REGULATION OF REDOX SIGNALING BY LIPID ELECTROPHILES IN BREAST CANCER

by

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ABSTRACT

A number of steps in breast cancer progression and metastasis are regulated by redox signaling pathways. Electrophilic lipids such as 15-deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) (15d-PGJ\(_2\)) are mediators of redox signaling pathways because of their ability to modify critical cysteine residues (thiols) in redox-sensitive proteins. In this thesis, we examine the effect of lipid electrophiles such as 15d-PGJ\(_2\) and others on redox signaling pathways in breast cancer. Furthermore, we develop new strategies to regulate cancer cell behavior in response to lipid electrophiles using three strategies: 1) through organelle-specific targeting of electrophiles 2) by exploiting the concentration-dependence of effects of electrophiles, and 3) utilizing electrophiles which modify alternate target proteins. We synthesized a novel mitochondrially-targeted analog of 15d-PGJ\(_2\) (mito-15d-PGJ\(_2\)) and found that it was more potent at initiating intrinsic apoptotic cell death and was less effective at upregulating the expression of the intracellular antioxidants heme oxygenase-1 and glutathione than untargeted 15d-PGJ\(_2\). In addition, we demonstrated that 15d-PGJ\(_2\), at sub-lethal concentrations, attenuated migration, stimulated focal adhesion disassembly, and caused extensive reorganization of the F-actin cytoskeleton. Moreover, we defined a role for the redox-sensitive p38 MAP kinase signaling pathway in mediating these effects. These results suggest a potential anti-metastatic activity of 15d-PGJ\(_2\). Finally, by comparing the biological responses of 15d-PGJ\(_2\) to a structurally related lipid electrophile, Pros-
taglandin A$_1$ (PGA$_1$), we showed that the effects of 15d-PGJ$_2$ on the F-actin cytoskeleton and cell migration were specific for 15d-PGJ$_2$ and cannot be recapitulated using PGA$_1$.

Taken together, our work now provides a basis for the use of these strategies to fine-tune biological responses to electrophiles, as well as deeper understanding of the role of redox signaling by lipid electrophiles in the regulation of breast cancer and metastasis.
DEDICATION

This thesis is dedicated to the breast cancer survivors in my life – Rochelle Kleinschrodt, Marilyn Kleinschrodt, and Sydney Dranka.
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I would like to take this opportunity to thank all of the people who have been instrumental in shaping my graduate career. To my mentor, Dr. Aimee Landar, thank you for taking me on as your first student and for all of your support over the last four years. It is difficult to put into words succinctly all that I have learned during my time with you at UAB. Our lessons reached well beyond the fundamentals of basic bench science, to mentoring, lab management, career development, and defining success in science. You’ve taught me to think beyond the confines of current dogma and to explore the creativity possible in science. With all that you’ve taught me in hand, I can confidently enter the scientific community knowing I am well-prepared for what lies ahead. To my close collaborator and nearly-second mentor, Dr. Darley-Usmar, your contribution to my scientific training has been invaluable. I find your approach to the scientific method and belief in scientific development as a "process" to be unique and refreshing as indicated by your successful trainees. I will take so much of this with me as I move on from UAB. To the remaining members of my committee, Drs. Dickinson, Doeller, and Frost, thank you for serving on my committee. Our interesting and challenging discussions over the last few years drove much of the work included in this thesis and provided the basis for new and exciting interpretations and future directions for my work. I am grateful for your contri-
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To the Landar lab, our “lab family” has grown quickly in the past year, but Michelle and Karina have been there since the beginning with me. Michelle, we’ve made a great team doing mito preps and 2D gels. I can’t count how many plates we’ve scraped together over the years to get enough material to make any sense of our data. Sitting next to each other (both in 342 and 347), our discussions between timepoints, data analysis, lipid preps, and everything else a lab mom has to take care of have been a highlight for me. We also have the best looking, most cheerful desks in the lab. Karina, your insight and hard-working attitude have made working with you a very fruitful effort. To the new members of the lab, Fen, Praveen, and Stephanie, I’m sorry I haven’t had the opportunity to work with you more though our short time together has been a pleasure. I look forward to seeing your fabulous work come out of the lab in the future! Finally, to our past lab member, Joo Yeun, your excellent work provided the basis for many of my pursuits, and I am grateful for the opportunity I’ve had to work with such a solid and efficient scientist (and that I never had to treat cells with 15d-PGJ$_2$ every 20 min for any length of time).

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together in Wisconsin. To my fellow Seahorsians, Blake, Brad, Gloria, and Jessica, only you know how challenging analyzing Seahorse data can really be. Best of luck in the future, and make sure to have your BOFA daily! To the other members of the lab, Yaozu, Sarah, Colin, Mi Jung, Balu, and those who have moved on from the lab, you all have contributed to making the environment one of exciting discussion and collaboration in which I have been blessed to work.

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reagents for these studies for which I am grateful. Additionally, a number of \textit{in vitro} studies included in this thesis were conducted in the laboratory of Dr. Richard Cohen. I believe science cannot be practiced in isolation, and without the help of this long list of generous, supportive scientists, this work would not have been possible.

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LIST OF ABBREVIATIONS

°C  Degree Celsius
15d-PGJ₂  15-deoxy-Δ₁²,₁⁴-Prostaglandin J₂
15-keto-PGE₂  15-keto-Prostaglandin E₂
15-PGDH  15-hydroxyprostaglandin dehydrogenase
15(R)-PGD₂  15(R)-Prostaglandin D₂
6x-His  6 histidine tag
ΔΨ  Mitochondrial membrane potential
ALDH  Aldehyde dehydrogenase
ANOVA  Analysis of variance
ANT  Adenine nucleotide translocator
BD-15d-PGJ₂  BODIPY conjugated 15-deoxy-Δ₁²,₁⁴-Prostaglandin J₂
BODIPY FL EDA  4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl ethylenediamine
BSA  Bovine serum albumin
bt-15d-PGJ₂  Biotin conjugated 15-deoxy-Δ₁²,₁⁴-Prostaglandin J₂
CO₂  Carbon dioxide
CAT  Catalase
cGMP  Cyclic guanosine monophosphate
cm  Centimeter
cm⁻¹  1/centimeter
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper zinc superoxide dismutase</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6’-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMEM-F12</td>
<td>1:1 ratio of Dulbecco’s modified Eagle’s medium and F-12 medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DP2</td>
<td>Prostaglandin D2 receptor 2</td>
</tr>
<tr>
<td>DTNB</td>
<td>5-5’-Dithio-bis(2-nitro-benzoic acid)</td>
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<tr>
<td>DTPA</td>
<td>Diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>EC50</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular consumption rate</td>
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<tr>
<td>EcSOD</td>
<td>Extracellular superoxide dismutase</td>
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<tr>
<td>EDC</td>
<td>Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EP</td>
<td>E-prostanoid receptor</td>
</tr>
<tr>
<td>EpRE</td>
<td>Electrophile reponse element</td>
</tr>
<tr>
<td>ER-α</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>ESI-MS</td>
<td>Electrospray mass spectrometry</td>
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<td>EtOH</td>
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<td>F-actin</td>
<td>Filamentous actin</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FCCP</td>
<td>Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>g</td>
<td>gram</td>
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<tr>
<td>GCL</td>
<td>Glutamyl cysteine ligase</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GFP-MB231</td>
<td>Lentiviral GFP expressing MDA-MB231 cells</td>
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<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>GST</td>
<td>Glutathione transferase</td>
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<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>Water</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HCl</td>
<td>Hydrogen chloride</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor-1 alpha</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>H-Ras</td>
<td>Harvey rat sarcoma viral oncogene homolog</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Hsp27</td>
<td>Heat-shock protein 27</td>
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<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
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<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>JC</td>
<td>JC murine mammary adenocarcinoma cells</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
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<tr>
<td>L</td>
<td>Liter</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>LOH</td>
<td>Lipid alcohol moiety</td>
</tr>
<tr>
<td>LOO’</td>
<td>Lipid peroxyl radical</td>
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<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mmol</td>
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</tr>
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<td>M⁻¹</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
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<tr>
<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCF10A</td>
<td>Human immortalized mammary epithelial cells</td>
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<td>MDA-MB231</td>
<td>MDA-MB231 human mammary adenocarcinoma cells</td>
</tr>
<tr>
<td>MEGM</td>
<td>Mammary epithelial cell growth medium</td>
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<td>Mito-15d-PGJ₂</td>
<td>Mitochondrially-targeted 15-deoxy-Δ⁰¹۸-Prostaglandin J₂</td>
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<td>Mito-PGE₂</td>
<td>Mitochondrially-targeted Prostaglandin E₂</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>mol</td>
<td>Mole</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NaBH₄</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<td>NEM</td>
<td>N-Ethylmaleimide</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>nsRNA</td>
<td>Non-silencing siRNA</td>
</tr>
<tr>
<td>$O_2^-$</td>
<td>Superoxide</td>
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<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>p-FAK</td>
<td>Phosphorylated focal adhesion kinase</td>
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<tr>
<td>PGA$_1$</td>
<td>Prostaglandin A$_1$</td>
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<tr>
<td>PGD$_2$</td>
<td>Prostaglandin D$_2$</td>
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<td>PGE$_1$</td>
<td>Prostaglandin E$_1$</td>
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<tr>
<td>PGE$_2$</td>
<td>Prostaglandin E$_2$</td>
</tr>
<tr>
<td>PGH$_2$</td>
<td>Prostaglandin H$_2$</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>$pK_a$</td>
<td>Acid dissociation constant</td>
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<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>pmol</td>
<td>Picomole</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethanesulphonylfluoride</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RLS</td>
<td>Reactive lipid species</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
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LIST OF ABBREVIATIONS (continued)

ROS  Reactive oxygen species
ROSI  Rosiglitazone
RPMI 1640  Roswell Park Memorial Institute medium
SDS  Sodium dodecyl sulfate
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFN  Sulforaphane
siRNA  Small interfering RNA
TBST-T  Tris buffered saline with Tween
TPMP  Methyltriphenylphosphonium
TPP+  Triphenylphosphonium
Tyr  Tyrosine
V  Voltage
VDAC  Voltage dependent anion channel
VEGF  Vascular endothelial growth factor
WASP  Wiskott-Aldrich Syndrome protein
WAVE  WASP verprolin homologous protein
XO  Xanthine oxidase
CHAPTER 1

INTRODUCTION

Breast cancer accounts for approximately 25% of all new cancer cases diagnosed among women annually and is the second leading cause of cancer-related death among women [1]. Ninety percent of all cancer-related deaths occur not as a result of the primary tumor, but of complications associated with metastasis, or the spread of cancer to secondary sites throughout the body. Moreover, survival rates are much higher when disease is localized at diagnosis compared with disseminated disease [1]. Thus, further understanding of the regulation of this complex process which ultimately results in metastatic lesion formation is crucial for the development of new anti-cancer treatment strategies.

REGULATION OF TUMORIGENESIS AND METASTASIS

Tumorigenesis is a multi-step process that is characterized by the accumulation of genetic mutations which ultimately lead to unregulated cell growth. In 2000, Hanahan and Weinberg addressed the “hallmarks of cancer” in a seminal review in the field, where, taking the current knowledge to date about genetic abnormalities in cancer, they outlined the acquired capabilities of cancer cells. They defined the hallmarks of cancer to be 1) limitless replicative potential, 2) sustained angiogenesis, 3) evasion of apoptosis, 4) self-sufficiency in growth signals, 5) insensitivity to anti-growth signals, and 6) tissue
invasion and metastasis [2]. Nearly all mutations in oncogenes and tumor suppressor genes converge on regulation or deregulation of one of these functional aspects of cancer.

More recently, an extension of the Hanahan and Weinberg “hallmarks of cancer” has been put forth. The revised hallmarks include a number of stress phenotypes in cancer including metabolic, proteotoxic, mitotic, oxidative, and DNA damage stress as well as the capability of evading immune surveillance [3]. The “hallmarks of cancer” are shown schematically in Figure 1-1. Multiple lines of evidence indicate that there is significant interplay between these cancer phenotypes. For example, angiogenic signaling and metabolic stress are both uniquely tied to hypoxic signaling. Gene transcription by the oxygen-sensing transcription factor hypoxia inducible factor-1 alpha (HIF-1α) coordinately regulates metabolic flux towards more glycolytic metabolism as well as stimulates the production of angiogenic mediators like vascular endothelial growth factor (VEGF; reviewed in [4]). Additionally, it is also becoming increasing clear that these stress phenotypes may be exploited to sensitize cancer cells to cell death. It is important to note that these hallmarks highlight the broad nature of the field of cancer research and represent important therapeutic targets for the treatment of cancer.

