation assay. As described (26), equal numbers of MDA-MB-231 cells treated with vehicle or MBQ-167 were seeded in ultra-low attachment plates (Corning) at a density of 500 cells per well in serum-free mammary epithelium basal medium (Lonza). Mammospheres were counted after 4 days of incubation in 0 or 250 nmol/L MBQ-167 at 37°C, 5% CO₂. Mammosphere-forming efficiency was calculated as the number of mammospheres divided by the number of cells seeded per well and expressed relative to vehicle controls.

Cell viability assays. As described (16), equal numbers of MDA-MB-231, GFP-HER2-BM, or MCF10A cells were incubated in 0 to 1 μmol/L MBQ-167 for 120 hours. The CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) was used according to the manufacturer’s instructions. This assay allows the quantification of the viability of both attached and detached cells in the same well. GI₅₀ was determined as 100 × (T – T₀)/(C – T₀) = 50 (T = the optical density of drug treatment after 120 hours, T₀ = the optical density at time zero, and C = the optical density of the untreated controls). Curves were fitted using the 4-parameter logistic nonlinear regression models in GraphPad Prism software.

Cell-cycle progression. MDA-MB-231 cells were incubated with 0 or 250 nmol/L MBQ-167 for 48 hours, and all cells (detached and attached) were stained with PI, as described (27). Cell-cycle stage was analyzed using a 4-color flow cytometer (FACSCalibur, BD Biosciences).

A total of 20,000 events were analyzed for each sample. List-mode files were collected using Cell Quest software 3.3 and analyzed using the Flow Jo software v10.7 (BD Biosciences).

Apoptosis assay. Apoptosis was measured using a Caspase-Glo3/7 Luminescence Assay Kit as per manufacturer’s instructions (Promega, Corp.), as described (17). Following treatment of equal numbers of cells with vehicle or MBQ-167 for 24 hours, Caspase-3/7 Glo reagent was added and incubated at room temperature for 60 minutes. Caspase-3/7 activities were determined by quantifying luminescence.

Annexin V staining. Apoptotic cells were detected by fluorescence microscopy of Annexin V/Cy3-18–stained cells as per manufacturer’s instructions (Sigma-Aldrich). Briefly, GFP-MDA-MB-231 cells grown on coverslips were treated with vehicle or 250 or 500 nmol/L MBQ-167 for 6 hours and stained with Annexin V/Cy3-18 in binding buffer (10 mmol/L HEPES/NaOH, pH 7.5, 0.14 mol/L NaCl, 2.5 mmol/L CaCl₂) for 15 minutes at room temperature. Coverslips were washed in binding buffer and fixed with 3.7% paraformaldehyde prior to fluorescence microscopy. Images were digitally acquired from an Olympus inverted fluorescence microscope.

Animal protocol. All animal studies were conducted under approved protocol #A8180112 Institutional Animal Care and Use Committee, in accordance with the NIH Guideline for the Care and Use of Laboratory Animals. Female athymic nu/nu mice, 4 to 5 weeks old (Charles River Laboratories, Inc.), were maintained under pathogen-free conditions in HEPA-filtered cages.

Tumor establishment. GFP-HER2-BM cells (~5 × 10⁵) in Matrigel (BD Biosciences) were injected at the fourth right mammary fat pad under isoflurane inhalation (1%–3% in oxygen using an inhalation chamber at 2 L/min) to produce orthotopic primary tumors, as described (17). After tumor establishment (1-week post-occlusion), animals were randomly divided into treatment groups (n = 6).

Administration of MBQ-167. Mice were treated with vehicle [12.5% ethanol, 12.5% Cremerophor (Sigma-Aldrich), and 75% 1× PBS, pH 7.4] or 1 or 10 mg/kg BW MBQ-167 by i.p. injection in a 100 μL volume 3× a week. Treatments continued until sacrifice at day 65.

Whole-body fluorescence image analysis. Mammary tumor growth was quantified as changes in the integrated density of GFP fluorescence, as described (28). Mice were imaged on day 1 of treatment administration and once a week thereafter for 65 days, using the FluorVivo Small Animal In Vivo Imaging System (INDEC Systems, Inc.). Tumor fluorescence intensities were analyzed using the ImageJ software (NIH, Bethesda, MD). Relative tumor growth was calculated as the integrated density of fluorescence of each tumor on each day of imaging relative to the integrated density of fluorescence of the same tumor on day 1 of treatment, as described (17). As suggested (29), optimal tumor growth was calculated as %ΔT/C = (ΔT/ΔC) × 100 when ΔT > 0, ΔT is the average tumor size on day 65.
treated mice – average tumor size on day 01 of treated mice. 8C is average tumor size on day 65 of control mice – average tumor size on day 01 of control mice. Tumor growth delay was calculated as the percentage by which the treated group tumor size is delayed in attaining a specified number of doublings (from day 1) compared with controls using: \( \left( \frac{T}{C} - 1 \right) \times 100 \), where \( T \) and \( C \) are the median times in days for treated and control groups to double in tumor size.

