Truncated Actin-Targeting Macrolide Derivative Blocks Cancer Cell Motility and Invasion of Extracellular Matrix

Bhavin V. Pipaliya, Daria N. Trofimova, Rebecca L. Grange, Madhu Aeluri, Xu Deng, Kavan Shah, Andrew W. Craig, John S. Allingham,* and P. Andrew Evans*

ABSTRACT: Cancer metastasis is a complex process involving highly motile tumor cells that breach tissue barriers, enter the bloodstream and lymphatic system, and disseminate throughout the body as circulating tumor cells. The primary cellular mechanism contributing to these critical events is the reorganization of the actin cytoskeleton. Mycalolide B (MycB) is an actin-targeting marine macrolide that can suppress proliferation, migration, and invasion of breast and ovarian cancer cells at low nanomolar doses. Through structure–activity relationship studies focused on the actin-binding tail region (C24–C35) of MycB, we identified a potent truncated derivative that inhibits polymerization of G-actin and severs F-actin by binding to actin’s barbed end cleft. Biological analyses of this miniature MycB derivative demonstrate that it causes a rapid collapse of the actin cytoskeleton in ovarian cancer cells and impairs cancer cell motility and invasion of the extracellular matrix (ECM) by inhibiting invadopodia-mediated ECM degradation. These studies provide essential proof-of-principle for developing actin-targeting therapeutic agents to block cancer metastasis and establish a synthetically tractable barbed end-binding pharmacophore that can be further improved by adding targeting groups for precision drug design.

INTRODUCTION

Cancer metastasis, in which malignant cells spread from the primary site to colonize distant organs, accounts for over 90 percent of cancer-related deaths. In many patients, metastasis has already occurred by the time they are diagnosed with primary cancer. Consequently, this mechanism represents one of the most significant challenges for successful cancer treatment. Cancers that overexpress human epidermal growth factor receptor 2 (HER2) are invasive, with high rates of metastasis and poor prognosis. HER2-driven metastasis is particularly prevalent in subsets of breast and ovarian cancers. Although many signaling pathways are implicated in metastasis, the engine driving metastasis is fuelled by rapid and dynamic actin polymerization.

In an analogous manner to microtubules, which are targeted by some of the best classes of natural product-derived cancer chemotherapeutics, the actin cytoskeleton is targeted by many natural products that elicit extremely potent cytotoxic effects in cancer cells. Unfortunately, clinical use of these agents is impeded by their low natural abundance, structural complexity, and their inability to discriminate between tumor cells and normal tissues. In a program directed toward identifying novel small molecules that target actin, we have focused on the design, synthesis, and biological evaluation of truncated analogues of the actin-depolymerizing natural product, mycalolide B (1, MycB), which was isolated from the marine sponge Mycale sp. MycB (1) is a macrolide toxin that suppresses motility and invasion of HER2+ breast (HCC1954) and ovarian (SKOV3) cancer cell lines at low nanomolar doses and halts cell growth completely at higher doses (200 nM). Like several other natural actin-targeting macrolides, MycB (1) consists of a structurally complex macrolactone (the “ring”) and a long aliphatic side chain (the “tail”) that terminates with an (E)-N-methyl-N-vinylformamide moiety (MVF) (Figure 1A). X-ray crystallographic analysis of actin–macrolide complexes, in combination with structure–activity relationship studies, indicate that the ring appears to be important for making the initial interaction with actin on a large and shallow hydrophobic surface that is exposed in both its globular (G-actin) and filamentous (F-actin) state (Figure 2). The tail component intercalates into the narrow hydrophobic cleft splitting subdomains 1 and 3 at the barbed end of actin, which forms a critical interface during actin

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subunit polymerization, and is the binding site for a myriad of actin-binding proteins.12,13