One hallmark of cancer described above is tissue invasion and metastasis. The process of metastasis is a highly-regulated, complex, but linear process, whereby cells in the primary tumor become invasive and spread into surrounding tissue. Cells can then gain access to the vasculature or lymph to be transported to secondary sites. There is also evidence that some metastatic cells will migrate along nerves (perineural spread) or between the endothelium and basement membrane, never entering the blood vessels themselves (extravascular spread) [5, 6]. In hematogenous metastasis, or spread via the vascu-
Evading apoptosis

Insensitivity to anti-growth signals

Self-sufficiency in growth signals

Tissue invasion & metastasis

Sustained angiogenesis

Evading immune surveillance

Limitless replicative potential

Functional phenotypes

Metabolic stress

Mitotic stress

Proteotoxic stress

Oxidative stress

DNA damage stress

Stress phenotypes

Figure 1-1: Hallmarks of cancer. The hallmarks of cancer as first described by Hanahan and Weinberg [2] are shown in red text. Later, Luo et al. [3] revised these hallmarks to include a number of stress phenotypes which are associated with cancer (shown in teal text). Additionally, these characteristics of cancer represent important therapeutic targets for the treatment of cancer.
lature, cells must survive the shear forces within the vessels and evade immune detection while being transported to secondary sites. Once at the secondary site, cells must arrest and proliferate to form a metastatic lesion [7]. A summary of this process is depicted in Figure 1-2. Because of the linear nature of this metastatic cascade, the ability to inhibit any step of this process will ultimately inhibit the formation of metastatic lesions and offers researchers a viable target for therapeutic intervention.

**MECHANISMS OF REDOX SIGNALING**

It is becoming clear that a number of steps in both tumorigenesis and metastasis are regulated by redox signaling. Before examining these pathways, first an overview of this type of signaling will be discussed. The primary mechanism by which redox signaling occurs is through the post-translational modification of critical cysteine residues (thiols) in redox-sensitive proteins. This modification can then change the structure and/or function of the modified protein and alter downstream signaling associated with that protein [8]. This is shown schematically in Figure 1-3A. There are multiple lines of evidence which demonstrate that redox signaling occurs in a regulated and specific manner and does not simply represent non-specific oxidative damage.

Conserved cysteine residues occur in almost all classes of proteins and in many cases are important for protein function [9-11]. For example, there are three cysteine residues in the active site of low-$K_m$ aldehyde dehydrogenase (ALDH) [12] which when oxidatively modified significantly inhibit enzyme activity [13]. It is for this reason that thiols are poised to mediate diverse redox signaling responses to multiple stimuli.
Figure 1-2: Schematic of metastatic cascade. The process begins with the formation of a primary tumor (a). Tumor cells become invasive and penetrate through the basement membrane, spreading into surrounding tissue (b). In hematogenous metastatic spread, tumor cells intravasate into nearby vessels for transport to secondary sites (c). Cells must survive shear forces and evade immune detection in the vasculature (d). Upon arrival at a secondary site, cells can adhere to the vessel wall and extravasate (e). Tumors cells then proliferate at secondary site forming metastatic lesions (f).
A primary mechanism by which redox signaling occurs is through the modification of protein cysteine residues. “X” denotes an oxidant or electrophilic compound. This cysteine modification can change the protein structure and/or function and, as a consequence, alter downstream signaling associated with the modified protein (A). The thiol group on cysteine residues can exist in a protonated or deprotonated (thiolate) form (B). Thiolate is much more sensitive to modification than the protonated thiol form.
Additionally, cysteine modification occurs in a specific manner. While cysteine is present in most proteins, it is not extremely abundant (approximately 1.9% of total amino acid composition), and only a small percentage of cysteine is susceptible to modification [14]. Susceptibility of cysteine residues to modification is dictated by a number of factors including $pK_a$ (acid dissociation constant) of the thiol, the accessibility of the thiol within the protein structure, subcellular localization, and the reactivity of the thiol-modifying agent. $pK_a$ of a specific thiol is defined as the pH at which 50% of that thiol will be deprotonated. Thus, a thiol having a $pK_a$ of 7.4 will be 50% deprotonated at physiological pH. Since deprotonated thiol (thiolate) is much more nucleophilic in nature, lower $pK_a$ thiols which are more likely to be deprotonated at physiologic pH are better targets for modification (Figure 1-3B) [15].

Localization of thiol residues, either within a protein or within the cell, also seems to be important in dictating their relative susceptibilities to modification; however, these factors are less well characterized. There is evidence demonstrating site-selective modification of cysteine residues within a single protein by two different reactive lipid species [16] though characterization of this type of regulation for a larger subset of proteins has not been examined to date. A summary figure of the concepts presented here which regulate susceptibility to thiol modification is shown schematically in Figure 1-4. The relative susceptibility to modification of proteins can be pictured as a bull’s-eye whereby those proteins that are the most sensitive to modification are in the center and those that are less sensitive to modification are on the outside of the bull’s-eye. Since multiple factors determine the relative susceptibility to modification, it is important to note that there is considerable interplay between these factors.
Susceptibility to thiol modification

Figure 1-4: Factors which determine susceptibility to thiol modification. Thiol residues have different susceptibilities to being modified by thiol reactive agents. This is shown schematically using a bull’s-eye whereby the thiols most sensitive are in the inner ring and those less sensitive are on the outer ring. Multiple factors impact on the relative susceptibility of a thiol to modification including $pK_a$, accessibility within a protein structure, and sub-cellular location.
Finally, the reactivity of the thiol-modifying agent itself imparts an important selective pressure for the sub-set of proteins (or subproteome) which will be modified. In general, as the reactivity of a thiol-modifying agent increases, its relative specificity for modifying a subproteome decreases. In the case of modification of thiols by electrophilic compounds, recently, some attempt has been made to quantitatively evaluate the relative reactivities of these electrophiles and to assign an electrophilic “softness” parameter to describe the ability to modify thiols [17]. Soft electrophiles more readily modify thiol residues and can modify higher pKₐ thiols. Moreover, electrophilic softness correlates with neurotoxic potency in a model of synaptosome function [17]. Similarly, the source of an electrophile often dictates its potential protein targets, as the site of generation may promote modification within a subcellular microdomain.

**SOURCES OF REDOX SIGNALING MEDIATORS**

Species capable of modifying redox signaling pathways can be derived from several sources such as the diet, environment, or endogenously through enzymatic or non-enzymatic processes [10, 18-21]. There are many intracellular sources of reactive oxygen and nitrogen species (ROS and RNS, respectively) that have been shown to be important mediators of redox signaling through their ability to modify thiols. More recently, it has become clear that a third class of reactive species, reactive lipid species (RLS), can also mediate redox signaling processes. Figure 1-5 shows thiol modification by ROS, RNS, and RLS.

Under physiological conditions, generation of ROS occurs through multiple regulated processes. ROS are enzymatically generated through the activity of enzymes like
Figure 1-5: Modification of thiol residues by ROS, RNS, and RLS in redox signaling processes. Oxidation of thiol residues by ROS can cause intramolecular disulfide bridge formation (a) or sulfenic (-SOH), sulfenic (-SO₂H) or sulfonic (-SO₃H) acid derivatives (b). Michael-type adduction of thiols by electrophilic lipids gives alkylation products (c). Thiols modification derived from RNS can also occur which results in S-nitrosothiol formation (d). These types of thiol modification have been shown to alter protein function and affect downstream signaling processes.
NADPH oxidases and xanthine oxidase (XO). Additionally, mitochondria are an important source of ROS. An estimated 2-4% of electrons passed down the respiratory chain during normal mitochondrial respiration “leak” and react with molecular oxygen to form superoxide anion ($O_2^•$) [22]. This “leak” can be modulated by a number of factors including altered electron flux into the chain, mitochondrial uncoupling, and changes in functionality of respiratory complexes [23].

Nitric oxide (NO) is a physiologically relevant RNS. Its production occurs through the activity of nitric oxide synthase (NOS) which catalyzes the conversion of L-arginine to L-citrulline and releases free NO. Canonical NO signaling occurs through the activation of soluble guanylate cyclase which causes production of cGMP and ultimately leads to vasorelaxation in the vasculature. Nitric oxide also binds to and inhibits cytochrome $c$ oxidase in the mitochondrial electron transport chain, and NO has been shown to be an important modulator of mitochondrial ROS generation through this mechanism [24]. Additionally, NO has been shown to modify thiols which results in the formation of relatively stable $S$-nitrosothiol modifications that have been identified in many biological systems [8].

Reactive lipid species are emerging as important mediators of redox cell signaling. RLS can be derived from both enzymatic and non-specific lipid oxidation pathways. Reactive oxygen and nitrogen species can promote the peroxidation of unsaturated lipids such as arachidonic acid which is found in high concentrations in cellular membranes. In the presence of oxygen, this rapidly forms lipid peroxyl radicals ($LOO^•$) which can cyclize with the hydrocarbon backbone to form cyclic peroxides and generate multiple reactive lipid species including families of isoprostanes and cyclopentenone compounds [20,
The electrophilic RLS derived from lipid peroxidation vary in their relative softness, or reactivity, and as such may show specific modification of cysteine residues or modify less reactive nucleophilic amino acids such as lysine and histidine.

Endogenously-generated reactive lipids can be formed through enzymatic pathways, most notably cyclo-oxygenase (COX), which catalyzes the controlled oxidation of lipids such as arachidonic acid [20, 27, 28]. The oxidized lipid product of COX, Prostaglandin H2 (PGH2), is the precursor for a family of lipid prostaglandins some of which are electrophilic in nature [27, 29]. The COX-dependent oxidation of arachidonic acid and dihomo-γ-linolenic acid, two biologically relevant membrane lipids, is shown schematically in Figure 1-6. Electrophilic COX metabolites (e.g. 15-deoxy-Δ12,14-Prostaglandin J2 and Prostaglandin A1; 15d-PGJ2 and PGA1 respectively) have been shown to modify thiol residues, participate in redox signaling pathways, and have been shown to have anti-cancer activity. Although cyclo-oxygenase-dependent lipid oxidation through the COX-2 isoform correlates with high grade, metastatic cancer lesions, it is thought that this is driven through Prostaglandin E2 (PGE2)-dependent stimulation of survival, proliferation, angiogenesis, and invasion, and not through 15d-PGJ2-dependent electrophilic signaling mechanisms. In fact, the balance of PGE synthase and PGD synthase is often also altered in invasive cancers such that PGE2 synthesis is predominant [30-32], and this also suggests that supplementation or restoration of endogenous electrophiles may have therapeutic benefits.

Multiple enzymatic systems are poised to shut-off the activity of redox signaling mediators in vivo. In the mitochondrial matrix, superoxide can be dismuted to the freely diffusible product hydrogen peroxide (H2O2) by the enzyme manganese superoxide dis-
Figure 1-6: Source and effects of cyclo-oxygenase-2 (COX-2) derived prostaglandins in cancer. Arachidonic acid can be metabolized by COX-2 to the precursor lipid, Prostaglandin H$_2$ (PGH$_2$). PGE synthase converts PGH$_2$ to Prostaglandin E$_2$ (PGE$_2$) while PGD synthase converts PGH$_2$ to Prostaglandin D$_2$ (PGD$_2$). 15-deoxy-$\Delta^{12,14}$-Prostaglandin J$_2$ (15d-PGJ$_2$) is form through non-enzymatic dehydration and isomerization of PGD$_2$. PGE$_2$ mediates many of the pro-tumorigenic processes observed in cancers with elevated COX-2 levels. PGE$_2$ can be further metabolized to a keto-derivative enzymatically. In contrast, many anti-cancer effects have been reported for 15d-PGJ$_2$. COX-2 dependent metabolism of dihomo-$$\gamma$$-linolenic acid generates an intermediate (Prostaglandin E$_1$; PGE$_1$) and subsequently Prostaglandin A$_1$ (PGA$_1$). Interestingly, similar anti-cancer effects have been reported for PGA$_1$ and 15d-PGJ$_2$ both of which are electrophilic in nature.
mutase (MnSOD). Hydrogen peroxide can diffuse into the cytosol and alter the activity of a number of redox-sensitive signaling proteins. This “signal” can then be turned off by the action of another antioxidant enzyme, catalase (CAT), which breaks down H\textsubscript{2}O\textsubscript{2} to water and O\textsubscript{2}. Cytosolic and extra-cellular isoforms of SOD, CuZnSOD and EcSOD respectively, dismutate superoxide from extra-mitochondrial sources. The family of glutathione peroxidases (GPx) of which there are 5 isoforms found in humans reduce hydroperoxides and hydrogen peroxide. A simplified diagram of sources of ROS, RNS, and RLS discussed here as well as enzymatic detoxification reactions is shown in Figure 1-7. Glutathione tranferases (GSTs) also play an important role in inactivating redox signaling by conjugating multiple reactive lipid species to glutathione [20] for export from the cell and excretion.