**Analysis of metastases**

Following sacrifice, lungs, kidneys, livers, bones, and spleens were excised and immediately stored in liquid N2. Stored organs were thawed and analyzed by fluorescence microscopy, as described (17).

**Liver enzyme assays**

Frozen stored livers were thawed and homogenized to measure alkaline phosphatase (ALP) and alanine transaminase (ALT) activities using colorimetric assay kits from Abcam and Cayman Chemicals respectively, as per manufacturer’s instructions.

**Statistical analysis**

Statistical analyses used Microsoft Excel and GraphPad Prism, and differences were considered statistically significant at \( P < 0.05 \).

**Results**

We previously characterized the Rac inhibitor EHop-016 with an IC\(_{50}\) of 1.1 \( \mu \)mol/L in highly metastatic breast cancer cell lines and determined that the carbazole fragment of EHop-016 was a key contributor to its activity. Additional observations from molecular docking studies suggested that EHop-016 binds to Rac in a bent U-shaped conformation (4, 16). This led to the synthesis of 9-ethyl-3-(5-phenyl-[1,2,3]triazol-1-yl)-9\( H \)-carbazole (MBQ-167; Fig. 1), which contains the essential carbazole group, and due to its ortho-substitution on a central triazole ring is forced to adapt the desired bent shape. In silico modeling predicts that MBQ-167 binds deeper into the putative binding pocket of Rac with potential H-bonding with Asn39, which is present in the switch region of both Rac and Cdc42 (Supplementary Fig. S1).

**MBQ-167 affects cancer cell polarity**

Human breast cancer cells were visualized by bright-field microscopy following MBQ-167 treatment. At \( \geq 100 \) nmol/L, starting at 6 hours, MBQ-167 induced a loss of polarity in metastatic breast cancer cells. Treatment with 500 nmol/L MBQ-167 for 24 hours resulted in approximately 95% cell rounding and detachment from the substratum in metastatic MDA-MB-231 cells (Fig. 2A, top). Moreover, MBQ-167 induced this phenotype in multiple mesenchymal cancer cell types including GFP-HER2-BM, MDA-MB-468, and Hs578t human breast cancer cells (data not shown), as well as MiaPaCa-2 pancreatic cancer cells, SKOV3 ovarian cancer cells, AGS and NCI-N87 gastric cancer cells, and SH-SYS5 neuroblastoma cells (Supplementary Fig. S2). On the other hand, noncancer mammary epithelial MCF10A and epithelial breast cancer MCF7 cells were resistant to MBQ-167 and remained polarized and attached to each other and the substratum (Fig. 2A, bottom).

To further investigate the effect of MBQ-167 on MDA-MB-231 cells, we performed immunofluorescence microscopy following 0 to 500 nmol/L MBQ-167 to detect actin dynamics (by rhodamine phalloidin) and focal adhesions (by anti-p-tyrosine and anti-vinculin). MBQ-167 rearranged the actin cytoskeleton and focal adhesions to result in loss of cell polarity and attachment to the extracellular matrix (ECM), with a marked reduction in both Rac-regulated lamellipodia/invadopodia and Cdc42-induced microspikes and filopodia (Fig. 2B and C). Moreover, in MBQ-167–treated cells, the focal adhesions were reduced from the cell edge and rearranged from the cytoskeleton to the center of the rounded detaching cells.

**MBQ-167 inhibits Rac and Cdc42 activation in metastatic cancer cells**

Because MBQ-167 disrupted the Rac and Cdc42-mediated cytoskeletal architecture, we investigated its potential to inhibit both Rac and Cdc42 activation in metastatic cancer cells. MDA-MB-231 and GFP-HER2-BM cells were treated for 24 hours with vehicle or MBQ-167, and the detached and attached cells (\( \geq 50\% \) for each population) were recovered and lysed immediately.

![Figure 1. Design and synthesis of MBQ-167.](image-url)

**Synthesis of MBQ-167.** Reaction conditions: (i) conc. H\(_2\)SO\(_4\), NaNO\(_2\), water \( 0\degree C \) to \( 5\degree C \), 1 hour; (ii) NaN\(_3\), \( 0\degree C \), 1 hour, 76%; (iii) THF, 3, 50\% \( 0\degree C \), 1 hour; (iv) NH\(_4\)Cl (aq), 86%.

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**Figure 1.** Design and synthesis of MBQ-167.

**Figure 2.** Immunofluorescence microscopy images of MBQ-167 effects on breast cancer cell morphology. (A) Bright-field images of MDA-MB-231 human breast cancer cells treated with vehicle or 500 nmol/L MBQ-167 for 24 hours. (B) Immunofluorescence microscopy images of MBQ-167 effects on MDA-MB-231 human breast cancer cell actin dynamics (rhodamine phalloidin) and focal adhesions (anti-p-tyrosine and anti-vinculin). (C) Immunofluorescence microscopy images of MBQ-167 effects on MDA-MB-231 human breast cancer cell microspikes and filopodia. (Top) Vehicle control; (Middle) 500 nmol/L MBQ-167; (Bottom) 500 nmol/L MBQ-167 + 10\% FBS.