While a recent total synthesis of MycB (1) was achieved in more than 50 total steps,14 the structural complexity and limited availability from natural sources (89 mg of MycB (1) was isolated from 1.8 kg of sponge)15 impeded its development as an anticancer agent. We and others have recognized that an appropriately designed and optimized synthetic analogue of the macrolide tail should inhibit G-actin polymerization, destabilize F-actin, and display similar levels of cytotoxicity to that exhibited by the natural product.16,17 Unfortunately, the simplified tail analogues that have been developed to date are substantially less potent, and their ability to inhibit cancer cell migration and invasion has not been evaluated.9,16−18

■ RESULTS AND DISCUSSION

Synthesis of Mycalolide B Analogues. Guided by the crystal structure of the actin−MycB complex (Figure 2, PDB ID: 6MGO), we sought to identify the MycB (1) pharmacophore that would become the focus for future precision drug design against the “actin addiction” of cancer metastasis. To accomplish this, we directed our efforts toward addressing the following questions: What design features are required to offset the binding free energy loss derived from the removal of the macrocycle and retain the ability to block actin polymerization and disrupt existing actin filaments? Does the actin-disrupting activity of the analogue suppress migration and invasion of metastatic cancer cells ex vivo? Is the analogue conducive to large-scale synthesis and functionalization with linkers for bioconjugation to ligand-targeted drug delivery systems?19,20

With these questions in mind, we prepared a panel of >30 MycB (1) “tail” analogues that explored variations of the C24 substituents, the C24 and C30 esters, the N-vinylformamide substituent, and the stereochemistry at C30 (see Table S2 in the Supporting Information for pertinent details). All analogues were evaluated biochemically for inhibition of G-actin polymerization and F-actin depolymerization. The synthetic strategy for the construction of the most potent truncated analogue (2) is highlighted in Figure 1B. This particular analogue was tested in HER2+ cancer cells for its effects on the actin cytoskeleton structure and functions related to cancer cell metastasis. By determining its cocystal structure with G-actin, we are able to provide a structural explanation for its activity and a framework for designing even more potent analogues.

The synthesis of 2 was initiated with the preparation of the C29−C34 fragment 7 in five steps from the lactate derived enantioselectically enriched ketone 321 (Scheme 1A). Paterson asymmetric anti-aldol reaction between the (E)-boron enolate derived from benzoyloxy ketone 3 with (R)-3-(benzoyloxy)-2-methylpropanal (4)22 in the presence of chlorodicyclohexylborane and dimethylethylamine afforded the anti-aldol adduct 5 with excellent yield and good diastereoselectivity (dr = 9:1 determined by 500 MHz 1H NMR).23,24 Methylation25 of the secondary alcohol of the secondary alcohol 5 with (CH3)3OBF4 (i.e., Meerwein’s salt) in the presence of proton sponge (MilliporeSigma) followed by a one-pot benzylation and ketone reduction using lithium borohydride afforded the diol 6 in 76% yield over two steps. Oxidative cleavage of the diol 6 with sodium periodate gave the corresponding aldehyde, which was treated with the vinyl organocerium reagent to afford the allylic alcohols 7 and 8 in 55% and 27% yield, respectively, over two steps. The undesired diastereomer 8 could be separated and converted to the desired diastereomer 7 via the following two-step sequence: Oxidation of the secondary alcohol 8 using Dess-Martin periodinane (DMP) followed by a stereoselective reduction with sodium borohydride in the presence of cerium(III) chloride furnished the desired secondary alcohol 7 in 80% yield and with significantly improved diastereoselectivity (dr = 9:1 determined by 500 MHz 1H NMR).

