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The Effects of Acetylenic Tricyclic Bis-(Cyano Enone) on Cell Migration

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Graduate Program in Pharmacology and Toxicology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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Abstract

Although cancer survival rates have significantly improved over the past few decades, the improvements are primarily due to early diagnosis and inhibiting cancer growth. Limited progress has been made in the treatment of cancer metastasis, which contributes to 90% of cancer related deaths, and therapeutic agents targeting the various aspects of metastasis are lacking. One potential approach is to utilize small pharmacological compounds to inhibit tumour cell motility, as a strategy against tumour cell migration, invasion, and metastasis. The acetylenic tricyclic bis-(cyano enone), TBE-31, has been shown to be a promising chemopreventative compound. However, its effects on cell migration are unknown. This thesis focuses on deciphering the molecular mechanisms TBE-31 utilizes to inhibit cell migration. I demonstrated that TBE-31 binds with cysteine 374 of actin, inhibits actin polymerization and stress fiber formation. These findings were applied to a model of epithelial-to-mesenchymal transition, a precursor event to metastasis, where I determined TBE-31 was able to inhibit the crucial rearrangement of cortical actin to form actin stress fibers, which prime tumour cells for migration. In addition to the actin cytoskeleton, I demonstrated that TBE-31 alters microtubule dynamics and organization. Microtubule-dependent trafficking was also shown to be disrupted by TBE-31, and the localization of the polarity proteins Rac1, IQGAP and Tiam1 were altered from the leading edge of migrating cells. Lastly, TBE-31 was shown to inhibit Rat2 and NIH3T3 fibroblast as well as H1299 non-small cell lung cancer tumour cell migration. Taken together, my work provides novel insights on the underlying mechanisms by which TBE-31 utilizes to inhibit cell migration and provide
important knowledge for developing therapeutic compounds that target tumour cell motility in metastasis.
Keywords

TBE-31

Acetylenic tricyclic bis-(cyano enone)

Synthetic triterpenoids

CDDO-Im

CDDO-Me

Actin cytoskeleton

Microtubule network

Cell polarity

Cell migration

Metastasis
Co-Authorship Statement

All synthetic triterpenoid compounds and TBE-31 used in Chapter 2, 3 and 4 were synthesized by Drs. T. Honda, and G.W. Gribble, and generously provided by M.B. Sporn from Dartmouth Medical School in Hanover, USA or T. Honda from Stoney Brook University in Stoney Brook, USA.

Chapter 2 of this thesis was previously published in the journal of Cancer Prevention Research in July 2014 (Cancer Prev Res 7, 727-737, (2014)).

This peer reviewed article is titled “The Acetylenic Tricyclic Bis(cyano enone), TBE-31 Inhibits Non–Small Cell Lung Cancer Cell Migration through Direct Binding with Actin.” The sources of reagents and plasmids are listed in the materials and methods section of the chapter and any additional items mentioned in the footnotes section. Drs. Akira Saito and Tadeshi Honda synthesized the TBE-55 and TBE-56 used in this paper and composed Figure 1, the schematic of compounds used in this paper. All other figures and experiments were carried out and repeated by Eddie Chan under the supervision of Dr. John Di Guglielmo at the University of Western Ontario in the department of Physiology and Pharmacology.

Chapter 3 of this thesis was published in the journal Biochimica et Biophysica Acta - Molecular Cell Research on January 13th 2016 (Biochim biophys Acta 1863, 638-649, (2016)).

This peer reviewed article is titled “The acetylenic tricyclic bis(cyano enone), TBE-31, targets microtubule dynamics and cell polarity in migrating cells.” The sources
of reagents and plasmids are listed in the materials and methods section of the chapter. Drs. Akira Saito and Tadashi Honda synthesized the TBE-55 and TBE-56 used in this paper. All figures and experiments were carried out and repeated by Eddie Chan under the supervision of Dr. John Di Guglielmo at the University of Western Ontario in the department of Physiology and Pharmacology.

Chapter 4 of this thesis is in preparation for submission

The sources of reagents and plasmids are listed in the materials and methods section of the chapter and the TBE-55 and TBE-56 used were synthesized by Drs. Akira Saito and Tadashi Honda. The computational docking analysis of TBE-31 and actin was carried out by Brennan Dirk, under the supervision of Dr. Jimmy Dikeakos at the University of Western Ontario, London, Canada. For Mass Spectrometry analysis, samples prepared by Eddie Chan were analyzed by Ms. Paula Pittock at the Biological Mass Spectrometry Laboratory at the University of Western Ontario. All other figures and experiments were carried out and repeated by Eddie Chan under the supervision of Dr. John Di Guglielmo at the University of Western Ontario in the department of Physiology and Pharmacology.
“May there never develop in me the notion that my education is complete but give me the strength and leisure and zeal continually to enlarge my knowledge.”

— Maimonides
Acknowledgments

The work in this would not have been possible without the support and guidance of many important individuals. I would like to first express my gratitude to my supervisor Dr. John Di Guglielmo. He has given me the opportunity and encouraged me to develop this project according to my own interests. My independence as a scientist is one of my strengths that I value the most and I have him to thank for that. He has been relentlessly optimistic in times when I was not, and his examples of positive attitude and perspective will be my lessons for many years to come. I could not have asked for a better mentor.

I would also like to express my gratitude towards members of my advisory committee, Dr. Moshmi Bhattacharya, Dr. Lina Dagnino, and Dr. Peter Chidiac for their thoughtful guidance and helpful discussion that directed my project and held my work to the highest standards. I would also like to thank Dr. Karen Liby and Dr. Peter Stathopoulos for their helpful discussion, encouragement and for recognizing me as a strong scientist during my periods of struggle and uncertainty.

I would also like to thank all past and present members of the Di Guglielmo Lab with whom I had the pleasure to work with, and call friends. Thank you for the countless assistance and support. I would like to thank in particular the three students who were present when I first started, Ciric To, Adrian Gunaratne, and Sarah McLean you treated me like a younger brother and it is only fitting I regard you as my lab family. There was never a dull moment working with you guys, fond memories of our shenanigans, food adventures, and laughter will be cherished for many years to come. A second thanks to
Ciric To for teaching me many of the fundamentals of working in the lab, and techniques and assays specific to working with triterpenoids.

Thank you to the many friends from neighboring labs including the Bhattacharya, Hammond, Feng, Chidiac, Stathopoulos, and Urquhart labs for their assistance, shared reagents, equipment and fun times outside of the lab.

Finally, I would like to thank my family for their support and showing me hard work and perseverance.
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## List of Abbreviations

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<tr>
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<th>Full name</th>
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<tbody>
<tr>
<td>+TIP</td>
<td>Microtubule plus end-tracking proteins</td>
</tr>
<tr>
<td>A549</td>
<td>adenocarcinomic human alveolar basal epithelial cells</td>
</tr>
<tr>
<td>ADF</td>
<td>actin depolymerizing factor</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
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<tr>
<td>Arp</td>
<td>Actin related proteins</td>
</tr>
<tr>
<td>ARPC</td>
<td>Actin-related protein complex subunit</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
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<td>ATPase</td>
<td>adenosine 5'-triphosphatase</td>
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<tr>
<td>AVA</td>
<td>Actin, VCA domain of n-Wasp, Arp2/3</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>b-TBE-31</td>
<td>biotin labeled TBE-31</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
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<tr>
<td>CaCl₂</td>
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<td>Cdc42</td>
<td>Cell division cycle 42</td>
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<td>CDDO</td>
<td>2-cyano-3, 2-dioxooleana-1,9-dien-28-oic acid</td>
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<td>CDDO-Im</td>
<td>CDDO-Imidazolide</td>
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<td>CDDO-Me</td>
<td>CDDO-Methyl ester</td>
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<td>CLASP</td>
<td>Cytoplasmic linker-associated protein</td>
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<td>Cytoplasmic linker protein 170</td>
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<td>CObl</td>
<td>Cordon-bleu protein</td>
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<tr>
<td>Cy2</td>
<td>Cyanine 2</td>
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<td>Cys</td>
<td>Cysteine</td>
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</tr>
<tr>
<td>DAPI</td>
<td>4', 6 diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DIAPH1 or DRF1</td>
<td>Diaphanous-related formin-1</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>EB-1</td>
<td>End binding protein 1</td>
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<td>E-cadherin</td>
<td>Epithelial Cadherin</td>
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<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EEA1</td>
<td>Early endosomal antigen 1</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
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<td>F-12K</td>
<td>Kaighn's Modification of Ham's F-12 Medium</td>
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<tr>
<td>F-actin</td>
<td>Filamentous-actin</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FH1 and FH2</td>
<td>Formin homology 1 and 2</td>
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<td>G-actin</td>
<td>Globular-actin</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating proteins</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GDI</td>
<td>Guanosine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>Gln</td>
<td>Glutamine</td>
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<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>Definition/Description</td>
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<tr>
<td>GTPase</td>
<td>Guanine triphosphatase</td>
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<tr>
<td>H1299</td>
<td>human non-small cell lung carcinoma cell line</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<td>HAT cells</td>
<td>Mv1Lu cells stably transfected with HA epitope-tagged TGFβ type II receptor</td>
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<td>Heme-oxygenase 1</td>
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<td>HPLC</td>
<td>High performance liquid Chromatography</td>
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<td>HRP</td>
<td>Horse radish peroxidase</td>
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<td>HSF1</td>
<td>Heat shock factor protein 1</td>
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<td>Hsp70</td>
<td>Heat shock protein-70</td>
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<tr>
<td>IC50</td>
<td>half maximal inhibitory concentration</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>Immune globulin G</td>
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<tr>
<td>IL</td>
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<td>IL1β</td>
<td>interleukin-1-beta</td>
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<td>IL6</td>
<td>interleukin-6</td>
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<td>Definition</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>IQGAP1</td>
<td>IQ motif containing GTPase activating protein 1</td>
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<tr>
<td>IκB</td>
<td>Inhibitor of kappa beta</td>
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<tr>
<td>JNK</td>
<td>c-jun NH2-terminal kinase</td>
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<td>kDa</td>
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<td>LC</td>
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<td>LC-MS</td>
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<td>LIM</td>
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<td>LIMK1</td>
<td>Lin11, Isl-1 &amp; Mec-3 domain kinase 1</td>
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<td>Lmod</td>
<td>Leiomodin</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>Lys</td>
<td>Lysine</td>
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<td>MAP</td>
<td>Microtubule-associated protein</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>mDia1</td>
<td>mammalian Dia1</td>
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<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
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<tr>
<td>Mes</td>
<td>2-(N-morpholino) ethanesulfonic acid</td>
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<td>Mg$^{2+}$</td>
<td>Magnesium ion</td>
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<td>MgCl2</td>
<td>Magnesium chloride</td>
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<td>MLC</td>
<td>myosin light chain</td>
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<td>MLCK</td>
<td>myosin light chain kinase</td>
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<td>MMP</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>MSB</td>
<td>Microtubule stabilization buffer</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule-organizing center</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Mv1Lu</td>
<td>Mink lung cells</td>
</tr>
</tbody>
</table>
NaCl Sodium chloride

NaHCO₃ Sodium biocarbonate

N-cadherin neural cadherin

NEAA Non-essential amino acids

NIH3T3 Mouse embryo fibroblasts

NPF Nucleation promoting factor

NQO1 NAD(P)H:quinone oxidoreductase 1

Nrf2 Nuclear factor erythroid 2-related factor 2

n-WASp Neural wiskott aldrich syndrome protein

PAK P21 activating kinase

Par6 Partitioning-defective 6

PB1 Phox and Bem 1

PBS Phosphate-buffered saline

PC12 Pheochromocytoma cell line

PDB Protein data bank

PDZ PSD-95/Dlg1/ZO-1 homology
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulphonylfluoride</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PT</td>
<td>Permeability transition</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>QToF</td>
<td>Quadrupole Time-of-flight</td>
</tr>
<tr>
<td>Rac</td>
<td>Rho-related C3 botulinum toxin substrate</td>
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<tr>
<td>Rat2</td>
<td>Rat fibroblasts (cell line)</td>
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<td>REDOX</td>
<td>Reduction oxidation</td>
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<td>Rho</td>
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<td>Rho GTPase</td>
<td>Rho guanine triphosphatase</td>
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<td>Ras homolog gene family, member A</td>
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<tr>
<td>RhoB</td>
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<tr>
<td>RhoC</td>
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<td>ROCK</td>
<td>RhoA-associated protein kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute 1640 medium</td>
</tr>
<tr>
<td>SCAR</td>
<td>suppressor of cAMP receptor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SH</td>
<td>Src homology</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<td>Smad</td>
<td>Mothers against decapentaplegic, drosophilia homolog</td>
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<td>TARP</td>
<td>translocated actin-recruiting phosphoprotein</td>
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<td>dicarbonitrile</td>
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<td>TEM</td>
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<td>TF</td>
<td>transcription factor</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>TGFβR I and II</td>
<td>Transforming growth factor beta receptor I and II</td>
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<td>T-lymphoma invasion and metastasis 1</td>
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<td>TMR</td>
<td>Tetramethylrhodamine</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
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<td>TNTE</td>
<td>Tris-NaCl-Tween-EDTA</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris-hydrochloric acid</td>
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<td>UVA</td>
<td>Ultraviolet A</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCA</td>
<td>Verprolin-cofilin-acidic</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WASP</td>
<td>Wiskott–Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP-family verprolin-homologous protein</td>
</tr>
<tr>
<td>WH2</td>
<td>WASP-homology 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>ZEB 1 and 2</td>
<td>Zinc finger E-box-binding homeobox 1 and 2</td>
</tr>
<tr>
<td>βPIX</td>
<td>PAK-interacting exchange factor β</td>
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Chapter 1

Introduction
1 Introduction

1.1 Cancer Metastasis

Cancer arises from cells that have undergone multiple genetic mutations that lead to the loss of normal cell behaviour $^{1,2}$. These mutations initially result in uncontrolled growth and proliferation. As the malignant cells continue to gain genetic mutations, a portion of these cells will gain the ability to migrate and invade adjacent tissue, and eventually disseminate to distant organs where they may establish new tumour colonies. These colonies are known as metastases, and are responsible for over 90% of human cancer related deaths $^{3-6}$. Cell migration is a key process in regulating metastasis, and to date effective drugs targeting metastasis are lacking $^{7-9}$.

1.2 Cell Migration: an Overview

Cell migration is a broad term used to describe the process that involves the translocation of cells from one location to another. This may occur in inert environments such as in soil, or on glass or plastic as is commonly seen in \textit{in vitro} experiments, or within complex multicellular organisms $^{10-12}$. Cells migrate in response to stimuli from their environment and this plays an essential role in many major physiological processes such as embryogenesis, growth, development, immune response and wound healing $^{10-12}$. However, abnormal cell migration can contribute to pathological conditions, such as autoimmune diseases, chronic inflammation and cancer $^{10-16}$.

Cell migration can be broken down into a set of component processes, which include front/rear polarization, protrusion, adhesion, translocation of the cell body, and
retraction of the rear (Figure 1.1). These processes are orchestrated by a network of both intracellular and extracellular signaling molecules and the cytoskeleton. Although it is helpful to think of these as separate, individual processes, in actual fact they flow seamlessly from one another or overlap each other. We begin with a cursory overview of the entire process before examining the major cellular components in more detail.

1.2.1 Polarization

Cell polarization is often thought of as the initial step in cell migration. Polarization in cell migration refers to the tendency of a migrating cell to have a distinct front or leading edge, and a rear or lagging end. This is often stimulated and reinforced by environmental cues. These cues can be chemotactic (induced by chemoattractants or morphogens), haptotactic (from varying concentrations of substrate), mechanotactic (breakdown of cell-cell contacts), electrotactic (induced by electric fields), durotactic (due to differences in pliability), or a combination of these. The microtubule network rearranges, polarity proteins are delivered to the leading edge, and actin polymerization begins.

1.2.2 Protrusion

Protrusions are the de novo formation of extensions in the plasma membrane in the direction of migration. The protrusion is primarily driven by the local polymerization of actin. Large broad, fan-like protrusions are formed by branched actin polymerization called lamellipodia or alternatively thin spike-like protrusions can be generated by
Figure 1.1 An overview of cell migration

Cell migration begins with i) polarization of the cytoskeleton towards the direction of migration. The polarity proteins initiate actin polymerization to generate ii) protrusions in the plasma membrane known as lamellipodia. New focal iii) adhesions underneath the lamellipodia adhere the membrane to the substratum. Meanwhile the focal adhesions at the rear disassemble and the iv) cell body translocates and the rear retracts.
Resting non-migratory state

i. Polarization

Front-rear polarity

Lamellipodium driven by actin polymerization

ii. Protrusion

Disassembly

New focal adhesion

iii. Adhesion

Contraction

iv. Cell Body Translocation and Retraction of the Rear
parallel bundles of actin called filopodia. These two forms of protrusions are thought to have different roles, with the former contributing primarily to forward movement and traction, and the latter providing mechanosensory and exploratory functions.  

1.2.3 Adhesion

Focal adhesions are mainly composed of integrin receptors, a large superfamily of heterodimeric receptors, which form specialized attachments sites wherein cells make contacts with the extracellular matrix (ECM). They are dynamic structures that form underneath the cell protrusions to provide traction to the substratum. They grow and shrink in size due to the recruitment and disassembly of proteins in response to mechanical forces. Focal adhesions are able to link the cytoskeletal network to the ECM, allowing cells to respond to the external environment through a feedback loop of, “inside-outside-inside,” signaling. Cellular contractility generated by the actomyosin cytoskeleton is transmitted to the ECM as traction forces by focal adhesions, and cells are also able to sense the local extracellular environment via focal adhesions.

1.2.4 Cell body translocation and retraction of the rear

Cell body translocation and retraction of the rear are the least well-understood processes in cell migration. Cell body translocation involves the coordinated contraction of the actomyosin cytoskeleton, and the microtubule network and motor proteins such as dynein help translocate the nucleus and other organelles. Meanwhile at the rear, mature focal adhesions are disassembled and contraction of the actomyosin cytoskeleton leads to retraction.
As the above steps—polarization, protrusion, adhesion, cell body translocation, and retraction of the rear—continue to occur in a cyclical fashion, the cell will translocate forwards.

1.3 Cell Migration: the role of Rho GTPases, actin and microtubules

Cell migration is a complex, multi-step process, involving careful orchestration and coordination between signaling molecules and the cytoskeleton. Polarity proteins establish and maintain a leading edge for proper directional movement, and help regulate the cytoskeleton. The actin cytoskeleton provides force for protrusion, and tension for retraction. While the microtubules establish a network for organelle translocation and protein delivery. These three components, their regulation, and their contributions to cell migration will be covered in greater detail below.

1.3.1 Polarization and the Rho GTPases

Rho GTPases belong to the Ras superfamily of small GTPases and are highly conserved throughout eukaryotes. To date, over 20 Rho GTPases have been identified, and it is thought that each one acts as a molecular switch to control distinct biochemical pathways. However only Rho (three isoforms, RhoA, RhoB, and RhoC), Rac (three isoforms, Rac1, Rac2, and Rac3) and Cdc42 have been studied extensively. Like all regulatory GTPases, these proteins are found in an inactive GDP-bound conformation, and in an active GTP-bound conformation. The interconversion between the two is
regulated by three types of regulatory proteins. Guanine nucleotide exchange factors (GEFs) activate GTPases by catalyzing the exchange of GDP for GTP, whereas GTPase-activating proteins (GAPs) increase the intrinsic GTP hydrolysis rate, thereby inactivating it (Figure 1.2A)\textsuperscript{23-25}. Guanine nucleotide dissociation inhibitors (GDIs) sequester the GDP-bound form of some GTPases in the cytosol, and prevent them from localizing to the membrane or being activated by GEFs \textsuperscript{23-25}. Rho GTPases can be activated directly or indirectly by various cell-surface receptors, including integrins, cadherins, cytokine receptors, receptor tyrosine kinases and G protein-coupled receptors\textsuperscript{26}. In the active GTP-bound conformation, Rho GTPases can interact with a range of effector proteins including kinases, actin regulators and adaptor proteins, leading to changes in cell behaviour. The spatiotemporal regulation of each Rho GTPase is therefore important to determine the outcome of its activity \textsuperscript{23-25}.

Cell migration is an inherently polarized process, with the molecular processes at the front different from those at the rear. Whether cell polarization is induced by a chemical gradient of soluble or membrane-bound chemoattractants, or by the localized activation of integrins, the recruitment and activation of the Rho GTPases, Cdc42 and Rac, at the leading edge are major events necessary for triggering cell polarity (Figure 1.2B)\textsuperscript{10-12,23-25,27}. The major targets for Rac and Cdc42 that mediate actin polymerization in protrusions are the Wiskott–Aldrich syndrome protein (WASP)/WASP-family verprolin-homologous protein (WAVE) family of actin related proteins 2 and 3 (Arp2/3) complex activators. Rac1 stimulates lamellipodial extensions by activating WAVE proteins, while Cdc42 binds to WASP proteins to stimulate Arp2/3. Cdc42 also affects
**Figure 1.2 Rho GTPases and cell migration**

**A)** Rho GTPases can exist in an active (GTP-bound) or an inactive (GDP-bound) confirmation. In the active state, they interact with target (effector) proteins to induce a cellular response. Most Rho GTPases have an intrinsic ability to hydrolyze the GTP to GDP and inorganic phosphate (P$_i$), but it is also promoted by Rho GTPase-activating proteins (GAPs). The exchange of GDP for GTP is promoted by Rho guanine nucleotide exchange factors (GEFs). In addition, when in the GDP-bound state, the Rho GTPases can interact with Rho GDP dissociation inhibitor (GDI), which sequester the complex and prevent reactivation.

**B)** During cell migration, external signals from the environment (for e.g. interaction with the ECM, or soluble signals) activate Cdc42, Rac1 and RhoA to establish front-rear polarity. Cdc42 binds to Par6, which activates aPKC. This promotes the local phosphorylation and inhibition of GSK3, leading to rearrangements of the microtubule network through microtubule-associated proteins (MAPs). Cdc42 and Rac1 activate WASP and WAVE respectively, which in turn promotes branched actin polymerization through Arp2/3 and promote lamellipodia formation. Cdc42 also acts on formin to promote stress fiber formation and filopodia at the leading edge. Similarly, RhoA activated formin, myosin II and ROCK to promote stress fiber formation and retraction at the rear.
polarity by localizing the microtubule-organizing center (MTOC) and Golgi apparatus in front of the nucleus, to orient the two towards the leading edge. This aids in the microtubule-mediated delivery of Golgi-derived vesicles to the leading edge, providing membrane and associated proteins for protrusion 1-3,9-11,13. These processes will be covered in greater detail in the following actin and microtubule descriptions.

1.3.2 The Actin Cytoskeleton

1.3.2.1 Structure

Actin is the most abundant protein in most eukaryotic cells 28, and is expressed in three main isoforms, α, β, and γ. There are three α-isoforms found in skeletal, cardiac and smooth muscles, while β- and γ- isoforms are expressed in non-muscle and muscle cells. The actin structure is highly conserved and the isoforms only differ by a few amino acids 28,29. Actin is found both in globular monomeric (G-actin) or polymeric filamentous (F-actin) forms. The 375 amino acid, 42 kDa protein, folds into two major α/β domains, also often respectively referred to as the outer and inner domains due to their locations in filamentous actin, or the small and large domains due to their apparent size difference observed by electron microscopy. Each domain can be further divided into two subdomains for a total of four domains 28,30. The polypeptide chain passes twice through the two major domains at residue Lys336 and at linker helix Gln137-Ser145, which functions as a hinge between these two domains. As a result, two clefts between the domains are formed. The upper cleft binds nucleotides and associated divalent cations (i.e. Mg2+), which form another important linkage between the two major domains. The lower cleft contains primarily hydrophobic residues and is important in mediating
longitudinal contacts between actin subunits in the filament. In addition, this hydrophobic cleft is the major binding site for most actin-binding proteins, and small molecules, and is thus often called the target binding cleft \(^{28,31}\). The actin monomers polymerize to form the actin filaments, which are constructed with two chains of actin that turn gradually around each other to form a two-chained long helix \(^{28}\).