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**Table 1.** Summary of MBQ-167 effects on breast cancer cell morphology.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>500 nmol/L MBQ-167</th>
<th>500 nmol/L MBQ-167 + 10% FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin dynamics</td>
<td>Intact</td>
<td>Disrupted</td>
<td>Disrupted</td>
</tr>
<tr>
<td>Focal adhesions</td>
<td>Intact</td>
<td>Disrupted</td>
<td>Disrupted</td>
</tr>
<tr>
<td>Microspikes and filopodia</td>
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<td>Disrupted</td>
<td>Disrupted</td>
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</tbody>
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**Figure 2B.** Immunofluorescence microscopy images of MBQ-167 effects on MDA-MB-231 human breast cancer cell actin dynamics (rhodamine phalloidin) and focal adhesions (anti-p-tyrosine and anti-vinculin). (A) Bright-field images of MDA-MB-231 human breast cancer cells treated with vehicle or 500 nmol/L MBQ-167 for 24 hours. (B) Immunofluorescence microscopy images of MBQ-167 effects on MDA-MB-231 human breast cancer cell actin dynamics (rhodamine phalloidin) and focal adhesions (anti-p-tyrosine and anti-vinculin). (C) Immunofluorescence microscopy images of MBQ-167 effects on MDA-MB-231 human breast cancer cell microspikes and filopodia. (Top) Vehicle control; (Middle) 500 nmol/L MBQ-167; (Bottom) 500 nmol/L MBQ-167 + 10\% FBS.
Equal amounts of protein were subjected to activation assays for Rac or Cdc42. Following treatment with 250 nmol/L MBQ-167 for 24 hours, the attached population of MDA-MB-231 cells demonstrated approximately 25% decrease in Rac activation, whereas the detached cells were more responsive with approximately 75% decrease (Fig. 3A). At earlier times (6 hours), treatment with 250 or 500 nm MBQ-167 induced a 10% to 20% inhibition in Rac activity in the attached cell population, whereas the detached population demonstrated approximately 40% to 50% inhibition (Supplementary Fig. S3). Similarly, Cdc42 activity was inhibited by 60% in the attached cells and 78% in the detached MDA-MB-231 cells following 250 nmol/L MBQ-167 for 24 hours (Fig. 3C). These results indicate that both Rac and Cdc42 activities are inhibited while the cells are still attached to the substratum but the more responsive cells get detached first. Incubation of MDA-MB-231 cells with 500 nmol/L MBQ-167 for 24 hours resulted in approximately 90% detachment of cells and a parallel decrease in Rac and Cdc42 activities, demonstrating that a majority of the cells were responsive to MBQ-167. Similarly, the GFP-HER2-BM highly metastatic breast cancer cell line responded to MBQ-167 by inhibition of Rac and Cdc42 activities significantly in the detached cell populations (Supplementary Fig. S4A and S4B). However, the nonmetastatic, more epithelial MCF7 cell line, which did not respond to MBQ-167 by the cell detachment phenotype, was also insensitive to MBQ-167 treatment in Rac inhibition (Supplementary Table S2). This may be due to differences in the Rac and Cdc42 GEFs that are expressed and activated in metastatic breast cancer cell lines.
(MDA-MB-231) compared with the less metastatic, more epithelial MCF7 cell line.

Next, as a measure of the specificity of MBQ-167 as a Rac/Cdc42 inhibitor, the IC\textsubscript{50} values for Rac and Cdc42 activation were also determined following 24 hours in MBQ-167 using combined attached and detached populations. Results show that MBQ-167 inhibits Rac 1/2/3 activity in the MDA-MB-231 cells with an IC\textsubscript{50} of 103 nmol/L and Cdc42 activity with an IC\textsubscript{50} of 78 nmol/L (Fig. 3D and E). Because the IC\textsubscript{50} for Rac inhibition by EHop-016 is 1.1 \textmu mol/L and Cdc42 inhibition is approximately 8 \textmu mol/L (16), MBQ-167 is 10× more potent than EHop-016 for Rac inhibition and 100× more potent for Cdc42 inhibition.

To indirectly determine the specificity of MBQ-167 for inhibiting Rac activation by GEFs, Rac activity was determined from our previously characterized MDA-MB-435Br cells expressing a control vector or constitutively active Rac1 (G12V) (2). However, MBQ-167 did not affect the Rac activity of this cell line expressing a Rac1 (G12V) (data not shown), indicating that the constitutive activation of Rac1 desensitizes the cells to inhibition by MBQ-167. We have also determined that MBQ-167 inhibits the interaction of Rac and Cdc42 with their GEFs by pull-down assays using a Rac1 or Cdc42 (G15A) mutant, as described (ref. 16; data not shown). Moreover, as demonstrated from activation assays for Rac, Cdc42, and Rho from attached and detached cell populations, MBQ-167 did not affect the related GTPase Rho activation in both cell populations of MDA-MB-231 and GFP-HER2-BM metastatic cancer cells (Supplementary Table S1 and Supplementary Figures S4A-C).