The synthesis of the C24−C28 fragment 12 commenced from the commercially available enantioselectically enriched homoallylic alcohol 9 (Scheme 1B). Benzylation of secondary alcohol 9 using sodium hydride and benzyl bromide gave the benzyl ether 10 in 87% yield. Ozonolysis of olefin 10 provided the aldehyde, which was subjected to the Leighton asymmetric crotylation using the chiral cis-crotylsiline generated in situ from (Z)-but-2-en-1-yltrichlorosilane and (1S,2S)-2-tert-butyl-6-(((2-(methylamino)cyclohexyl)amino)methyl)phenol (L9) in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), to furnish the homoallylic alcohol 11 in excellent yield and diastereoselectivity (dr ≥ 19:1 determined by 500 MHz 1H NMR).26 The secondary alcohol 11 was methylated and the benzyl ether oxidatively cleaved to enable the protection of the
resulting secondary alcohol as the TBS ether 12 for selective deprotection later in the synthesis. The construction of the two fragments set the stage to examine the cross-coupling and the completion of the MycB truncated analogue 2 (Scheme 2). The fragments 7 and 12 were subjected to a Type II alkene cross-metathesis using 10 mol % Hoveyda-Grubbs 2nd generation catalyst to furnish the cross-coupled product, 27−29 which was reduced with diimide, generated in situ from 2,4,6-triisopropylbenzenesulfonyl hydrazide 30,31 and triethylamine, to afford the secondary alcohol 13 in 65% yield over two steps. Yamaguchi esterification of the secondary alcohol 13 using the mixed anhydride derived from (R)-tetrahydrofuran-2-carboxylic acid and 2,4,6-trichlorobenzoyl chloride in the presence of 4-dimethylaminopyridine (DMAP), followed by hydrogenolysis of the terminal benzyl ether, furnished the primary alcohol 14 in excellent yield (87% over two steps). The key vinyl iodide 15 was then prepared via a three-step sequence in 70% overall yield. Dess-Martin periodinane mediated oxidation of the primary alcohol 14 to the aldehyde, followed by Takai olefination 32,33 with anhydrous CrCl2 and iodoform, and subsequent cleavage of the TBS ether with 3 N hydrochloric acid, afforded the required vinyl iodide 15. The formamide moiety was then installed using a copper(I)-catalyzed Goldberg coupling of vinyl iodide 15 with N-ethylformamide in the presence of 1,10-phenanthroline and cesium carbonate in dimethylacetamide at elevated temperature. 35 Yamaguchi esterification of the C24 secondary alcohol with the mixed anhydride obtained from 4-azidobutyric acid, followed by azido reduction using Lindlar’s catalyst under an atmosphere of hydrogen furnished the primary amine 2 in 64% yield over three steps. Hence, the synthesis of the truncated analogue 2 was accomplished from the enantiomerically enriched ketone 3 in 11.7% overall yield via this convergent and modular sequence.

**Biochemical and Biological Activity of MycB Analogues.** A quantitative analysis of the inhibition of G-actin polymerization by analogue 2 was carried out using the pyrene−actin fluorescence assay, with MycB (1) as a positive control. The polymerization of 9 μM skeletal muscle G-actin was monitored by the increase in fluorescence over the duration of 600 s after the addition of actin polymerization buffer at 50 s. Representative traces of actin polymerization in the presence of increasing concentrations of MycB (1) or analogue 2 are shown in Figure 3A,B. These data show that analogue 2 caused a...
concentration-dependent decrease in both the rate and the final yield of the polymerized actin. The inhibitory concentration of analogue 2 required to decrease the rate of G-actin polymerization by half (IC$_{50}$) was 200 ± 15 nM, while the IC$_{50}$ for MycB (1) was 20 ± 15 nM (Figure S1). Thus, although the molecular weight and number of stereocenters in analogue 2 are approximately half that of MycB (1), it retains substantial inhibitory effects on actin polymerization.