### 1.3.2.2 Actin Polymerization

The first step in actin polymerization is nucleation, which is defined as the formation of a stable multimer of actin monomers. This occurs slowly, and is the rate-limiting step in polymerization due to the relative instability of actin dimer intermediates and the activity of actin monomer sequestering proteins that suppress spontaneous nucleation in cells \(^{32}\). However, once a nucleus is formed, actin polymerization proceeds quickly at the barbed fast growing *plus* end, and more slowly at the pointed *minus* end. The ATPase activity of actin increases upon incorporation into the filament, and causes the spontaneous hydrolysis of the bound ATP to ADP. This destabilizes the filament and the ADP-actin dissociates and undergoes nucleotide exchange to revert back to ATP-actin. This in turn provides more actin monomers for polymerization at the positive end. The constant recycling of actin results in actin treadmilling (Figure 1.3A) \(^{32-34}\).

The rates of nucleation and treadmilling of pure actin are far too slow to account for the rates of cell migration, and a number of actin binding proteins are responsible for facilitating nucleation and promote elongation \(^{34}\). Filament nucleators bear little overall
Figure 1.3 The actin cytoskeleton and cell migration

A) Actin treadmilling. i) Formation of actin oligomers are unfavourable. ii) Once an actin nucleus is formed, the association of monomers proceeds quickly. The plus (+) end grows much faster than the minus (-) end. iii) Hydrolysis of ATP and the dissociation of phosphate destabilizes the actin and causes depolymerization. Dissociated ADP-actin undergoes nucleotide exchange, and the resulting ATP-actin recycles back to the plus end. iv) Rates of treadmilling are greatly increased by the actin depolymerizing factor (ADF)/Cofilin family and Profilin. ADF/Cofilin facilitate the depolymerization of ADP-actin, and Profilin facilitates nucleotide exchange of ADP-actin to ATP-actin.

B) Fascin bundles actin filaments to produce actin stress fibers.

C) When active, the actin-related protein 2/3 (Arp2/3) complex binds to the side of pre-existing actin filaments to provide nucleation sites for actin, which results in a branched array of actin filaments.

D) At the leading edge, stress fibers form spike-like protrusions in the plasma membrane known as filopodia, and the branched actin forms a broad fan-like protrusion of plasma membrane called the lamellipodia. Stress fibers are also present in the cell body and aid in retraction of the rear.
resemblance to one another, but all include the WASP-homology 2 (WH2) domain for interacting with actin. Known nucleators include Arp2/3 complex and its large family of nucleation promoting factors, Spire, CObl, VopL/VOpF, TARP and Lmod 28,34. Elongation factors also include a wide variety of formins—which are modular proteins characterized by the presence of two conserved domains, formin homology 1 and 2 (FH1 and FH2), as well as Ena/VASP 28,34. Actin depolymerizing factor (ADF) also known as coflin, can induce ADP-actin depolymerization at the minus end 35,36, while profilin facilitates the nucleotide exchange of ADP-actin to ATP 37. The combined effect of ADF and profilin enhances the rate of actin treadmilling by 125-fold alone 34,38. A number of other actin binding proteins also help regulate the temporal and spatial organization of the overall actin cytoskeleton. There are three main types of actin-based structures found within the cell: filopodia, lamellipodia, and stress fibers (Figure 1.3D)39.

1.3.2.3 Branched Actin and the Lamellipodia

During cell migration, the localization of Rac1 and Cdc42 activate the family of WASP proteins, which includes the ubiquitous neural-Wasp (N-WASP), and suppressor of cAMP receptor (SCAR)/WAVE isoforms 1, 2 and 3. These proteins in turn activate Arp2/3. Arp2/3 is a complex of seven conserved subunits including the two actin related proteins Arp2 and Arp3, and ARPC1, ARPC2, ARPC3, ARPC4 and ARPC5 34,40. The Arp2/3 activators help bring actin monomers close in proximity to the Arp2/3 complex, once incorporated into an actin filament the complex provides a new nucleation point for a daughter filament, which elongates at 70 degrees from the mother filament, thus forming a branched array of actin filaments (Figure 1.3C) 40,41. During cell migration, this
branched actin network forms the basis of the lamellipodia, the broad fan-like plasma membrane projection that provides the primary form of forward locomotion.

### 1.3.2.4 Unbranched Actin; Filopodia and Stress Fibers

In contrast to branched actin, unbranched actin (also known as linear, or processive actin) assembly contribute to creating actin structures such as filopodia, stress fibers and actin arcs. Cofilin can sever existing actin filaments to provide more barbed ends to provide sites for actin polymerization and Fascin can bundle actin filaments to promote the formation of filopodia—the spike-like projections that function as mechanosensory, and exploratory devices. RhoA directly promotes stress fiber assembly through its effectors, RhoA-associated protein kinase (ROCK) and the formin mDia1 (mammalian Dia1; also known as DIAPH1 and DRF1). mDia1 promotes the polymerization of long parallel actin filaments, whereas ROCK inhibits cofilin through activation of LIM domain kinase 1 (LIMK1), ensuring that the forming actin filaments do not get severed. Stress fibers provide structure, and during cell migration, these actin stress fibres are found to associate with focal adhesions, and help provide a contractile force to retract the lagging end of the cell. Actin arcs are similar to actin stress fibers in that they also provide structure, but do not directly attach to focal adhesions, and display a periodic α-actinin-myosin pattern which contribute to their arc structure and give them the ability to contract.

### 1.3.3 The Microtubule Network

Microtubules (MTs) are noncovalent polymers of the protein tubulin found in all dividing eukaryotic cells and in nearly all differentiated cell types. During cell division, a
large dynamic array of MTs called the mitotic spindle facilitates the physical separation of chromosomes and serves to orient the plane of cleavage. In non-dividing cells, MTs organize the cytoplasm, position the nucleus and organelles, and serve as the primary structural components of the flagella and cilia\textsuperscript{47,48}. During cell migration MTs help establish and maintain the spatial and temporal coordination of migratory signals and events\textsuperscript{27,47,49}.

1.3.3.1 Microtubule structure and dynamics

Microtubules are composed of heterodimers made up of $\alpha$ and $\beta$-tubulin monomers, which are approximately 50\% identical to each other at the amino acid level and have a molecular mass of approximately 50 kDa\textsuperscript{47}. These heterodimers are aligned in a polar head-to-tail fashion to form protofilaments and they associate laterally to form a 25 nm wide hollow cylindrical microtubule (Figure 1.4A, B and C). The heterodimers associate in the same direction and generate a polarized filament with a distinctive minus and plus end. In a cell, the minus end is anchored to the microtubule organizing center (MTOC), while the plus end is free to associate with heterodimers to further elongate\textsuperscript{47,50}. This polymerization is regulated by the hydrolysis of $\beta$-tubulin bound GTP (GTP bound to $\alpha$-tubulin is non-exchangeable and is never hydrolyzed), which occurs with a delay after the tubulin heterodimer has been incorporated into the microtubule end. As a result the newly formed microtubule tip contains a cap of GTP-tubulin, which helps stabilize the structure, whereas the shaft of the microtubule is composed of GDP-tubulin and is intrinsically less stable. In the presence of the cap a microtubule continues to grow, and a loss of the cap leads to rapid microtubule shrinkage, termed catastrophe. For this
Figure 1.4 The microtubule network and cell migration

A) Microtubules are composed of subunits of tubulin heterodimers formed from tightly linked α- and β- tubulin monomers. The GTP molecule in the α-tubulin does not hydrolyze to GDP, however the GTP molecule in β-tubulin does, which plays an important role in filament dynamics.

B) The tubulin heterodimers align in a head-to-tail fashion to generate a polarized protofilament.

C) The protofilaments associate laterally to form a 25 nm wide hollow cylindrical tube known as a microtubule.

D) Microtubules are under a state of dynamic instability, characterized by polymerizing (left) and depolymerizing microtubules (right). GTP-tubulin is incorporated at polymerizing microtubule ends. The GTP is quickly hydrolyzed soon after polymerization and phosphate is released, resulting in a GDP-tubulin shaft which is intrinsically less stable than the GTP-tubulin cap. If a GTP-tubulin cap is not maintained by incoming GTP-tubulin, the GDP-tubulin shaft depolymerizes (termed catastrophe). As GTP-tubulin is replenished, the microtubule can transit back to polymerization (rescue). The microtubules infrequently pause in a state, neither gaining nor losing tubulin subunits and these tubulin heterodimers lie within a fully formed cylinder (bottom).

E) During cell migration, microtubules polarize, and maintain front-rear polarity by coordinating specific cellular activities, including the transport of membrane vesicles to the leading edge.
reason, the microtubules are under a constant state of ebb and flow termed dynamic instability, which is dependent on capping proteins and monomer concentration (Figure 1.4D). The nature of this instability helps the microtubule distribute in the cell during cytoskeletal reorganization and undergo a, “search and capture,” process where the tip of the microtubule shrinks and grows to explore the extracellular space until capturing an organelle or microtubule associate protein\textsuperscript{47,50}.

1.3.3.2 Regulation and function in cell migration

Microtubules have long been implicated in cell migration, however the mechanism of their involvement has remained unclear, and has even been considered controversial at times. Most studies have concluded that microtubules play a positive role in cell migration by regulating actin polymerization, transporting membrane vesicles to the leading edge and/or facilitating the turnover of focal adhesions\textsuperscript{49,51-53}. In general, their greatest contribution is to maintain a polarized intracellular organizations and correctly coordinate the position of specific cellular activities (Figure 1.4E)\textsuperscript{27,49,54}.

The local activation of Cdc42 is a crucial early event in cell migration leading to the polarization of the microtubule network. Cdc42 can be activated by a wide variety of polarity cues\textsuperscript{55}. In a scratch-induced migration assay, integrin engagement leads Src- and Scrb-dependent activation of the protein βPIIX, which in turn acts as Cdc42 specific GEF. βPIIX also contributes to the recruitment of Rac1, but is activated independently\textsuperscript{55-57}. Cellular redistribution of Cdc42 can also be initiated by soluble ligands acting as membrane bound or membrane permeable chemoattractants. This mirrors the establishment of an intracellular phosphoinositide gradient established by the activation
of PI3K at the front and PTEN at the back. Once activated, Cdc42 binds and recruits Par6, and activates atypical protein kinase C (aPKC), which in turn promotes the local phosphorylation and inactivation of GSK3 kinases. GSK3 is a constitutively active serine/threonine kinase that regulates several +TIPs (microtubule plus-end tracking proteins, a subclass of microtubule-associated proteins (MAPs)) and stabilizes a number of MAPs. GSK3β can phosphorylate stathmin and help establish a gradient of stathmin activity, which promotes microtubule growth at the leading edge and depolymerization at the rear. The inhibition of GSK3β at the leading edge also promotes the recruitment of APC. APC functions to help promote microtubule growth, but also microtubule capture to the leading edge via the enhanced binding of IQGAP1 to Clip-170.

1.4 Metastasis Cascade

Cell migration requires a complex orchestration of numerous proteins, and not surprisingly, the dysregulation of any of these can lead to pathological diseases. In the later stages of cancer, cell migration is exploited to allow tumour cells to spread to new areas of the body in a process called metastasis. Metastasis is responsible for 90% of all cancer related deaths, yet it remains the most poorly understood component of cancer pathogenesis. The basic steps of metastasis include the invasion of tumour cells into the local tissue, intravasation into the lymph or blood system, dissemination, extravasation, and colonization at distant organs (Figure 1.5). These steps will be covered in greater detail below.
Figure 1.5 The metastasis cascade

During metastasis progression, tumour cells acquire a more invasive phenotype through a process termed epithelial-to-mesenchymal transition and exit their primary site of growth and invade into the surrounding extracellular matrix (local invasion). When the cancer cells reach the blood vessels, they can enter into the circulatory system (intravasation) and distribute throughout the body (dissemination). Eventually the tumour cells will arrest at a distant site, exit the circulation and invade the microenvironment of a foreign tissue (extravasation). At the foreign site, the cancer cells must adapt to survive and thrive (micrometastasis) before developing into full colonies (macrometastasis).
Normal epithelium → Primary tumour formation → Local invasion

Extravasation

Dissemination and arrest at distant site

Intravasation

Micrometastasis → Metastatic colonization → Metastatic disease
1.4.1 Local Invasion and Epithelial-to-Mesenchymal Transition

Local invasion involves the entry of cancer cells that have resided within a well-confined primary tumour into the surrounding tumour-associated stroma, and then into the adjacent tissue parenchyma. The first barrier to invasion is the basement membrane, a specialized extracellular matrix component that plays a vital role in organizing the epithelial tissue by separating the epithelium from the stroma. Epithelial cells are highly organized and form sheet-like layers that are held together by complexes such as tight junctions, adherens junctions and desmosomes. In addition, they display apical-basal polarity and are attached to the basement membrane. The precisely controlled tissue architecture of normal epithelium keep the epithelial cells relatively immobile and serve as an intrinsic barrier for tumour cell migration and invasion\textsuperscript{14-16,63,64}.

In order to overcome this and other obstacles for invasion, carcinoma cells may co-opt a cell-biological program known as epithelial-to-mesenchymal transition (EMT) (Figure 1.6) EMT is normally a critical process for healthy embryonic morphogenesis where cells transition between epithelial and mesenchymal states in a highly plastic and dynamic manner\textsuperscript{65}. EMT programs are orchestrated by a set of pleiotropically acting transcription factors (TFs)—including Slug, Snail, Twist, ZEB1, and ZEB2—which trigger the entrance into a mesenchymal state by suppressing expression of epithelial markers and inducing the expression of other markers associated with mesenchymal cells\textsuperscript{14,63,65}. Cell-cell junctional proteins, like E-cadherin, which normally tethers epithelial cells into sheets, are repressed, and N-cadherin, which is associated with mesenchymal and migratory cells, are up-regulated\textsuperscript{66}. The actin cytoskeleton rearranges from the cell cortex to form actin stress fibers, and the overall apical-basal polarity is lost.
Figure 1.6 Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is an important process during development, but also marks an important event during tumour progression where cells become more invasive and metastatic. Key events include the disruption of cell-cell junctional complexes—such as tight junctions, adherens junctions and desmosomes—the loss of apical-basal polarity and a restructuring of the actin cytoskeleton from cortical actin to actin stress fibers. EMT also involves a shift in expression of epithelial markers (for e.g., E-cadherin and ZO-1) to cells that express mesenchymal markers such as N-cadherin and vimentin. Cells that have undergone EMT display an enhanced migratory capacity and increased invasive potential. The reverse process, mesenchymal-to-epithelial transition (MET), may also occur.
Epithelial-to-Mesenchymal Transition

- Tight-Junction Dissociation
- Adherens junctions and Desmosome dissociation
- Loss of apical-basal polarity
- Cytoskeleton Reorganization
- Migration and Invasion

Epithelial Cell → Actin → Mesenchymal Cell

**Epithelial Markers**
- E-Cadherin
- Cortical Actin
- Claudins
- Occludins
- ZO-1

**Mesenchymal Markers**
- N-Cadherin
- Actin Stress Fibers
- Vimentin
- Matrix metalloproteases

**Characteristics**
- Cobblestone
- Non-motile
- Non-invasive
- Apical-Basal Polarity

**Characteristics**
- Elongated
- Motile
- Invasive
- Front-Rear Polarity

EMT

EMT effectors
- Growth Factors (i.e., TGFβ)
- Cytokines

MET
Consequently the precise architecture of normal epithelium is lost, and the cells begin to exhibit multiple mesenchymal attributes, including heightened migratory and invasive potential \(^{14,63,65}\). In addition, the Snail and ZEB factors induce the transcription of metalloproteases that then degrade the basement membrane, enabling local cell invasion \(^{14,65}\).

After undergoing EMT and dissolving the basement membrane, carcinoma cells migrate and invade into the stroma. Here the carcinoma cells encounter a variety of tumour-associated stromal cells. The resultant composition of these associated stromal cells is governed largely by the state of tumour progression. As the primary tumour progression proceeds, the stroma becomes increasingly “reactive” which is to mean that it acquires many of the characteristics associated with wound healing or chronic inflammation \(^{63,67}\). Components of the tumour associated stroma can include fibroblasts, myofibroblasts, endothelial cells, adipocytes, and various bone marrow-derived cells—including mesenchymal stem cells, macrophages and other immune cells \(^{63,68}\). Crosstalk between the tumour cells and the stroma enhance the aggressive behaviours of carcinoma cells through various types of signaling. Therefore, there is a bi-directional interaction that occurs between the tumour cells and the nearby stroma, the carcinoma stimulates the formation of an inflamed stroma, and the stroma reciprocates enhancing the malignant traits of the carcinoma, establishing a self-amplifying positive feedback loop \(^{63,68,69}\).

1.4.2 Intravasation

Entry into the stroma provides opportunities for the tumour cells to access the systemic circulation and disseminate to distant sites. Intravasation involves locally migratory and invasive carcinoma cells entering the lumen of either the lymphatic or
blood vessels. Although intravasation into the lymphatic vessels occurs and is important as a prognostic marker for cancer progression, intravasation into the hematogenous circulation appears to represent the major mechanism by which metastatic carcinoma cells disseminate, and this will be the primary focus in the following section.

Intravasation requires cancer cells to cross the endothelium, a process that is known as transendothelial migration (TEM). This can occur through paracellular TEM, which involves the disruption of junctions between adjacent endothelial cells, which allows the tumour cells to squeeze between them or transcellular TEM, which involves the migration of cancer cells directly through the endothelial cell body. Although most studies have shown paracellular TEM in vitro, there is evidence suggesting they can also use a transcellular route. Thus far, it is not clear which route is used by cancer in vivo, or if the route is dependent on the vasculature or cancer type.

The major barrier for paracellular TEM is the presence of endothelial cell junctions. In vitro it has been shown that cancer cells induce endothelial cell junction opening by secreting various factors that activate signaling pathways in endothelial cells. For example, the carcinoma cells or the tumour associated stroma, such as macrophages, produce VEGF or TGFβ1, which can disrupt cell junctions based on VE-cadherin-β-catenin complexes and induce endothelial cell junction opening, thus facilitating cancer cell TEM. Other cancers may influence endothelial cell signaling by stimulating the Rho GTPases to facilitate TEM by causing an increase in actomyosin contractility, and stress fiber-mediated tension on adherens junctions, causing an opening. Similarly, endothelial RhoA is activated by lung cancer attachment to endothelial cells, which
activate ROCKs, leading to increased stress fiber assembly, and tight-junction disruption, to again facilitate cancer cell TEM \(^{73,77}\).

Recent studies have provided some evidence to show that tumour cells may migrate through the endothelial cell body instead of only through paracellular route. During transcellular intravasation, the Ca\(^{2+}\)-calmodulin complex in an endothelial cell activates myosin light chain (MLC) kinase (MLCK) at the site of cancer cell attachment. This induces a local phosphorylation of MLC and to actomyosin contraction, which leads to rapid actin cytoskeletal and membrane remodelling which creates a transient pore-like structure for the cancer cell to cross through the endothelial cell \(^{73,76,77}\).

Intravasation can occur in blood vessels normally present near the primary tumour, or through new blood vessels formed within the local microenvironment of the tumour via the process of neoangiogenesis, which can be initiated by VEGF from the carcinoma cells or tumour associated stroma. In contrast to the normal blood vessels, this neovasculature generated in response to carcinoma cells is often described as tortuous, prone to leakiness and in a state of continuous reconfiguration \(^{63,78}\). This weakened vasculature is thought to facilitate tumour cell intravasation more easily.

### 1.4.3 Survival and dissemination through the circulation

Once the carcinoma cells have successfully intravasated into the lumen of blood vessels, they can disseminate widely through the venous and arterial circulation. However they must survive a variety of stresses in order to reach distant organs \(^{63}\). For example, in the absence of integrin-dependent adhesion to the extracellular matrix,
epithelial cells would normally undergo anoikis—a form of apoptosis triggered by the loss of anchorage to substratum. Some of the signaling events in anoikis must be repressed in the carcinoma cells. In addition, the tumour cells must overcome the damage incurred by the hemodynamic shear forces generated in the blood stream, and predation by cells of the innate immune system.

1.4.4 Arrest and Extravasation

Once lodged in the microvasculature of distant organs, the circulating tumour may form a microcolony that eventually causes the surrounding vessel to rupture, thereby placing tumour cells in contact with the tissue parenchyma. Alternatively, the carcinoma cells may once again cross the endothelium by undergoing TEM as outlined above. This may superficially resemble the steps of intravasation, but the process can in fact be quite a bit more challenging. During extravasation the carcinoma cells no longer have the co-opted tumour associated stroma to help provide signals, and the advantages provided by the tortuous and leaky neovasculature at the intravasation site are absent.

1.4.5 Metastatic Colonization

Once extravasated the carcinoma cells must survive in a foreign microenvironment, which can differ greatly from the site of the primary tumour formation. Differences may include the types of stromal cells, extracellular matrix constituents, available growth factors, cytokines, and even the microarchitecture of the tissue itself. For these reasons, the disseminated cancer cells are, at least initially, poorly adapted to their new surroundings. As a result, the tumour cells may suffer slow attrition over periods of
weeks and months, or persist as microcolonies in a state of long-term dormancy, neither gaining nor losing overall cell number 63,82.

1.5 Targeting Metastasis

Despite the natural barriers, challenges, and the high rates of attrition that accompany the steps of metastasis, overt metastases eventually do arise, and they almost invariably represent the source of terminal disease 63,70. The suppression of cancer metastasis is an urgent therapeutic need, yet most existing drugs inhibit only cancer cell proliferation 7-9. Therefore identifying therapeutic agent(s) that may target any of the various cell processes involved in metastasis will be crucial in reducing cancer mortality.

1.6 Synthetic Triterpenoids

1.6.1 Terpenes

Terpenes are a large and diverse class of organic compounds that function as biosynthetic building blocks within nearly every living creature. Terpenes are derived from units of isoprene, which has the molecular formula C₅H₈, therefore the basic molecular formula for terpenes are multiples of (C₅H₈)n. This is known as the isoprene rule or the C₅ rule. Terpenes may be classified by the number of isoprene units in the molecule, and a prefix in the name indicates the number of terpenes units needed to assemble the molecule. One single isoprene unit or C₅ (hermiterpenes), C₁₀ (monoterpenes), C₁₅ (sesquiterpenes), C₂₀ (diterpenes), C₂₅ (sesterpenes), C₃₀ (triterpenes), C₄₀ (tetraterpenes), >C₄₀ (polyterpenes) 83-86. Strictly speaking terpenes are
hydrocarbons, upon modification, oxidation, or addition of functional groups the terpene is then known as a terpenoid, therefore a C\textsubscript{30} triterpene with functional groups would be called a triterpenoid. These terpenoids comprise the largest group of natural plant products, with over 20,000 known members \cite{86,87}.