MBQ-167 inhibits Rac and Cdc42 downstream effectors

To investigate the effect of MBQ-167 on Rac/Cdc42 signaling, we investigated its effect on the major Rac/Cdc42 downstream effector PAK. The phosphorylation status of several PAK residues was analyzed by Western blotting, as a measure of its activity. At 250 nmol/L, 24-hour treatment with MBQ-167 inhibited PAK1 and PAK2 phosphorylation at the T423/T402 and S199/S192 residues in the detached population of MDA-MB-231 cells. Except for PAK1\textsuperscript{T423}, phosphorylation of all of these residues was significantly decreased in the attached population as well. Even though PAK1\textsuperscript{T423} phosphorylation was not inhibited in the attached cells, the reduction in the homologous PAK2 phosphorylation sites indicates a preferential inhibition of PAK2 in the attached cells (Fig. 4A and B). Interestingly, MBQ-167 induced a dramatic increase in the phosphorylation of the PAK1\textsuperscript{S164} (Fig. 4A). However, overall PAK activity is inhibited by MBQ-167 because the activating phosphorylation (Y507/T508) of the direct PAK substrate LIMK and the inactivating phosphorylation (S3) of coflin (actin depolymerization factor), a downstream effector of LIMK, were both decreased following MBQ-167

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Figure 4.
The effect of MBQ-167 on signaling downstream of Rac and Cdc42. A, The effect of MBQ-167 on PAK1 and PAK2 phosphorylation as measured by Western blotting for p-PAK1 (T423)/p-PAK2 (T402), p-PAK1 (S199)/p-PAK2 (S192), and p-PAK1 (S144)/p-PAK2 (S140) levels in MDA-MB-231 cells after 24 hours of treatment in 0 or 250 nmol/L MBQ-167. Data for separate attached (Att) and detached (Det) populations are shown. Left, representative Western blot analyses (n = 3). Right, relative PAK activity following 24 hours in 0 or 250 nmol/L MBQ-167. Data for separate attached (A) and detached (D) populations are separated, lysed, and equal protein used for Western blotting. Representative Western blot analysis of total or p-LIMK1/2 (Y507/T508) following 24 hours in 0 or 250 nmol/L MBQ-167 (n = 2) is shown. C, Representative Western blot analysis of total or p-cofilin (S3) of equal amounts of total protein lysates following 4, 12, or 24 hours in 250 nmol/L MBQ-167 (n = 3). Separated attached (A) and detached (D) populations are shown for 12 and 24 hours of MBQ-167 treatment. D, Effect of MBQ-167 on Stat3 phosphorylation and expression. Representative Western blot analysis is shown for p-STAT3 (Y705) and total STAT3 expression in GFP-HER2/C6 cells after 24-hour treatment with vehicle or 100, 200, or 500 nmol/L MBQ-167. Representative Western blot analysis (left) and quantification (right). n = 3, *P < 0.05. Error bars represent ±SEM. E and F, Effect of MBQ-167 on cell migration. E, Effect of MBQ-167 on MDA-MB-231 cellular migration as measured by a Transwell assay. Images are representative of three independent experiments. The graph below shows quantification of 20 microscopic fields per treatment per experiment of PI-stained cells that migrated to the underside of the membrane through 8-μm diameter pores (n = 3, **P < 0.05). Error bars represent ±SEM. F, Effect of MBQ-167 on cell migration in a scratch assay. MDA-MB-231 cells plated at equal density were subjected to a scratch in the center and treated with MBQ-167 at 0, 250, or 500 nmol/L. Micrographs were digitally acquired at 0 and 24 hours and the distance of the scratch quantified for each treatment and presented relative to the distance at time 0. Results are an average of two technical replicates and two biologic replicates for each treatment ±SD. *P < 0.05, **P < 0.01. G, Effect of MBQ-167 on mammosphere-forming efficiency in MDA-MB-231 cells. MDA-MB-231 cells treated with 0 or 250 nmol/L MBQ-167 were subjected to mammosphere assays for 4 days. Cells were treated with MBQ-167 only once before placing on the mammosphere medium. Mammosphere-forming efficiency was calculated as the percentage of the number of mammospheres divided by the number of cells seeded per well. n = 3; ***P < 0.001. Error bars represent ±SD.
treatment. The decrease in coillin phosphorylation was evident after 12 hours following 250 nmol/L MBQ-167 (Fig. 4C), indicating activation of coillin, which accounts for the observed actin cytoskeletal restructuring (Fig. 2B). Moreover, in the MCF7 cell line, which did not respond to MBQ-167 by cell detachment or inhibition of Rac activation, MBQ-167 also did not affect PAK activity (Supplementary Fig. S5).