There are also families of thioredoxins [33], glutaredoxins [34], and peroxiredoxins [35] that enzymatically turn over modified thiol resides and return them to the reduced or unmodified state. It is therefore clear that redox signaling pathways can be specifically activated by numerous stimuli, and redox mediators are generated in a regulated manner. This type of signaling can, in turn, be terminated by the activity of the enzymes described above, or in some cases, degradation and turnover of the modified protein [36].

**ELECTROPHILES AS REDOX SIGNALING MEDIATORS**

The focus of this work is the role of lipid electrophiles in regulating redox cell signaling in breast cancer. Figure 1-8 shows examples of electrophiles that can be derived from sources such as diet, environment, or endogenously through enzymatic or non-enzymatic processes. They are also arranged from left to right based on increasing rela-
Figure 1-7: Endogenously-derived redox signaling mediators. There are several sources of reactive oxygen, nitrogen, and lipid species which play an important role in regulating redox signaling. Mitochondria are recognized as both a source and target of reactive species. In addition, nitric oxide synthases (NOS), xanthine oxidase (XO) and NADPH oxidases are important sources of enzymatically generated RNS and ROS. Reactive lipid species can be derived secondarily to ROS/RNS production through lipid peroxidation pathways. Multiple enzymatic pathways exist to terminate signaling associated with these molecules including superoxide dismutases (MnSOD, CuZnSOD, and EcSOD), catalase and glutathione peroxidases (GPx).
Figure 1-8: Specificity versus reactivity of environmental and dietary electrophiles. Shown pictorially are a number of environmental and dietary electrophiles and the sources from which they may be derived. The electrophiles are loosely ordered left to right by increasing reactivity. The less reactive electrophiles more readily modify only the most nucleophilic amino acid, cysteine, whereas the more reactive electrophiles modify multiple nucleophilic residues including cysteine, histidine, and lysine. Importantly, an inverse relationship between reactivity and specificity for cysteine modification exists. Electrophiles which modify cysteine preferentially are thought to participate in the regulation of redox signaling pathways whereas exposure to more reactive electrophiles is associated with predominantly deleterious effects.
tive reactivity. Dietary electrophiles such as sulforaphane, found in cruciferous vegetables, and curcumin which is found in curry dishes have been shown to have potent anti-inflammatory properties and are thought to have potential health benefits [18, 19]. More reactive electrophiles like acrolein, a component of cigarette smoke, are associated with predominantly deleterious effects. As discussed previously, less reactive electrophiles demonstrate more specificity for cysteine modification whereas more reactive electrophiles can modify other nucleophilic amino acids such as lysine and histidine; however, cysteine modification appears to be necessary for the regulation of redox signaling mechanisms.

Our group is particularly interested in the electrophilic cyclopentenone prostaglandin 15d-PGJ2 which modifies primarily cysteine residues through a Michael-type addition (Figure 1-9) [37]. In the context of cancer, 15d-PGJ2 has garnered much interest because of its ability to inhibit angiogenesis, cause growth arrest, and induce cell death in several cancer cells lines [38-41].

There are two basic mechanisms that have been proposed to explain the biological actions of 15d-PGJ2. First, 15d-PGJ2 has been proposed as the endogenous ligand for peroxisome proliferator activated receptor (PPAR) gamma (PPARγ). PPARs are ligand-inducible transcription factors which belong to the nuclear hormone receptor superfamily [42, 43]. New evidence suggests they may also play a role in oncogenesis, as they modulate proliferation and apoptosis and are expressed in many human tumors including breast [44]. The second mechanism of action by which 15d-PGJ2 alters cellular signaling pathways is through the post-translational modification of redox-sensitive signaling molecules as mentioned above. There are multiple protein targets of 15d-PGJ2 which can
Figure 1-9: Thiol modification by 15d-PGJ$_2$ through Michael-type addition. Thiolate readily reacts with the electrophilic carbon centers of 15d-PGJ$_2$ which are present due to the $\alpha,\beta$-unsaturated carbonyl moieties leading to the formation of stable covalent lipid protein adducts.
mediate diverse biological responses. We have termed this group of proteins the electrophile responsive proteome [45]. This latter mechanism likely underlies the pleiotropic effects of 15d-PGJ₂ reported in the literature [46].

In this thesis, we will also compare the effects of 15d-PGJ₂ to a second lipid electrophile, PGA₁. PGA₁ also modifies thiol residues by a similar mechanism as 15d-PGJ₂, and potent anti-cancer effects have also been reported for this compound [47-49].

**REDOX REGULATION OF CANCER AND METASTASIS**

In the context of cancer, multiple aspects of cell survival and proliferation have been shown to be redox regulated. At the level of transcriptional regulation, a number of transcription factors contain redox-sensitive cysteine residues in their DNA binding sites including the AP-1 family member c-Jun, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and HIF-1α, and the activity of these transcription factors has also been linked to regulation of proliferation and survival pathways in cancer (reviewed in [9]). For example, glutathionylation of c-Jun inhibits its DNA binding activity [50]. Additionally, modification of Cys62 of the p50 subunit of NF-κB through reversible oxidation [51], glutathionylation [52], and S-nitrosothiol formation [53] inhibits NF-κB-dependent gene transcription.

Cytoprotective signaling pathways also have important components which are redox-regulated. The induction of cytoprotective, intracellular antioxidants can occur through the modification of multiple cysteine residues on the cytosolic protein Kelch-like ECH-associating protein 1 (Keap1). Keap1 sequesters the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in the cytosol; however, when Keap1 is modified,
Nrf2 is released to translocate to the nucleus and activate the transcription of several gene targets including the intracellular antioxidant heme oxygenase-1 (HO-1) and the enzymes responsible for the synthesis of glutathione (GSH) [54, 55]. Upregulation of intracellular antioxidants is potentially undesirable in the context of cancer since it is well known that induction of phase II enzymes is associated with resistance of cancer cells to multiple therapeutic modalities including chemotherapy and ionizing radiation [56, 57].

Cell survival pathways are also extensively modulated by redox signaling pathways. Oxidative inactivation of the tumor suppressor phosphatase and tensin homolog (PTEN) has been shown to occur and results in enhanced PI3K/Akt signaling and promotes cell survival [58]. Moreover, some apoptotic machinery, in particular the caspases, has active-site cysteine residues which have been shown to be S-nitrosated under basal conditions. Denitrosation is required for full activation and execution of the apoptotic pathway in response to some stimuli [59, 60]. From these studies and others, it is clear the redox signaling pathways are integral to regulating basic cancer signaling pathways and may represent important therapeutic targets.

Additionally, it is clear steps in the metastatic cascade are also redox-regulated. Early in the metastatic cascade, cancer cells become migratory and invade into the surrounding tissue eventually gaining access to the vasculature. Extra-cellular matrix remodeling often occurs in this process through the activity of matrix metalloproteinases (MMPs) [61]. The role of mitochondrially-generated ROS in the regulation of MMP expression and activity is well established in the literature [62-65]. Additionally, in later steps of the metastatic cascade, angiogenesis is crucial for the survival and growth of lesions at secondary sites. Interestingly, expression of the pro-angiogenic factor VEGF has
been shown to also be linked to mitochondrial ROS, and its levels can be modulated by mitochondrially-targeted antioxidants [66]. Taken together, these results suggest there are redox-sensitive signaling pathways controlling basic processes required for metastasis. Points of redox-regulation of metastasis are summarized in Figure 1-10 though this is not an exhaustive list of possible redox regulation in this context.

**OPPOSING EFFECTS OF 15d-PGJ2 IN CANCER**

In a therapeutic context, 15d-PGJ2 has been proposed as a potential anti-cancer agent because of its ability to regulate redox-sensitive aspects of angiogenesis, growth arrest, and cell death in several cancer cell lines through the covalent modification of proteins [67, 68]. It has been shown to inhibit angiogenesis through the suppression of inflammatory enzymes and cytokines, and this occurs through the direct modification of key components of the NF-κB signaling pathway. Specifically, the potent anti-inflammatory effects of 15d-PGJ2 are attributed to the covalent modification the p50 subunit of NF-κB by 15d-PGJ2 which results in the inhibition of its DNA binding activity [69]. More recently, Kim et al. demonstrated the covalent modification of estrogen receptor alpha (ER-α), a finding that has important implications for the treatment of hormone responsive cancers. In this study, it was found that 15d-PGJ2 modifies two cysteine residues within the COOH-terminal zinc finger of ER-α, and this modification inhibits its DNA binding activity, decreases target gene expression, and inhibits proliferation [70]. The cell death effects of 15d-PGJ2 have been reported to occur through a pleiotropic mechanism involving activation of PPARγ and through interactions with mitochondrial proteins which lead to the activation of apoptosis [46, 71].
Figure 1-10: Redox modulation of the metastatic cascade. It is becoming increasingly clear that multiple steps in the metastatic cascade can be regulated by redox signaling mechanisms. Cellular processes including migration, invasion, survival, adhesion, proliferation, and angiogenesis which all play important roles at different points in successful metastasis have redox-regulated components.
Conversely, an emerging literature suggests that there are a number of cancer-promoting effects of 15d-PGJ₂. Recent work has identified 15d-PGJ₂ as a chemical inactivator of the tumor suppressors LKB1, PTEN, and p53 [72-74]. Moreover, modification of Harvey rat sarcoma viral oncogene homolog (H-Ras) by 15d-PGJ₂ has been shown to increase proliferation of a number of different cell types including cervical cancer cells [75]. Additionally, it has been shown that modification of thiol residues on Keap1 induces the synthesis of intracellular antioxidants such as HO-1. HO-1 induction, in turn, has been shown to upregulate both the pro-angiogenic factor VEGF and MMPs which results in enhanced migratory potential of breast cancer cells [76, 77]. It is well recognized that these redox signaling pathways may be important therapeutic targets; however, the major limitation has been the inability to selectively target the specific redox signaling pathways which regulate the anti-cancer effects of electrophiles. These diverse biological effects of 15d-PGJ₂ are summarized in Figure 1-11. Moreover, a strategy allowing for the selection of the anti-tumor effects of 15d-PGJ₂ while minimizing the pro-tumor effects would enhance the therapeutic potential of 15d-PGJ₂ in cancer. This will be discussed further in this thesis.

COMPARTMENTALIZATION OF 15d-PGJ₂ PROTEIN TARGETS

Evidence clearly supports the fact that protein targets of 15d-PGJ₂ in the electrophile responsive proteome are found in different compartments in the cell including the cytosol and mitochondria [69, 78]. For example, it has been shown that modification of thiol residues on the cytosolic protein Keap1 induces the synthesis of intracellular antioxidants whereas the modification of mitochondrial protein thiols promotes permeability.
Figure 1-11: Biological responses of 15d-PGJ₂ in cancer. 15d-PGJ₂ has attracted considerable interest as a potential cancer therapeutic because of its ability to inhibit angiogenesis, cause growth arrest, and induce apoptosis. More recently, however, a number of cancer promoting effects of 15d-PGJ₂ have been reported including inhibition of tumor suppressors, induction of antioxidant expression, and proliferation. A focus of this thesis is to investigate strategies to fine-tune the biological responses to lipid eletrophiles in an effort to enhance their anti-tumor activities.
transition which can lead to cell death [71]. These findings led us to examine whether
electrophilic modification of proteins within specific sub-cellular compartments can be
used to fine-tune the biological responses to electrophiles. A strategy of this sort may
prove to be efficacious in designing electrophile-based therapeutic agents with increased
specificity.

**DEVELOPMENT OF MITOCHONDRIAL THERAPEUTICS**

Mitochondrial targeting can be achieved by exploiting the bioenergetic properties
of the organelle. Actively respiring mitochondria generate a membrane potential through
the translocation of protons from the matrix across the inner mitochondrial membrane.
This results in a net negative charge in the matrix. This membrane potential can drive the
accumulation of delocalized, lipophilic cations into the matrix (Figure 1-12). A number
of synthetic lipophilic cations can be used to target a compound to the mitochondrion;
however; the best characterized is triphenylphosphonium (TPP+) [79]. Dr. Michael Mur-
phy has used this approach extensively to target antioxidants to the mitochondrion (e.g.
mito-Vitamin E) [80]. He has also used this technology to target the mitochondrial elec-
tron carrier and antioxidant, ubiquinone, to the mitochondrion as a therapeutic for the
treatment of Friedrichs Ataxia, a neurodegenerative disease of mitochondrial origin. This
study is currently in clinical trial, and mitochondrially-targeted derivatives have been
shown to be both safe and bioavailable in humans [81, 82].