1.6.2 Triterpenoids and oleanolic acid

Triterpenoids are formed through the cyclization of the 30-carbon squalene, and result in protostanes, lanostanes, holostanes, cycloartanes, dammaranes, euphanes, tirucallanes, tetranortriterpenoids, quassinoids, lupanes, oleananes, friedelanes, ursanes, hopanes, isomalabaricanes, and saponins. These triterpenoid carbon frameworks are cyclized by members of the oxidosqualene cyclase family, which has expanded greatly in plants \cite{88}.

Oleanolic acid and its isomer ursolic acid are triterpenoid compounds that exist widely in plants, and have provided the basis of many traditional folk medicine due to their anti-inflammatory and hepatoprotective abilities. Studies on oleanolic acid confirmed that this triterpenoid does indeed have beneficial effects in numerous diseases, including chemically-induced liver injuries and cancer \cite{89-93}. However, these natural triterpenoids were found to have poor bioavailability and relatively weak biological activity, thus modification and synthetic versions of oleanolic acid have been made to improve on these shortcomings \cite{94,95}. 
1.6.3 Synthetic Oleanane Triterpenoids

More than 300 derivatives of oleanolic acid have been made by the Gribble laboratory (Dartmouth, USA, New Hampshire) and these molecules have been screened for their abilities to inhibit the *de novo* synthesis of the inflammatory enzyme nitric-oxide synthase (iNOS)\(^{96,97}\). With the rationale being that inflammation is a causative factor for the pathogenesis of many human diseases, including cancer. Of the initial candidate screen, the 46\(^{th}\) triterpenoid prepared (TP-46) in Gribble laboratory was identified as the first, “hit,” in their iNOS assay. Subsequent refinement and modification lead to the creation of TP-151 (“CDDO”) (2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid), which was over 10,000 fold more potent than TP-46, and approximately 400,000 times more potent than oleanolic acid in their iNOS assay. Furthermore, it was found that by modifying the C-28 site on CDDO and replacing it with different functional groups, more potent synthetic oleanolic acid derivatives could be developed, two of which are CDDO-Imidazolide (CDDO-Im) and CDDO-Methyl ester (CDDO-Me) (Figure 1.7A)\(^{96,97}\).

As a group, these synthetic oleanane triterpenoids are multifunctional molecules in cell culture assays. In the nanomolar-to-micromolar range, they have been shown to suppress inflammation, activate cytoprotective pathways, induce differentiation, inhibit proliferation and induce apoptosis\(^{95,97-103}\).

1.7 Tricyclic Compounds

During the development of CDDO, structure-activity studies revealed that the 2-cyano-1-en-3-one in ring A and a 9(11)-en-12-one in ring C are responsible for high potency of CDDO. They reasoned that perhaps the entire oleanane skeleton might not be
Figure 1.7 Synthetic oleanane triterpenoids and tricyclic compounds

A) The base structure of the synthetic oleanolic acid derivatives, CDDO, CDDO-Im, and CDDO-Me. Highlighted in red are the enone moieties that contribute to the compounds extreme potency compared to oleanolic acid.

B) The base structure for the acetylenic tricyclic bis-(cyano enone) compounds, TBE-31, TBE-55 and TBE-56. Note the similarity in rings A and C when compared to the synthetic oleanolic acid derivatives.
A) Synthetic Oleanane Triterpenoids

- CDDO
- CDDO-Me
- CDDO-Im

R$_1$ = COOH
R$_1$ = COOCH$_3$
R$_1$ = COO-Imdazolide

B) Acetylenic tricyclic bis-(cyano enones)

- TBE-31
- TBE-55
- TBE-56

R$_1$ = CH$_3$
R$_1$ = CH$_2$OH
R$_1$ = CH$_2$-Biotin
necessary for its potency, and therefore focused their attention on generating a new class of tricyclic compounds (tricyclic-bis-enone derivatives (TBEs)) which have the same A, B, and C rings of CDDO $^{104,105}$. In designing the tricyclic compounds, the medicinal chemists have aimed to avoid some of the limitations of the synthetic oleanane triterpenoids, in which only a single functional group at C-17 could be modified, and an expensive 11 step synthesis required to convert oleanolic acid into CDDO. In contrast, the TBEs could be entirely synthetic, and thus are not hindered by limited functional groups or by steric hindrance and the new compounds may have more structural diversity, better stability, enhanced pharmacokinetic and pharmacodynamics profiles compared to the synthetic oleanane triterpenoids $^{104-106}$.

An early TBE-compound, TBE-9, was found to be nearly equivalent to CDDO in potency, both in *in vitro* assays inhibiting activity against production of nitric oxide by interferon-$\gamma$ in mouse macrophages, and in *in vivo* studies using mouse peritoneal inflammation induced by thioglycollate and interferon-$\gamma$. $^{97,104,105}$ The design, synthesis and biological evaluation of new TBE analogues was continued by the Gribble laboratory. The acetylenic tricyclic bis-(cyano enone), TBE-31 [($\pm$)-(4aα,8aα,10aβ)-1,2,4a,6,8a,9,10,10a-octahydro-8a-ethyl-1,1,4a-trimethyl-2,6-dioxophenanthrene-3,7-dicarbonitrile], synthesized from cyclohexanone as a starting material, was again found to be significantly more potent than CDDO (Figure 1.7B)$^{106,107}$.

### 1.7.1.1 TBE-31 - The acetylenic tricyclic bis-(cyano enone)

Since the compounds design and conception in 2007, TBE-31 has become an attractive candidate to be used as a lead compound. Different groups have assessed its
ability to suppress chronic inflammation, act as a chemopreventative agent and even its ability to serve as an anti-depressant \textsuperscript{106}.

Liby \textit{et al.} in 2008, showed that nanomolar concentrations of TBE-31 were able to suppress the induction of the inflammatory protein, iNOS, activate phase 2 cytoprotective enzymes \textit{in vitro} and \textit{in vivo}, block cell proliferation, and induce differentiation of leukemia cells. When TBE-31 was orally administrated to rats, it significantly reduced formation of aflatoxin-DNA adducts, and decreased the size and number of aflatoxin-DNA preneoplastic hepatic lesions in rats by $>90\%$. This work also suggested TBE-31 may interact with DTT and thus proteins that contain reactive cysteine residues, such as Keap1 \textsuperscript{108}.

The Keap1/Nrf2/ARE pathway regulates the ability of eukaryotic organisms to adapt and survive under various conditions of oxidative, electrophilic, and inflammatory stress by signaling the expression of a network of more than 100 genes, many of which encode for cytoprotective (“phase 2”) proteins. Under basal conditions, this pathway does not operate at its maximal capacity, but can be induced by a wide variety of different classes of small molecules. One universal property of these inducers is their capacity to modify sulfhydryl groups by alkylation, oxidoreduction or disulfide formation \textsuperscript{109-111}. Much of the work by Dinkova-Kostova and her group has focused on this pathway as a target for TBE-31. They demonstrated spectroscopically that both of the cyano enone functional groups of TBE-31 react with Keap1 and activate transcription of cytoprotective genes. By designing monocyclic compounds resembling fragmented rings A or C of TBE-31 (Figure 1.7B), they were able to demonstrate that ring C had a greater contribution as a Michael acceptor (i.e. greater ability to react with cysteines). Although,
most importantly, the presence of both rings held together in the configuration of TBE-31 resulted in exceptionally high inducer potency towards the Keap1/Nrf2/ARE pathway.

Further studies focused on in vivo applications of TBE-31 and the Keap1/Nrf2/ARE pathway. Long-term (five days per week for four weeks) topical applications of small (200 nM) quantities of TBE-31 were found to cause robust systemic induction of the Keap1/Nrf2/ARE pathway and decrease 6-ithoguanine nucleotide—which is a metabolite of azathioprine, a highly effective anti-inflammatory and immunosuppressive agent used in organ transplantation and autoimmune disease, but which profoundly increases the risk for development of unusually aggressive cutaneous squamous cell carcinoma—incorporation in DNA of skin, blood, and liver of azathioprine-treated mice, indicating extraordinary bioavailability and efficacy. In addition, TBE-31 at nanomolar concentrations protected cells that do contain 6-ithoguanine in their genomic DNA against oxidative stress caused by UVA radiation through the induction of the Keap1/Nrf2/ARE pathway. Thus, TBE-31 lowers two of the risk factors that contribute to the development of skin carcinogenesis associated with thiopurine therapies. This was confirmed in further experiments where the dosage of TBE-31 was also optimized. Topical TBE-31 (40 nmol/mouse, 2 days a week) was administered to mice while simultaneously administering azathioprine (1mg/kg/d in the drinking water) 2 weeks before starting an irradiation schedule, which consisted of chronic suberythemal solar simulated UV radiation (comprised of 2 J/cm² UVA and 90 m J/cm² UVB). After biweekly exposures for 15 weeks, irradiation was discontinued and tumour development was evaluated during the subsequent 16 weeks. TBE-31 was administered throughout these 31 weeks. The tumour incidences were not significantly
different between the control and the TBE-31 treated groups, but tumour multiplicity (tumour number) and tumour volume were all significantly lower. With the control group having 9.5 tumours per mouse with an overall average volume of 225 mm³ per mouse, compared to 5.3 tumours with an overall average volume of 41.9 mm³ per mouse for the TBE-31-treated group ¹¹³. In addition, in similar experiments the topical application of TBE-31 was shown to reduce the upregulation of 3 important inflammatory markers, IL6, IL1β and cyclooxygenase-2 by 55-, 24-, and 30% respectively, in the skin of UV radiation-induced inflammation in mice. This protective effect was largely absent in Nrf2-KO mice ¹¹³. Overall, Dinkova-Kostova and her group demonstrate that TBE-31 effectively activate the Keap1/Nrf2/ARE pathway, reduce inflammation and reduce tumour burden.

Other studies have explored TBE-31 and the Keap1/Nrf2/ARE outside of a chemopreventative context. Nrf2 activators have anti-depressant effects in animal models of depression. One study showed TBE-31 was able to potentiate nerve growth factor induced neurite outgrowth in PC12 cells in a concentration dependent manner. This effect was lost in Nrf2 siRNA treated cells. Furthermore, oral administration of TBE-31 to mice significantly attenuated an increase in serum levels of tumour necrosis factor-α (TNF-α) after administration of lipopolysaccharide (LPS). Lastly, in their LPS induced model of depression, they found TBE-31 to lower signs of depression in the tail suspension and forced swimming tests used to assess behaviour in mice. Therefore, TBE-31 may also be a potential therapeutic drug for inflammation-related depression ¹¹⁴. Aside from the Keap1/Nrf2/ARE pathway, TBE-31 has also been found to be able to upregulate Hsp70
in an HSF1-dependent manner in mouse embryonic fibroblasts (MEFs), and this has been shown to play a role in protection against peroxynitrite-induced cytotoxicity\textsuperscript{115}.

The success of TBE-31 has prompted medicinal chemists to continue refining the process of generating TBE-31. Steps in the synthesis have been improved to increase the efficiency and yield of TBE-31\textsuperscript{116,117}. Moreover, a method for generating $^{13}\text{C}_2^{15}\text{N}_2$-labeled TBE-31 and use in liquid chromatography-mass spectrometry (LC-MS) has been established, which will be useful for quantification of cell, tissue and plasma levels of TBE-31\textsuperscript{118}. Lastly, a biotin conjugated TBE-31 has been generated for use as a tool for the isolation of protein targets of TBE-31 (Figure 1.7B)\textsuperscript{119}.

### 1.8 CDDO-Im, CDDO-Me and cell migration

Much of the current literature on the class of synthetic oleanolic acid derivatives and TBE-31 has been focused on their anti-inflammatory properties as outlined above. Although this is important work and has shown their potential use as chemopreventative agents through their anti-inflammatory properties, it has not address their effects on metastasis. Studies in the Di Guglielmo lab have begun exploring other important properties of these compounds, namely the cytoskeletal altering effects of the synthetic oleanolic acid derivatives and their effects on cell migration—an important component of metastasis. More specifically, CDDO-Im was found to alter microtubule dynamics by disrupting the microtubule capping protein, Clip-170. Microtubule-dependent processes were also altered, including the dispersal of EEA1-positive endosomes from the perinuclear region of CDDO-Im treated cells. Furthermore, CDDO-Im disrupted the localization of IQGAP1, PKC$\zeta$, Par6, and TGF$\beta$ receptors from the leading edge of
migrating cells and inhibited TGFβ-dependent cell migration (Figure 1.8A top)\textsuperscript{120}. In addition, CDDO-Im and CDDO-Me were also found to associate with Arp3, a subunit of the Arp2/3 complex, which is necessary for branched actin polymerization and the formation of the lamellipodia in migrating cells. CDDO-Im and CDDO-Me were found to inhibit Arp3 localization at the leading edge, abrogate cell polarity, and inhibit Arp2/3-dependent branched actin polymerization (Figure 1.8A bottom)\textsuperscript{121}. The effects of CDDO-Im and CDDO-Me on the microtubule network, cell polarity and the actin cytoskeleton are all believed to contribute to inhibiting cell migration, thus making the synthetic triterpenoids potential anti-metastatic drugs \textsuperscript{120,121}.

1.9 Rationale and Hypothesis

Metastatic disease accounts for over 90% of cancer mortalities and there is an urgent need for therapeutic agents. Modulating or inhibiting any of the various steps that contributes to metastasis will greatly reduce incidences of cancer mortality. We have previously studied synthetic oleanolic acid derivatives for their abilities to impair cell migration—a process that is heavily utilized in multiple steps during the metastatic cascade. The newer tricyclic compounds or TBEs resemble the synthetic oleanolic acids, and contain the same active regions responsible for their potency (Figure 1.8B). TBE-31 has been demonstrated to be particularly potent in anti-inflammatory assays compared to the synthetic triterpenoids, but their effects on cell migration have not yet been studied. Given the structural similarities between the synthetic oleanolic derivatives and TBE-31, we hypothesize that TBE-31 will inhibit cell migration by targeting components of the
Figure 1.8 The effects of the synthetic oleanane triterpenoids and tricyclic compounds on cell migration

A) A summary of the effects of CDDO-Im on cell migration. Top, CDDO-Im was found to alter microtubule dynamics by disrupting the microtubule capping protein, Clip-170, disrupt microtubule dynamics and organization, and cause a loss of polarity proteins to the leading edge. Bottom, CDDO-Im was found to inhibit Arp2/3-dependent branched actin polymerization at the leading edge. Note filopodia are unaffected as their formation is independent of Arp2/3.

B) The effects of TBE-31 on cell migration are currently unknown.
cytoskeleton. Therefore, we propose to examine the effects of TBE-31 on the components of the cytoskeleton necessary for cell migration. More specifically, we aim to:

1) Characterize the effect(s) of TBE-31 on the actin cytoskeleton and cell migration.

2) Characterize the effect(s) of TBE-31 on the structure and dynamics of the microtubule network and cell migration.

3) Determine how TBE-31 interacts with actin.

In doing so, we will broaden our understanding of the molecular biology of TBE-31 and their role in cell migration and help advance the development of the tricyclic compounds and anti-metastatic agents in general.
1.10 References


   10.1038/nrc1886 (2006).


119 Saito, A. *et al.* Synthesis and biological evaluation of biotin conjugates of (+/-)-(4bS,8aR,10aS)-10a-ethynyl-4b,8,8-trimethyl-3,7-dioxo-3,4b,7,8,8a,9,10,10a-octahydro-phenanthrene-2,6-dicarbonitrile, an activator of the Keap1/Nrf2/ARE pathway, for the isolation of its protein targets. *Bioorganic & medicinal chemistry letters*, doi:10.1016/j.bmcl.2013.08.058 (2013).


Chapter 2

The acetylenic tricyclic bis-(cyano eneone), TBE-31 inhibits non-small cell lung cancer cell migration through direct binding with actin

A version of this chapter has been published in Cancer Prev Res 7, 727-737, (2014).
2 Chapter 2

2.1 Chapter Summary

The migratory and invasive potential of the epithelial-derived tumour cells depends on epithelial-to-mesenchymal transition (EMT) as well as the reorganization of the cell cytoskeleton. Here we show that the tricyclic compound TBE-31 directly binds to actin and inhibits linear and branched actin polymerization \textit{in vitro}. Furthermore, we observed that TBE-31 inhibits stress fiber formation in fibroblasts as well as in non-small lung cancer cells during TGFβ-dependent EMT. Interestingly, TBE-31 does not interfere with TGFβ-dependent signaling or changes in E-cadherin and N-cadherin protein levels during EMT. Finally, we observed that TBE-31 inhibits fibroblast and non-small cell lung tumour cell migration with an IC$_{50}$ of 1.0 and 2.5 µM, respectively. Taken together, our results suggest that TBE-31 targets linear actin polymerization to alter cell morphology and inhibit cell migration.
2.2 Introduction

Cell migration is essential in numerous physiological processes such as embryogenesis, immune response, cell differentiation and cell renewal. However, it is also important in the later stages of cancer metastasis, where cell migration and invasion allow cancer cells to establish secondary tumour sites, which accounts for over 90% of cancer related deaths [1].

Epithelial cells are normally non-motile, organized in an apical-basal polarity, and multiple cell-to-cell adhesions help assemble epithelial cells into a sheet-like formation [2-5]. In order for epithelial based tumours to detach from the primary tumour and migrate, the cells will have to dissociate these cell-to-cell contacts in a process known as the epithelial-to-mesenchymal transition (EMT) [2-5]. These biochemical changes, among others, liberate the epithelial cells and enable them to assume a much more mesenchymal-like phenotype and prime them for cell migration and invasion [2-5].

Cell migration begins with an initial protrusion of the cell membrane. Newly forming cell adhesions at the front attach the protrusions to the substratum and provide traction, while the focal adhesions at the rear disassemble and contractions help retract the tail. As this occurs repeatedly the cell translocates across the substratum [6-8]. This process relies on the localization of polarity proteins and the reorganization of different components of the cytoskeleton, which consists of the microtubule network, intermediate filaments and the actin cytoskeleton. Although all the cytoskeletal components act in concert, it is the actin cytoskeleton that provides the force necessary for translocation. The localization of Rac1 or Cdc42 towards the leading edge with other polarity proteins is a key regulatory event that stimulates actin polymerization and results in membrane
protrusion towards the direction of migration. G-actin subunits polymerize to form actin filaments creating filopodia and lamellipodia. Cdc42 and formins regulate actin polymerization to form long unbranched bundles of actin to form filopodia, which are thought to act as sensors that can probe for external cues. Rac1 activates actin related proteins 2 and 3 (Arp2/3), causing it to bind to preexisting bundles of actin and promote branched actin polymerization to form lamellipodia, which are broad sheet like protrusions that drive the cell forwards 6-8. Bundles of actin also form stress fibers to give a cell its shape and work with focal adhesions and myosin to produce contractile forces in cell migration 9,10. Since EMT and cell migration are necessary processes for tumour cell metastasis, chemotherapeutic drugs that target various aspects of these processes are continuously sought.

Triterpenoids are a family of naturally occurring compounds biologically synthesized in plants by the cyclization of squalene. They are a chemically diverse family with over 20,000 known compounds 11,12. Although oleanolic acid is of particular interest because of its anti-inflammatory and anti-tumourigenic properties, these potencies are relatively weak 11-14. Continuous synthetic modifications on oleanolic acid led to 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), which has much higher anti-inflammatory and cytoprotective potency than oleanolic acid, as well as its imidazolide (CDDO-Im) and its methyl ester (CDDO-Me) 11-16. They have been demonstrated to effectively inhibit cytokines from inducing the formation of inducible nitric oxide synthase in macrophage cells and inducing phase 2 cytoprotective enzymes, both of which help prevent a cancer causing environment. In addition, they were found to inhibit proliferation, induce differentiation, and induce apoptosis in cancer cells in vitro. They
have also been shown to inhibit carcinogen induced primary tumour growth, orthotopic and ectopic tumour formation, as well as lung metastasis in multiple experimental animal models 11,12,17-22.

Recently, we have observed that CDDO-Im and CDDO-Me have a number of effects on the cytoskeleton, leading to the interference of physiological processes that depend on them 23,24: CDDO-Im was observed to disrupt the microtubule network, displacing the polarity proteins IQGAP1 and Rac1 from the leading edge of migrating cells to abrogate cell polarity 23. Branched actin polymerization depends on the binding of actin related protein 2/3 (Arp2/3) to preexisting bundles of actin to provide a nucleation site and allow for branches of actin to polymerize, and CDDO-Im and CDDO-Me were both found to inhibit the actions of Arp2/3 24. Together these actions potently inhibit cell migration 23,24.

Structure-activity studies on the parental pentacyclic compounds have revealed that the 2-cyano-1-en-3-one in ring A and a 9(11)-en-12-one in ring C are responsible for the potency of these compounds. Therefore new tricyclic compounds, termed tricyclic bis(enone) compounds (TBEs), were generated and which have great potential for structural diversity, robust pharmacokinetic and pharmacodynamic profiles 25-27. In the pool of semisynthetic triterpenoids and synthetic TBEs, TBE-31 (Figure 2.1B) is one of the most potent compounds 25-27. The oral administration of TBE-31 resulted in a profound and dose–dependent induction of the cytoprotective enzymes, NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferases in the stomach, skin, and liver of mice, and indicated excellent oral bioavailability 28. Also, long-term topical daily applications of TBE-31 caused a robust systemic induction of the
Figure 2.1 Chemical structures of pentacyclic triterpenoids and the acetylenic tricyclic bis-(cyano enone), TBE-31.

A) The basic pentacyclic triterpenoid structure. CDDO-Im, which contains an imidazolide group, is used in this study.

B) The acetylenic tricyclic bis-(cyano enone), TBE-31 and below is shown the biotinylated TBE-31, TBE-56.
A) Triterpenoids

CDDO: \( R = \text{OH} \)
CDDO-Me: \( R = \text{OMe} \)
CDDO-Im: \( R = \)

B) TBE-31
TBE-55
Keap1/Nrf2/ARE pathway and decreased 6-thioguanine incorporation in DNA of skin, blood, and liver of azathioprine–treated mice, indicating extraordinary bioavailability and efficacy 29. Furthermore, TBE-31 is orally highly protective against aflatoxin–induced liver cancer in rats 30.

To examine protein targets of TBE-31, a biotin conjugate of TBE-31, TBE-56, which is obtained from TBE-55, was designed and synthesized 31. In the present study, we investigated the effect(s) of TBE-31 on the actin cytoskeleton, and have determined that unlike the CDDO analogues, TBE-31 binds directly to actin to inhibit polymerization, and by doing so, TBE-31 also inhibits stress fiber formation during EMT as well as NSCLC tumour cell migration. Therefore, TBE-31 is an exciting prospect for multi-targeted inhibition of metastatic potential.
2.3 Methods

2.3.1 Cell Culture, Antibodies and Reagents

Rat2 and NIH 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s Medium (DMEM). A549 lung adenocarcinomas were cultured in Kaighn's Modification of Ham's F-12 Medium (F-12K) and H1299 human non-small cell lung carcinoma cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640). All cells were cultured in a 37°C incubator with 5% CO₂, and all media was supplemented with 10% fetal bovine serum (FBS) unless otherwise stated.

AlexaFluor 555 conjugated phalloidin (A34055) was purchased from Invitrogen. Anti-Smad2/3 (BD610843), anti-E-Cadherin (BD610182), anti-N-Cadherin (BD610921) and anti-Paxillin (BD610051) were purchased from BD transduction Laboratories (Mississauga, Ontario). Anti-Phospho-Smad 2 (AB3849) was purchased from Millipore. Cytochalasin D (C8273), Anti-Arp3 (A5979) was purchased from Sigma-Aldrich. NeutrAvidin Agarose beads (29200) was purchased from Thermo Scientific. Biotin Azide (PEG4 carboxamide-6-Azidohexanyl Biotin) (B10184) was purchased from Life Technologies. CDDO-Im and TBE-31 compounds were provided by Dr. Michael B. Sporn (Dartmouth, NH). The purified actin (AKL99), Arp2/3 (RP01), VCA domain of WASP (VCG03) and actin polymerization kits (BK003) were purchased from Cytoskeleton Inc.
2.3.2 Immunofluorescence microscopy

In all immunofluorescence microscopy studies, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.25% Triton X-100 for 5 min, blocked in 10% FBS, and immunostained overnight with the appropriate primary antibodies. All images were taken with an Olympus IX81 inverted epifluorescence microscope.