Rac activity has also been shown to directly stimulate the activity of the transcription factor, STAT3 (30). As seen in Fig. 4D and Supplementary Fig. S6A, MBQ-167 decreased STAT3 activity following 24-hour exposure in both the attached and detached populations of MDA-MB-231 and GFP-HER2-BM cells. However, MBQ-167 did not affect MAPK activities, either p38-MAPK or the p42/44 MAPK, as well as Akt activities as demonstrated by Western blotting with phospho-specific antibodies (Supplementary Fig. S7).

MBQ-167 inhibits cell migration and mammosphere formation

As Rac/Cdc42 and its downstream effector PAK directly regulate cell migration, we investigated the effect of MBQ-167 on MDA-MB-231 cell migration. The detached and attached cell populations were recovered following 18 hours in MBQ-167 and equal numbers of cells (vehicle-treated and MBQ-167-treated attached and detached populations) were used for a Transwell assay for 6 hours. This short incubation time is not sufficient for MDAMB-231 cell division (doubling time of ~38 hours) or inhibition of cell viability (Supplementary Fig. S8B and S8C). Therefore, the assay only measures the efficiency of cell migration.

MBQ-167 treatment reduced directed migration of the attached MDA-MB-231 cell population by approximately 60% to 70% at 250 and 500 nmol/L. In the detached population, MBQ-167 (250 and 500 nmol/L) inhibited cell migration by approximately 90% in a statistically significant manner (Fig. 4E). In the more metastatic GFP-HER2-BM cell line, 250 and 500 nmol/L MBQ-167 inhibited cell migration by 80% to 90% in both attached and detached cells (Supplementary Fig. S6B). These results were confirmed in a wound-healing assay where 250 and 500 nmol/L MBQ-167 treatment for 24 hours resulted in statistically significantly approximately 80 and 90% inhibition of wound closure, respectively (Fig. 4F).

STAT3 and Rac activities have been implicated in enhanced breast cancer stem cell–like properties and therapy resistance (31, 32). Therefore, we evaluated the capacity of MBQ-167 to target cancer stem cell populations using a mammosphere formation assay. Addition of MBQ-167 once for 4 days reduced the mammosphere-forming efficiency of MDA-MB-231 cells by approximately 50% (Fig. 4C).

MBQ-167 inhibits cell survival

MBQ-167 induces a phenotype characterized by cell rounding, loss of lamellipodia, and eventual detachment from the surface substratum (Fig. 2). Therefore, we tested the potential of MBQ-167 to induce anoikis: apoptosis due to dissolution of integrin-mediated cell to ECM attachments (33). It should be emphasized that the metastatic cancer cells that detach in response to MBQ-167 following 24-hour treatment are viable, as evidenced by Trypan blue exclusion from live cells (Supplementary Fig. S8A). These detached cells also have the capacity for regrowth when replated without MBQ-167 (data not shown). As shown in Supplementary Fig. S8B, MDA-MB-231 cells are 100% viable at concentrations ≤300 nmol/L for 24 hours. At 24-hour MBQ-167 treatment, approximately 75% of MDA-MB-231, GFP-HER2-BM, and MCF7 breast cancer cells, as well as the MCF10 mammary epithelial cells, are viable even at 5 μmol/L MBQ-167 (Supplementary Fig. S8C). Prolonged treatment for 48, 96, and 120 hours with MBQ-167 results in cell detachment from the substratum and loss of cell viability (Supplementary Fig. S8B).

Figure 5A shows an MTT assay following MBQ-167 treatment for 120 hours for the metastatic cancer cells MDA-MB-231 and GFP-HER2-BM and the noncancer mammary epithelial cells MCF10A, when we obtained almost 100% cell death for all cell types at high concentrations (1000 nmol/L) of MBQ-167. This assay includes both detached and attached cells in the case of the metastatic cancer cells. MBQ-167 at 120 hours decreased the viability of MDA-MB-231 and GFP-HER2-BM cells with a GI50 of 110 and 150 nmol/L, respectively. However, the GI50 for the MCF10A epithelial cells at 350 nmol/L MBQ-167 was approximately 3× higher (Fig. 5A). It should be noted that MBQ-167 inhibits Rac and Cdc42 activities with IC50 values in the approximately 100 nmol/L range at 24 hours when MDA-MB-231 and GFP-HER2-BM, MCF7, and MCF10 cells are still viable (Supplementary Fig. S8B and S8C). Next, we determined whether the effect of MBQ-167 on cell viability is due to cell-cycle arrest by flow cytometry. As shown in Fig. 5B, MBQ-167 significantly arrested the cell cycle of MDA-MB-231 cells in the G2–M phase.