Based on these findings, we have synthesized a mitochondrially-targeted analog
of 15d-PGJ₂ (mito-15d-PGJ₂) by conjugating the TPP⁺ moiety to 15d-PGJ₂. This ap-
proach will allow us to not only define the mechanism by which mitochondrial proteins
Figure 1-12: Mitochondrial targeting of electrophiles. The lipophilic cation triphenylphosphonium (TPP+) can be conjugated to any number of therapeutic molecules to direct their accumulation within mitochondria. TPP+ conjugated compounds accumulate first into the cytosol due to the plasma membrane potential and then accumulate within the mitochondrial matrix because of the significant polarization of the mitochondrial inner membrane which results from electron transport.
regulate the biological effects of this lipid electrophile, but also open new avenues for the development of mitochondrially-targeted electrophilic lipid therapies for the treatment of disease.

**SUMMARY**

The goal of this thesis work is to develop new strategies to regulate cancer cell behavior in response to lipid electrophiles. To this end, we have examined how cellular behavior can be controlled by 1) organelle-specific targeting of electrophiles, 2) exploiting the concentration-dependence of effects of electrophiles, and 3) utilizing electrophiles which modify different sub-proteomes.
CHAPTER 2

METHODS

INTRODUCTION

This chapter summarizes the methods utilized to conduct the experiments described in this thesis. We first describe the synthesis and characterization of novel tagged derivatives of prostaglandins which were used primarily in Chapter 3. In addition, we describe the functional assays used to assess biological responses to electrophiles including measurements of cytotoxicity, intracellular antioxidant capacity, processes important for cell motility, and cellular bioenergetics. Finally, we investigate the protein targets of the electrophiles which mediate the biological responses we have characterized. This was accomplished using one and two dimensional SDS-PAGE and Western blotting techniques as well as a candidate protein target approach focusing on H-Ras.

MATERIALS

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO) unless otherwise noted. Prostaglandin E₂ (PGE₂), Prostaglandin A₁ (PGA₁), 15-deoxy-Δ¹²,¹⁴-Prostaglandin J₂ (15d-PGJ₂), Rosiglitazone (ROSI), and 15(R)-Prostaglandin D₂ (15(R)-PGD₂) were purchased from Cayman Chemical (Ann Arbor, MI). BODIPY FL EDA was purchased from Molecular Probes (Eugene, OR). Alexa Fluor® 633 Phalloidin and Texas Red Phalloidin were purchased from Invitrogen (Carlsbad, CA). The kinase
inhibitors SB203580 (p38 inhibitor), LY294002 (PI3K inhibitor), and FR180202 (ERK inhibitor) were purchased from Calbiochem (San Diego, CA). 2-amino-ethyl-triphenylphosphonium bromide was provided by Dr. B. Kalyanaraman.

CHARACTERIZATION OF TAGGED LIPID REAGENTS

A strategy which has been extensively employed for the detection of electrophilic lipid modified proteins is the incorporation of chemical tags onto the lipid. In our laboratory, we have previously added fluorophore and affinity tags to 15d-PGJ₂ for utilization in intracellular localization studies and identification of lipid modified proteins respectively [14, 83]. The fluorophore BODIPY FL EDA and biotinylated derivatives of 15d-PGJ₂ (BD-15d-PGJ₂ and bt-15d-PGJ₂) were synthesized using the method previously described [83]. The commercially available product, EZ-link 5-(biotinamido)pentylamine (Pierce, Rockford, IL) was used for the synthesis of bt-15d-PGJ₂. The tagging reagents used both contain amine functional groups which allows for the tagging of the terminal carboxylic acid group of 15d-PGJ₂. This is achieved through a carbodiimide-mediated condensation reaction which results in the formation of an amide bond between the lipid and the tag with the loss of water. This synthesis reaction can be adapted for tagging of other carboxylic acid containing lipids or for use with other tags which have available amine groups. Structures of all parent and tagged lipid derivatives used in these studies are shown in Figure 2-1.

We have recently developed a series of mitochondrial-targeted lipid derivatives utilizing a similar tagging strategy. Murphy et al. have pioneered a novel strategy for intracellular targeting of compounds to the mitochondrion [84]. The conjugation of a de-
Figure 2-1: Structures of parent and modified lipids used in these studies. Electrophilic carbons are denoted by asterisks.
localized, lipophilic cation to a compound of interest directs its accumulation within the mitochondrion and is sustained by the mitochondrial membrane potential [79, 85]. This strategy has been employed to direct therapeutic antioxidants to the mitochondrion [82, 84, 86, 87]. Importantly, these mitochondrially-targeted therapeutics have also been shown to be safe and orally bioavailable in humans [81, 86]

**Synthesis of mitochondrially-targeted lipids**

In order to target lipids to the mitochondria, we have conjugated the lipophilic cation triphenylphosphonium (TPP+) to lipids of interest. The procedure is a modification of a previously reported method [14, 83]. For the synthesis of mitochondrially-targeted 15d-PGJ₂, the reaction mixture consisted of 2 mg 15d-PGJ₂, 2 mg 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC; Pierce), and 2 mg 2-aminoethyl-triphenylphosphonium bromide in 80% acetonitrile / 19% ethanol / 1% water. The reaction was incubated 18 h at room temperature with constant mixing. The product was purified by reversed phase HPLC using a C18 Luna column (Phenomenex, Torrance, CA) with a linear gradient from 10% acetonitrile / 0.24% acetic acid / 90% water to 95% acetonitrile / 5% water. Shown in Figure 2-2 are the HPLC chromatograms for unmodified 15d-PGJ₂ (Fig. 2-2A) and the reaction mixture for 15d-PGJ₂ and amino-ethyl-triphenylphosphonium (Fig. 2-2B). The eluate was monitored at the characteristic absorbance of the cyclopentenone at 306 nm [88]. As indicated, the parent 15d-PGJ₂ elutes as a single peak (Fig. 2-2A) with a retention time of 27 min, whereas in the reaction mixture, this peak is not detectable and is replaced with two peaks (“a” and “b” in Fig. 2-2B) at 18-20 min. To further identify the products of the reaction, fractions were collected to
Figure 2-2: HPLC of 15d-PGJ$_2$ and mito-15d-PGJ$_2$. Pure 15d-PGJ$_2$ was injected onto a C18 semi-preparative column and separated by HPLC using the same solvent gradient used to purify mito-15d-PGJ$_2$ as described in Methods (A). Mito-15d-PGJ$_2$ reaction mixture was purified by HPLC (B) and the predominant peak (peak “b”) was found to correspond to the product. Mito-PGE$_2$ was eluted using a solvent gradient as described in Methods (C) and peak “a” was confirmed to contain the product.

capture the products in each peak separately. After extracting the lipid with chloroform, solvent was evaporated under a constant stream of nitrogen gas, and the final product reconstituted in ethanol. Peak “a” corresponded to a partial reaction product with 15d-PGJ2 (results not shown). Peak “b” was confirmed to be the expected product, mito-15d-PGJ2 using electrospray mass spectrometry (ESI-MS).

Prostaglandin E2 which is structurally related to 15d-PGJ2, yet not electrophilic was used as a control in these studies. We designed and synthesized the mitochondrially targeted derivative of PGE2 using the same strategy of conjugation to TPP\(^+\) described above (mito-PGE\(_2\)). The product was purified by reversed phase HPLC with a linear gradient from 10% acetonitrile / 90% water to 95% acetonitrile / 5% water. The eluate was monitored at the characteristic absorbance for the TPP\(^+\) moiety at 225 nm for mito-PGE\(_2\). Figure 2-2C shows a representative HPLC trace with the predominant peak (“a”) corresponding to the product. The addition of the TPP\(^+\) moiety was confirmed by ESI-MS, and the concentration was measured by absorbance at 268 nm by using the extinction coefficient for TPP\(^+\) of 3,000 M\(^{-1}\) cm\(^{-1}\) [84].

**Mass spectrometric analysis of synthesized lipids**

Product integrity and purity for the products mito-15d-PGJ\(_2\) and mito-PGE\(_2\) was confirmed by ESI-MS and MS/MS (Fig. 2-3 and 2-4). Due to the positive charge conferred by the TPP\(^+\) moiety, mito-15d-PGJ\(_2\) is observed at its exact mass [M]\(^+\) with an m/z value of 604.3 (Fig. 2-3A), and similarly, mito-PGE\(_2\) is observed at an m/z value of 640.3 (Fig. 2-3B). Shown in Figure 2-4 are the MS/MS spectra for mito-15d-PGJ\(_2\) (Panel A) and the predicted ion fragments that we propose are derived from the parent structure
Figure 2-3: Electrospray mass spectrometric verification of mitochondrial-targeted lipids. Electrospray mass spectrometry (ESI-MS) verification of mito-15d-PGJ$_2$ (A) and mito-PGE$_2$ (B) in positive ionization mode with the expected [M]$^+$ values are shown. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society. http://www.biochemj.org
Figure 2-4: MS/MS verification of mito-15d-PGJ₂. Representative MS/MS of mito-15d-PGJ₂ (A) and fragments of the molecule that can be ascribed to the triphenylphosphonium moiety, linker, and lipid (B) are shown. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society. http://www.biochemj.org
(Panel B). We could not detect the uncharged fragments in either positive or negative ion mode presumably due to their hydrophobic and uncharged character.

IUPAC nomenclature for the compounds used in this study is as follows: 15d-PGJ2 (11-oxo-prosta-5Z,9,12E,14E-tetraen-1-oic acid), mito-15d-PGJ2 (11-oxo-prosta-5Z,9,12E,14E-tetraen-1-amido-ethyl triphenyl phosphonium), and mito-PGE2 (9-oxo-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-amido ethyl triphenyl phosphonium).

**UV/Visible spectral analysis of 15d-PGJ2 and mito-15d-PGJ2**

To both assess the integrity of the products and determine their concentration, visible absorption spectroscopy was used. To achieve this, the extinction coefficient for each wavelength (250-350 nm) was calculated for 15d-PGJ2 by using a known concentration diluted in ethanol (Fig. 2-5A). The same procedure was performed for methyltriphenylphosphonium (TPMP; Fig. 2-5B). The extinction coefficient for the compounds at all wavelengths was then determined. The simulated spectra of the product, mito-15d-PGJ2, was generated by adding the extinction coefficients calculated for TPMP and 15d-PGJ2, assuming a 1:1 molar ratio of the triphenyl phosphonium moiety to the lipid. Finally, the simulated spectrum was compared to the actual spectrum obtained using the absorbance spectrum for mito-15d-PGJ2 (Fig. 2-5C) and found to be essentially identical. Taken together, these data demonstrate that the conjugation at carbon 13 in the cyclopentenone ring of the parent compound is unmodified by the addition of amino-ethyl-triphenylphosphonium. In all subsequent experiments, the concentration of mito-15d-PGJ2 was calculated using the absorbance at 306 nm and an extinction coefficient at 306 nm of 12,000 M⁻¹ cm⁻¹ [88].
Figure 2-5: Representative UV-Vis spectra of 15d-PGJ₂, the triphenyl phosphonium moiety (TPMP), and the synthesized compound, mito-15d-PGJ₂. The extinction coefficient for each compound was calculated using measured absorbances as described in Methods. Extinction coefficient versus wavelength for 15d-PGJ₂ (A), TPMP (B), and simulated (red trace) superimposed on the measured spectra for mito-15d-PGJ₂ (C) are shown. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society.

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CELL CULTURE

Multiple cell lines were used to examine the effects of lipid electrophiles in vitro including JC murine mammary adenocarcinoma cells, MDA-MB231 human mammary adenocarcinoma cells, MDA-MB231 cells stable expressing green fluorescent protein (GFP; GFP-MB231), MCF10A human immortalized mammary epithelial cells, and wild-type murine embryonic fibroblasts (MEFs) and MEFs null for nuclear factor erythroid 2-related factor 2 (Nrf2). JC cells (purchased from ATCC, Manassas, VA) and MDA-MB231 cells (a gift from Dr. Danny Welch) were cultured in RPMI 1640 media (Media-tech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA). GFP-MB231 cells (also a gift from Dr. Danny Welch) were cultured in DMEM-F12 media supplemented with 5% FBS, non-essential amino acids (Media-tech), and L-glutamine (Invitrogen). MCF10A immortalized human mammary epithelial cells (ATCC) were cultured in Mammary Epithelial Cell Growth Medium (MEGM) supplemented with MEGM SingleQuots (Lonza, Walkersville, MD). Murine embryonic fibroblasts (MEFs) derived from wild-type and Nrf2−/− mice as previously described [89] were cultured in 10% FBS DMEM media supplemented with 50 μM β-mercaptoethanol. Cultures were maintained in 5% CO₂ and humidified in a 37°C incubator. All experiments using adenocarcinoma cells were performed at 0.5% FBS in aforementioned medias at ~50% confluence unless otherwise noted. Experiments utilizing MCF10A cells and MEFs were conducted at confluence in MEGM or 0.5% FBS in DMEM respectively with aforementioned supplementation.
For experiments, cells were cultured in 6-well cluster plates except for bioenergetic and mitochondrial membrane potential measurements, in which case specialized Seahorse Bioscience culture plates or 12-well cluster plates respectively were used. For lipid exposures in 6-well cluster plates, 1 mL media was used, and a 10 μM addition of lipid was then equivalent to approximately 160 pmols lipid/μg protein.