2.3.3 Scratch Assays and Immunofluorescence Microscopy

H1299 or NIH 3T3 cells were grown to confluence, and the monolayer was scratched with a pipette tip. DMSO, and varying concentrations of TBE-31 and CDDO-Im were added to their appropriate media, and incubated at standard conditions. For scratch assays, bright field images were obtained at 0 h with an Olympus IX81 microscope and the same coordinates were reimaged after 18 h (H1299 cells) or 12 h (NIH 3T3 cells). The extent of cell migration was measured by finding the difference in width of the scratch between 0 h and final time point. To generate videos, an Olympus IX81 inverted microscope was fitted with a custom chamber which allowed us to maintain a temperature of 37°C and an atmosphere containing 5% CO₂. Bright field images were collected at regular 15 min intervals over the duration of the experiment. For immunofluorescence studies, Rat2 cells were scratched and given 4 h to polarize, before incubating in media containing DMSO, 1.0 µM of TBE-31, or 1.0 µM of CDDO-Im for 2 h. The cells were then fixed, permeabilized and immunostained with anti-Arp3 antibody, phalloidin for filamentous actin, and DAPI for the nuclei.
2.3.4 Subconfluent Cell Migration

Subconfluent NIH3T3 or H1299 cells were grown and treated with DMSO, 1 \( \mu M \) TBE-31 or 0.5 \( \mu M \) CDDO-Im. Time-lapse observations were made using an Olympus IX81 inverted microscope equipped with a custom chamber which allowed us to maintain a temperature of 37°C and an atmosphere containing 5% CO\(_2\). Bright field images were collected at 10 min intervals over 18 h. Distance of migration was determined by tracking the positions of cell nuclei over 18 h using the MtrackJ plugin\(^{32}\) for imageJ software\(^{33}\).

2.3.5 In Vitro Actin Polymerization

In vitro actin polymerization experiments were carried out using a modified version of the protocol supplied by Cytoskeleton Inc. Purified pyrene labeled actin was resuspended and incubated in general actin buffer provided by the Cytoskeleton Inc. for 1 h on ice to depolymerize any actin oligomers followed by microcentrifugation at 4 °C for 30 min. Two \( \mu M \) actin (Actin) or actin in the presence of 100 nM VCA domain of n-WASp protein and 13 \( \mu M \) Arp2/3 complex (AVA) were incubated with DMSO, or 100 \( \mu M \) of TBE-31 for 15 min on ice, before adding the actin polymerization buffer and warming the mixtures to 37°C to initiate polymerization. The change in fluorescence was measured with a Wallac Victor3 V plate reader.

As a control, actin was fully polymerized in the absence of treatment (30 min) and the fluorescence intensity was measured. DMSO, 100 \( \mu M \) TBE-31 or 100 \( \mu M \) CDDO-Im was then added and the fluorescence intensity was immediately re-measured to ensure that these molecules do not interfere or quench the fluorescence intensity.
2.3.6 Actin Stress Fiber Repolymerization

A subconfluent layer of NIH 3T3, H1299 or Rat2 cells grown on coverslips incubated in media containing 5 μM of cytochalasin D for 30 min at 37°C. Afterwards, the cells were rinsed with clean media to remove cytochalasin D, before reincubating in media containing either DMSO, 1 μM TBE-31, or 1 μM CDDO-Im. At various time points after reintroducing the new media, the cells were fixed permeabilized, and labeled for actin using AlexaFluor 555-conjugated phalloidin.

2.3.7 Affinity Pull-downs

Rat2 lysates incubated with DMSO, biotin, TBE-55, or TBE-56 at 4°C for 2 h, followed by incubating with NeutrAvidin beads for 1 h at 4°C to precipitate the proteins interacting with the biotin conjugate, TBE-56. Afterwards, the beads were thoroughly washed, and 2X Laemmli sample prep buffer was added. These samples were then subjected to SDS-PAGE, and silver staining was performed. Proteins that were uniquely stained in the TBE-56 samples were excised from the gel, trypsinized and analyzed by electrospray mass spectrometry. To confirm our results, the same pull-down experiments from above were performed with cell lysates or purified actin proteins, immunoblotting with anti-actin was performed after subjecting the samples to SDS-PAGE. For competitive binding studies, additional samples were added where TBE-31 or TBE-55 were incubated with lysates 1 h before incubating with TBE-56, followed by the same steps as above.
2.3.8 Epithelial-to-mesenchymal transition

A549 cells were incubated with 0.2% FBS F-12k media for 4 h, before the addition of 200 pM TGFβ was added for 48 h at 37°C to initiate an epithelial-to-mesenchymal transition. For immunofluorescence studies the cells were grown on coverslips and fixed, permeabilized and immunostained with monoclonal paxillin antibody, and AlexaFluor 555 conjugated phalloidin for filamentous actin after the 48 h treatment. For western blot analysis, the cells were lysed, subjected to SDS-PAGE and immunoblotted for E-Cadherin, N-Cadherin, Smad2/3, Phospho-Smad2 or actin.
2.4 Results

A hallmark of epithelial derived tumours is unregulated cell growth, and in many cases, the later stage of tumour progression is that cells undergo metastasis to migrate away from the primary tumour site and invade distant organs to establish secondary tumours \(^2,^{34-37}\). Therefore the development of chemotherapeutic agents that can inhibit different aspects of metastasis, including EMT and cell migration, will play a critical role in reducing in the rate of cancer mortality. EMT and cell migration are dependent on the reorganization of the cytoskeleton, and the actin cytoskeleton has been proposed to be a feasible target in tumour metastasis \(^38\).

2.4.1 TBE-31 does not alter Arp3 localization in polarized cells

Cell migration is dependent on the proper localization of proteins involved in maintaining cell polarity (for e.g., Cdc42/Rac1 and IQGAP1) as well as proteins involved in the formation of the lamellipodia \(\text{via} \) Arp2/3-dependent branched actin polymerization at the leading edge of migrating cells \(^6-8,^{39-41}\). We previously demonstrated that CDDO-Im displaces Arp2/3 from the leading edge, reduces branched actin polymerization and inhibits cell migration \(^23,24\). TBE-31 contains a similar structural core as the pentacyclic triterpenoids (Figure 2.1) and has been shown to induce the Keap1/Nrf2/ARE pathway in a similar fashion as the pentacyclic compounds \(^28,29\). However, the effect(s) of TBE-31 on the cytoskeleton remain unknown. We therefore used immunofluorescence microscopy to examine if TBE-31 would target the cytoskeleton by first assessing if it targets the Arp2/3 complex and displaces it from the leading edge of migrating cells as
efficiently as CDDO-Im. Briefly, Rat2 fibroblasts were grown to confluence, scratched and allowed to polarize before being treated with DMSO, 1 \(\mu\)M TBE-31 or 1 \(\mu\)M CDDO-Im. The cells were then immunostained for Arp3 and filamentous actin, followed by processing for immunofluorescence microscopy (Figure 2.2A). We observed that 88 ± 3% of the cells treated with DMSO exhibited Arp3 localization at the leading edge, while CDDO-Im reduced leading edge staining of Arp3 to 53 ± 18% (Figure 2.2), consistent with our previous observations \(^{24}\). Interestingly, TBE-31 did not alter leading edge staining of Arp3 compared to the cells treated with vehicle (Figure 2.2). This was intriguing because the core of the CDDO-Im molecule (similar to the TBE-31 core) was modeled to fit into the Arp3 inhibitory groove \(^{24}\), similar to the \textit{bona fide} Arp3 inhibitor, CK-869 \(^{42}\). The lack of displacement of Arp3 from the leading edge in cells treated with TBE-31 suggests that the compound is not associating and interfering with the subcellular localization of Arp3. Therefore, in order to assess the binding of TBE-31 with Arp3 and/or other cytoskeletal proteins, we next carried out a pull-down approach.

2.4.2 TBE-31 binds to actin directly

To determine the binding partners for TBE-31, cell lysates were incubated with either DMSO or TBE-56, followed by precipitation with NeutrAvidin beads. Proteins associating with TBE-56 were separated on SDS-PAGE, processed and analyzed by mass spectrometry (Figure 2.3A). Although we previously used this approach to identify that CDDO-Me associated with Arp3 \(^{24}\), we were unable to detect an association between TBE-56 and Arp3. The main cytoskeletal protein that was identified to associate with TBE-56 was actin and we further analyzed this interaction by immunoblot analysis.
Figure 2.2 TBE-31 does not alter Arp3 localization to the cell periphery

A) Confluent monolayers of Rat2 fibroblasts were scratched and incubated at 37°C in DMEM supplemented with 10% FBS for 4 hours to allow them to polarize. Cells were then incubated for 2 h in media containing DMSO, 1 µM CDDO-Im, or 1 µM TBE-31. The cells were then fixed, permeabilized and immunostained with Arp3 (green), AlexaFluor 555 phalloidin (red) for filamentous actin and DAPI (blue) for the nuclei. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 40x magnification. Bar = 10 µm. The arrows highlight staining of Arp3 at the cell periphery.

B) Experiments were carried out as described in panel A, quantitated and graphed for the number of cells containing leading edge staining for Arp3 (n=3±SD, *p<0.05).
Figure 2.3 TBE-31 binds to actin

A) Rat2 fibroblasts were incubated with either 10 μM biotin, TBE-55 or biotinylated-TBE-31 (TBE-56), lysed, and incubated with NeutrAvidin-agarose beads. SDS-PAGE and silver staining were performed and proteins that were uniquely stained in TBE-56-treated samples were excised from the gel, trypsinized, and analyzed by electrospray mass spectrometry. Protein identifications are indicated on the right of the gel. Non-specific binding labeled as NS. (n=3)

B) Rat2 lysates or purified actin were incubated with either DMSO, TBE-55, or biotinylated-TBE-31 (TBE-56) at 4°C for 2h, followed by incubating with NeutrAvidin beads for 1h at 4°C to precipitate the proteins interacting with TBE-56. Afterwards, the beads were thoroughly washed, and sample prep buffer was added. These samples were then subjected to SDS-PAGE, followed by immunoblotting for actin. Shown here is a representative immunoblot (n=3).

C) To perform competitive binding studies similar experiments were performed as described in Panel B. Additional samples were add that had been pretreated with increasing concentrations of TBE-31 or TBE-55, before incubating with biotinylated TBE-31 (TBE-56). Shown here is a representative immunoblot (n=3)
(Figure 2.3B). Briefly, Rat2 lysates were incubated with DMSO, TBE-55 or TBE-56, precipitated with NeutrAvidin beads and processed for immunoblotting with actin antibodies and we observed that TBE-56 associated with actin in cell lysates (Figure 2.3B, left panel). In order to assess if this interaction was direct, we carried out the pull-down assay using purified actin and observed similar results (Figure 2.3B, right panel), suggesting that TBE-56 does indeed bind directly to actin and not through an associated binding partner. Furthermore, we performed competitive binding assays where Rat2 lysates were pretreated with either DMSO, or increasing concentrations of TBE-31 or TBE-55, before incubating with TBE-56. We found that by increasing the concentrations of TBE-31 or TBE-55 in the pretreatment, actin was unable to be precipitated by the biotin conjugate, TBE-56 (Figure 2.3C).

These results suggested that biotinylated TBE-31 is able to associate with actin but not Arp3. We next assessed if the tricyclic compound would have an effect on linear vs. actin polymerization in vitro.

### 2.4.3 TBE-31 inhibits actin polymerization

Actin is a globular multifunctional protein that binds end-to-end to form filaments, which can provide a basis for contractile forces, cell protrusions or structure. We examined the effects of TBE-31 on actin dynamics by assessing in vitro polymerization of actin (Figure 2.4A). Briefly, purified pyrene labeled actin was incubated on ice to depolymerize any actin oligomers. Actin was incubated in the
Figure 2.4 TBE-31 inhibits actin polymerization and stress fiber formation

A) Purified pyrene-labeled actin was polymerized in the presence of DMSO (vehicle), 5 μM cytochalasin D (Cyto. D; top panel), 100 μM CDDO-Im (middle panel) or 100 μM TBE-31 (bottom panel). For branched actin polymerization 100 nM VCA domain of n-WASp protein and 13 μM Arp2/3 complex in addition to the Actin (AVA). Actin polymerization was measured by pyrene fluorescence and graphed as fluorescence intensity (arbitrary units) versus time (min). (n=3 ± SEM). Note: All the conditions in the graphs were carried out together and the results are separated into three graphs for the sake of clarity. The control conditions (gray lines) are shown in each graph to indicate the differences in vehicle vs. compound treated samples.

B) To ascertain that the addition of TBE-31 or CDDO-Im would not quench pyrene-actin fluorescence intensity, pyrene actin polymerization was carried out as described in the Materials section, measured (-) and then DMSO, TBE-31 or CDDO-Im were added to the samples and re-measured (+) for fluorescence intensity. Note that the fluorescence intensity did not appreciably change after vehicle or drug addition.

C) A subconfluent layer of NIH 3T3 cells were grown on coverslips and incubated in DMEM supplemented with 5 μM cytochalasin D at 37°C for 30 min to depolymerize the actin cytoskeleton. Afterwards, the cells were rinsed with PBS to remove the previous treatments. Media supplemented with DMSO, 1 μM TBE-31, or 1 μM CDDO-Im was then reintroduced to the cells while the actin stress fibers repolymerized in the absence of cytochalasin D. The cells were then fixed, permeabilized and immunostained with AlexaFluor 555 phalloidin to visualize actin filaments (red), and DAPI (blue) for the nuclei. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 40x magnification (n=3). Bar=20 μm.

D) Quantitation of cells with actin stress fibers (inset; white arrows) was carried and graphed (bottom panel; n=3± SEM, *p<0.05). Quantified cells were differentiated from cells containing cortical actin staining (inset; yellow arrowhead).

E) A subconfluent layer of Rat2 fibroblasts were grown on coverslips and incubated in
media supplemented with 5 μM cytochalasin D at 37°C for 30 min to depolymerize the actin cytoskeleton. Afterwards, the cells were rinsed with PBS to remove the previous treatments. Media supplemented with DMSO, 1 μM TBE-31, or 1 μM CDDO-Im was then reintroduced to the cells while the actin stress fibers re-polymerized in the absence of cytochalasin D. The cells were then fixed, permeabilized and immunostained with AlexaFluor 555 phalloidin to visualize actin filaments (red), and DAPI (blue) for the nuclei. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 40x magnification (n=3). Bar=20 μm. Inset: Representative areas were magnified to demonstrate actin stress fibers (white arrows) or areas of branched actin polymerization (blue arrowheads). Note that TBE-31 treatment reduced the length of stress fibers but did not inhibit branched actin polymerization. However, CDDO-Im treated Rat2 fibroblasts contained actin stress fibers comparable to vehicle-treated cells but contained reduced branched actin polymerization.
presence of DMSO (vehicle), TBE-31, CDDO-Im or cytochalasin D (CD) for 15 min on ice, before adding the actin polymerization buffer and warming the mixtures to 37°C to initiate polymerization. To induce branched actin polymerization, Arp2/3, and the VCA domain of n-WASp was added in addition to the Actin (AVA). By recording the change in fluorescence intensity, we measured the rate of polymerization. Cytochalasin D is a well-established mycotoxin that reversibly binds with globular actin monomers to prevent polymerization. As expected, cytochalasin D inhibited the rate of linear and branched actin polymerization (Figure 2.4A, top panel). CDDO-Im inhibited the Arp2/3 mediated branched actin polymerization and had little effect on the polymerization rate of linear actin (Figure 2.4A, middle panel). This was expected, as we have previously demonstrated that CDDO-Im only acts on Arp2/3 to inhibit Arp2/3 mediated branched actin polymerization. Interestingly, TBE-31 was able to inhibit both branched and linear actin polymerization (Figure 2.4A, bottom panel). Furthermore, TBE-31 also reduced the total overall amount of polymerized actin by approximately 40%, suggesting that unlike cytochalsin D, which is capable of depolymerizing filamentous actin, TBE-31 may be sequestering monomeric actin and preventing it from being incorporated into growing filamentous actin fibers. Finally, to ascertain that the addition of the tri- or penta-cyclic compounds was not interfering with the fluorescence of the pyrene actin in the assay, we allowed actin to fully polymerize and then incubated the polymerized actin in the presence or absence of DMSO (control) CDDO-Im or TBE-31 (Figure 2.4B). We observed that the addition of the tri or penta-cyclic compounds did not quench the fluorescent signal.
Taken together, these results are intriguing because CDDO-Im and CDDO-Me were found to inhibit cell migration by blocking branched actin polymerization \(^{24}\), however TBE-31 may affect cell migration by targeting general actin polymerization. We therefore next assessed the effect TBE-31 would have an effect to actin polymerization in cells.

Actin polymerization in cell culture was also examined in parallel with our \textit{in vitro} experiments by examining actin stress fiber formations in NIH 3T3 fibroblasts. Briefly, NIH 3T3 fibroblasts were incubated for 30 min in media containing cytochalasin D to completely depolymerize the actin stress fibers. Afterwards, cytochalasin D was removed and the cells were incubated in media containing DMSO, TBE-31, or CDDO-Im while the actin cytoskeleton re-polymerized. At various time points, the cells were fixed, permeabilized, and labeled for filamentous actin using phalloidin. Immediately after cytochalasin D treatment, actin filaments were completely depolymerized, causing the cells to round up and lose their polarized morphology (Figure 2.4C, top panel). However, after removing the cytochalasin D and incubating cells in the presence of DMSO or CDDO-Im, the actin re-polymerized and formed stress fibers. Interestingly, TBE-31-treated cells did not reestablish elongated stress fibers (Figure 2.4C, top panel). Closer examination showed that only short, cortical stress fiber formation was occurring (Figure 2.4C, inset). Quantitation of these cells for cells with actin stress fibers \textit{vs.} cells without stress fibers supported our observations. By two hours DMSO, CDDO-Im, and TBE-31 treated cells had 47 ± 1%, 40 ± 3% and 39 ± 5% of cells with stress fibers formed respectively (Figure 2.4D). By six hours DMSO and CDDO-Im treated cells had 69 ± 3 % and 73 ± 2% recovery of stress fibers, while TBE-31 treated cells had lagged behind
with only 43 ± 4%. Similar results were obtained using Rat2 fibroblasts (Figure 2.4E). These results further support the idea that TBE-31 inhibits actin polymerization and predict that stress fiber formation would be inhibited in tumour cells undergoing epithelial to mesenchymal transition.

2.4.4 TBE-31 inhibits TGFβ-dependent actin stress fiber formation during epithelial-to-mesenchymal transitions

During epithelial-to-mesenchymal transitions, the actin cytoskeleton rearranges from a cortical alignment associated with cell-cell junctions into actin stress fibers, which are associated with cell migration. We therefore investigated whether TBE-31 would have an effect on the actin-dependent changes that occur during EMT. Briefly, non-small cell lung cancer (NSCLC) A549 cells were incubated in serum-free medium or medium containing TGFβ for 48 h, and either DMSO or TBE-31. Afterwards, the cells were fixed, permeabilized and immunostained with paxillin antibody and phalloidin to visualize filamentous actin. Under bright field microscopy we observed a cobble stone phenotype associated with epithelial cells in the cells that were not stimulated with TGFβ, regardless of the addition of DMSO or TBE-31 (data not shown). These cells only demonstrated cortical actin staining with little to no stress fibers, which is another characteristic of epithelial cells (Figure 2.5A). Conversely, the cells stimulated with TGFβ in the presence of DMSO demonstrated an elongated, and spindle like phenotype and under immunofluorescence microscopy these cells also demonstrated proper stress fiber formation (Figure 2.5A), all of which are associated with a more mesenchymal phenotype. TGFβ stimulation in the presence of TBE-31 resulted in less stress fiber
Figure 2.5 TBE-31 inhibits TGFβ-dependent actin stress fiber formation during NSCLC tumour cell EMT

A) Serum-starved A549 cells were incubated in the presence or absence of TGFβ and with DMSO or 1 µM TBE-31 for 48 h at 37°C. The cells were then fixed, permeabilized, and immunostained with monoclonal paxillin antibody (green), and AlexaFluor 555 conjugated phalloidin (red) for filamentous actin. Shown here are representative immunofluorescence images at 40x magnification (n=3). Bar = 10 µm. Insets highlight area of interest and indicate actin stress fibers (white arrows) vs. cortical actin staining (yellow arrowhead).

B) Serum-starved A549 cells were incubated in media supplemented with 200 pM TGFβ and either DMSO or 1 µM TBE-31 for 30 min at 37°C. Cells were lysed prior to TGFβ stimulation, and 0.5 h, 1.5 h and 4.5 h after stimulation. Lysates were subjected to SDS-PAGE and immunoblotted for p-Smad2, and Smad2 (n=4).

C) A549 cells were serum-starved for 4 h, before incubating in media containing 200 pM TGFβ and either DMSO, 1 µM TBE-31, or varying concentrations of cytochalasin D for 48 h. Cells were subsequently lysed, subjected to SDS-PAGE and immunoblotted for E-Cadherin, N-Cadherin, or Actin (n=3).
formation and elongation; instead they displayed the cobblestone phenotype associated with epithelial cells (Figure 2.5A). These results suggest that TBE-31 inhibits the actin related changes in EMT induced by TGFβ.

In order to assess if the inhibition of actin stress fiber formation in the A549 cells was due to an inhibition of the EMT process, we examined TGFβ signal transduction by examining Smad2 phosphorylation to ensure TBE-31 was not interfering with TGFβ signaling. Briefly, A549 cells were serum starved for 4 h before treatment with DMSO, TBE-31 or varying concentrations of cytochalasin D for 30 min. Afterwards, TGFβ was added to the media and was allowed to incubate for another 30 min before being lysed, subjected to SDS-PAGE, and immunoblotted for P-Smad2, Smad2 and actin. We observed that regardless of the pharmacological treatment, TGFβ was able to induce the phosphorylation of Smad2, suggesting that none of our treatments affected the TGFβ signaling pathways (Figure 2.5B).

To further verify proper TGFβ signaling, we examined other biochemical changes that mark EMT. E-cadherin is the major component of epithelial adherens junctions, which form a belt-like structure around the cells to tether adjacent cells into an immobile sheet-like formation. During EMT the expression of E-cadherin becomes down regulated, which liberates the cells from one another. Conversely the expression of N-Cadherin, an adhesion molecule associated with highly migratory mesenchymal cells, becomes up regulated. This is known as the cadherin switch and is a major hallmark of EMT 2-5. These changes were examined by treating A549 cells with TGFβ for 48 h in the presence or absence of DMSO, TBE-31 or cytochalasin D, subjected to SDS-PAGE and immunoblotted for E-Cadherin and N-Cadherin (Figure 2.5C). Regardless of treatment,
TGFβ stimulation led to a decrease in E-cadherin expression, while N-cadherin expression increased. This suggested that a proper cadherin switch was occurring in the presence of TGFβ in vehicle, TBE-31 or cytochalasin D-treated cells and further reconfirms that TBE-31 does not have an effect on TGFβ signaling, or gene transcription associated with EMT.