To evaluate whether cell-cycle arrest was accompanied by an increase in apoptosis, we measured the activity of the effector caspase-3/7 in whole-cell populations (both attached and detached). A dose-dependent increase was observed for caspase-3/7 activity in both MDA-MB-231 and GFP-HER2-BM cell lines after 24 hours in MBQ-167 (Fig. 5C, left). To determine whether MBQ-167 induces anoikis, the relative levels of caspase-3/7 activities were analyzed in the attached and detached MDA-MB-231 cell populations following 24 hours at 250 nmol/L MBQ-167. There was a significant 15-fold increase in caspase-3/7 activities in the detached population compared with the attached population of MDA-MB-231 cells (Fig. 5C, right). In Supplementary Fig. S9, we validate the effect of MBQ-167 on apoptosis by showing increased Annexin V staining in MDA-MB-231 cells following 250 or 500 nmol/L MBQ-167. In 500 nmol/L MBQ-167, cells also demonstrated the classic blebbing associated with apoptosis. Finally, to explore the effect of MBQ-167 on mitochondrial apoptosis, we analyzed the expression of the prosurvival proteins Bcl-2, Bcl-xL, and Mcl-1 by Western blotting. We found a significant decrease in the expression of prosurvival proteins after 24 hours with 250 nmol/L MBQ-167 (Fig. 5D).

MBQ-167 inhibits mammary tumor progression in nude mice

To test the effect of MBQ-167 on mammary tumor progression, nude mice were used to establish mammary fat pad tumors from GFP-HER2-BM cells. One week following mammary tumor establishment, the mice were treated 3 × a week with 0, 1, or 10 mg/kg BW MBQ-167 by intraperitoneal injection for 65 days. The vehicle-treated mice demonstrated a linear increase in tumor growth, whereas MBQ-167–treated mice demonstrated a statistically significant reduction in tumor growth (Fig. 6A). At sacrifice, 1.0 mg/kg BW of MBQ-167 resulted in approximately 80% reduction in tumor growth and the 10 mg/kg BW MBQ-167 treatment resulted in approximately 95% reduction in tumor growth. As EHop-016 only exerts approximately 40% reduction...
of tumor growth at 10 mg/kg BW (17). MBQ-167 is $10 \times$ more effective than EHop-016 (Fig. 6A).

The optimal percentage change in tumor size, which takes into account the individual tumor growth for each treatment, showed that the tumors from mice treated with 1 mg/kg BW MBQ-167 demonstrated a 58% growth change compared to controls (100%), whereas tumors from mice treated with 10 mg/kg BW MBQ-167 demonstrated only a 9% increase in tumor size (Supplementary Table S3). These data indicate that even though there was no tumor regression during the time of study, there was a drastic reduction in tumor growth in the 10 mg/kg BW MBQ-167–treated mice.
Figure 6.
In-vivo efficacy of MBQ-167. Mammary fat pad tumors were established in nude mice by inoculating $5 \times 10^5$ GFP-HER2-BM cells. Following 1 week, mice were treated with vehicle control or 1.0 or 10.0 mg/kg BW MBQ-167 3 x a week by i.p. injection. A, Left, Representative excised tumors following 0, 1.0, or 10.0 mg/kg BW MBQ-167. Right, Average relative tumor growth from fluorescence in situ images up to 65 days following 0, 1.0, or 10 mg/kg BW MBQ-167 (3 x a week; $n = 6$). B, Representative fluorescence micrographs of lungs, spleens, and kidneys from vehicle or MBQ-167-treated mice following necropsy. C, Mouse weights from 1 to 65 days. D and E, Liver enzyme activities following MBQ-167 treatment. Following necropsy, livers were harvested, lysed, and subjected to ALP activity (D) or ALT activity assays (E). $n = 4$. Error bars represent ±SEM.
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when the tumor growth delay was quantified, the control mice doubled in 8 days, and the MBQ-167–treated mice demonstrated similar doubling times for both treatments (10 and 11 days). However, at the second doubling (22), there was a delay in tumor growth of the MBQ-167–treated mice, where the tumors from control-treated mice reached 2 in 14.5 days, whereas the tumors from 1 and 10 mg/kg BW–treated mice were similar to each other by reaching 2 in 30 days. By the third doubling, there was also a disparity between the 2 MBQ-167 treatments, where the tumors from control mice reached 2 in 27 days, while the 1 mg/kg BW MBQ-167–treated tumors took 57 days to reach the same size, and the 10 mg/kg BW–treated tumors never reached 2 in tumor growth. Similarly, only the control tumors reached 2 in 33 days, whereas the tumors from both MBQ-167–treated (1 and 10 mg/kg BW) mice never reached this size. This result demonstrates a significant inhibition in tumor growth initiated after 24 days of MBQ-167 treatment (Fig. 6A, Supplementary Table S4). This drastic reduction in tumor growth following MBQ-167 treatment resulted in no metastases to all of the organs tested (Fig. 6B).

The mice from this study were also examined once a week for potential toxicity. The mice treated with vehicle or MBQ-167 did not show any significant weight loss or phenotypic changes during the 65-day study (Fig. 6C). At necropsy, livers were harvested, lysed, and subjected to liver enzyme assays as a test for potential toxic effects. Figure 6D shows that MBQ-167 does not affect ALP activity in the livers of MBQ-167–treated nude mice (1 and 10 mg/kg BW). However, the liver ALT levels were significantly increased by 10 mg/kg BW MBQ-167 treatment, indicating a potential metabolism of MBQ-167 at higher concentrations (Fig. 6E).