**ASSESSMENT OF CELL VIABILITY**

Cell viability in response to lipid electrophiles was assessed using multiple methods including propidium iodide (PI) and Annexin V flow cytometry, caspase 9 activation, lactate dehydrogenase (LDH) release, and the MTT assay. Rationale for use of each method is given where appropriate in subsequent chapters.

**Flow cytometric analysis of apoptosis and necrosis**

Apoptosis and necrosis were measured after treatment for 16 h by flow cytometric analysis using an Annexin V FITC Apoptosis Detection Kit (Calbiochem). Briefly, treated cells were trypsinized then incubated with PI and FITC conjugated Annexin V. Fluorescence was measured using a BD LSR II flow cytometer.

**Caspase 9 activation**

After exposure to electrophiles, cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and then pro-caspase 9 levels were detected by Western blot (described in more detail in *Western blotting section*). Pro-caspase 9 is cleaved to its active form upon activation of the intrinsic apoptosis pathway. Cleavage of pro-caspase
9 is indicative of activation of caspase 9 and intrinsic apoptosis signaling [90]. Caspase 9 activation was used in conjunction with other indices of apoptosis.

**Lactate dehydrogenase release from cells**

For LDH release assays, after lipid exposure, aliquots of media from treatment wells were taken, and cells were harvested by scraping in PBS lysis buffer containing 0.1% Triton X-100. After centrifugation of cell lysates to remove debris, LDH activity in supernatant and media aliquots was measured spectrophotometrically by monitoring the oxidation of NADH (0.3 mM) at 340 nm. LDH release to the media was used as an indicator of cell death; however, it does not discriminate between late apoptosis and necrosis, especially at later time points.

**MTT viability assay**

Cell viability was also measured by MTT assay as described previously [91], with the following modifications. After treatment, the treatment media was replaced with media containing 0.4 mg/mL thiazoyl blue tetrazolium, and the cells were incubated at 37°C for an additional 2 h. The media was then removed, and the resulting formazan crystals were solubilized in Dimethyl sulphoxide (DMSO), and the absorbance was read at 550 nm.

**MEASUREMENT OF COLONY FORMATION CAPACITY**

Colony formation was measured after treatment with indicated concentrations of 15d-PGJ2. Cells from experimental dishes were trypsinized and collected. All cells from
each dish were then centrifuged, resuspended in fresh medium, and counted. Cells were then plated in 6 well plates at low density (100-200 cells per well), and clones were allowed to grow for 14 days in complete medium in the presence of 0.1% gentamycin. Cells were subsequently fixed with 70% ethanol and stained with Coomassie blue for analysis of clonogenic cell survival as previously described [92]. This assay measures both the ability of cells to re-adhere to tissue culture plastic after treatment and their ability to proliferate upon adherence.

**MEASUREMENT OF CELLULAR MIGRATION**

Cells were grown to confluence in 6 well plates, and then scratched with the narrow end of a sterile pipet tip. Medium was immediately changed to remove floating cells and was replaced with treatment media. The width of the scratch was measured at four points in each well after initial wounding, and then cells were incubated for 8 h at 37°C in a CO₂-incubator. After 8 h, the scratch width was measured again, and the ability of the cells to migrate into the cell-free zone (relative motility) was expressed as the normalized percent change in the width of the scratch after 8 h compared to control treatment.

**DETERMINATION OF FOCAL ADHESION DISASSEMBLY**

Focal adhesions were assessed using interference reflection microscopy. Cells were plated on glass coverslips, allowed to attach and then grown 24 h before being serum starved (0.5% FBS RPMI 1640) for 30 min prior to indicated treatments. Cells were then fixed in 3% glutaraldehyde (Sigma, St. Louis, MO) for 30 min at 37 °C, rinsed, and mounted on glass slides. Slides were imaged using interference reflection microscopy
using a modified inverted Zeiss microscope as described previously [93]. Cells containing > 6 focal adhesions were scored as positive by an observer (Manuel A. Pallero) without prior knowledge of sample conditions. 300 cells/cover slip were scored for each treatment group in triplicate.

**USE OF FLUORESCENCE CONFOCAL MICROSCOPY**

**Phosphorylated focal adhesion kinase immunofluorescence**

Relative levels of phosphorylated focal adhesion kinase (p-FAK) were determined using immuno-fluorescence techniques. JC cells were plated on glass coverslips, treated as indicated, and fixed using paraformaldehyde (3.7%) for 10 min. Cells were then rinsed twice with PBS and permeabilized with 0.1% Triton X-100 (v/v) in PBS. Cells were blocked with 5% normal goat serum (Vector Labs, Burlingame, VT) and 0.3% Triton X-100 (v/v) in PBS for 60 min at room temperature. Cells were incubated in p-FAK (Tyr 397) primary antibody (Cell Signaling, Danvers, MA) at 1:100 in antibody dilution buffer (1% BSA (w/v) and 0.3% Triton X-100 (v/v) in PBS) overnight at 4°C. Alexa Fluor® 488 conjugated goat anti-rabbit secondary antibody (1:500, 1 h; Invitrogen) was applied, and then coverslips were mounted on glass slides using Vectashield Hard Set Mounting Medium containing DAPI (Vector Laboratories). Phospho-FAK foci were visualized using confocal fluorescence microscopy on a Leica DMIRBE laser scanning confocal microscope with excitation from a 488 nm laser line and emission detection suitable for fluorescein. Nuclei were imaged using a UV laser line and emission detection suitable for DAPI.
Visualization of F-actin cytoskeleton

The F-actin cytoskeleton was visualized using a fluorescently labeled phallotoxin. JC cells were plated on glass coverslips, treated, fixed, and permeabilized as described above. Next, cells were incubated with blocking solution (PBS containing 1% BSA, w/v) for 30 min followed by application of 2 units of Alexa Fluor® 633 Phalloidin or Texas Red Phalloidin (Invitrogen) for 30 min at room temperature in blocking solution. F-actin imaging was performed using either a 591 nm or 633 nm laser line for excitation and emission detection suitable for Texas Red or Alexa Fluor® 633 respectively. In p-FAK and F-actin costaining experiments, Alexa Fluor® 633 Phalloidin was co-incubated with secondary antibody. All confocal images represent single sections and were manipulated using linear histogram correction in Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA).

DETERMINATION OF GLUTATHIONE LEVELS

Total glutathione (glutathione + glutathione disulfide) was determined in cell lysates treated as indicated using the recycling assay described previously [94]. Briefly, after treatment, cells were lysed in 10 μM Diethylenetriamine pentaacetic acid (DTPA) and 0.1% Triton X-100 in PBS, pH 7.4. Total glutathione in lysates was determined spectrophotometrically by monitoring the reduction of 5-5’-Dithio-bis(2-nitro-benzoic acid) (DTNB) at 412 nm. Values were normalized to protein content assayed by the Bradford method (Bio-Rad protein assay kit, Hercules, CA).
WESTERN BLOT ANALYSIS

Cell lysate proteins were separated by 10% or 12.5% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Protein amounts were quantitated by the method of Bradford (Bio-Rad), and equivalent amounts of protein were loaded. Uniform protein loading was verified by Ponceau S (0.1% Ponceau S w/v in 5% acetic acid v/v) or Amido Black (0.1% Napthol Blue w/v in 7% acetic acid v/v) staining of the membranes, which showed no significant differences in protein levels on the blots among samples. The membranes were blocked with 5% nonfat milk/TBS-T solution for 1 h at room temperature, and then incubated with primary antibody overnight at 4°C or for 3 h at room temperature. Antibody incubation conditions are as follows: anti-heme oxygenase (HO-1; 1:1000; Stressgen, Ann Arbor, MI), anti-caspase 9 (1:1000; Cell Signaling), anti-voltage dependent anion channel (VDAC; 1:3000; Mitosciences), anti-β-actin (1:1000; Cell Signaling), anti-cytochrome c oxidase subunit I (1:1000; Mitosciences), anti-focal adhesion kinase (FAK; 1:1000, Cell Signaling) and H-Ras (1:1000; Novus Biologicals, Littleton, CO). After washing with TBS-T, membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. Membranes then were developed using SuperSignal West Dura chemiluminescence substrate (Pierce) and imaged using a CCD camera imaging system (Alphalnnotech San Leandro, CA).

MEASUREMENT OF MITOCHONDRIAL FUNCTION AND EXTRACELLULAR ACIDIFICATION USING EXTRACELLULAR FLUX TECHNOLOGY

To measure mitochondrial function in intact cells, a Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA) was used [95].
This instrument detects changes in oxygen levels and pH in the media surrounding adherent, cultured cells. This is achieved by creating a transient 7 μL microchamber in which small changes in oxygen and pH can be measured sensitively. The optimal seeding density of MDA-MB231 cells needed to obtain a measurable oxygen consumption and extracellular acidification rates (OCR and ECAR respectively) was established, and both OCR and ECAR show a linear response with respect to cell number (Figure 2-6). For subsequent experiments, a seeding density of 40,000 cells per well was selected to allow both potential increases and inhibition of OCR and ECAR to be assessed within the linear range of the instrument.

To assess cellular bioenergetics in intact MDA-MB231 cells, we utilized a previously described mitochondrial function protocol which is schematically represented in Figure 2-7A. After a baseline OCR is established (first 3 measurements), oligomycin, FCCP, and Antimycin A are injected sequentially. The OCRs determined after each injection are then used to calculate a number of the mitochondrial function parameters. The addition of oligomycin results in a decrease in OCR, and the difference between the basal OCR and oligomycin-inhibited OCR can be ascribed to the mitochondrial activity utilized for ATP synthesis. The remaining OCR can be ascribed to all processes which allow ion movement across the mitochondrial inner membrane and is collectively termed proton leak. Next, the maximal mitochondrial function that can be sustained in the cells with endogenous substrates is assessed by the addition of the proton ionophore, FCCP. As expected, this stimulates oxygen consumption, as OCR is no longer constrained by the proton gradient in the mitochondria. The difference between the basal OCR and this maximal rate is termed the reserve capacity of the mitochondrial function in cells under these
Figure 2-6: Optimization of MDA-MB231 cells for use in extracellular flux analysis. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) from MDA-MB231 cells. Cells were seeded at increasing densities (20,000-60,000) in specialized microplates, and the basal OCR (A) and ECAR (B) were measured. Seahorse XF24 Analyzer protocol included 2 min mixing, 2 min waiting, and 3 min measurement times for each measurement. Values represent means ± s.e.m., n = 3-4.
Figure 2-7: Assessment of mitochondrial function using extracellular flux analysis. A schematic diagram demonstrating the use of specific inhibitors to determine the sites of cellular oxygen consumption is shown (A). Sequential injections of oligomycin, FCCP, and Antimycin A are used to define the mitochondrial function parameters of basal OCR (“Basal”), ATP-linked OCR (“ATP”), processes which allow for ions movement across the inner mitochondrial membrane collectively termed proton leak (“Proton”), maximal respiration (“Maximal”), reserve respiratory capacity (“Reserve”), and OCR consumption independent of Complex IV (“Other”) for MDA-MB231 cells are shown (B). Extracellular acidification (ECAR) is measured concomitantly (C).
conditions. Finally, the complex III inhibitor, Antimycin A, is added to assess the oxygen consumption at cytochrome c oxidase, the terminal member of the electron transport chain. The small remaining OCR can be ascribed to oxygen consumption which occurs independently of Complex IV and likely represents partial reduction of oxygen resulting in reactive oxygen species (ROS) formation.

Using this assay, substantial insight can be made into the bioenergetic profile of intact cells. A single assay allows for the calculation of six mitochondrial parameters 1) Basal OCR, 2) Maximal OCR, 3) oxygen consumption linked to ATP production (“ATP”), 4) oxygen consumption due to ion movement across the mitochondrial inner membrane, i.e. Proton Leak (“Proton”), 5) the reserve respiratory capacity (“Reserve”), and 6) oxygen consumption which occurs independently of Complex IV (“Other”) (Figure 2-7B). ECAR is measured concomitantly and is an indicator of glycolytic flux since glycolysis results in lactate formation and excretion leading to acidification of the unbuffered media surrounding the cells (Figure 2-7C). All values are normalized to protein levels assayed at the completion of the XF24 protocol using the Bradford method (Bio-Rad).