To assess the ability of analogue 2 to disrupt preassembled actin polymers, we monitored the decrease in the fluorescence of 0.2 μM pyrene-F-actin immediately after the addition of increasing concentrations of MycB (1) or analogue 2. A comparison of the concentration–response curves in Figure 3C,D indicates that MycB (1) is a more potent F-actin depolymerization agent than analogue 2. This outcome reinforces previous findings that the macrocyclic ring is a major contributor to the F-actin-severing capability of barbed-end binding natural products like MycB (1). However, all concentrations of analogue 2 clearly stimulate rapid F-actin fluorescence decay within seconds of addition, indicating that some of the F-actin depolymerization activity of analogue 2 involves filament severing. Moreover, the relative amount of analogue 2 required to decrease the pyrene fluorescence to the same baseline level as MycB (1) was similar to the associated G-actin polymerization inhibition. These data show that, although less potent, a synthetic barbed-end binding analogue can elicit the same bioactivities as the natural product.

On the basis of our recent finding that MycB (1) treatment led to rapid disruption of F-actin cytoskeleton in SKOV3 cells, we also examined the ability of analogue 2 to disrupt F-actin in SKOV3 cells. Upon treatment of SKOV3 cells with analogue 2 at 1 μM or above for 1 h, cortical F-actin and membrane projections began to collapse and large F-actin aggregates began to form, which is consistent with the outcome with 0.1 μM MycB (1) (Figure 4). Smaller effects on F-actin were observed upon treatment with analogue 2 at a 0.1 μM dose, although some aggregates were detectable near the cell cortex. This indicates that analogue 2 enters the cell quickly and can disrupt intact F-
actin polymers in cancer cells, leading to loss of their typical motile phenotype, such as a prominent leading-edge projection.

To test the longer-term effects of analogue 2, we performed a dose response assay of SKOV3 cell invasion using a modified wound closure assay with an overlay of extracellular matrix (ECM) proteins (Matrigel). Treatment with analogue 2 at 5 or 10 μM caused significant inhibition of SKOV3 cell invasion over 48 h (Figure 5A). At the higher dose, treatment with analogue 2 resulted in comparable suppression of cell invasion to that of MycB (1) at 1 μM (Figure 5B).

Since cancer cells require F-actin-based protrusions with extracellular matrix degradation activity (invadopodia) to close the wound area in these invasion assays, we performed assays to measure the effect of analogue 2 and MycB (1) on invadopodia activity in SKOV3 cells. Plating of SKOV3 cells on coverslips coated with DQ-collagen, a quenched form of fluorescent collagen, the invadopodia-derived matrix metalloproteinases (MMP) can digest DQ-collagen, leading to increased fluorescence (ECM degradation). Confocal images from these assays revealed ECM degradation in vehicle-control-treated cells, but not in those treated with an MMP inhibitor (Figure S2). Treatments with MycB (1) or analogue 2 led to F-actin disruption and loss of ECM degradation (Figure S2). Quantification of these results revealed that the treatment with analogue 2 at doses as low as 0.1 μM significantly reduced ECM degradation by SKOV3 cells (Figure 5C). Together, these data provide the first demonstration of potent antimetastatic activity by a truncated actin-targeting natural product analogue.

Crystal Structure of Analogue 2 Bound to Actin. To provide a structural explanation for these activities of analogue 2, and to provide a framework for designing more potent analogues, we crystallized analogue 2 bound to G-actin and determined its X-ray crystal structure to a resolution of 1.7 Å (Figure 6A). The data clearly demonstrate that the analogue 2 forms an extended conformation within the cleft separating actin subdomains 1 and 3, which mimics most barbed-end binding actin toxins. This site is where the DNase I loop of the lower longitudinal actin subunit would bind during F-actin polymerization.

The actin−2 binding interface is formed by 12 residues that make hydrophobic contacts along the central aliphatic region of analogue 2, and by two water-bridged hydrogen bonds between the terminal N-ethylformamide group and amino acid residues Ile136 and Ala170 deep within the cleft (Figure 6B and Figure S3). The (R)-tetrahydrofuran ester side chain at C30 also interacts with a network of water molecules that bridge to the side chain hydroxyl group of Tyr169. At the other end of analogue 2, the terminal amino group of the C24 ester side chain sits above a large hydrophobic patch on actin, where the ring of most barbed-end-targeting macrolide toxins binds. However, the polar amino terminus of analogue 2 projects away from actin to hydrogen bond with nearby surface waters. By extending this positively charged terminal amino group, it may be possible to establish a new interaction with a negatively charged region (Glu334) at the entrance of the barbed end cleft, mimicking either the salt bridge or H-bond formed by the F-actin-severing proteins twinfilin and cofillin, respectively. Functionalization of the terminal amino group with a linker for bioconjugation to cancer-cell-specific monoclonal antibodies could also be explored to focus the antimetastatic activity of this compound on tumor cells.