2.4.5 TBE-31 inhibits cell migration

We next focused our attention on cell migration, another actin-dependent process that follows EMT in metastasis. Actin polymerization provides the main driving force in cell migration, and we have previously shown that the triterpenoids CDDO-Im and CDDO-Me inhibit the Arp2/3 mediated branched actin polymerization by binding to Arp2/3 and decrease the rate of cell migration. Since we have demonstrated that TBE-31 is able to bind to actin directly and modify its polymerization into stress fibers, we next assessed if it could inhibit cell migration as well. To assess this, we used H1299 human non-small cell lung carcinoma cells as well as migrating fibroblasts (NIH 3T3 cells), which exhibited robust migration in our scratch assays (Figure 2.6). In brief, H1299 or NIH 3T3 cells were grown to confluence and scratched to stimulate migration. The cells were then incubated in either DMSO, or varying concentrations of TBE-31 or CDDO-Im. We observed that after 18 h, DMSO treated H1299 cells had migrated on average 432 ± 25 µm, whereas the NIH 3T3 cells had migrated 545 ± 30 µm after 12 h. Overall, TBE-31 or CDDO-Im treated cells demonstrated a dose-dependent decrease in cell migration (Figure 2.6A, graphs) (Video 2-1). More specifically, the IC₅₀ of TBE-31 for H1299 cells was found to be 2.5 µM and 1 µM for the NIH 3T3 cells.
Figure 2.6 TBE-31 inhibits NSCLC tumour cell migration

A) Top panel; Confluent monolayers of H1299 lung tumour cells were scratched and incubated at 37°C in media containing either DMSO, 2 µM TBE-31 or 1 µM CDDO-Im for 18 h. Shown are representative bright field microscopy images taken with an Olympus IX81 microscope at 10x magnification at 0 and 18h post-incubation. Bottom panel; Scratch assays were carried out as described above using varying concentrations of TBE-31 or CDDO-Im and the relative cell migration was quantitated using ImagePro software (n=4±SD). *P<0.05.

B) Top panel; Confluent monolayers of NIH 3T3 fibroblasts were scratched and incubated as described in panel A. Shown are representative bright field microscopy images taken with an Olympus IX81 microscope at 10x magnification at 0 and 12h post-incubation (n=3). Bottom panel; Scratch assays were carried out as described above using varying concentrations of TBE-31 or CDDO-Im and the relative cell migration was quantitated using ImagePro software (n=3±SD). *P<0.05.

C) Subconfluent H1299 or NIH 3T3 cells were incubated with vehicle (DMSO), 1 µM TBE-31 or 0.5 µM TCDDO-Im and imaged over 18 hours. Using Image J software, the tracking patterns of migrating single cells (multi-color plots) were measured and graphed. The average distance migrated by the H1299 or NIH 3T3 cells are shown on the right (n=3 ± SD). *P<0.05.

Video 2.1. TBE-31 inhibits NSCLC tumour cell migration

Confluent monolayers of H1299 lung tumour cells were scratched and incubated at 37°C in media containing either DMSO (top), 1 µM TBE-31 (middle) or 1 µM CDDO-Im (bottom) for 18 h. Representative bright field microscopy images were taken at 10x magnification every 15 min using an Olympus IX81 microscope to create a movie.

Video 2.2. TBE-31 inhibits NIH 3T3 cell migration

Single cell tracking of NIH 3T3 cells was carried out using time-lapse microscopy with an Olympus IX81 inverted microscope equipped with a custom chamber which allowed
us to maintain a temperature of 37°C and an atmosphere containing 5% CO₂. Bright field images were collected at 10 min intervals over 18 h. Distance of migration was determined by tracking the positions of cell nuclei over 18 h using the MtrackJ plugin (32) for imageJ software (33).
The IC$_{50}$ for CDDO-Im was found to be approximately 1 µM, for both cell lines, similar to our previous results using Rat2 fibroblasts$^{24}$. 

To confirm that TBE-31 and CDDO-Im were indeed inhibiting the migration of both tumour and fibroblast cell lines, we carried out single cell tracking studies of H1299 or NIH 3T3 cells (Figure 2.6B). Briefly, subconfluent populations of cells were incubated with media containing vehicle (DMSO), CDDO-Im or TBE-31 and imaged for 18 hours. The migration tracks as well as the average distance travelled were calculated and graphed (Figure 2.6B). (Video 2-2). Consistent with the scratch assays, we observed that both TBE-31 and CDDO-inhibited NIH 3T3 and H1299 cell migration at low micromolar concentrations.

Overall, our results show that TBE-31 associates and inhibits actin polymerization, resulting in reduced tumour cell stress fiber formation and cell migration.
2.5 Discussion

Although the overall effect of TBE-31 on cell migration are not dissimilar from CDDO-Im and CDDO-Me (the compounds it was derived from) the effects on actin polymerization are strikingly different: CDDO-Im and CDDO-Me were shown to only inhibit Arp2/3 dependent branched actin polymerization and did not have an appreciable effect on linear actin polymerization. This CDDO-Im-dependent or CDDO-Me-dependent inhibition of Arp2/3 activity or perhaps branched actin formation was also associated with an absence of polarity proteins at the leading edge of migrating cell. The differences in TBE-31 and CDDO compound in their abilities to target linear vs. branched actin polymerization may also effect polarity protein localization and activity and will be investigated in future studies. In addition, CDDO-Im demonstrated the ability to alter microtubule dynamics and organization. Future studies will also examine the effects of TBE-31 on the microtubule network and the possible impact it may have on microtubule-dependent processes such as protein trafficking, cell signaling, cell division, mitosis, and cell migration.

The enormous biological diversity of cancer has led to limited promise for therapies that target single signaling molecules. It has been suggested that strategic combinations of agents targeting against the most critical alterations in cancer will be needed, or simply the use of more unspecific agents that modulate several relevant targets simultaneously. Not surprisingly, drug discovery has moved towards investigating multi-target drugs in the last decade, in a large part due to the development of cancer therapeutics. TBE-31 is an attractive compound for cancer prevention due to its antioxidative, anti-inflammatory response capabilities by inducing the Keap1/Nrf2/ARE pathway, and
now inhibiting cell migration by targeting actin. The parallels and contrasts between TBE-31 and the CDDO analogs may lend well to combinational therapies with cytotoxic drugs. The possibility for these and other synergistic effects between TBE-31 and drugs already used in the clinic will provide us with future avenues of research.
2.6 Footnotes

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2.7 References


31 Saito, A. *et al.* Synthesis and biological evaluation of biotin conjugates of (±)-(4bS,8aR,10aS)-10a-ethynyl-4b,8,8-trimethyl-3,7-dioxo-3,4b,7,8,8a,9,10,10a-octahydro-phenanthrene-2,6-dicarbonitrile, an activator of the Keap1/Nrf2/ARE pathway, for the isolation of its protein targets. *Bioorganic & medicinal chemistry letters* 23, 5540-5543, doi:http://dx.doi.org/10.1016/j.bmcl.2013.08.058 (2013).


Chapter 3

The acetylenic tricyclic bis-(cyano enone), TBE-31, targets microtubule dynamics and cell polarity in migrating cells
A version of this chapter has been published in *Biochim biophys Acta* **1863**, 638-649, (2016).
Chapter 3

3.1 Chapter Summary

Cell migration is dependent on the microtubule network for structural support as well as for the proper delivery and positioning of polarity proteins at the leading edge of migrating cells. Identification of drugs that target cytoskeletal-dependent cell migration and protein transport in polarized migrating cells is important for understanding the cell biology of normal and tumour cells and can lead to new therapeutic targets in disease processes. Here, we show that the tricyclic compound TBE-31 directly binds to tubulin and interferes with microtubule dynamics, as assessed by end binding 1 (EB1) live cell imaging. Interestingly, this interference is independent of in vitro tubulin polymerization. Using immunofluorescence microscopy, we also observed that TBE-31 interferes with the polarity of migratory cells: The polarity proteins Rac1, IQGAP and Tiam1 normally localized at the leading edge of DMSO-treated migrating cell, but were observed to be in multiple protrusions around the cell periphery of TBE-31-treated cells. Finally, we observed that TBE-31 inhibits the migration of Rat2 fibroblasts with an IC$_{50}$ of 0.75 µM. Taken together, our results suggest that the inhibition of cell migration by TBE-31 may result from the improper maintenance of cell polarity of migrating cells.
3.2 Introduction

Epithelial cells are normally organized with apical-basal polarity, and multiple cell-to-cell junctions and adhesions maintain the barrier function in non-motile sheet-like formations. During epithelial-to-mesenchymal transition (EMT), epithelial cells undergo changes in the signaling pattern that define cell shape. The cell-to-cell junctions and apical basal polarity are lost, the cytoskeleton reorganizes, and the overall motility of the cells is enhanced. EMT is an essential process during organ development as well as in wound healing. However, during cancer progression, EMT acts as a precursor event to metastasis, unmasking the migratory and invasive potential of the tumour cells. Like EMT, cell migration is necessary in normal physiological conditions, such as embryogenesis, immune response, and wound healing, but during cancer metastasis cell migration and invasion allow cancer cells to establish secondary tumour sites, which accounts for over 90% of cancer related deaths.

Cell migration begins with an initial protrusion of the cell membrane at the leading edge of the cell. Nascent adhesions attach the protrusion to the substratum, while mature focal adhesions at the rear disassemble. This occurs repeatedly to translocate the cell across the substratum. These events rely on the dynamic nature and coordination between the actin cytoskeleton and the microtubule network. Tubulin heterodimers, formed by α and β subunits, polymerize to form microtubules, slender filamentous tubes that emanate from the microtubule-organizing center (MTOC). The microtubule network is highly dynamic, constantly polymerizing and depolymerizing, and is tightly regulated, both spatially and temporally. During cell migration the microtubule network reorganizes to establish a structural skeleton for front-rear polarity. Polarity proteins such
as Rac1 and Cdc42 are asymmetrically trafficked to the leading edge via Rab5 positive endosomal vesicles along microtubules\textsuperscript{11-13}. This stimulates the rearrangement of the actin cytoskeleton, and initiates polymerization to provide the membrane necessary for protrusion\textsuperscript{7-10}. Since cell migration is necessary for tumour cell metastasis and cancer progression, chemotherapeutic agents that target aspects of this process are continually sought.

The semi-synthetic oleanane triterpenoids are a class of multifunctional compounds that were observed to inhibit cell migration by targeting microtubule dynamics as well as actin related protein 2/3 (Arp2/3)-dependent branched actin polymerization\textsuperscript{14,15}. Analysis of these pentacyclic compounds identified that the 2-cyano-1-en-3-one in ring A and a 9(11)-en-12-one in ring C are responsible for drug action (Figure 3.1A). Therefore new tricyclic compounds, termed tricyclic bis(enone) (TBE) compounds were generated. TBEs have great potential for structural diversity as well as more robust pharmacokinetic and pharmacodynamic profiles\textsuperscript{16-18}. Of these compounds, TBE-31 (Figure 3.1A) was shown to be most potent in \textit{in vitro} anti-inflammatory assays\textsuperscript{16-18} and we previously determined that TBE-31 binds directly to actin to inhibit linear actin polymerization\textsuperscript{19}. TBE-31 inhibits stress fiber formation during EMT as well as non-small cell lung cancer tumour cell migration\textsuperscript{19}. Interestingly, the mechanism(s) that TBE-31 utilizes to inhibit cell migration are distinct from the pentacyclic triterpenoids, despite having common predicted reactive rings\textsuperscript{14,15,19}. Therefore, the identification and understanding of which proteins associate and are modulated by TBE-31 will be of great benefit for developing anti-cancer agents. In the
present study, we investigated the effect(s) of TBE-31 on the dynamics of the microtubule network and the positioning of polarity proteins in migrating cells.
3.3 Materials and Methods

3.3.1 Cell Culture, Antibodies and Reagents

Rat2 fibroblasts were cultured in Dulbecco’s modified Eagle’s Medium (DMEM). Mv1Lu mink lung cells were cultured in minimal essential medium (MEM) supplemented with 1% non-essential amino acids (NEAA). Mv1Lu cells stably transfected with HA epitope-tagged TGFβ type II receptor (HAT cells) were cultured in MEM containing 1% non-essential amino acids and 0.3 mg/mL Hygromycin B (Invitrogen # 10687-010). All cells were cultured in a 37°C incubator with 5% CO₂, and all media was supplemented with 10% fetal bovine serum (FBS) unless otherwise stated.

Monoclonal anti-Rac1 (cat. # 610650) and anti-EEA1 (cat. # 610457) antibodies were purchased from BD Biosciences (Mississauga, Ontario). Polyclonal anti-IQGAP1 (H-109; cat. # sc-10792), anti-HA (Y-11; cat. # sc-805), polyclonal anti-Tiam1 (cat. # sc-872), and polyclonal anti-Rab 5 (FL-215; cat. # sc-28570) were purchased from Santa Cruz Technology (Santa Cruz, CA.). Monoclonal anti-tubulin (2.1, cat. # T-4026) was purchased from Sigma-Aldrich. Monoclonal anti-active Rac1-GTP (cat. # 26903) was purchased from New East Biosciences. Nocodazole and Paclitaxel were purchased from Sigma-Aldrich. NeutrAvidin Agarose beads were purchased from Thermo Scientific. Biotin Azide (PEG4 carboxamide-6-Azidohexanyl Biotin) (cat. # B10184) was purchased from Life Technologies. The tubulin polymerization kit (cat. # BK006P) and purified tubulin (cat. # T240) were purchased from Cytoskeleton Inc. TBE-31, CDDO-Im were used as previously described (16). The biotinylated derivative of TBE-31 (TBE-56) and the non-biotinylated control (TBE-55) were synthesized as previously described (Figure 3.1A)²⁰.
3.3.2 Affinity Pull-downs and Mass Spectrometry

Rat2 lysates were prepared by incubating cells with TNTE lysis buffer (150 mM NaCl, 0.5 % Triton X-100, 25 mM Tris-Cl [pH 7.4], 5 mM EDTA) for 30 min at 4 °C and centrifuged (15,000 g_{av}) for 10 min to remove cellular debris. The lysates were then incubated with 0.1% DMSO (vehicle control), 10 μM biotin, 10 μM TBE-55, or 10 μM TBE-56 at 4°C for 2 h, followed by incubating with NeutrAvidin beads for 1 h at 4°C to precipitate the proteins interacting with the biotin conjugate, TBE-56. Afterwards, the beads were thoroughly washed, and 2X Laemmli sample prep buffer was added. These samples were then subjected to SDS-PAGE, and silver staining was performed. Proteins that were uniquely stained in the TBE-56 samples were excised from the gel, trypsinized and analyzed by electrospray mass spectrometry using a Waters, Q-ToF Global mass spectrometer equipped with a Z-spray source and run in positive ion mode. The peptides were identified using MASCOT software. To confirm our results, pull-down experiments were carried out as described above, using cell lysates or 1 μg purified tubulin, followed by immunoblotting with anti-tubulin (1:1000) antibodies. For competitive binding studies, additional samples were pre-incubated with TBE-31 or TBE-55 for 1 h before pull-down studies were performed.

3.3.3 Subcellular Fractionation

Fractions containing the cytoskeleton were separated from those containing detergent-solubilized membranes and cytosol by the method described by Contin et al. \(^{21}\). Briefly, Mv1Lu cells were incubated for 2 h in control medium or media containing 10
μM nocodazole, 1 μM TBE-31 or 1 μM CDDO-Im and then rinsed with microtubule stabilization buffer (90 mM Mes (pH 6.7), 1 mM EGTA, 1 mM MgCl₂, 10% (v/v) glycerol) that had been preheated to 37 °C. Cells were then lysed with microtubule stabilization buffer containing 10 μM paclitaxel, 0.5% Triton X-100, and protease inhibitors for 4 min at 37 °C. The solubilized fractions were then collected. To collect the remaining cellular structures containing the cytoskeleton, SDS-PAGE sample buffer was added to the culture dishes. Following scraping and passaging through a syringe, the fractions containing the cytoskeleton were collected. To analyze the partitioning of cytoskeletal proteins, fractions containing the soluble proteins or the cytoskeleton were subjected to SDS-PAGE and immunblotted with monoclonal anti-tubulin (1:1000) antibody.

3.3.4 Immunofluorescence Microscopy

In all immunofluorescence studies, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.25% Triton X-100 for 5 min, blocked in 10% FBS, and immunostained overnight with anti-Rac1 (1:100), anti-Tiam1 (1:100), anti-EEA1 (1:100) or anti-IQGAP (1:100) antibodies. Following incubation with Alexafluor-488 or -555 labeled secondary antibodies (1:250 dilution) and DAPI staining, cells were imaged using an Olympus IX81 inverted epifluorescence microscope.
3.3.5 Leading Edge Immunofluorescence Microscopy

For immunofluorescence studies examining proteins at the leading edge, Rat2 fibroblasts were grown to confluence on glass coverslips, and scratched with a pipette tip. They were then allowed to elongate and polarize for 4 h, before incubating in media containing DMSO, 1.0 μM of TBE-31, or 1.0 μM of CDDO-Im for 2 h. The cells were then fixed, permeabilized and immunostained with the primary and secondary antibodies as described above. For experiments where cells were probed using TBE-56 (or the TBE-55 control), the cells were left untreated before being fixed, permeabilized and immunostained with the appropriate primary antibodies.

3.3.6 Scratch Assays

Rat2 fibroblasts were grown to confluence, and the monolayer was scratched with a pipette tip. The cells were then incubated with DMSO (control) or varying concentrations of TBE-31 or CDDO-Im. An Olympus IX81 inverted microscope was fitted with a custom chamber maintaining a temperature of 37°C and an atmosphere containing 5% CO₂. Bright-field images were collected over 16 h. The extent of cell migration was measured by finding the difference in width of the scratch at 0 time and 16 h in triplicate. For washout and recovery experiments, the media was replaced with drug free media, and the cells were imaged for a further 24 h.
3.3.7 EB1 Movies

Mv1Lu cells were transfected with GFP-tagged EB1 using PolyJet transfection reagent (100688) from FroggaBio as per the manufacturer’s protocol. The cells were then incubated for 48 h before incubating them with media containing DMSO (control) or 1 mM TBE-31. The fluorescent EB1 “comets” were imaged in live cells using an Olympus IX81 microscope as described above and quantitated using the Fiji tracking tool for ImageJ. EB1 comets (≥ 1000 comets/cell) were imaged and the distances travelled were quantitated from 10 cells/condition/experiment and graphed (N=3 ± SEM).

3.3.8 TGFβ Receptor Endocytosis

Receptor internalization studies were carried out as previously described. Briefly, HAT cells expressing HA-tagged TGFβ type II receptor (TβRII) were incubated with anti-HA antibody for 2 h at 4 °C, followed by Alexafluor-555 (1:500) secondary antibody. Cells were then washed and further incubated at 37 °C for 1 h with DMSO, 1 mM TBE-31, or 1 mM CDDO-Im. Afterwards, the cells were fixed, permeabilized and immunostained with anti-EEA1, secondary antibodies and DAPI.

3.3.9 In Vitro Tubulin Polymerization

In Vitro tubulin polymerization experiments were carried out according to the standard protocols supplied by Cytoskeleton Inc. In brief, 2 mg/ml of purified tubulin was polymerized in the presence of either 10 μM TBE-31, 10 μM CDDO-Im, 3 μM taxol, or 3 μM nocodazole, diluted in the tubulin polymerization buffer provided by
Cytoskeleton Inc at 37°C 1h. The buffer contained a fluorescent reporter, which allowed us to measure the rate of polymerization by measuring the change in fluorescence with a Wallac Victor3 V plate reader (Perkin-Elmer). All samples were done in duplicates for each experiment.

3.3.10 Statistical analysis

All quantitation carried out in this study was analyzed using one-way ANOVA. Statistical significance (p<0.05) for all quantitation (n≥3 ± SEM) is indicated with an asterisk (*).
3.4 Results

In situ invasion of epithelial tumour cells relies on processes such as EMT and cell migration \(^{6,24-27}\). Therefore the development and characterization of chemotherapeutic compounds that target different steps of metastasis, and in particular cell migration, may play an important role in curtailing tumour-related death. We recently showed that the tricyclic compound, TBE-31, is capable of inhibiting actin polymerization \(^{19}\), however, the mechanism(s) that this compound utilizes to inhibit cell migration has not been fully characterized. We therefore set out identify TBE-binding proteins using various control and biotinylated tricyclic compounds (Figure 3.1A).

3.4.1 TBE-31 binds to tubulin directly

To identify TBE-56-binding proteins, we incubated cell lysates with biotin, a non-biotinylated derivative of TBE-31, TBE-55 (control), or TBE-56 (a biotinylated derivative of TBE-55), followed by precipitation with NeutrAvidin beads (Figure 3.1B). The TBE-56-bound proteins were separated on SDS-PAGE, processed and analyzed by mass spectrometry (Figure 3.1B). Although we previously identified several TBE-binding proteins using this approach \(^{19}\), this new analysis extended the identification of novel cytoskeletal binding partners, including myosin-9, alpha-actinin, zyxin, and vimentin (Figure 3.1C). However, the largest number of peptides identified in the analysis corresponded to alpha and beta tubulin—the basic building blocks of microtubules. This therefore led us to investigate this interaction in greater detail through immunoblotting analysis. Briefly, Rat2 lysates were incubated with DMSO, TBE-55 or
Figure 3.1 Tricyclic compounds directly associate with tubulin

A) Structures of the triterpenoid and tricyclic compounds used in the study.

B) Rat2 fibroblast lysates were incubated with 10 µM biotin, TBE-55 or TBE-56 at 4°C for 2h, followed by incubating with NeutrAvidin beads for 1h at 4°C to precipitate the proteins interacting with TBE-56. SDS-PAGE and silver staining were performed and the bands that were uniquely stained in the TBE-56-precipitated sample were excised from the gel, trypsinized and analyzed by electrospray mass spectrometry. Dashes indicate the bands that were analyzed.

C) Table of proteins identified to precipitate with TBE-56. The predicted molecular mass, identification, protein coverage, and number of peptides that were present in the analysis are shown. Note that tubulin was present in multiple bands from the analysis (indicated in boxes).

D) Rat2 cell lysates (left panel) or purified tubulin protein (right panel) were incubated with 10 µM TBE-55 (control) or 10 µM TBE-56 (biotinylated TBE) at 4°C for 2h, followed by incubating with NeutrAvidin beads for 1h at 4°C to precipitate the proteins interacting with TBE-56. The beads were then washed, subjected to SDS-PAGE and immunoblotted with anti-beta (β) tubulin antibodies. The migration of tubulin is indicated on the left of each panel.

E) Rat2 cell lysates were incubated with 0.1% DMSO (−), 10 µM biotin, 10 µM TBE-55 (control) or 10 µM TBE-56 (biotinylated TBE) at 4°C for 2 h, followed by incubating with NeutrAvidin beads for 1 h at 4°C to precipitate proteins interacting with TBE-56. The beads were then washed, subjected to SDS-PAGE and immunoblotted with antibeta-tubulin (α-beta-tubulin) or anti-EB1 (α-EB1) antibodies. The migration of tubulin or EB1 is indicated on the left of each panel.

F) Competitive binding studies were carried out similarly to the experiments described in Panel D. Additional samples were added that had been preincubated with the indicated
concentrations of TBE-31 or TBE-55, before incubating with TBE-56. Shown here is a representative immunoblot for tubulin.