We have noted that high levels of FCCP inhibit mitochondrial respiration presumably due to the loss of the ability to accumulate respiratory substrates. Accordingly, oligomycin and FCCP concentrations which elicit maximal effects were optimized (Figure 2-8). Concentrations of 0.3 μg/mL oligomycin and 1 μM FCCP were used for all additional experiments in MDA-MB231 cells. Also of note, for lipid exposures in Seahorse culture plates, 250 μL media was used, and a 10 μM addition of lipid was approximately
Figure 2-8: Concentration-dependent effects of oligomycin and FCCP on OCR. MDA-MB231 cells were seeded (40,000 cells/well) in specialized microplates. Basal OCR and OCR after the injection of increasing concentrations of oligomycin (A) or FCCP (B) were measured. Seahorse XF24 Analyzer protocol included 2 min mixing, 2 min waiting, and 3 min measurement times for each measurement. Values represent means ± s.e.m., n = 3-4, * p < 0.05 compared to vehicle control (EtOH), ** p < 0.01 compared to vehicle control (EtOH).
125 pmols lipid/µg protein, a similar amount of lipid per microgram protein when compared to exposure in 6-well cluster plates.

**MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL (ΔΨ)**

The mitochondrial inner membrane electrochemical potential (Δψ) was assessed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen). JC-1 accumulates and aggregates in mitochondria based on ΔΨ and fluoresces in the red range. If ΔΨ is lower, JC-1 does not accumulate and aggregate, and the monomers fluoresce in the green range. For this assay, cells grown in 12-well culture plates were treated as described in the figure legends, and JC-1 (7.4 µM) was added directly to MDA-MB231 cells in culture medium and incubated for 30 min. Next, cells were washed with PBS, and red/green fluorescence was measured using a fluorescence plate reader. Data are expressed as the ratio of red to green fluorescence where a lower ratio represents mitochondrial membrane depolarization.

**EXPERIMENTAL METASTASIS MODEL**

The effect of 15d-PGJ2 on metastasis in vivo was assessed using an experimental metastasis assay as described previously [96]. Nine to ten mice per experimental group were used, and 4 experimental groups were examined: untreated control, vehicle control, 15d-PGJ2, and 15d-PGJ2 reversed. GFP-MB231 cells were pretreated with 15d-PGJ2 (1 µM, 4 h; “15d-PGJ2” group) or vehicle control (0.1% EtOH, 4 h; “vehicle control” group) prior to being lifted from tissue culture plates, and a cell suspension was prepared for injection. A second set of cells were treated with 15d-PGJ2 (1 µM, 4 h) then treatment me-
dia was replace for complete media for an additional 12 h (“15d-PGJ2 reversed” group) to allow for reversal of 15d-PGJ2-induced alterations in the F-actin cytoskeleton and focal adhesions (data not shown). Cell suspensions were prepared in Hank’s Buffered Salt Solution for injection. Five hundred thousand cells in 0.2 mL were injected into the lateral tail vein of each animal. A treatment scheme is shown in Figure 2-9. After 6 weeks, metastasis to the lung was assessed by scoring the number of macroscopic lesions per lung. Animals which had fewer than 40 metastases per lung were eliminated from the analysis. Animals were maintained under the guidelines of the National Institutes of Health and the University of Alabama at Birmingham. The protocol was approved by the institutional Animal Care and Use Committee (conducted under Dr. Danny Welch’s protocol). Food and water were provided ad libitum.

DETECTION OF MODIFICATION OF KEAP1 AND β-ACTIN BY BT-15d-PGJ2

Modification of Kelch-like ECH-associated protein 1 (Keap1) and actin by bt-15d-PGJ2 was assessed using affinity purification techniques using neutravidin beads. Pulldown protocols were optimized for detection of each protein of interest.

Detection of bt-15d-PGJ2 modified Keap1

To determine the extent of modification of Keap1 by 15d-PGJ2, JC cells were treated with increasing concentrations (0.3-20 μM) of bt-15d-PGJ2 for 4 h. After the treatment, cell lysates were prepared in 1% Triton X-100 in Tris-HCl (10 mM) lysis buffer. Biotinylated proteins were affinity precipitated using 100 μL of a 50% slurry of Neutravidin beads (Pierce) which were pre-washed with 20 mM Tris-HCl (pH 7.4, 6
Figure 2-9: Treatment scheme for *in vivo* experimental metastasis experiment. GFP-MB231 cells were used to examine the effect of 15d-PGJ$_2$ on experimental metastasis. Cells were treated *ex vivo* prior to injection into athymic female mice. Untreated cells were used as control (A). Vehicle control cells were treated with EtOH (0.1%) for 4 h prior to injection (B). For 15d-PGJ$_2$ exposure, cells were treated for 4 h (1 μM) prior to injection (C). For the last treatment group, cells were exposed to 15d-PGJ$_2$ for 4 h (1 μM), and then treatment media was replaced with complete media for an additional 12 h to allow for time for the reversal of effects of 15d-PGJ$_2$ (D).
times). Cell lysates (6 mg protein) containing protease inhibitor cocktail were added to the beads and incubated for 3 h at room temperature with rotation. Beads were then washed with 600 µl 0.1 M glycine (pH 2.8, 6 times) followed by 600 µl 20 mM Tris Base (pH 10, 6 times) and then 600 µl 20 mM Tris-HCl (pH 7.4) to neutralize the beads. Samples were then prepared for analysis by heating the beads to 80°C for 10 min in 80 µl of 5x sample buffer (0.5 M Tris, 20% SDS, 50% Glycerol, 1% Bromophenol blue, pH 6.8) containing β-mercaptoethanol to release the biotin labeled proteins. Samples were centrifuged at 12,500 rpm for 10 min at 4°C, and supernatants used for analysis. Proteins were separated by SDS-PAGE, and transferred to nitrocellulose membranes at 100V for 2 h and membranes were then blocked with 5% skim milk in TBS-T. After blocking, membranes were incubated with a polyclonal primary antibody against Keap1 (E-20 1:1000 dilution in 5% skim milk in TBS-T; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by a HRP-conjugated donkey anti-goat secondary antibody (Santa Cruz; 1:1000 dilution in 5% skim milk in TBS-T). Membranes were developed by chemiluminescence using SuperSignal West Dura substrate and sequential images taken with quantitation only performed on bands which had not reached saturation using an AlphaInnotech imager.

Detection of bt-15d-PGJ\textsubscript{2} modified β-actin

To determine the modification of β-actin by 15d-PGJ\textsubscript{2}, JC cells were treated as described above. After treatment, cell lysates were prepared in N-Ethylmaleimide (NEM; Pierce) containing lysis buffer (10 mM NEM, 1% Triton X-100 in PBS) for 1 h in order to alkylate sulfhydryls and prevent auto-oxidation during the sample processing. β-Mercaptoethanol was added to quench the excess NEM. Cell lysates were incubated with
sodium borohydride (10 mM NaBH₄ in 5 mM NaOH) overnight to reduce the carbonyl group on the pentene ring in order to stabilize the lipid adducts on proteins. Biotinylated proteins were affinity precipitated using 100 µL of a 50% slurry of Neutravidin beads (Pierce, Rockford, IL) which were pre-washed with 20 mM Tris-HCl (pH 7.4, 6 times). Cell lysates (3 mg protein) containing protease inhibitor cocktail were added to the beads and incubated for 3 h at room temperature with rotation. Proteins were separated by SDS-PAGE, and transferred to nitrocellulose membrane at 100V for 2 h and membranes blocked with 5% skim milk in TBS-T. Membranes were incubated with a polyclonal primary antibody against β-actin (Cell Signaling; 1:1000 dilution in 5% skim milk in TBS-T), followed by a HRP-conjugated donkey anti-rabbit secondary antibody (Amer-sham; 1:1000 dilution in 5% skim milk in TBS-T). Membranes then were developed using SuperSignal West Dura chemiluminescence substrate (Pierce) and imaged using an Alphalnnotech imager.

MITOCHONDRIAL FRACTIONATION AND PROTEIN ADDUCT DETECTION

To determine intracellular localization of stable lipid-protein adducts, a cell fractionation protocol was utilized. Following experimental incubations, MDA-MB231 cells were harvested by scraping in ice-cold HMIM buffer (300 mM sucrose, 20 mM Tris-HCl, 2 mM EGTA, pH 7.35 at 4ºC) containing protease inhibitors and PMSF (1 mM). Cell suspensions were then homogenized used 18 passes with a dounce homogenizer powered by a drill press [97]. Intact cells and nuclei were removed by centrifugation at 1,000 x g for 10 min at 4ºC. The supernatant containing the mitochondria was then centrifuged at 15,000 x g for 10 min at 4ºC. The supernatant from this spin was removed and aliquoted
as the cytosolic fraction. The mitochondrial pellet was washed with 1 mL HMIM buffer and centrifuged again at 15,000 X \( g \) for 10 min at 4°C. The mitochondrial pellet was re-suspended in 40 \( \mu \)L HMIM buffer and protein measurements for all fractions were measured using the Lowry method. Validation of the fractionation protocol used demonstrates substantial enrichment of mitochondria as evidenced by the enrichment of the mitochondrial proteins VDAC and cytochrome \( c \) oxidase subunit I in the mitochondrial fraction compared to the cytosolic fraction (Figure 2-10A,B). Citrate synthase activities were also measured using standard spectrophotometric methods as previously described \[98, 99\]. Citrate synthase activity was enriched in the mitochondrial fraction (Figure 10-9B).

For detection of bt-15d-PGJ\(_2\) and mito-15d-PGJ\(_2\) adducts in fractionated samples, after blocking for 1 h, the membranes were probed using an anti-TPP\(^+\) antibody (1:10,000; received from Dr. Michael P. Murphy) and streptavidin conjugated to HRP (1:10,000; GE Healthcare, Piscataway, NJ) in TBS-T then developed using ECL\(^+\) chemifluorescence detection and imaged using a Typhoon fluorescence detector (GE Healthcare).

2D PROTEOMIC ANALYSIS OF MODIFIED PROTEOMES

Following experimental incubations, MDA-MB231 lysates were analyzed by two dimensional isoelectric focusing-SDS-PAGE. Total cell lysates (50 \( \mu \)g) were separated in the first dimension on a pH 3-10 gradient (IPG strips, Bio-Rad) followed by resolution on 12.5% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose membranes as described previously \[55\]. After blocking for 1 h, the membranes were probed as described above for detection of bt-15d-PGJ\(_2\) and mito-15d-PGJ\(_2\) adducts, respectively.
Figure 2-10: Confirmation of mitochondrial fractionation. Mitochondrial fractions from MDA-MB231 cells were prepared as described in Methods. VDAC and COX subunit I protein levels were determined in homogenate, mitochondrial, and cytosolic fractions using Western blot analysis (A) and quantification of Western blots and the activity of the mitochondrial matrix enzyme citrate synthase are expressed as fold enrichment over homogenate (B). Representative Western blot images are shown. Values represent means ± s.e.m., n = 3. ** p < 0.01 compared to homogenate.
Membranes were developed as described above using an Alphalnnotech imaging camera, and total protein was detected using Deep Purple stain (GE Healthcare).

**KNOWLEDGE OF H-RAS USING RNA-SILENCING**

A siRNA approach was used to decrease protein levels of our candidate protein target of 15d-PGJ2, H-Ras. All siRNA constructs used in this study are commercially available and were purchased from Qiagen (Valencia, CA). JC cells were treated with siRNA constructs directed against murine H-Ras or a murine non-silencing control construct (nsRNA) complexed with Lipofectamine 2000 at a final concentration of 20 μM in OptiMEM media (Gibco, Carlsbad, CA). Cells were transfected for 8 h, and then transfection media was removed and replaced with complete RPMI media for an additional 40 h prior to any experimental manipulation. H-Ras knockdown using this method was confirmed by Western blot analysis for H-Ras protein (described in *Western blot analysis*).

**STATISTICAL ANALYSIS**

Data reported are means ± s.e.m. for n ≥ 3, as indicated in figure legends. Statistical significance was evaluated by Student’s t-test or analysis of variance (ANOVA) among the groups using GraphPad Prism 4. The minimum level of significance was set at p < 0.05. Bonferroni’s or Tukey’s Multiple Comparisons test was used for post-hoc analysis of significance between groups.
CHAPTER 3
MITOCHONDRIAL TARGETING OF 15d-PGJ2 INCREASES APOPTOTIC EFFICACY VIA REDOX SIGNALING MECHANISMS

INTRODUCTION

Electrophilic compounds have attracted great interest as potential therapeutic agents in several fields including cancer, cardiovascular disease, and neurodegeneration [42, 67, 68]. As discussed previously in Chapter 1, their primary mechanism of action occurs through their ability to covalently modify key proteins in redox cell signaling pathways [37, 71, 100]. In the therapeutic context, electrophiles are particularly interesting because they are innately pleiotropic in nature since they can modify multiple redox signaling pathways simultaneously. This property of electrophiles underpins their potential as therapeutics; however, the major limitation has been the inability to selectively target the specific redox signaling pathways which regulate the beneficial effects of electrophiles. In this chapter, we will investigate the potential of using organelle specific targeting of an electrophile to alter the biological responses in cancer.