Analogue 2 also forms unique hydrophobic interactions with Leu346 through the N-ethyl substituent on the N-vinyl-formamide and with Thr148 via the (R)-tetrahydrofuran ester moiety in place of the 2,3-di-O-methylglycerate found in MycB (1). These novel features could contribute to the potent biochemical and cell biological effects of this compound by enhancing the disruption of protein−protein contacts rather than by improving the affinity for actin. Specifically, the protrusive (R)-tetrahydrofuran ester moiety could provide
additional steric hindrance that prevents the addition of actin protomers at the barbed end during filament assembly (Figure 6C). This is an important consideration that other studies have not taken into account during analogue design. Hence, we speculate that improving $K_d$ alone may be insufficient to improve the activity.

**CONCLUSIONS**

In summary, we have developed a concise, divergent, and scalable route to a bioactive and synthetically tractable analogue of MycB (1). Notably, this route permitted the assessment of the effect of structural variations at C24, C30, and the MVF, which identified a combination of tail modifications that lead to the potent inhibition of actin polymer formation and disruption of F-actin stability. Gratifyingly, this analogue is active in SKOV3 cells and rapidly disrupts the actin cytoskeleton, causing cells to round up at low concentrations of analogue 2. The crystal structure of analogue 2 bound to actin suggests that some of the features of analogue 2 that differ from MycB (1) are located at positions that do not contact actin directly, but instead face toward the solvent. Design, synthesis, and functional analysis of new analogues that explore modifications at these positions are currently underway.

**EXPERIMENTAL SECTION**

**Proteins and Reagents.** Actin and pyrene actin were purchased from Cytoskeleton Inc. (Denver, CO). Mycalolide B was purchased from Wako Pure Chemical Industries Ltd., Japan, and stored at $-20^\circ$C.

**Pyrene–G-actin Polymerization Assay.** A mixture of rabbit skeletal muscle G-actin and pyrene–G-actin (2:1 ratio) was diluted in G-buffer (2 mM Tris-HCl, 0.2 mM CaCl$_2$, and 0.8 mM Na$_2$CO$_3$) to a final concentration of 9 μM, incubated on ice for 30 min, and further centrifuged for 1 h at 130000g at $+4^\circ$C. Then 63 μL of this mixture was mixed with 1.4 μL of DMSO, MycB (1), or analogue 2 (0–1000 nM) in a fluorometer cuvette and incubated at 25 $^\circ$C for 50 s. Actin polymerization reactions were initiated by the addition of 7 μL of actin polymerization buffer (100 mM Tris-HCl (pH 7.5), 500 mM KCl, 20 mM MgCl$_2$, and 10 mM ATP) to a pyrene–actin–toxin mixture. Fluorescence emission at 406 nm was monitored by using a Fluorolog fluorimeter (Jobin Yvon Horiba) with an excitation wavelength of 365 nm for a period of 600 s. The rate of polymerization was calculated as an increase in the fluorescent intensity between 300 and 350 s and divided at 50 s. The polymerization rate of actin in the absence of the toxin was normalized to 100%. The rate of polymerization in the presence of the toxin was expressed as a percentage relative to the no-toxin value and plotted as a function of concentration. The IC$_{50}$ value was deduced as the concentration of the toxin at which a 50% rate of polymerization was observed. All experiments were performed in triplicate and presented as mean ± standard error (SEM).