G) Purified tubulin (1 µg/ml) was incubated with 10 µM TBE-56 as described in panel A. Additional samples were added that had been preincubated with the indicated concentrations of biotin or TBE-31 before incubating with TBE-56. Shown here is a representative immunoblot for beta-tubulin.
D) TBE-55: - + - Input (10%) TBE-56: - - + tubulin- α-tubulin Cell Lysates

TBE-55: - + - Input (10%) TBE-56: - - + tubulin- α-tubulin Purified Tubulin

E) TBE-55: - - + - TBE-56: - - - + Biotin: - + - - Input (5%)

Cell Lysates

Tubulin- α-tubulin

EB1- α-EB1

F) TBE-31: - - - - - 10μM 100μM - - -
TBE-55: - - 100μM - - - - - 10μM 100μM Input (10%) TBE-56: - - - 1μM 10μM 10μM 10μM 10μM 10μM

Biotin: - 100μM - - - - - - -

Cell Lysates

Tubulin- α-tubulin

G) TBE-31: - - + 10μM 100μM
TBE-56: - - 10μM 10μM 10μM Input (5%) Biotin: - 10μM - - -

Purified Tubulin

Tubulin-
TBE-56, precipitated with NeutrAvidin beads, processed for immunoblotting and probed with tubulin antibodies (Figure 3.1D). As expected, we observed that TBE-56 associated strongly with tubulin in cell lysates, whereas the negative TBE-55 control did not (Figure 3.1D, left panel). To assure that the TBE-tubulin interaction was not occurring through a secondary protein (for example a microtubule +TIP protein) we repeated the experiment and immunoblotted the precipitated TBE-56 for the presence of the +TIP protein, EB1 (Figure 3.1E). We observed that tubulin, but not EB1, associated with TBE-56. Furthermore, we also assessed TBE-56 association with purified tubulin, and observed results similar to our cell lysate studies (Figure 3.1D, right panel), suggesting that the interaction between TBE-56 and tubulin is indeed direct and not through an intermediate binding partner. Finally, we carried out competitive binding assays to assess if the parental TBE-31 compound could compete with the TBE-56-tubulin interaction (Figure 3.1F). Briefly, cell lysates were pretreated with either DMSO, or increasing concentrations of TBE-31 or TBE-55, before performing TBE-56 pull-downs. We found that by increasing the concentrations of TBE-31 or TBE-55 in the pretreatment, tubulin was hindered from precipitation by TBE-56 in a dose-dependent manner (Figure 3.1F), suggesting that TBE-31 itself is indeed able to associate with tubulin. To confirm that TBE-31 associates with tubulin directly, we repeated these experiments using purified tubulin and observed similar results (Figure 3.1G). Having found that tricyclic compound TBE-31 associates with tubulin, we next assessed if TBE-31 would have an effect on tubulin polymerization and/or the cellular microtubule network.
3.4.2 TBE-31 alters microtubule organization and dynamics

We first investigated the ability of TBE-31 to inhibit tubulin polymerization in vitro (Figure 3.2A). In the presence of either the vehicle (DMSO) or TBE-31, tubulin was able to fully polymerize within one hour of incubation. That is, TBE-31 does not alter in vitro tubulin polymerization. As positive and negative controls, we also assessed tubulin polymerization in the presence of either nocodazole or paclitaxel, bona fide depolymerizing and stabilizing agents, respectively. As expected, tubulin was unable to polymerize in the presence of nocodazole and the rate of polymerization was increased in the presence of paclitaxel (Figure 3.2A). Finally, as another control, we assessed in vitro tubulin polymerization in the presence of the pentacyclic compound, CDDO-Im. Since we had previously demonstrated that this compound interferes with microtubule dynamics in cells, we expected that CDDO-Im would alter in vitro tubulin polymerization (Figure 3.2A). Interestingly, this did not occur (Figure 3.2A) and therefore raises the possibility that triterpenoid and/or tricyclic compounds may alter microtubule dynamics in cells but not via the rate of tubulin polymerization/depolymerization directly. Thus, we attempted to assess the ability of TBE31 or CDDO-Im to alter tubulin depolymerization in cells.

We assessed microtubule depolymerization by subcellular fractionation studies in drug treated cells by isolating the polymerized cytoskeleton from the soluble monomers. After subjecting cytoskeletal or soluble fractions to SDS-PAGE and immunoblotting, we observed that tubulin remained in the polymerized cytoskeletal fractions in DMSO, CDDO-Im or TBE-31-treated cells (Figure 3.2B). However, when
Figure 3.2 TBE-31 inhibits microtubule dynamics independent of tubulin polymerization.

A) Purified tubulin was polymerized in the presence of 10 µM TBE-31, 10 µM CDDO-Im, 3 µM paclitaxel, or 3 µM nocodazole as described in Materials and Methods. Tubulin polymerization was measured and graphed as the change in fluorescence intensity (arbitrary units) over time (min).

B) Mv1Lu cells were incubated in DMSO (vehicle), 1 µM TBE-31, 1 µM CDDO-Im, or 10 µM nocodazole (Nocod.; positive control) for 2 h at 37°C and then subjected to lysis at 37 °C to separate soluble proteins (S) from the cytoskeleton (C). The subcellular fractions were then subjected to SDS-PAGE analysis followed by immunoblotting for tubulin.

C) Subconfluent Mv1Lu cells were grown on coverslips and incubated in either DMSO, 2 µM TBE-31, or 2 µM CDDO-Im for 2 h at 37°C. The cells were then fixed permeabilized and immunostained with monoclonal tubulin antibody (green) and DAPI (blue) for the nuclei. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 60x magnification. The insets highlight the organization of the microtubule network. Bar = 10 µm.

D) Subconfluent Mv1Lu cells transiently expressing GFP-EB1 were incubated in either DMSO or 2 µM TBE-31 and imaged using an Olympus IX81 fluorescent microscope equipped with a cell chamber. GFP-EB1 mobility was visualized over 2.5 min. The starting point of EB1 comet movement (top panels) is shown. Insets were magnified to visualize comet tail morphology (middle panels). Comet tail migration over 2.5 min. was visualized (colored lines in bottom panels) and the track lengths were measured and graphed. Shown are the mean ± SEM (n=3). Blue to red color bar represents shorter to longer tracks, respectively.
A) 

Fluorescence Intensity (Arbitrary Units) 

![Graph showing fluorescence intensity over time for different conditions.

B) 

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C) 

Images showing cellular structures under different treatments:

- **DMSO**
- **TBE-31**
- **CDDO-Im**
cells were incubated with the microtubule-depolymerizing agent, nocodazole, the majority of the tubulin segregated with the soluble cellular components (Figure 3.2B). Although these results were consistent with the *in vitro* assays, we continued our analysis of TBE-31 or CDDO-Im on microtubule dynamics because of the crucial role that the microtubule network plays in cell migration by providing a cellular structure as well as a network for vesicular trafficking \(^7,8,11\).

We examined the cellular microtubule network by assessing the general morphology using immunofluorescence microscopy. Briefly, Mv1Lu cells were treated with DMSO, TBE-31, or CDDO-Im, and immunostained with a tubulin antibody to visualize the microtubule network. In the DMSO-treated cells, microtubules emanated from the microtubule-organizing center (MTOC) in a straight and linear fashion, while the CDDO-Im demonstrated a high degree of curvature and a tortuous pattern, which is consistent with our previous findings (\(^14\); Figure 3.2C). Interestingly, the microtubule organization in TBE-31 treated cells was observed to be in a crosshatched pattern, that was distinct from DMSO and CDDO-Im treated cells (Figure 3.2C). Together these results suggest that TBE-31 alters the organization of the microtubule network, but not necessarily the rates of growth or retraction. We furthered our investigation by next examining microtubule-dependent dynamics.

End binding protein 1 (EB1) localizes to the microtubule plus ends where it modulates their dynamics and interaction with organelles \(^11,28,29\). We assessed the rate of microtubule growth as well as the microtubule persistence by transfecting GFP tagged EB1 in Mv1Lu cells and visualizing them over time to observe microtubule dynamics in the presence of our compounds (Figure 3.2D). In DMSO treated control cells, we
observed that EB1-labeled ends of growing microtubules gave the appearance of rapidly growing oval (longer tail) comet-like streaks that radiated outwards smoothly from the MTOC in an organized and structured manner (Figure 3.2D; Video 3-1). However, TBE-31 treated cells demonstrated shorter, rounded comet-like streaks, consistent with slower growing microtubules. Furthermore, close examination of movies gave the appearance of multiple MTOCs in TBE-31 treated cells, whereas a single major MTOC was observed in control cells. This multi-MTOC and overall less organized movement pattern was consistent with the cross-hatched microtubule pattern demonstrated in the immunofluorescence microscopy analysis (Figure 3.2D; Video 3-2). To confirm that the EB1 tracks were less organized in the TBE-31-treated cells we carried out quantitation and observed that the EB1 tracks in DMSO treated cells averaged 8.5±1 µm/track, whereas TBE-31-treated cells contained significantly shorter EB1 tracks, averaging 5.8 ± 0.6 µm/track (Figure 3.2D, bottom graph).

3.4.3 TBE-31 disrupts microtubule-dependent trafficking

Having shown that TBE-31 affects microtubule organization and dynamics, we sought to investigate processes that are dependent on proper microtubule function; the positioning of the early endosome compartment to the peri-nuclear region of the cell. In brief, Mv1Lu cells were incubated in media containing DMSO, 1 µM TBE-31, or 1 µM CDDO-Im for 2h at 37°C. The cells were then immunostained with early endosome antigen 1 (EEA1) antibody for the early endosome and DAPI for the nuclei (Figure 3.3A). We then scored the number of cells that demonstrate a peri-nuclear staining
Video 3-1 and 3-2 EB-1 Movies

Subconfluent Mv1Lu cells transiently expressing GFP-EB1 were incubated in either DMSO or 2 µM TBE-31 and imaged using an Olympus IX81 fluorescent microscope equipped with a cell chamber. GFP-EB1 mobility was visualized over 2.5 min.
Figure 3.3 TBE-31 affects trafficking and cellular distribution of EEA1-positive endosomes

A) Subconfluent Mv1Lu cells were grown on coverslips and incubated in DMSO, 1 µM TBE-31, or 1 µM CDDO-Im for 2 h at 37°C. The cells were then fixed, permeabilized, and immunostained with EEA1 antibody (green) for the early endosome and DAPI (blue) for the nuclei. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 60x magnification. The insets highlight the early endosome compartments. Bar = 10 µm.

B) Immunofluorescence microscopy was carried out as described in panel A and the number of cells with peri-nuclear EEA1 staining was quantitated and graphed. Shown are the mean ± SEM (n=3; * P \leq 0.05).

C) Mv1Lu cells stably expressing HA-tagged type II TGFβ receptors (TβRII) were incubated at 4 °C with anti-HA antibodies, followed by Alexa555-labelled secondary antibodies (red). The cells were then incubated at 37 °C in the absence (top panel; Control) or presence of 1 µM TBE-31 (middle panel) or 1 µM CDDO-Im (bottom panel) for 1 h. Cells were then fixed, permeabilized, and immunostained with anti-EEA1 (EEA1; green) antibodies. Areas of interest (insets) are magnified and are shown below each panel. The contour (dotted line) of each cell was added to indicate the relative position of TβRII and EEA1 from the cell periphery. Bar = 10 µm.

D) Immunofluorescence microscopy was carried out as described in panel C and the number of cells with peri-nuclear TGFβ receptor staining was quantitated and graphed. Shown are the mean ± SEM (n=3; * P \leq 0.05).
D) Cells with perinuclear TGFβ receptors (%)

Control  TBE-31  CDDO-Im
pattern versus cells with a dispersed EEA1 staining pattern. In the DMSO treated cells 80.7 ±1.0% of the cells displayed peri-nuclear staining, whereas TBE-31 and CDDO-Im treated cells displayed only 60.3 ±1.3% and 39.4 ±0.6% peri-nuclear staining, respectively (Figure 3.3B). We next examined what implications this may have on endocytosis and protein trafficking. To do so, we used the well-characterized transforming growth factor beta (TGFβ) receptor (TGFβR) model that utilizes microtubules to deliver TGFβRs to the early endosome 31.

Cell surface TGFβRs, undergo endocytosis and traffic to the early endosome where they can interact and phosphorylate Smad proteins to propagate TGFβ signaling 23,32,33. To evaluate the ability of TGFβR microtubule-dependent trafficking to the early endosome, we used an immunofluorescence microscopy based approach. Briefly, cells stably expressing HA-tagged type II TGFβR (TβRII) were treated with DMSO, TBE-31 and CDDO-Im and then incubated at 4°C to inhibit endocytosis and to allow the labeling of cell surface receptors. The cells were then incubated at 37°C which reinitiated receptor endocytosis and trafficking to the early endosome. We then performed standard immunofluorescence microscopy and probed for EEA1 to visualize the early endosome in addition to the previously labeled TGFβR. We observed that TGFβRs in vehicle (DMSO)-treated cells internalized and co-localized to EEA1-positive vesicles in the peri-nuclear region of the cell (Figure 3.3C). Interestingly, TBE-31 or CDDO-Im cells demonstrated less peri-nuclear trafficking of TGFβRs, however the receptors still co-localized with EEA1-positive vesicles near the cell periphery (Figure 3.3D). Taken together, our results suggest that TBE-31 alters microtubule dynamics, and impairs
protein trafficking—are important in cell polarity and migration\(^7\)\(^{-11}\). As such, we next sought to explore the effects of TBE-31 on these processes.

### 3.4.4 TBE-31 alters front-rear polarity in migrating cells

Cell migration is dependent on the proper localization of proteins involved in maintaining cell polarity (for e.g., Rac1 and IQGAP1)\(^8\)\(^{-10},\(^{34-36}\), which are dependent on early endosome trafficking (Rab5 positive vesicles for Rac1)\(^11\)\(^{-13}\). We previously demonstrated that CDDO-Im displaces IQGAP1, and Rac1 from the leading edge of migrating Rat2 fibroblasts and inhibits cell migration\(^14\),\(^15\). Here we examined if the effects of TBE-31 on the microtubule network and protein trafficking would also lead to impaired cell polarity. Delivery of Rac1 has been shown to be dependent on Rab5 function, so Rab5 vesicles were examined in a similar manner as our analysis of the EEA1 compartment. However, Rab5 distribution in TBE-31-treated cells was similar to that of control cells (treated with DMSO), while CDDO-Im treated cells did exhibit a higher degree of Rab5 vesicle placement near the cell periphery (Figure 3.4).

We next examined the localization of IQGAP1 and Rac1 in migrating cells (Figure 3.5). Briefly, Rat2 fibroblasts were grown to confluence before an artificial wound was introduced by scratching the cells. The cells were allowed to polarize for 4h before being treated with DMSO, TBE-31 or CDDO-Im. Following immunostaining for IQGAP1 and Rac1, the cells were processed for immunofluorescence microscopy (Figure 3.5A). We observed that 66.9 ± 5.8% of the cells treated with DMSO were elongated and had IQGAP staining at the leading edge—a phenotype indicative of proper polarization.
Figure 3.4 TBE-31 does not affect cellular distribution of Rab5-positive endosomes

Subconfluent Mv1Lu cells were grown on coverslips and incubated in 0.1% DMSO, 1 µM TBE-31, or 1 µM CDDO-Im for 2 h at 37°C. The cells were then fixed permeabilized and immunostained with Rab5 antibody (green) for the early endosome and DAPI (blue) for the nuclei. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 60x magnification. The insets highlight the early endosome compartments. Bar = 10 µm.
Figure 3.5 TBE-31 alters cell polarity

A) Confluent monolayers of Rat2 fibroblasts were scratched and incubated at 37°C in DMEM supplemented with 10% FBS for 4 hours to allow them to polarize. Cells were then incubated for 2 h in media containing DMSO, 1 µM TBE-31, or 1 µM CDDO-Im. The cells were then fixed, permeabilized and immunostained with monoclonal Rac1 antibody (green), polyclonal IQGAP1 antibody (red) and DAPI (blue) for the nuclei. Shown here are representative immunofluorescence images taken with an Olympus IX81 microscope at 40x magnification. Bar = 10 µm.

B) Immunofluorescence microscopy was carried out as described in panel A and the number of cells containing an elongated morphology and one IQGAP1-positive leading edge, cells containing multiple IQGAP1-positive protrusions or cells lacking IQGAP1 staining at the cell surface were quantitated and graphed. Shown are the mean ± SEM (n=3; * P≤0.05).
and migration. In contrast, only 36.1 ± 4.1% of CDDO-Im-treated cells demonstrated proper polarization (Figure 3.5B). Furthermore, the majority, 49.7 ± 1.0%, of CDDO-Im-treated cells showed no elongation, as well as an absence of IQGAP1 from the leading edge, which is consistent with our previous findings 14. TBE-31 was also observed to affect polarity, with only 42.0 ± 5.3% of cells showing IQGAP1 localization at the leading edge. Interestingly, unlike CDDO-Im or DMSO treated cells, TBE-31 treated cells exhibited IQGAP1 localization at multiple areas around the cell periphery, causing the formation of multiple protrusions in 43.5 ± 2.5% of cells (Figure 3.5B).

The observation that TBE-31 induced multiple Rac1/IQGAP-positive protrusions in cells prompted us to investigate whether these protrusions contained active Rac1. We therefore examined the multiple protrusions in the TBE-31 cells using an active-Rac1 antibody that only recognizes Rac1 when it is in its GTP-bound form (Figure 3.6A). The experimental design was similar to the one described above: A monolayer of cells were scratched and allowed to polarize before treating with various compounds. The cells were then immunostained with Rac1 and anti-active Rac1-GTP. In the DMSO control, Rac1 at the single protrusions co-localized with active-Rac1-GTP. Interestingly, TBE-31-induced multiple protrusions contained active (GTP-bound) Rac1 (Figure 3.6B), thus confirming that the Rac1 was indeed active in multiple regions of TBE-31 treated cells. To further confirm that cellular regions that were labeled with the active-Rac1 antibody indeed contained GTP-bound Rac1, we further analyzed if there was the Rac1 activator, Tiam1, co-localization (Figure 3.6B). Indeed, we found co-localization of Tiam1 and active-Rac1 in single protrusions of control cells, multiple protrusions in TBE-31 treated
Figure 3.6 TBE-31 redistributes Rac1 activity to multiple areas of the plasma membrane

**A)** Confluent monolayers of Rat2 fibroblasts were scratched and incubated at 37°C in DMEM supplemented with 10% FBS for 4 hours to allow them to polarize. Cells were then incubated for 2 h in media containing 0.1% DMSO, 1 µM TBE-31, or 1 µM CDDO-Im. The cells were then fixed, permeabilized and immunostained with an antibody that recognizes GTP-bound Rac1 (Active-Rac1; green), a Rac1 antibody (Rac1; red) and DAPI (blue) for the nuclei. Shown are representative immunofluorescence images. Areas of interest (insets) are magnified and are shown below each panel. Bar = 10 µm.

**B)** Confluent monolayers of Rat2 fibroblasts were scratched and incubated at 37°C in DMEM supplemented with 10% FBS for 4 hours to allow them to polarize. Cells were then incubated for 2 h in media containing DMSO, 1 µM TBE-31, or 1 µM CDDO-Im. The cells were then fixed, permeabilized and immunostained with a polyclonal antibody that recognizes GTP-bound Rac1 (Active-Rac1; green), a monoclonal Tiam1 antibody (Tiam1; red) and DAPI (blue) for the nuclei. Shown are representative immunofluorescence images. Areas of interest (insets) are magnified and are shown below each panel. Bar = 10 µm.
cells, and a lack of membrane localization in CDDO-Im-treated cells (Figure 3.6B). Taken together, our results suggest that TBE-31 can disrupt the front-rear polarity in migrating fibroblasts, in a manner distinct from CDDO-Im. TBE-31 induces the formation of multiple protrusions that contain active Rac1, whereas CDDO-Im reduces active Rac1 localization at the membrane.

### 3.4.5 TBE-31 inhibits cell migration

A single dominant leading edge is associated with directionally persistent intrinsic cell migration, and enhanced directed cell migration.

Therefore multiple protruding lamellipodia may result in random intrinsic migration and reduce the capacity for directed cell migration. We therefore next sought to examine the effects of TBE-31 on directed cell migration. In brief, a confluent monolayer of Rat2 fibroblasts was scratched and incubated in media containing either DMSO, varying concentrations of TBE-31, or CDDO-Im. Bright-field images were taken with an inverted microscope at 0 h and 16 h. The initial and final width of the scratch was measured, and the difference was calculated to determine the distance migrated. We observed that with increasing concentrations of TBE-31 or CDDO-Im the distance of migration decreased in a dose-dependent manner (Figure 3.7A). More specifically, quantitation demonstrated that both compounds exhibited similar inhibition of cell migration and above 0.75 μM, TBE-31 and CDDO-Im were able to reduce cell migration by approximately 60.0 ± 7.7% and 86.0 ± 2.6% percent, respectively (Figure 3.7B). CDDO-Im and TBE-31 have been previously shown to induce apoptosis, albeit at higher concentrations. In order to assess if the decrease in cell migration may be due to possible cytotoxic effects, we performed trypan blue
Figure 3.7 TBE-31 inhibits cell migration.

A) Confluent monolayers of Rat2 fibroblasts were scratched and incubated at 37°C in DMEM supplemented with 10% FBS, containing either DMSO, 1 μM TBE-31 or 1 μM CDDO-Im for 16 h. Shown are representative brightfield microscopy images taken with an Olympus IX81 microscope at 10x magnification at 0 and 16 h post-incubation.

B) Scratch assays were carried out as described in panel A with cells incubated with increasing concentrations of TBE-31 or CDDO-Im. Relative cell migration was quantitated using ImagePro software and graphed. Shown are the mean ± SEM (n=3; *=P≤0.05).
exclusion tests. We found that TBE-31 nor CDDO-Im was cytotoxic up to concentrations of 1.0 µM in Rat2 fibroblasts (data not shown). Furthermore, wash out studies indicated that cells recover from CDDO-Im or TBE-31 incubation to fill in the gap (data not shown).

Taken together, our results suggest that TBE-31 targets the microtubule cytoskeleton and establishes multiple leading edges around migrating cells, which leads to decreased cell migration.
3.5 Discussion

We have previously demonstrated that TBE-31 targets linear actin polymerization to alter cell morphology and inhibit cell migration \(^{19}\). In this study we continued to examine the effects of TBE-31 on cell migration with a focus on the microtubule network and observed that TBE-31 binds to tubulin directly and disturbs the dynamics of the microtubule network. Identifying the potential binding sites of TBE-31 on tubulin will allow for the characterization of how this class of compound inhibits microtubule dynamics. Based on previous studies, the interaction of triterpenoid and tricyclic compounds with target proteins has been shown to occur via association with reactive cysteine residues \(^ {38-42}\). We are currently assessing if this is the case of TBE-31 binding to tubulin. Furthermore, studying the impact of TBE-31 on different microtubule-dependent trafficking processes will be of great interest. Indeed, endocytosis and recycling play an important role in the regulation of integrin turnover and redistribution—especially during dynamic processes such as cell migration and invasion. The way in which focal adhesion proteins are trafficked is also recognized to influence their function and dictate the polarized distribution \(^ {43,44}\). Finally, the multiple leading edges visualized in TBE-31-treated cells begs the question if the effects are global or local. Is cell polarity preserved and/or is there a defect in control of number of projections, without necessarily eliminating polarity? Addressing these questions will help identify the different mechanisms that these chemopreventative multi-target drugs use to inhibit cell migration.
3.6 Footnotes

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3.7 References


Saito, A. *et al.* Synthesis and biological evaluation of biotin conjugates of (+/-)-(4bS,8aR,10aS)-10a-ethynyl-4b,8,8-trimethyl-3,7-dioxo-3,4b,7,8,8a,9,10,10a-octahydro-phenanthrene-2,6-dicarbonitrile, an activator of the Keap1/Nrf2/ARE pathway, for the isolation of its protein targets. *Bioorganic & medicinal chemistry letters*, doi:10.1016/j.bmcl.2013.08.058 (2013).