Electrophiles can be derived from several sources including diet, environment, or endogenously through enzymatic or non-specific lipid peroxidation processes [10, 21]. One of the best characterized electrophilic lipids generated enzymatically through cyclooxygenase (COX) is 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂). This electrophilic lipid has been proposed as both a potential anti-cancer and anti-inflammatory agent because of its ability to regulate redox-sensitive aspects of angiogenesis, growth arrest, expression of
inflammatory cytokines and cell death through the covalent modification of proteins [67, 69, 70, 101]. Conversely, an emerging literature suggests that there are a number of cancer promoting effects of 15d-PGJ$_2$ which include inactivation of tumor suppressors, stimulation of proliferation, and induction of cytoprotective intracellular antioxidants [74, 75, 77].

Evidence clearly supports the fact that protein targets of 15d-PGJ$_2$ in the electrophile responsive proteome are found in different compartments in the cell including the cytosol and mitochondria, and this is consistent with reports that 15d-PGJ$_2$ acts by pleiotropic mechanisms [69, 71]. In work pioneered by Murphy et al., a novel strategy for intracellular targeting of compounds to the mitochondrion has been developed. The conjugation of a delocalized, lipophilic cation to a compound of interest directs its accumulation within the mitochondrion which is sustained by the mitochondrial membrane potential [84, 85]. Based on these previous studies suggesting that electrophilic modification of cytosolic targets are protective [55] while modification of mitochondrial proteins may regulate apoptosis [71], we hypothesized that targeting an electrophile to the mitochondria would enhance the apoptotic effects of this compound while limiting the induction of intracellular antioxidants.

To test this hypothesis, we synthesized a novel mitochondrially-targeted analog of 15d-PGJ$_2$ (mito-15d-PGJ$_2$; shown schematically in Figure 3-1) and examined its effects on apoptotic cell death and induction of intracellular antioxidants in comparison to 15d-PGJ$_2$ in MDA-MB231 human breast cancer cells. We found that mito-15d-PGJ$_2$ was more potent at initiating intrinsic apoptosis than 15d-PGJ$_2$. Additionally, mito-15d-PGJ$_2$ was less effective at upregulating Kelch-like ECH-associated protein 1 (Keap1)-
Figure 3-1: Mitochondrial targeting of 15d-PGJ$_2$. 15d-PGJ$_2$ was conjugated to a triphenyl phosphonium (TPP$^+$) moiety to direct its accumulation within mitochondria. Based on the properties of TPP$^+$, it is expected that TPP$^+$ conjugated 15d-PGJ$_2$ (mito-15d-PGJ$_2$) will accumulate first into the cytosol based on the plasma membrane potential (5-10x) and then into the mitochondrial matrix approximately 100-500x relative to the amount added for treatment.
dependent antioxidant expression (heme oxygenase-1 and glutathione; HO-1 and GSH respectively) and caused profound defects in mitochondrial bioenergetics and mitochondrial membrane depolarization when compared to 15d-PGJ2. A mitochondrially-targeted analog of the non-electrophilic lipid prostaglandin E2 (mito-PGE2) and the targeting moiety methyltriphenylphosphonium (TPMP) did not recapitulate these effects. Taken together, these results demonstrate for the first time the feasibility of activating specific redox signaling pathways with an electrophile by selectively targeting specific compartments within the cell.

RESULTS

**Cytotoxicity of 15d-PGJ2 and mito-15d-PGJ2**

It is well established that lipid electrophiles at low concentrations induce phase II cytoprotective enzymes but at higher levels initiate cell death [54]. For example, the parent compound in these studies, 15d-PGJ2, has been shown to induce apoptotic cell death in a number of cancer cell lines at doses ranging from 5-50 μM [102-104]. To determine the relative cytotoxicity of mito-15d-PGJ2, MDA-MB231 cells were treated with increasing concentrations (0.3-30 μM) of either mito-15d-PGJ2 or 15d-PGJ2 for 16 h and cytotoxicity was assessed using flow cytometric analysis after staining with propidium iodide (PI) and Annexin V. Mito-15d-PGJ2 treatment resulted in a greater decrease in viable cells (those that are not positive for either Annexin V or PI) at lower concentrations when compared to 15d-PGJ2 (Figure 3-2A). A very small percentage of cells underwent early or late apoptosis after exposure to 15d-PGJ2 whereas predominately early and late apoptosis were observed with exposure to mito-15d-PGJ2 (Figure 3-2B,C). In contrast, 15d-
Figure 3-2: Effect of 15d-PGJ$_2$ and mito-15d-PGJ$_2$ on cell viability. Viability of MDA-MB231 cells after exposure to 15d-PGJ$_2$ or mito-15d-PGJ$_2$ (0.3-30 μM, 16 h) was determined by flow cytometry using PI and Annexin V staining. The percentage of cells which are viable (PI negative and Annexin V negative, B), early apoptotic (PI negative and Annexin V positive, C), late apoptotic (PI positive and Annexin V positive, D), and necrotic (PI positive and Annexin V negative is shown. Ethanol (EtOH) was used as a vehicle control. Results represent means ± s.e.m., n = 3. * p < 0.05 compared to EtOH vehicle control. ** p < 0.01 compared to EtOH vehicle control. # p < 0.05 compared to 15d-PGJ$_2$. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society. http://www.biochemj.org
PGJ₂ induced predominantly necrotic cell death at 30 μM whereas this represented only 3-10% of the cells treated with mito-15d-PGJ₂ across this dose range (Figure 3-2D).

As a further confirmation that mito-15d-PGJ₂ causes apoptotic cell death, MDA-MB231 cells were treated with 10 μM 15d-PGJ₂ or mito-15d-PGJ₂ for 4 h, then cell lysates were probed for procaspase 9, a protein which is cleaved upon activation of the intrinsic (mitochondrial) apoptotic signaling. Staurosporine (1 μM) was used as a positive control for caspase activation and resulted in partial cleavage, or activation, of procaspase 9. Treatment with 15d-PGJ₂ also resulted in a partial cleavage of procaspase 9 while mito-15d-PGJ₂ caused complete cleavage of this apoptotic precursor (Figure 3-3). Mito-PGE₂ and TPMP were used to ensure that these effects could not be attributed to either the triphenylphosphonium moiety or the lipophilic prostanoid structure, and neither compound has an effect on the levels of procaspase 9. This result suggests that the proapoptotic effect of mito-15d-PGJ₂ is due to the electrophilic nature of the compound. Taken together these data show that mito-15d-PGJ₂ induces cell death primarily through apoptosis more robustly than 15d-PGJ₂, and is consistent with the hypothesis that mitochondrial-targeting biases the mechanism of cell death towards apoptosis over necrosis.

**Induction of the intracellular antioxidants by 15d-PGJ₂ and mito-15d-PGJ₂**

It has been shown that the modification of critical thiols in the cytosolic protein Keap1 by 15d-PGJ₂ results in translocation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) to the nucleus and leads to subsequent transcription of genes under the control of the Electrophile Response Element (EpRE) [55, 105]. Such genes include HO-1 and subunits of glutamyl cysteine ligase (GCL) which control the
Figure 3-3: Effect of 15d-PGJ₂ and mito-15d-PGJ₂ on caspase 9 activation. Caspase 9 activation in MDA-MB231 cells treated with 10 µM 15d-PGJ₂, mito-15d-PGJ₂, mito-PGE₂, or TPMP was assessed using Western blot analysis of Procaspase 9 and quantified. Staurosporine (1 µM) was used as a positive control. Ethanol (EtOH) was used as a vehicle control. Equivalent amounts of protein were loaded for each sample and equal protein loading was confirmed after transfer to nitrocellulose membranes using Ponceau S staining. A representative Western blot is shown. Results represent means ± s.e.m., n = 3. * p < 0.05 compared to EtOH vehicle control. ** p < 0.01 compared to EtOH vehicle control. # p < 0.05 compared to 15d-PGJ₂. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society. http://www.biochemj.org
production of GSH [106]. Accordingly, we next investigated the effect of 15d-PGJ2 and mito-15d-PGJ2 on the induction of HO-1 and GSH. MDA-MB231 cells were treated with increasing concentrations (0.3-10 μM) of 15d-PGJ2 or mito-15d-PGJ2 for 4 h and cell lysates were analyzed for HO-1 protein levels. 15d-PGJ2 caused a dose-dependent increase in HO-1 protein from 0.3-3 μM and a slight decrease at higher concentrations (Figure 3-4A) similar to the biphasic dose response we have previously described [54]. However, mito-15d-PGJ2 induced HO-1 to a lesser extent. TPMP (10 μM) was used as a control for the effect of the triphenylphosphonium moiety and had no effect on HO-1 levels.

As an additional readout of the effect of these electrophiles on the Keap1/Nrf2 system, GSH was also measured by treating MDA-MB231 cells (4 h) with increasing concentrations (0.3-10 μM) of 15d-PGJ2 or mito-15d-PGJ2. Compound containing media was removed to attenuate cytotoxicity, and cells were incubated for 16 h to allow time for the \textit{de novo} synthesis of GSH. Similar to the result with HO-1, 15d-PGJ2 treatment resulted in a significant increase in total GSH levels; however, mito-15d-PGJ2 was unable to induce GSH (Figure 3-4B).

**Nrf2 dependence of the regulation of antioxidant expression and cell death**

We have previously reported that the induction of HO-1 by 15d-PGJ2 can be inhibited by modification of mitochondrial protein thiols [54]. To test the possibility that mito-15d-PGJ2 is functioning through a similar mechanism, cells were pretreated with mito-15d-PGJ2 for 4 h then 15d-PGJ2 was added to stimulate HO-1 and GSH levels. Mito-15d-PGJ2 does not prevent 15d-PGJ2 induction of HO-1 indicating that it is not inhibiting the mitochondrial component required for HO-1 synthesis (Figure 3-5A). Interestingly, in the
Figure 3-4: Differential induction of HO-1 and GSH by 15d-PGJ2 and mito-15d-PGJ2. MDA-MB231 cells were treated with increasing concentrations (0.3-10 μM) of 15d-PGJ2 and mito-15d-PGJ2. Cells were treated for 4 h then HO-1 protein levels were determined using Western blot analysis and quantified (A). Ethanol (EtOH) was used a vehicle control. Equivalent amounts of protein were loaded for each sample and equal protein loading was confirmed after transfer to nitrocellulose membranes using Ponceau S staining. A representative Western blot is shown. Cells were treated for 4 h then compound containing media was removed and replaced with 0.5% FBS RPMI 1640 for an additional 16 h. Cell lysates were then analyzed for total GSH content and values were normalized to protein (B). Results represent means ± s.e.m., n = 3. ** p < 0.01 compared to EtOH vehicle control. # p < 0.05 compared to 15d-PGJ2.

combined treatment group, mito-15d-PGJ₂ pretreatment enhances HO-1 induction by 15d-PGJ₂. These data are consistent with the known ability of HO-1 to be induced by a number of mechanisms some of which appear to be independent of Keap1/Nrf2 [107]. In contrast, the induction of GSH appears to require Nrf2 and consistent with this finding, pretreatment with mito-15d-PGJ₂ does not prevent 15d-PGJ₂-dependent induction of GSH (Figure 3-5B).