Discussion

Herein, we describe the biochemical characterization of MBQ-167, which inhibits Rac and Cdc42 activation. Rac and Cdc42 are pivotal signaling intermediates whose dysregulation has been implicated in oncogenic transformation, cancer progression, metastasis, and multiple diseases (1, 6, 34). Recent studies, including our own, have shown that targeting Rac and Cdc42 has potential for metastatic cancer therapy. However, the current small-molecule inhibitors of Rac and Cdc42 are only effective at micromolar concentrations (4, 16, 17, 22).

The aim of this study was to develop a new, more potent inhibitor of Rac and Cdc42 with an IC50 < 1.0 μmol/L. Accordingly, we report that the novel derivative MBQ-167 shows improved efficacy in metastatic breast cancer cells by inhibiting Rac activity with an IC50 of 103 nmol/L and Cdc42 with an IC50 of 78 nmol/L. This concentration-dependent response of breast cancer cells to MBQ-167 demonstrates that MBQ-167 is specifically inhibiting the biochemical activation of the Rac and Cdc42. However, MBQ-167 did not inhibit the Rac activity of cells expressing dominant active Rac1 (G12V) indicating that MBQ-167 is specifically inhibiting Rac1 activation. Moreover, our data show that MBQ-167 does not affect the activation of the related GTPase Rho. Therefore, we expect this new molecule to be useful as a tool for probing Rac and Cdc42 function in responsive cell types.

We also found that MBQ-167 was an effective inhibitor of the Rac and Cdc42 downstream effector PAK. Interestingly, MBQ-167 induced an increased autophosphorylation of S144 (PAK1), the activation of which is not essential but contributes to the activity of the PAK domain (35). This result may be due to a feedback mechanism compensating for Rac/Cdc42 inhibition. Nevertheless, phosphorylations in the PAK kinase domains, as well as the PAK effectors LIMK and coflin, a potent regulator of actin filament dynamics during cell migration (36), were significantly inhibited by MBQ-167. Therefore, we conclude that overall, MBQ-167 inhibits PAK activity, contributing to a reduction in actin cytoskeletal extensions and cell migration. As Rac and Cdc42 also regulate Wiskott–Aldrich syndrome protein (WASP) family members that contribute to actin dynamics (37, 38), MBQ-167 may exert additional inhibitory effects on the cytoskeleton.

Furthermore, Cdc42 regulates cell polarity through the polarity protein partitioning defective proteins (PAR6, 3), which stabilize microtubules during directed migration (39). Using a haploid derivative of the yeast strain BY4741, where the essential gene Cdc42 was knocked out conditionally via a tetracycline-inducible promoter (40), we show that MBQ-167 exerts a similar phenotype to the cells with reduced Cdc42 expression. Supplementary Fig. S10 demonstrates that Cdc42 knockout abolishes cell polarity where the yeast buds (daughter cells) are not aligned symmetrically with the mother cells. A similar nonpolar effect was also observed on yeast cell budding in the presence of MBQ-167. This mutant phenotype was more pronounced in the yeast cells with both Cdc42 knockout and MBQ-167 treatment demonstrating that MBQ-167 may inhibit the highly conserved yeast Cdc42 to regulate cell polarity. As expected, MBQ-167 treatment also enhanced the growth inhibitory effects of Cdc42 and Cdc42. The regulation of microtubule dynamics by Rac and Cdc42 activities is also critical for cell-cycle progression, where Cdc42, and thus PAK, controls mitotic spindle formation and cell-cycle progression in G2–M (41, 42). Therefore, the observed MBQ-167–mediated metastatic breast cancer cell-cycle arrest in the G2–M phase may be a consequence of Rac/Cdc42/PAK inhibition by MBQ-167.

We also show that the decreased Rac/Cdc42/PAK activities, cell viability, loss of cell polarity, and detachment from the substrate in response to MBQ-167 is limited to cancer cells that have undergone epithelial-to-mesenchymal transition (EMT) but not to epithelial cancer or noncancer cells. This selective response to MBQ-167 may be due to the differential expression and activities of Rac and Cdc42 GEFs in different breast cancer cell lines (43), where only a subset of the approximately 80 known Rac and Cdc42 GEFs is expected to be expressed and activated in the metastatic breast cancer cell lines that were investigated. Moreover, the currently available Rac/Cdc42 inhibitors also inhibit only a subset of Rac/Cdc42 GEFs. For instance, NSC23766 inhibits only Tiam-1 and Tri activation of Rac, whereas EHOP-016 is a specific inhibitor of the Vav/Rac interaction (13, 16). Therefore, MBQ-167 may inhibit only a subset of Rac/Cdc42 GEFs that are preferentially expressed/activated in the more metastatic mesenchymal-like cancer cells lines. We are currently investigating the specific GEFs inhibited by MBQ-167.