Chapter 4

Acetylenic tricyclic bis-(cyano enone) interacts with actin through cysteine-374 to interfere with actin polymerization
4 Chapter 4

4.1 Chapter Summary

The migratory and invasive potential of tumour cells relies on the actin cytoskeleton. In the past we have demonstrated TBE-31 inhibits actin polymerization and here we further examine the exact binding between TBE-31 and actin. We demonstrate that iodoacetamide, a cysteine (Cys) alkylating agent, interferes with the ability of TBE-31 to interact with actin. In addition, computational analysis identified Cys 217, Cys 272, Cys 285 and Cys 374 as potential TBE-31 binding sites. Using mass spectrometry analysis we determined 1 molecule of TBE-31 binds with 1 molecule of actin. We mutated the cysteines of actin to alanine and performed a pull-down analysis with a biotin labeled TBE-31 and demonstrated that by mutating Cys 374 to Alanine the pull-down significantly decreased association between TBE-31 and actin, suggesting that TBE-31 binds to Cys 374. A characterization of the eGFP-C374A-Actin in NIH3T3 cells showed reduced stress fiber formation, suggesting Cys 374 is necessary for efficient polymerization or incorporation into filamentous actin. Lastly, transwell migration assays were conducted with NIH3T3 cells, and the number of cells that migrated through the transwells was significantly reduced. Taken together or results suggest TBE-31 binds to Cys 374 of actin to inhibit actin polymerization and may potentially be the mechanism wherein TBE-31 utilizes to inhibits cell migration.
4.2 Introduction

Cell migration is essential for numerous physiological processes such as embryogenesis, immune response, cell differentiation, wound healing, and cell renewal. However, it is also co-opted in the later stages of cancer. During metastasis, tumour cell migration and invasion are exploited to allow cells to disseminate to distant sites and establish secondary tumours. Metastasis results in nearly 90% of cancer related deaths.

Cell migration begins with an initial protrusion of the plasma membrane. These protrusions attach to the substratum through newly formed focal adhesions to provide traction, while the mature focal adhesions at the rear or lagging end of the cell disassemble to aid in retraction of the rear. As this process repeatedly occurs, the cell translocates across the substratum. The initial steps of cell migration rely on the localization of polarity proteins to establish the front or leading edge of the cell and promote reorganization of the cytoskeleton towards the direction of migration. The entire cytoskeleton acts in concert for proper cell migration, but actin polymerization provides the primary force for generating the cellular protrusions. The localization of Rac1 and Cdc42 to the leading edge initiates signaling cascades to activate actin-binding proteins and stimulate actin polymerization. Formin, and formin homologs increase polymerization rates by facilitating monomer addition to the barbed end of actin filaments and generally contribute to forming linear filamentous actin arrays. During cell migration, these linear or unbranched actin arrays form long, spike-like, protrusions known as filopodia, which probe the environment and act as sensors. In contrast, Rac1 activates actin related proteins 2 and 3 (Arp2/3), promoting them to bind to pre-existing actin filaments and act as additional sites of polymerization. This generates a
rapidly polymerizing branched array of actin, which allows formation of lamellipodia, which are broad, fan-like, protrusions that drive the cell forwards \(^1\text{-}^3,^9\).

The actin cytoskeleton is a potential target for tumour cell migration, an important aspect of metastasis; however there are still no actin targeting compounds used in chemotherapy \(^10,^11\). We have previously demonstrated that TBE-31 binds to actin, disrupts actin polymerization, and inhibits tumour cell migration \(^12\). In this study we aim at identifying the binding site(s) in actin implicated in interactions with TBE-31, thus allowing a better understanding of its mechanism(s) for inhibiting actin polymerization.

TBE-31 contains \(\alpha\beta\)-unsaturated carbonyl groups in their A and C rings. These functional groups suggest that TBE-31 may undergo Michael addition with nucleophilic targets containing reactive sulfhydryl residues (Figure 4.1). Indeed, previous studies demonstrated TBE-31 reacts directly with the sulfhydryl groups of dithiothreitol (DTT), suggesting TBE-31 may interact with proteins through reactive cysteines \(^13\text{-}^16\). Further research, demonstrated spectroscopically that both the cyano enone functions on ring A and C of TBE-31 react with cysteine residues in Keap1, resulting in the transcriptional activation of cytoprotective genes \(^15,^17,^18\). However, no specific cysteine residue(s) were identified in those studies. With these previous findings in mind, we hypothesized TBE-31 interacts with the cysteine residues of actin. In this study we use \textit{in silico} docking simulations, mass spectrometry and mutational analyses approaches to determine that cysteine 374 on actin is an important contributor of its interactions with TBE-31.
Figure 4.1 TBE-31 proposed mechanism of interaction with cysteines

The αβ-unsaturated carbonyl groups (highlighted in red) undergo Michael addition reaction with the sulphydryl groups (SH) of cysteines forming covalent adducts.
4.3 Materials and Methods

4.3.1 Cell Culture, Antibodies and Reagents

Rat2 and NIH 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s Medium (DMEM). Both cell lines were cultured in a 37°C humidified incubator with 5% CO2, and medium was supplemented with 10% fetal bovine serum (FBS) unless otherwise stated.

AlexaFluor 555-conjugated phalloidin (A34055) and anti-GFP antibodies (for immunofluorescence) (A6455) were purchased from Invitrogen. NeutrAvidin Agarose beads (29200) were purchased from Thermo Scientific. Anti-GFP (632381) for immunoblotting was purchased from Clonetech. Iodoacetamide was purchased from Bioshop (#IOD 500). Polyjet for transfection was purchased from FroggaBio (SL100688). CDDO-Im and TBE-31 compounds were provided by Dr. Michael B. Sporn (Dartmouth, NH). The plasmid encoding eGFP-Actin was a generous gift from Dr. Lina Dagnino (University of Western Ontario, ON, Canada).

4.3.2 Affinity Pull-downs

One mg of Rat2 protein lysates in 1 ml were incubated with 10 μM TBE-56 (biotinylated TBE-31 or b-TBE-31) at 4°C for 16 h, followed by incubating with 25 μl of NeutrAvidin beads for 1 h at 4°C to isolate the proteins interacting with the biotin conjugate. Afterwards, the beads were thoroughly washed, and 30 μl of 2X Laemmli sample preparation buffer was added. These samples were then subjected to denaturing polyacrylamide gel electrophoresis, and transferred to membranes that were probed with
anti-actin or anti-GFP antibodies. For studies utilizing iodoacetamide to block cysteines before pulldown analysis, the lysates were incubated with various concentrations of iodoacetamide for 4h on ice before incubating with TBE-56 and proceeding as above. For studies isolating the eGFP-Actin and the cysteine to alanine mutants, the plasmids were transfected into Rat2 cells for 24h before lysis. It was necessary in these experiments to include 1M Tris-Cl in the lysis buffer to ensure all samples contained monomeric actin irrespective of which individual cysteine to alanine mutant was analyzed.

4.3.3 Computational Analysis

The crystal structure of a complex between actin and cytochalasin D was obtained from the protein data bank (PDB code 3EKS) \(^{19}\). Cytochalasin D was removed and docking simulations were carried out using SwissDock \(^{20}\). All cysteines were assessed to verify if they are solvent exposed and the number of binding sites for TBE-31 and their affinities were recorded (appear as $\Delta G$).

4.3.4 Mass Spectrometry Analysis

Actin purified from human platelets was purchased from Cytoskeleton Inc (aphl99). This actin incubated with either 50-fold molar excess of TBE-31, iodoacetamide or the equivalent volume of DMSO, and was processed for mass spectrometry analysis. Mass spectra were generated using a QTof Micro mass spectrometer (Waters) equipped with a Z-spray source and run in positive ion mode with an Agilent 1100 HPLC used for LC gradient delivery. For LC-MS/MS, the protein was
cleaved with either trypsin or chymotrypsin and analysis in MASCOT combined both set of fragments to improve overall coverage of the protein.

4.3.5 Mutagenesis
Cysteine (C) residues in eGFP-labelled human actin (Addgene) were mutated to alanine (A) using the Quickchange mutagenesis kit (Agilent Technologies) according to manufacturer guidelines. All mutants were transformed into a XL1 Blue strain of *Escherichia coli*, amplified, purified by the Qiagen Miniprep Kit (Qiagen), and their sequences were verified at the London Regional Genomics Centre (London, ON, Canada).

4.3.6 Immunofluorescence microscopy
In all immunofluorescence microscopy studies, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.25% Triton X-100 for 5 min, blocked in 10% FBS, and immunostained overnight with the appropriate primary antibodies. All images were taken with an Olympus IX81 inverted epifluorescence microscope. Quantitations for cells with actin stress fibers were quantitated into three categories, strong stress fibers, weak stress fibers, and none. Quantitations were carried out in the green channel for GFP.
4.3.7 Transwell cell migration assays.

NIH3T3 cells were transfected with equal amounts of plasmids encoding either the eGFP-Actin or eGFP-Actin-C374A. After 24h, the cells were serum starved for 4h prior to being seeded onto the top of a Transwell chamber (Costar). A total of 50,000 cells per condition were seeded and allowed to migrate toward the bottom chamber, which contained medium supplemented with 10% serum for 18h. The upper and lower chamber also contained either DMSO (vehicle) or TBE-31 (0.5 μM). In parallel, 50,000 cells from each condition were seeded onto coverslips in medium containing 10% serum to monitor transfection efficiency, or in serum free medium containing either DMSO or 0.5 μM TBE-31, to monitor cell viability. After 18 h, the cells on the upper side of the Transwell membrane were removed with a cotton swab, and the cells that had migrated through the insert (i.e. on the underside of the Transwell membrane) were fixed with 4% paraformaldehyde. Cells on both the Transwell membrane and coverslips were stained with DAPI (4’,6’-diamidino-2-phenylindole) and mounted onto glass slides. Images were acquired using an IX81 inverted microscope (Olympus). Ten representative fields were acquired at ×100 magnification (approximate field of view of 59, 000 μm²) and quantified by counting the blue nuclei, or green fluorescent cells.

4.3.8 Statistical analysis

All quantitation carried out in this study was analyzed using one-way ANOVA. Statistical significance (p<0.05) for all quantitation is indicated with an asterisk (*).
4.4 Results

4.4.1 Iodoacetamide interferes with TBE-31 binding to actin

TBE-31 has been suggested to interact with proteins through cysteine residues via a Michael addition\textsuperscript{14,17}. To verify whether this is true of actin, we used iodoacetamide—a cysteine alkylating agent—to pretreat lysates before using in pull-down assays to assess if there would be an effect on TBE-31 binding to actin. In brief, Rat2 lysates were incubated with increasing concentrations (1-10 mM) of iodoacetamide, prior to a subsequent incubation with biotin labeled TBE-31 (TBE-55) and drug-bound complexes were isolated using NeutrAvidin-agarose beads. The samples were then processed for SDS-PAGE, and transferred to membranes that were probed for actin (Figure 4.2A). Concentrations of iodoacetamide of 5 mM and 10 mM significantly inhibited the pull-down of actin by the biotin labeled TBE-31 by 50.64 ± 9.68\% (P≤0.01) and 66.50 ± 8.87\% (P≤0.001) respectively (Figure 4.2B), thus supporting the notion that TBE-31 binds to actin through cysteine thiol groups.

4.4.2 TBE-31 is predicted to bind to cysteines using computer simulation analyses

To complement the above findings, we next sought to use computational simulation of the potential cysteines in actin that TBE-31 was likely to bind. SwissDock (Swiss Institute of Bioinformatics)\textsuperscript{20} was used to model TBE-31 binding to the crystal structure of actin (3EKS)\textsuperscript{19} acquired from the protein databank (PDB) server. The actin structure was originally determined from actin crystalized in the presence of
Figure 4.2 Iodoacetamide interferes with TBE-31 binding

**A)** Rat2 fibroblast lysates were incubated with a 0-10 mM iodoacetamide for 1h, and then incubated with 10 µM biotin labeled TBE-31 (TBE-56) overnight before precipitating with NeutrAvidin-agarose beads. The samples were then subjected to SDS-PAGE and immunoblotted for Actin. Shown are representative immunoblot (n=3).

**B)** Experiments carried out as described in Panel A were quantitated and graphed by dividing the amount of Actin pulled-down relative to the iodoacetamide free control. Error bars represent the mean +/- SEM. **P< 0.01, *** P<0.001.
A) Iodoacetamide

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Actin</th>
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</thead>
<tbody>
<tr>
<td>- 1 mM</td>
<td></td>
</tr>
<tr>
<td>5 mM</td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td></td>
</tr>
</tbody>
</table>

Pull-down: TBE-56

Input (5%)

α-Actin

B) Relative Actin Pull-down

<table>
<thead>
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<th>Iodoacetamide [mM]</th>
<th>Relative Actin</th>
</tr>
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<tbody>
<tr>
<td>-</td>
<td>1.00 (1.0)</td>
</tr>
<tr>
<td>1 mM</td>
<td>1.00 (1.0)</td>
</tr>
<tr>
<td>5 mM</td>
<td>0.60 (0.20)</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.40 (0.10)</td>
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</table>

**p < 0.01, ***p < 0.001
cytochalasin D (a *bona fide* actin depolymerizing agent), however for our analysis the cytochalasin D was removed. Of the > 40 potential TBE-31-actin interacting regions, the majority of the putative binding sites were predicted to reside in the same cleft that is targeted by cytochalasin D. More importantly, association of TBE-31 in this cleft positions this drug to interact with Cys 374, at the C-terminus of actin (Figure 4.3A). Other potential cysteines include Cys 217, Cys 272, and Cys 285, with binding affinities calculated to be $\Delta G$ -7.5, $\Delta G$ -6.8, $\Delta G$ -6.3 and $\Delta G$ -6.2 kcal/mol respectively (Figure 4.3B and 4.3C). With these potential targets in mind, we next sought to verify which cysteines were important for TBE-31 binding using a mass-spectrometry approach and mutational pull-down analyses.

### 4.4.3 Mass Spectrometry Analysis

We previously showed TBE-31 interacting with purified actin through an *in vitro* pulldown approach \(^1\). Although the actin preparation used in this approach was 99% pure, there is a possibility that the interaction of TBE-31 and actin may occur through an intermediate actin-binding protein. We therefore attempted to assess if TBE-31 binding to actin was direct and if so, determine the stoichiometry of the interaction. In brief, purified actin was incubated with a 50-fold molar excess of, TBE-31, iodoacetamide as a positive control and an equivalent volume of DMSO as a vehicle control. Afterwards, the samples were subjected to Liquid Chromatography Mass Spectroscopy analysis (LC-MS), and the average masses of the complexes analyzed were measured. The average
Figure 4.3 Modelling TBE-31 interaction with actin

A) Cytochalasin D bound Actin PDB file (3EKS) (right image) was used in SwissDock to model binding sites between TBE-31 and actin (left image)

B) Actin PDB file (3EKS) with TBE-31 bound, and cysteines highlighted

C) Summary of information for the cysteines on actin
A) 

B) 

C) 

<table>
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<tr>
<th>Cysteine</th>
<th>$\Delta G$ (kcal/mol)</th>
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<th>Solvent Exposed</th>
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<tr>
<td>17</td>
<td>-</td>
<td>-</td>
<td>✗</td>
<td>-</td>
</tr>
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<td>-6.8</td>
<td>n/a</td>
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<td>257</td>
<td>-</td>
<td>-</td>
<td>✗</td>
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<td>✓</td>
<td>Cytochalasin D Cleft</td>
</tr>
</tbody>
</table>
mass of actin (Figure 4.4A) was determined to be 41661.52 ± 0.91 Da. The iodoacetamide treated samples (Figure 4.4C) contained additional species at molecular masses of 41719.56 ± 0.21 Da and 41776.32 ± 0.75 Da, which correspond to the addition of one (+57 Da) or two (+114 Da) iodoacetamide molecules after forming a Michael addition. Similarly, incubation with TBE-31 (Figure 4.4B) resulted in the appearance of a species with molecular mass of 41992.39 ± 0.64 Da (+330.38 Da), which corresponds to the addition of one molecule of TBE-31. These results confirm that TBE-31 binds to actin, and the presence of only one shifted molecular mass peak suggests that only one molecule of TBE-31 binds to one molecule of actin.

We next sought to determine which cysteine TBE-31 is interacting with. Purified actin was incubated with DMSO, and TBE-31 as above, and then digested with trypsin or chymotrypsin. The fragment sizes were determined by tandem Mass Spectroscopy (MS-MS) and the data were analyzed to find fragments with an increase in mass corresponding to the molecular weight of TBE-31. By combining the predicted fragments from the chymotrypsin and trypsin analyses, the overall coverage for actin (Figure 4.5A) was greater than 90%. However, although the overall coverage was high, fragments containing the surface exposed cysteines were relatively underrepresented. Indeed, there were only two fragments containing the last cysteine (Cys 374; Figure 4.5B). Since this analysis yielded inconclusive results and any potential binding sites observed in TBE-31-shifted actin fragments were also observed in the DMSO control, it was necessary to develop another method to determine which cysteine(s) on actin were responsible for interacting with TBE-31.
Figure 4.4 Mass Spectrometry analysis reveals TBE-31-Actin interaction

A) Mass of actin protein after incubation with DMSO as determined by liquid chromatography followed by mass spectrometry.

B) Mass of actin protein after incubation with 50-fold molar excess TBE-31 as determined by liquid chromatography followed by mass spectrometry.

C) Mass of actin protein after incubation with 50-fold molar excess iodoacetamide as determined by liquid chromatography followed by mass spectrometry.
Figure 4.5 LC-MS/MS analysis of TBE-31-actin interaction

A) Sequence coverage (blue) of actin (grey) after incubation with 50-fold molar excess TBE-31 or DMSO vehicle control, obtained by LC-MS/MS merged analysis of the peptide mixture obtained after separate trypsin and chymotrypsin digest.

B) Cropped view of analysis from above to highlight the cysteines.

C) Legend for post-translational modifications, with TBE-31 highlighted in red
4.4.4 Mutation of cysteine-374 interferes with TBE-31 binding to actin

A series of vectors encoding actin forms with cysteine to alanine mutation at positions 217 (C217A), 257 (C257A), 272 (C272A), 285 (C285A), and 374 (C374A) were generated, which includes all but one of the cysteines in actin. Based on our above analysis, double mutants were also generated at cysteine 272 and 374 (C272A/C374A); and cysteine 285 and 374 (C285A/C374A; Figure 4.6A). Each plasmid was sequenced to verify the intended mutations were present. These vectors were then transfected into Rat2 fibroblasts, and cell lysates were processed for pull-down analyses (Figure 4.6B) as done previously. Using this approach, we observed that significantly less (60.61±9.02%) C374A actin associated with the biotinylated-TBE-31, compared to the wild-type actin control (Figure 4.6C). Similarly, actin containing double cysteine mutants (that had C374A mutation in common) showed a comparable reduction as the single C374A mutant, thus suggesting that cysteine 374 is important for actin interaction with TBE-31.

4.4.5 Cysteine-374 is necessary for efficient actin polymerization

We next characterized actin containing the cysteine-374 mutation. We first assessed its ability to contribute to stress fibers in migrating cells. In brief, eGFP-Actin, eGFP-Actin-C257A, eGFP-Actin-C272A or eGFP-Actin-C374A was overexpressed in NIH3T3 cells, fixed and probed with phalloidin and anti-GFP antibodies. Using immunofluorescence imaging we observed robust stress fibers in the eGFP-Actin WT or mutant overexpressing cells (Figure 4.7A). Indeed, the eGFP-Actin was incorporated into
Figure 4.6 Cysteine to alanine mutation on cysteine 374 interferes with TBE-31 interaction

A) Schematic of eGFP-Actin (top), and the cysteine (C) to alanine (A) mutants generated

B) Rat2 fibroblasts were transfected with the indicated plasmids and lysed. One milligram of lysate then incubated with 10 μM of biotin labeled TBE-31 (TBE-56) for 16h before being pulled-down with NeutrAvidin-agarose beads. The samples were then processed and subjected to SDS-PAGE and immunoblotted for green fluorescent protein. Shown here are representative immunoblots (n=5).

C) Quantitations of the above experiments were carried out by dividing the amount of GFP-Actin pull down by the input and expressed as a relative amount compared to the WT pulldown (n=5). Error bars represent SEM. **P<0.01.
Figure 4.7 eGFP-C374A-actin demonstrates poor stress fiber incorporation

A) NIH3T3 cells were transfected with GFP-Actin, or GFP-Actin-C374A. After 48h the transfected cells were processed for immunofluorescence imaging and probed with phalloidin (red) for actin stress fibers, and anti-GFP (green) for GFP. Scale bars represent 10 microns.

B) Quantitation of experiments carried out as described in Panel A were carried out for cells containing either strong, weak or no stress fibers. GFP-ActinC257A, and GFP-Actin-C272A were included as controls. Errors bars represent the mean ± SEM. P<0.001
the stress fibers, indicating that overexpression of actin did not alter stress fiber formation. However, the eGFP-Actin-C374A overexpressing cells mostly had an absence of stress fibers. Quantitation of cells containing either strong, weak or no stress fibers (Figure 4.7B) was then carried out. The eGFP-Actin expressing cells contained roughly equal proportions of all three stress fiber forms; 30.68 ± 1.61% of cells had strong stress fibers, 33.47 ± 1.49% of cells had weak stress fibers, and 35.85 ± 1.38% of cells had no stress fibers. The eGFP-Actin-C374A expressing cells contained significantly fewer cells with strong stress fibers, and a significantly greater number of cells with no stress fibers; with only 6.96 ± 0.86% and 70.62 ± 5.56% respectively. eGFP-Actin-C257A and eGFP-Actin-C272A expressing cells were also quantitated, but neither showed significant difference for any of the categories when compared to the eGFP-Actin wild type control. This suggests cysteine 374 is important for actin polymerization and stress fiber formation.

These findings were supported by the literature. Cysteine 374 in actin may be involved in the formation of actin dimers, and post-translational modifications that normally occur on cysteine 374 are associated with lower rates of polymerization and weakening of actin filaments. Therefore, the mechanism of action for TBE-31 on actin polymerization, and possibly cell migration, could potentially be interrogated using eGFP-Actin-C374A in a cell overexpression model.
4.4.6 Transwell Cell Migration Assays

We previously demonstrated that TBE-31 interacts with actin and inhibits cell migration. In brief, NIH3T3 cells were transfected with vectors encoding eGFP-Actin or eGFP-Actin-C374A, and 50,000 of these cells were seeded into Transwell inserts after 24h. The top chamber contained serum free medium, whereas the bottom chamber contained either serum free medium, or medium supplemented with 10% FBS as a chemoattractant. DMSO or TBE-31 (0.25 or 0.5 \( \mu \)M) was added to both upper and lower chambers. The cells were given 18h to migrate before fixing the membrane, staining with DAPI and mounting for immunofluorescence analysis (Figure 4.8A).