**Pyrene–F-actin Depolymerization Assay.** A mixture of rabbit skeletal muscle G-actin and pyrene–G-actin (2:1) was diluted to 5 μM in A-buffer (100 mM Tris-HCl (pH 7.5), 500 mM KCl, 20 mM MgCl$_2$, and 10 mM ATP) and incubated for 1 h at room temperature to form F-actin. Toxin solutions (96 μL) containing a range of concentrations of MycB (1) or analogue 2 in buffer F (10 μM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM MgCl$_2$, 1 mM ATP, 0.2 mM DTT, and 0.2 mM CaCl$_2$) were added to 4 μL of the F-actin solution. Fluorescence emission at 406 nm was monitored with an excitation wavelength of 365 nm for a period of 900 s. All experiments were performed in triplicate.

**Cell Culture.** The SKOV3 cell line was obtained from American Type Culture Collection and authenticated by STR profiling. The cell line was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Multicell) supplemented with 10% fetal bovine serum (FBS, Multicell) and 1% antibiotic-antimycotic solution (Multicell) and cultured in a humidified incubator at 37 °C with 5% CO$_2$.

**Spinning Disc Confocal Microscopy.** SKOV3 cells were seeded on glass coverslips, coated with human FN, and allowed to attach and spread for 24 h prior to treatments with media, media + 1% DMSO (vehicle), 100 nM MycB (1), or 100–5000 nM analogue 2 for 1 h. Cells were then fixed with 4% PFA, permeabilized in 0.2% Triton X-100 and stained with TRITC-Phalloidin (1:400) to visualize the actin cytoskeleton and DAPI (1:400) to identify the nuclei. Images were acquired in red and blue channels using a Quorum WaveFX-X1 spinning disc confocal system (Quorum Technologies Inc., Guelph, Canada) in the Queen’s University Biomedical Imaging Centre. Confocal micrographs were merged, and scale bars were added using Metamorph software.

**Cell Invasion Assay.** Directional cell invasion measurements were performed on SKOV3 cells. Cells (2.5 × 10$^4$) were seeded in triplicate in a 96-well ImageLock plate (Essen BioScience). At approximately 90% confluence a scratch was induced using the IncuCyte Woundmaker (a 96-well wound-making tool, Essen BioScience). After
wounding and the removal of nonadherent cells, wells were overlaid with 5% Matrigel and incubated at 37 °C for 30 min to allow the Matrigel to solidify. DMEM containing the indicated doses of MycB (1) or analogue 2 was added to each well. The plate was then inserted into the IncuCyte Zoom Live Cell Analysis System at 37 °C and imaged every 2 h for 48 h. The percentage of wound closure was determined through IncuCyte Zoom Scratch Wound Analysis software package.

ECM Degradation Assay. To test the effects of MycB (1) and analogue 2 on ECM degradation by invadopodia, SKOV3 cells (2 × 10⁴) were seeded on #1.5 acid-washed glass coverslips that were coated with a mixture of Matrigel and DQC-Collagen (8:1). Eight hours later, cells were treated with DMSO control, MycB (1) (25 nM), or analogue 2 (0.1 or 1 μM) for 4 h. MMP inhibitor-ilomastat (GM6001, MedChem Express, 10 μM) was added as a positive control for inhibition of MMP-mediated cleavage of DQ-collagen by invadopodia. The formation of green dots represents the number of invadopodia. The amount of green fluorescence per field in confocal micrographs was analyzed and quantified using Image J software.