In addition, the relative insensitivity of epithelial-like cells to MBQ-167 may be because the hemidesmosomes in epithelial cells are primarily regulated by α6β4-integrin–mediated attachments to the intermediate filament cytoskeleton, which are not directly regulated by Rac and Cdc42. In contrast, the focal adhesions in mesenchymal cells, which are regulated by multiple integrin subunits to form attachments with the actin cytoskeleton,
are under Rac/Cdc42/PAK regulation (42, 44). Therefore, the observed reduction and reorganization of focal adhesions in MBQ-167-treated cancer cells may reflect the inhibition of the Rac/Cdc42/PAK–regulated integrin-mediated focal adhesion assembly at the cell leading edge.

Focal adhesions are not only important for directed migration, disruptions in proper regulation of cell adhesion to the ECM can result in anoikis, apoptosis induced by inadequate or inappropriate cell–matrix interactions (45). In this context, Rac1 has been shown to confer anoikis resistance (46). Our data with caspase assays and reduction in prosurvival BCl2 homology proteins validate the hypothesis that MBQ-167 acts as an anticancer agent by inducing anoikis. Data show that only the detached breast cancer cells respond to MBQ-167 increased caspase-3/7 activities, indicating that cell detachment precedes apoptosis signaling, as would be predicted during anoikis. Moreover, we show that MBQ-167 selectively decreases the viability of cancer cell lines that have undergone EMT, without affecting the noncancer cell line MCF10A. This cell line specificity could be due to differences in the dependence on Rac/Cdc42/PAK signaling and the accompanying integrin engagement and focal adhesion assembly, in the more migratory mesenchymal cells compared with the epithelial cells. In addition, MBQ-167 may have similar effects in multiple other cancers, including a number of ovarian, gastric, pancreatic, and neuroblastoma cell lines that have undergone EMT. Since EMT is associated with more stem cell–like properties, therapy resistance, and disease recurrence (47), MBQ-167 has the potential to reduce therapy resistance. Moreover, the fact that MBQ-167 is effective against the KRAS-mutant MiaPaCa-2 cell line demonstrates its ability to target oncogenic RAS–dependent cancers.

MBQ-167 also inhibits STAT3 phosphorylation, a Rac-regulated transcription factor shown to be active in several cancers (48). As STAT3 activity increases the expression of several genes involved in cell-cycle progression, its decrease in activity may contribute to the observed cell-cycle arrest by MBQ-167 treatment. Importantly, STAT3 transcriptionally regulates all 3 of the prosurvival BCL-2 family genes analyzed in this study (49). Furthermore, several reports show that cancer stem cell–like properties are dependent on STAT3 activity (50). Accordingly, MBQ-167 decreases the mammosphere-forming efficiency of MDA-MB-231 cells by approximately 50%. These results suggest that MBQ-167 may be further effective as an anticancer therapeutic by targeting cancer stem cell–like populations; however, more studies are needed to demonstrate that MBQ-167 specifically inhibits cancer stem cell activity.

Finally, we show that MBQ-167 reduces mammary fat pad tumor size starting as early as 3 weeks following treatment, with a 91% reduction by 2 months at a nontoxic concentration of 10 mg/kg BW. The drastic reduction in mammary tumors growth also resulted in a 100% inhibition of metastases to all organs tested, probably because less cells were shed by the small tumors. As evidenced by the in vitro data, the reduced tumor size in response to MBQ-167 treatment is predicted to be due to inhibition of Rac/Cdc42/PAK signaling ultimately leading to a loss in cell viability, growth, and polarity causing the cells to detach from the tumor and undergo anoikis. As we did not observe any metastases in mice treated with MBQ-167, any cells detached from the primary tumor probably go through anoikis and do not survive in the circulation.

Taken together, we have shown that MBQ-167 is an effective Cdc42 and Rac inhibitor that significantly decreases downstream signaling and cancer-promoting cell functions to ultimately reduce mammary tumor growth with 10× more potency than our first described Rac inhibitor EHop-016. However, the effects of MBQ-167 on the metastatic cancer cell phenotype, where the cells detach from the substratum to ultimately undergo apoptosis by anoikis mechanisms, may be due to the additional effects of MBQ-167 on integrin signaling or alternate mechanisms, which will be explored in future investigations. Nevertheless, the dramatic effect of MBQ-167 on mouse mammary tumor growth warrants further development of MBQ-167 as an anticancer therapeutic.

Disclosure of Potential Conflicts of Interest
E. Hernandez-O’Farrill, C. Vlaar, S. Dharmawardhane, and Linette Castillo-Pichardo have a conflict of interest due to provisional patent application: 1,5-disubstituted 1,2,3-triazoles compounds and method of using the same. Ser. No.: 62/328,282, Filing Date: April 27, 2016.

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Characterization of a Dual Rac/Cdc42 Inhibitor MBQ-167 in Metastatic Cancer

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