For each condition, the fraction of all cells that migrated through the insert was first determined from the number of DAPI-stained nuclei (Figure 4.8B). For cells expressing eGFP-Actin, the DMSO control treatment showed an average of 627.8 ± 52.8 cells that had migrated per field, whereas with the 0.25 \( \mu \)M or 0.5 \( \mu \)M TBE-31 treatment, cell number decreased to 559.38 ± 59.9 and 322.8 ± 57.5 cells per field, respectively. Similarly, the cells expressing eGFP-Actin-C374A treated with DMSO control, had an average of 606.9 ± 65.0 cells per field, whereas the 0.25 \( \mu \)M or 0.5 \( \mu \)M TBE-31 treatment again reduced the total number of cells that migrated through the insert to 511.7 ± 38.2 and 284.6 ± 44.5, respectively.

The total number of transfected cells was also determined by scoring GFP-expressing cells (Figure 4.8C). Interestingly, the proportion of cells expressing either eGFP-Actin or eGFP-Actin-C374A was not affected by TBE-31 compared to cells not expressing eGFP-labeled actin. Furthermore, cells expressing either form of actin responded in a similar way to TBE-31 treatment. These results show that TBE-31 does
Figure 4.8 Transwell cell migration with eGFP-Actin and eGFP-Actin-C374A

A) NIH3T3 cells were transfected with eGFP-Actin or eGFP-Actin-C374A. After 24h, the cells were serum starved for 4h before seeding into the transwells. The top chamber contained serum free media, while the bottom contained 10% serum. Either DMSO, or TBE-31 (1mM) was added into both compartments. After 18h of cell migration, the membranes were excised and prepared for immunofluorescence imaging. Images were taken at 100x magnification.

B) Quantitation of the entire population of cells by counting the nuclei in the experiment above. Errors bars represent the mean ± SEM. *P<0.05, **P<0.01 compared to the DMSO control. (n=4)

C) Quantitation of the eGFP-Actin or eGFP-Actin-C374A population by counting cells fluorescing green in the experiment above. Errors bars represent the mean ± SEM.. (n=4)
inhibit cell migration as we have shown in the past with other techniques. However, overexpressed actin, regardless of whether it can bind to TBE-31 or not, confers some resistance towards TBE-31-dependent inhibition of migration.
4.5 Discussion

In this study we demonstrated that iodoacetamide, a cysteine alkylating agent, interferes with the ability of TBE-31 to interact with actin. Computational analysis highlighted 4 cysteines as putative binding targets for TBE-31. Two approaches were used to validate these potential binding targets, a mass spectrometry, and a mutagenesis based approach. With the mass spectrometry approach, we were able to successfully demonstrate that incubation of actin with TBE-31 was able to form a new mass corresponding to the mass of actin and TBE-31 together. The absence of a second peak corresponding to the mass of two TBE-31 molecules and actin, suggests only one molecule of TBE-31 binds per molecule of actin. The same conditions were submitted for tandem MS/MS to identify which cysteine TBE-31 is interacting with, but the results were inconclusive due to a lack of coverage of peptide fragments containing Cys 374. Thus, a mutagenesis approach was used instead to determine which cysteine TBE-31 interacts with. A pull-down analysis was used with a panel of cysteine to alanine mutations, and the eGFP-Actin-C374A showed a significant reduction in pull-down by biotin labeled TBE-31. Further analysis of this C374A mutant showed impaired incorporation of this actin form into stress fibers, with the majority of cells containing either weak or no eGFP-Actin-C374A stress fibers. This suggested that this actin mutant would exhibit impaired incorporation into actin microfilaments. Transwell migration assays were conducted and the number of cells migrating through the transwells was significantly reduced by TBE-31. Taken together, these results suggest that TBE-31 interacts with actin through Cys 374 to inhibit actin polymerization and this may be a mechanism wherein TBE-31 utilizes to inhibit cell migration.
Our findings showing impaired incorporation of eGFP-Actin-C374A into stress fibers is consistent with the literature. Cys 374 has been reported to have an important role in establishing intermonomer contacts and may stabilize the interstrand relation in the actin filament. Furthermore, redox modification of Cys 374 has also been linked to intramolecular disulfide bond formation, decreased polymerization rates, increased critical concentrations and filament weakening. Indeed, S-(cysteine-374)glutathionyl actin was found to form filaments of low mechanical stability. An analysis of tetramethylrhodamine(TMR)-labeled actin at Cys 374 showed TMR-actin polymerized in very short filaments, which were easily destabilized by ATP hydrolysis. The critical concentration for polymerization of TMR-actin were found to be an order of magnitude higher than that of unlabeled actin. In addition the interaction of actin with a number of actin regulating proteins was profoundly altered by TMR labeling. Interestingly, the modification of Cys 374 has been implicated in the regulation of the actin cytoskeleton reorganization during cell adhesion and cell spreading, which are important processes in cell migration. Therefore the notion of TBE-31 interacting with actin through Cys 374 to impair actin polymerization and inhibit cell migration is consistent with the literature.

Interestingly, the overexpression of eGFP-Actin or eGFP-Actin-C374A reduced the inhibitory effects of TBE-31 on cell migration, even though only the wild-type eGFP-Actin should associate efficiently with TBE-31. In my previous studies (Chapters 2 and 3) we showed that TBE-31 has many putative binding targets. We have examined the effects of TBE-31 on the microtubule network, vesicle trafficking, and front-rear polarity, and demonstrated that TBE-31 disrupts all of these. Perhaps cell migration cannot be
fully recovered from this multi-targeted drug by simply overexpressing eGFP-Actin-C374A, because it is inhibiting cell migration through multiple proteins. However, since actin microfilaments are essential for cell migration, and modification of actin at Cys 374 negatively effects actin polymerization, further studies on the effects of TBE-31 on the actin cytoskeleton are warranted. Our previous studies (Chapter 2) have shown that stress fiber formation in the presence of TBE-31 results in short filaments, which is consistent with the analysis of TMR-actin. It may be interesting to examine the stability of the TBE-31 modified actin filaments by using electron microscopy or measuring ATP hydrolysis by the filament as done previously by Conchaudron et al.

A purified source of actin, and actin containing the cysteine to alanine mutations will open many avenues of experiments. We previously sought to generate a purified source of GST-actin and the cysteine mutants. We successfully excised the actin sequence from our eGFP vectors, and inserted them into a pGEX-4T-1 GST vector. They were then successfully expressed in BL21 competent cells, lysed and the GST tagged protein was precipitated with glutathione beads. However, despite using multiple approaches the elution of the protein from the beads was not successful. The approaches included using thrombin to cleave the protein from the GST, gradients of Triton X-100, guanidine and urea—strong denaturing agents, glutathione, and high salt concentrations. Only by using a combination of the above was a small amount of protein eluted and its activity seemed compromised—most likely due to improper refolding after using the denaturing agents. Future attempts at purifying actin and the cysteine mutants may be more fruitful by using a polyhistidine-tag instead of a GST-tag, and a baculovirus/SF9 cell expression system. Once the purified protein is obtained, in vitro actin
polymerization assays can be performed to perhaps demonstrate a dampened effect by TBE-31 in the cysteine mutants. In addition, mass spectrometry analysis can be used with the mutants to show changes (or lack of) in the whole protein mass shift, or fragment labeling.

Tandem MS/MS was not conclusive in determining which cysteine on actin TBE-31 interacts with. The relatively low amount of TBE-31 labeled actin, compared to unlabeled actin in the samples, was especially problematic considering the poor coverage of Cys 374. We attempted to address this by analyzing actin cleaved with chymotrypsin, or trypsin. Future experiments using other endoproteinases (for e.g., AspN) may generate peptides that yield better coverage. In addition, a bottom-up approach for analysis has been proposed where a synthesized N-terminal fragment containing Cys 374 could be labeled with TBE-31 and analyzed by Mass Spectroscopy. This can be used to test if the fragment and TBE-31 can withstand the ionization energy of MS/MS and be amenable for analysis.

Overall, this study provides novel insight on the underlying mechanisms by which TBE-31 utilizes to inhibit cell migration. It was demonstrated that TBE-31 interacts with actin through Cys 374, which is necessary for efficient actin polymerization and stress fiber formation. Thus making TBE-31 an attractive candidate for targeting the actin cytoskeleton and tumour cell migration in metastasis.
4.6 References


Chapter 5

Summary and Conclusions
5 Chapter 5

5.1 Summary

Cancer arises from cells that have undergone multiple genetic mutations that lead to the loss of normal cellular behaviour \(^1,2\). These mutations initially result in uncontrolled growth and proliferation. As the malignant cells continue to gain genetic mutations, some cells will gain the ability to migrate and invade adjacent tissue, and disseminate to distant organs where they may establish new tumour colonies. These colonies are known as metastases, and are responsible for over 90% of human cancer related deaths \(^3-6\). Cell migration is a key process in promoting metastasis, and to date effective drugs targeting metastasis are lacking \(^6-10\).

Synthetic oleanolic acid agents have received notable attention in recent years for their multiple anti-tumourigenic effects, which include inducing apoptosis, reducing inflammation, and inhibiting proliferation of tumour cells. Studies in our laboratory have also demonstrated that the specific synthetic oleanolic acid derivatives, CDDO-Im and CDDO-Me, target the cytoskeleton and inhibit cell migration \(^11,12\). More recently, medicinal chemists have synthesized a class of three ring compounds known as TBEs. They resemble the CDDO compounds and contain the same active regions responsible for their potency. TBE-31 was found to be particularly potent in anti-inflammatory assays, and many studies have outlined the effects of TBE-31 on the KEAP1/Nrf2 pathway. However their effects on cell migration were entirely unknown. Given their structural similarities to the CDDO compounds, I hypothesized that TBE-31 will inhibit cell migration by targeting components of the cytoskeleton. The three specific aims I designed to address this hypothesis were to:
1) Characterize the effect(s) of TBE-31 on the actin cytoskeleton and cell migration.

2) Characterize the effect(s) of TBE-31 on the structure and dynamics of the microtubule network and cell migration.

3) Determine how TBE-31 interacts with actin.

Data from experiments designed to address aims 1, 2, and 3 are presented in chapters 2, 3, and 4 respectively, and are briefly summarized below.

Arp2/3 is involved in the actin polymerization and the formation of the lamellipodia in cell migration. CDDO-Im and CDDO-Me have been shown to interact with Arp3, inhibit Arp2/3 activity and localization to the leading edge. In chapter 2, I determined that, unlike CDDO-Im or CDDO-Me, TBE-31 does not alter Arp3 localization to the leading edge. Using TBE-56 (biotin labeled TBE-31), I identified a number of putative protein binding partners. Of the identified proteins, actin was of particular interest as it is provides the force for generating cellular protrusions and establishing lamellipodia, key events for cell migration. The interaction between TBE-31 and actin was further validated and the results obtained suggested TBE-31 is able to associate with actin. I then demonstrated that TBE-31 is able to inhibit actin polymerization using an in vitro assay and inhibit actin stress fiber formation in NIH 3T3 fibroblasts, which is entirely unique effect from CDDO-Im or CDDO-Me. These findings were then applied to the model of epithelial-to-mesenchymal transition (EMT), a
precursor event to cancer metastasis. TGFβ was used to induce EMT in A549 lung cancer cells, and the rearrangement of cortical actin to actin stress fibers was inhibited by TBE-31. Lastly I demonstrated using scratch assays, and single cells, that TBE-31 is effective in inhibiting H1299 lung cancer cell, and NIH 3T3 cell migration for the first time.

In chapter 3, I identified tubulin to bind to TBE-31. However, unlike actin, TBE-31 was not found to inhibit the polymerization of tubulin, but instead it was found that TBE-31 alters the organization of the microtubule network and its dynamics. Microtubule-dependent processes were then investigated and TBE-31 was shown to disrupt microtubule-dependent trafficking. I then demonstrated that TBE-31 alters front-rear polarity in migrating cells, in a manner distinct from CDDO-Im. TBE-31 treated cells were observed to form multiple protrusions of active Rac1, whereas CDDO-Im reduces active Rac1 localization at the membrane. Since a single dominant leading edge is necessary for efficient directed cell migration, I then demonstrated that TBE-31 inhibited cell migration of Rat2 fibroblasts.

In chapter 4, the focus was on better understanding the biochemical interaction between actin and TBE-31 to determine the mechanism(s) in which TBE-31 utilizes to inhibit actin polymerization and cell migration. Previous work has suggested that TBE-31 may undergo Michael addition with nucleophilic targets containing reactive sulfhydryl groups (thiols)\textsuperscript{14,15}. However, no previous work has identified any specific cysteines on any proteins. By blocking the thiol groups on actin with a cysteine alkylating agent, the interaction between TBE-31 and actin was inhibited. Thus suggesting TBE-31 interacts with actin through cysteine thiol groups. This finding was supported by data from computational simulations. Furthermore, cysteine 374 was identified as a potential
candidate. Using mass spectrometry, it was determined that one molecule of TBE-31 interacts with one molecule of actin. LC/MS-MS was used to try to determine the exact cysteine(s) TBE-31 interacts with, however due to limitations of the technique this was not possible. Instead a pull-down approach was utilized with wild-type or mutated GFP-actin. I determined that TBE-31 interacts with actin through cysteine 374, marking this as the first ever identified target at the amino acid level. Characterization of the GFP-actin-C374A, demonstrated that cysteine 374 is necessary for efficient actin stress fiber formation. This suggests it may important for actin polymerization, which is supported by past research by others 16-19. The final experiment of this chapter attempted to interrogate the exact mechanism of action for TBE-31 on cell migration by overexpressing GFP-actin-C374A. Although these results confirmed TBE-31 inhibits cell migration using a third technique, they were not able to clearly demonstrate a recovery effect by overexpression, as the control GFP-actin reduced the effects of TBE-31 as well. However, the notion of TBE-31 interacting with actin through cysteine 374 to impair actin polymerization and inhibit cell migration is consistent with other studies on cysteine 374 modification on actin 20,21.

5.2 Limitations and Future Experiments

In this thesis, I demonstrated that TBE-31 interacts with the thiol groups of reactive cysteines, thus TBE-31 is a multi-targeting drug. Indeed, in chapter 2 and 3, a number of putative binding targets for TBE-31 were identified. Due to the multi-targeted nature of this drug, it was difficult to attribute specific phenotypic changes to specific targets. For instance, since the microtubule network and the actin cytoskeleton are
intimately linked, it is difficult to attribute the mislocalization of the polarity proteins specifically to the microtubule network alone. Future studies examining the effects of TBE-31 on other proteins, and other processes should keep this caveat in mind. For this reason, it was helpful to study the proteins in isolation in in vitro polymerization assays. Indeed, this approach was useful for the heavily mechanistic nature of my studies, and was valuable for narrowing down the mechanisms by which TBE-31 inhibits cell migration. However, it is important to note and remember for future studies, that these assays may not fully reflect what happens in vivo. In the cell, the cytoskeleton is tightly regulated in time and space by a large number of signaling, scaffolding and binding proteins. One of the limitations encountered in this study was the inability to silence the expression of actin or tubulin as they are fundamental to other processes outside of cell migration, such as cell division and providing structure to the cell. In order to recapitulate the effects of TBE-31 on these proteins in cell migration, it may be necessary to silence their regulatory proteins instead. For instance, formin and profilin which enhance general actin polymerization rates, can be silenced in lieu of actin. Similarly, another limitation of this study was the inability to properly overexpress GFP-actin-C374A. Due to the nature of the mutant, only low-to-medium levels of expression were tolerated before the actin cytoskeleton was compromised. This may explain why a recovery effect was not observed when GFP-actin-C374A was overexpressed in the transwell migration assays carried out in chapter 5. Future studies may need to resort to using an in vitro polymerization assays with a purified source of actin-C374A instead. The C374A mutant would be expected to have lower intrinsic rates of polymerization, but since TBE-31 does not interact with the mutant, it is expected that this rate of
polymerization will be unaffected by TBE-31. Thus determining the specific mechanism in which TBE-31 inhibits actin polymerization.

A major focus of my thesis was on the effects of TBE-31 on cell migration. Multiple approaches were used to assess this, including scratch assays, single cell migration, and transwell migration assays. It is important to identify the caveats and limitations of each technique as used in these studies. Scratch assays may be influenced by rates of cell proliferation. The studies addressed this by tracking single cells in sub confluent migration assays. However a caveat of this technique is that it is dependent on the cell type’s inherent predilection for exploring the environment, since no stimulus is present to induce cell migration. Transwell assays permit chemotaxis and is an excellent model for directed cell migration, but because the cells are usually fixed and stained in order to be quantified, they are endpoint assays. They cannot provide kinetic data as easily as the scratch and subconfluent cell migration assays do. Therefore this assay cannot distinguish between cells that have migrated a great distance versus a short distance, and can only discern between cells that have or have not migrated through the pores of the membrane. Therefore, the studies in this thesis utilized all three of these techniques to complement one another and minimize the above limitations to generate robust data to support the hypothesis.

Complementary future experiments may can include the use of the μ-Slide Chemotaxis (ibidi) system. In brief, two reservoirs are connected by a narrow observation area. The cells under investigation are seeded into the observation area, one reservoir is filled with regular media, while the other reservoir is filled with media containing a chemoattractant, which results in a gradient of the chemoattractant in the observation
area. This technique would allow for full observation over the course of the experiment—which would allow the collection of kinetic data—would not be as prone to influence from cell division, and still benefit from inducing directed cell migration with a chemotactic gradient.

It is also important to note that cell motility during the metastasis cascades includes cell migration and cell invasion. Although the two processes share similarities, it is important to note differences. During invasion, instead of lamellipodia, invadopodia form to mediate the migration and degradation of the extracellular matrix with matrix metalloproteases (MMP)\textsuperscript{24}. The invadopodia is comprised of an actin-filament rich core and a protein complex composed of integrins and integrin-associated proteins like vinculin, talin and paxillin\textsuperscript{24}. In addition, scaffolding proteins such as Tks5 have also been identified to be specific to invadopodia\textsuperscript{25}. Therefore, cell invasion is dependent on migration, but may require different mechanics\textsuperscript{24,26}. My studies have demonstrated TBE-31 as an effective inhibitor of cell migration, and warrant future experiments to include complementary models for cell invasion. The transwell migration assays from this thesis can easily be adapted to study invasion. A standard technique in the field is to coat the transwell inserts with Matrigel (Corning)\textsuperscript{27}, a solubilized basement membrane preparation, before seeding cell in the top chamber. In addition, another common technique is to seed the cells into a bed of Matrigel to study three dimensional cell culturing\textsuperscript{28}. Invasive cell lines will form protrusions to invade into the surrounding Matrigel. Lastly, a mixture of Matrigel and cells can be seeded into the μ-Slide Chemotaxis system outlined above to monitor chemotaxis directed cell invasion over time. Since TBE-31 targets the actin and microtubule cytoskeleton—components that are
also fundamental to cell invasion—it is expected that TBE-31 would inhibit invasion as well.

The *in vitro* cell migration techniques used in this thesis, and the *in vitro* cell migration and invasion assays proposed for future experiments above are valuable tools for initial studies and characterizing the effects of TBE-31. However, they may not be fully reflective of cell migration and invasion *in vivo*. For instance, cells utilize a greater range of protrusive structures such as blebs, lobopodia and pseudopods to squeeze through the extracellular matrix *in vivo* instead of forming only lamellipodia. The varying substratum of the extracellular matrix may provide multiple haptotactic cues, and multiple chemotactic agents can originate from various directions, thus further complicating the establishment of polarity. The promising results from my thesis justify the extension of my findings into animal models for future experiments. More specifically, the cancer cells can be transplanted into immunocompromised mice. A group of mice would receive intraperitoneal injections of TBE-31 suspended in saline while another group will receive equal injections of DMSO suspended in saline. After a period of time, organs can be harvested and examined for surface nodules, or subjected to histology for examination of metastatic burden. Studies such as these would examine metastasis as a whole, and be a more specific indication of how effective TBE-31 inhibits cell migration and invasion to reduce metastasis. Based on what has been demonstrated in this thesis, it would be expected that TBE-31 would inhibit tumour cell migration and invasion to reduce metastasis burden to distant organs.

During the study of TBE-31 on the cytoskeleton and cell migration, it was interesting to observe how TBE-31 has effects on the cytoskeleton unique from CDDO-
Im and CDDO-Me. In chapters 2 and 4, I demonstrated that TBE-31 binds to actin to inhibit polymerization, while other studies have shown CDDO-Im and CDDO-Me target Arp2/3 to inhibit the induction of branched actin polymerization. Interestingly, the general effect of inhibiting cell migration was observed with all three. Perhaps by targeting actin polymerization in two different manners critical for cell migration, better specificity and greater potency can be achieved with a combination of these two class of compounds. Future studies may benefit from examining this possible synergistic effect.

5.3 Significance and Conclusion

Metastasis accounts for over 90% of cancer related deaths. Significant progress has been made in understanding metastasis, however there is still a severe lack of therapies targeting the numerous processes involved. Most of the available therapies primarily focus on cancer growth. One potential approach is to target tumour cell motility as a strategy against tumour cell migration, invasion, and metastasis.

The synthetic triterpenoids and tricyclic compounds have gained attention in the recent years for their anti-inflammatory properties. Previous studies have shown the synthetic oleanolic acid derivatives have anti-migratory properties however the effects of TBE-31 on cell migration were completely unknown until now. My work has gleaned insight on how TBE-31 modulates various aspects of the cytoskeleton and polarity, in a fashion entirely unique from CDDO-Im or CDDO-Me. More specifically I demonstrated for the first time that TBE-31 targets the actin cytoskeleton, microtubule network, and polarity proteins, and inhibits tumour cell migration (Figure 5.1). This makes TBE-31 an
attractive candidate for targeting tumour cell motility in cancer metastasis, and warrants further investigation. In addition, this knowledge will be important for developing more efficacious and specific tricyclic compounds and synthetic triterpenoids for targeting cancer metastasis.
Figure 5.1 The effects of the synthetic oleanane triterpenoids and tricyclic compounds on cell migration

A) A summary of previous findings on the effects of CDDO-Im on cell migration. Top, CDDO-Im was found to alter microtubule dynamics by disrupting the microtubule capping protein, Clip-170, disrupt microtubule dynamics and organization, and cause a loss of polarity proteins to the leading edge. Bottom, CDDO-Im was found to inhibit Arp2/3-dependent branched actin polymerization at leading edge. Note filopodia unaffected as its formation is independent of Arp2/3.

B) A summary of the effects of TBE-31 on cell migration found in this thesis. Top, I determined TBE-31 directly binds to tubulin and interferes with microtubule dynamics and the polarity proteins Rac1, IQGAP and Tiam1 were found to localize to multiple conflicting locations around the cell periphery, resulting in the loss of a single dominant protrusion. Bottom, I determined TBE-31 binds to cysteine 374 of actin—a critical cysteine for filamentous actin stability—and inhibit actin polymerization. All of which I believe contribute to inhibiting efficient cell migration.
5.4 References


Appendices

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Chan E, and Di Guglielmo GM. The emerging role of synthetic triterpenoids in inhibiting cell migration. Cancer Cell & Microenvironment. (Invited review – manuscript in preparation)

Soares IN, Chan E, Thai B, and Di Guglielmo GM. Targeting PKM2 in cancer cells with covalent modifying drugs. (Manuscript in preparation)
Poster Presentations:


Chan E, Saito A, Honda T and Di Guglielmo GM. The acetylenic tricyclic bis-(cyano enone) interacts with cysteine residues on actin to inhibit non-small cell lung cancer cell migration, Windsor Cancer Research Group 2nd Biennial Conference, Windsor, ON (November 22nd, 2014) (Top Poster Award)


Chan E, Di Guglielmo GM. The Effects of the Synthetic Triterpenoid Acetylenic Tricyclic bis-(Cyano Enone), Department of Physiology and Pharmacology Annual Research Day, University of Western Ontario, London, ON. (November 8th, 2011) (Top Poster Award)