Crystallization of the Actin—MycB and Actin–2 Complex. MycB (1) was mixed with 10 mg/mL G-actin from rabbit muscle in G-buffer (5 mM Tris (pH 8), 0.2 mM CaCl₂, 0.2 mM ATP, and 0.5 mM DTT) at a 1:1 molar ratio. Analogue 2 and Latrunculin B (LatB) were mixed with G-actin in G-buffer at a 2:1:1 molar ratio. Mixtures were incubated on ice for 30 min. Crystals of the actin–MycB complex that were suitable for X-ray diffraction data collection grew in 3 days from 2 μL of sitting drops containing the actin–MycB complex in a 1:1 ratio with a precipitant solution containing 0.1 M sodium cacodylate/HCl (pH 6.5), 20% polyethylene glycol (PEG) 8000, and 0.2 M magnesium acetate at 293 K. Prior to diffraction data collection, crystals were transferred into a cryoprotectant composed of 0.1 M sodium cacodylate/HCl (pH 6.5), 20% PEG 8000, 0.2 M magnesium acetate, and 22% ethylene glycol (EG) and were then flash-cooled in liquid N₂. Crystals of the actin–2–LatB complex grew in 25 days from 2 μL of hanging drop containing a 1:2:1 ratio of a complex with a precipitant solution of 0.1 M Tris-HCl (pH 8.5) and 25% PEG 3350. Cryoprotectant was composed of 0.1 M Tris-HCl (pH 8.5), 27% PEG 3350, and 20% EG.

X-ray Diffraction Data Collection and Structure Determination. Diffraction data were collected from a single crystal at beamline 08ID-1 of the Canadian Light Source (Saskatoon, Canada) at 100 K and was indexed, integrated, and scaled with HKL2000. The actin–MycB complex structure was solved by molecular replacement from chain a of the actin–ulapualide A structure (PDB accession code IS22) with Phaser. The actin–2–LatB complex structure was solved by molecular replacement with the actin–MycB structure (PDB accession code 6MGO) with Phaser. The structures were refined with Refmac5 and manually optimized using Coot. During model building and refinement of the actin–MycB structure, it was discovered that the C5–C6 double bond was reduced and that C5 was linked to a sulfur. Data processing and refinement statistics are summarized in Table S1.

Coordinates and structure factors of actin–MycB and actin–2–LatB have been deposited in the Protein Data Bank with Accession Codes 6MGO and 6W7V, respectively.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c12404.

Experimental procedures, spectral data, supplementary figures, and copies of ¹H and ¹³C NMR spectra for all compounds (PDF)

AUTHOR INFORMATION

Corresponding Authors

John S. Allingham — Department of Biomedical & Molecular Sciences, Queen’s University, Kingston ON K7L 3N6, Canada; Email: allinghj@queensu.ca

P. Andrew Evans — Department of Chemistry, Queen’s University, Kingston ON K7L 3N6, Canada; Xiangya School of Pharmaceutical Sciences, Central South University, Changsha, Hunan 410013, P.R. China; orcid.org/0000-0001-6609-5282; Email: andrew.evans@chem.queensu.ca

Authors

Bhavin V. Pipaliya — Department of Chemistry, Queen’s University, Kingston ON K7L 3N6, Canada; orcid.org/0000-0003-2680-2178

Daria N. Trofimova — Department of Biomedical & Molecular Sciences, Queen’s University, Kingston ON K7L 3N6, Canada; orcid.org/0000-0002-5451-0765

Rebecca L. Grange — Department of Chemistry, Queen’s University, Kingston ON K7L 3N6, Canada

Madhu Aeluri — Department of Chemistry, Queen’s University, Kingston ON K7L 3N6, Canada

Xu Deng — Cancer Biology & Genetics Division, Queen’s Cancer Research Institute, Kingston ON K7L 3N6, Canada; Xiangya School of Pharmaceutical Sciences, Central South University, Changsha, Hunan 410013, P.R. China; orcid.org/0000-0001-7683-1626

Kavan Shah — Cancer Biology & Genetics Division, Queen’s Cancer Research Institute, Kingston ON K7L 3N6, Canada

Andrew W. Craig — Cancer Biology & Genetics Division, Queen’s Cancer Research Institute, Kingston ON K7L 3N6, Canada

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.0c12404

Author Contributions

B.V.P., D.N.T.; R.L.G., and M.A. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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