It has been established that activation of the Keap1/Nrf2 system by low levels of electrophilic stress results in increased resistance to cytotoxic levels of reactive oxygen and nitrogen species. It is then possible that the failure of mito-15d-PGJ₂ to activate the EpRE may contribute to its cytotoxicity. To test this, two different experimental strategies were employed. Firstly, MDA-MB231 cells were co-incubated with a cell death-inducing concentration of mito-15d-PGJ₂ (10 μM) and a concentration of 15d-PGJ₂ which increases antioxidant expression (3 μM). After 16 h treatment, cell death was assessed by LDH release and was unchanged by co-treatment with 15d-PGJ₂ (Figure 3-6A). This is consistent with the early induction of the apoptotic process before significant induction of phase II cytoprotective enzymes can occur. Secondly, wild-type and Nrf2⁻/⁻ murine embryonic fibroblasts were treated with 10 μM 15d-PGJ₂ or mito-15d-PGJ₂ and cell death was assessed. If the cytotoxicity is attenuated by activation of the Keap1/Nrf2 system, we would expect Nrf2 null cells to be sensitized to the electrophiles; however, we found that cytotoxicity of 15d-PGJ₂ and mito-15d-PGJ₂ is independent of Nrf2 levels in the cell (Figure 3-6B).
Figure 3-5: Effect of mito-15d-PGJ$_2$ on 15d-PGJ$_2$-induced increases in HO-1 and glutathione levels. MDA-MB231 cells were pretreated with 1 μM mito-15d-PGJ$_2$ for 4 h then 1 μM 15d-PGJ$_2$ was added for an additional 4 h. HO-1 protein levels were determined using Western blot analysis and quantified (A). Ethanol (EtOH) was used a vehicle control. Equivalent amounts of protein were loaded for each sample and equal protein loading was confirmed after transfer to nitrocellulose membranes using Ponceau S staining. A representative Western blot is shown. Cells were treated as described above then compound containing media was removed and 0.5% FBS RPMI 1640 was added for an additional 16 h. Cell lysates were then analyzed for total GSH content and values were normalized to protein (B). Results represent means ± s.e.m., n = 3-6. * p < 0.05 compared to EtOH vehicle control. ** p < 0.01 compared to EtOH vehicle control. # p < 0.05 compared to 15d-PGJ$_2$. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society. [http://www.biochemj.org](http://www.biochemj.org)
Figure 3-6: Nrf2-dependence of the regulation of cell death by mito-15d-PGJ₂.
Viability of MDA-MB231 cells incubated alone or in combination with 10 μM mito-
15d-PGJ₂ and 3 μM 15d-PGJ₂ for 16 h was determined by LDH release (A). Nrf2
wild-type and null murine embryonic fibroblast were treated with 15d-PGJ₂ or mito-
15d-PGJ₂ (3 and 10 μM) for 16 h then LDH release was measured (B). Ethanol was
used as a vehicle control. Results represent means ± s.e.m., n = 3-6. * p < 0.05
compared to EtOH vehicle control. ** p < 0.01 compared to EtOH vehicle control. # p
< 0.05 compared to 15d-PGJ₂. Reproduced with permission from Diers AR, Higdon
AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar
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Protein modification by bt-15d-PGJ2 and mito-15d-PGJ2 differ

The data thus far demonstrating the different biological properties of 15d-PGJ2 and mito-15d-PGJ2 suggest that the proteins modified by the electrophilic lipids will also differ. To test this, MDA-MB231 cells were incubated with biotin tagged 15d-PGJ2 (bt-15d-PGJ2) or mito-15d-PGJ2 (10 μM) for 1 h, and the protein lysates separated by 2D-IEF-SDS-PAGE followed by western blotting and detection for protein-lipid adducts using the triphenylphosphonium (TPP⁺) antibody or streptavidin conjugated to horseradish peroxidase (HRP). The patterns of protein stains for all treatments showed no significant differences (result not shown). Control cells not treated with electrophilic lipid showed no major positive reactions with the exception of one spot on the biotin blot (Figure 3-7) which we ascribe to an endogenous biotin containing carboxylase [14]. Figure 3-8 shows detection of approximately 63 proteins positive for biotin (panel A) and approximately 32 proteins positive for TPP⁺ (Panel B). The images were selected to minimize differences in potential sensitivity between the two detection techniques by selecting images with the same intensity for a common spot (indicated by an arrow on Figure 3-8) The overlay of these two images was achieved using the PDQuest proteomics software (Figure 3-9A) and shows red spots that are predominantly reactive with bt-15d-PGJ2 and green spots with mito-15d-PGJ2, and the proteins that react with both lipids are yellow and some are indicated with arrows. In Figure 3-9B, these data are summarized and indicate that 10 proteins are common targets of both electrophilic lipids, with 22 unique to mito-15d-PGJ2 and 53 unique to 15d-PGJ2. It was not possible to unequivocally overlay the protein adduct maps with the protein stain to identify these targets since many of these proteins are present in the cell at low abundance [14].
Figure 3-7: Background reactivity of Streptavidin-HRP and TPP$^+$ antibody on 2D-IEF-SDS-PAGE. Cell lysate proteins from MDA-MB231 cells were resolved and transferred to nitrocellulose membranes. Vehicle control (EtOH, 1 h) treated cell lysates were probed using Streptavidin-HRP (A) and TPP$^+$ antibody with HRP-conjugated secondary antibody (B) to determine background reactivity. Representative Western blots are shown. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society. http://www.biochemj.org
Figure 3-8. Visualization of bt-15d-PGJ2 and mito-15d-PGJ2 modified proteins by 2D-IEF-SDS-PAGE. MDA-MB231 cells were treated (10 μM, 1 h) with bt-15d-PGJ2 (A) or mito-15d-PGJ2 (B) and protein adducts were detected using Streptavidin-HRP and TPP+ antibody respectively. Representative Western blots are shown. The images were selected to minimize differences in potential sensitivity between the two detection techniques by selecting images with the same intensity for a common spot indicated by the arrow. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society.

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Figure 3-9: Common and unique protein targets of \( \text{bt-15d-PGJ}_2 \) and \( \text{mito-15d-PGJ}_2 \). A merged image of the Western blots in Figure 3-8 is shown. \( \text{bt-15d-PGJ}_2 \) spots are red, \( \text{mito-15d-PGJ}_2 \) spots are green, and common spots are yellow (A). Arrows designate some of the common spots detected. Analysis of spot quantity was determined and common and unique spots from \( \text{bt-15d-PGJ}_2 \) and \( \text{mito-15d-PGJ}_2 \) membranes are shown schematically (B). Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society.

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**15d-PGJ2 and mito-15d-PGJ2 localize to mitochondria and affect mitochondrial membrane potential**

To assess the relative localization and formation of protein adducts by the lipid electrophiles, mitochondrial fractions were isolated from cells treated with bt-15d-PGJ2 or mito-15d-PGJ2. Validation of the fractionation protocol used demonstrates substantial enrichment of the mitochondrial proteins in the mitochondrial fraction compared to the cytosolic fraction (Methods, Figure 2-10).

Protein adduct formation was assessed in fractionated samples from bt-15d-PGJ2 and mito-15d-PGJ2 treated cells using 1D-SDS-PAGE. Consistent with our previous results, bt-15d-PGJ2 localizes to the mitochondria and forms stable protein adducts [78]. Mito-15d-PGJ2 protein adducts are also enriched in the mitochondrial fraction (Figure 3-10A). Mitochondrial purity was also confirmed by voltage dependent anion channel (VDAC) enrichment in the mitochondrial fraction. Protein adducts in each fraction were quantified and expressed as fold increase in protein adducts in the mitochondrial fraction compared with the homogenate (Figure 3-10B). These data demonstrate that there is an approximately 2-fold increase in bt-15d-PGJ2 protein adducts in the mitochondria compared to the homogenate. With targeting to the mitochondria using TPP+ conjugation, the enrichment of protein adducts in that fraction is further enhanced to approximately 3-fold.

We next investigated whether the modification of mitochondrial proteins by lipid electrophiles altered mitochondrial function by determining the effect of 15d-PGJ2 and mito-15d-PGJ2 on mitochondrial membrane potential (Δψ). MDA-MB231 cells were treated for 4 h with increasing concentrations (1-10 μM) of 15d-PGJ2 or mito-15d-PGJ2
Figure 3-10: Localization of 15d-PGJ$_2$ and mito-15d-PGJ$_2$ to the mitochondrion. MDA-MB231 cells were treated with 10 μM bt-15d-PGJ$_2$ (bt-15d) or mito-15d-PGJ$_2$ (Mito-15d) for 1 h then mitochondrial (Mito) and cytosolic fractions were prepared from total homogenate (Homog) and resolved using 10% SDS-PAGE. Adduct formation was detected using an anti-TPP$^+$ antibody (for mito-15d-PGJ$_2$) or streptavidin-HRP (for bt-15d-PGJ$_2$). ECL$^+$ coupled to horseradish peroxidase was used to detect chemiluminescence protein adducts (A). VDAC protein levels were also determined using Western blot analysis to confirm mitochondrial preparation purity. Protein adducts were quantified and expressed as a percent of homogenate (B). Ethanol (EtOH) was used as a vehicle control. Results represent means ± s.e.m., n = 3-6. * p < 0.05 compared to EtOH vehicle control. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society. http://www.biochemj.org
then JC-1 fluorescence was assessed. Treatment with 15d-PGJ2 resulted in a dose-dependent decrease in the mitochondrial membrane potential. However, mito-15d-PGJ2 caused a more significant depolarization of the mitochondrial membrane (Figure 3-11). TPMP was used as a control for the effect of the triphenylphosphonium moiety and did not cause mitochondrial membrane depolarization.

**Measurement of bioenergetic function after exposure to 15d-PGJ2 and mito-15d-PGJ2 using extracellular flux technology**

To assess cellular bioenergetics in intact MDA-MB231 cells, the Seahorse Bioscience XF24 extracellular flux analyzer was used to determine the rates of change in both pH and oxygen in the media surrounding the cells [95, 108]. We have utilized a mitochondrial function protocol which is discussed in the Methods which allows for the assessment of multiple mitochondrial bioenergetic parameters including 1) Basal oxygen consumption rate (OCR), 2) Maximal OCR, 3) oxygen consumption linked to ATP production (“ATP”), 4) oxygen consumption due to ion movement across the mitochondrial inner membrane, i.e. Proton Leak (“Proton”), 5) the reserve respiratory capacity (“Reserve”), and 6) oxygen consumption which occurs independently of Complex IV (“ROS”). Extracellular acidification rate (ECAR) which is measured concomitantly and is an indicator of glycolytic flux is also established.

Using this protocol, mitochondrial function was measured in confluent MDA-MB231 cells which were first exposed to 15d-PGJ2, mito-15d-PGJ2 or mito-PGE2 (10 μM, 30 min). The lipids were then removed and the media was replaced with unbuffered DMEM for 1 h prior to the measurement of mitochondrial parameters. There was no significant effect of 15d-PGJ2 or mito-PGE2 on basal or maximal respiration; however, pro-
Figure 3-11: Effect of 15d-PGJ\(_2\) and mito-15d-PGJ\(_2\) on mitochondrial membrane potential (\(\Delta\Psi\)). MDA-MB231 cells were exposed to increasing concentrations (1-10 \(\mu\text{M}\)) of 15d-PGJ\(_2\), mito-15d-PGJ\(_2\), or TPMP for 4 h, and then \(\Delta\Psi\) was assessed using JC-1. Ethanol was used as a vehicle control. Data are represented as the ratio of red/green fluorescence. Values represent means ± s.e.m., * \(p < 0.05\) compared to EtOH vehicle control. ** \(p < 0.01\) compared to EtOH vehicle control. # \(p < 0.05\) compared to 15d-PGJ\(_2\). Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society.

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found decreases in basal and maximal OCR were seen with mito-15d-PGJ2 treatment (Figure 3-12A,B respectively). The analysis of the different components of OCR were also calculated and shown (Figure 3-12C). It is clear that 15d-PGJ2 may have a slight effect on mitochondrial function, whereas mito-15d-PGJ2 substantially suppresses all bioenergetic parameters. It is important to note that at this time point apoptosis has not yet been activated (Figure 3-12D) demonstrating that these mitochondrial bioenergetic defects precede apoptosis. These data show that mito-15d-PGJ2 modifies mitochondrial function whereas 15d-PGJ2 has a minimal effect on any of the bioenergetic parameters assessed.

In addition to the measurement of OCR, the ECAR, a surrogate marker of glycolysis, was also determined (Figure 3-13). Treatment with either 15d-PGJ2 or mito-PGE2 resulted in no change in ECAR; however, after a 30 min exposure to mito-15d-PGJ2, ECAR is significantly increased. Taken together with the OCR results, these data demonstrate that compared to 15d-PGJ2, mito-15d-PGJ2 impairs mitochondrial function and increases glycolytic flux, and this may represent a compensatory mechanism to overcome lost mitochondrial function.

**SUMMARY**

It is clear that electrophiles modify signaling proteins in both the cytosol and mitochondrion which results in diverse cellular responses including cytoprotective effects and, at high doses, cell death. These findings led us to the hypothesis that targeting electrophiles to specific compartments in the cell can be used to fine-tune their biological effects. In this chapter, we have examined the feasibility of activating specific redox signal-
Figure 3-12: Effect of 15d-PGJ₂, mito-15d-PGJ₂, and mito-PGE₂ on mitochondrial function. MDA-MB231 cells were treated for 30 min with of 15d-PGJ₂, mito-15d-PGJ₂, or mito-PGE₂ (10 μM) in specialized microplates then bioenergetic function was assessed using the Seahorse XF24 Analyzer. Basal oxygen consumption rate (OCR; A) and maximal OCR (B) are shown. The proportion of maximal respiration used for ATP-linked respiration (ATP), proton leak (Proton), and ROS formation (ROS), and reserve respiratory capacity (Reserve) was calculated from OCR measured after sequential injections of oligomycin (0.3 μg/mL), FCCP (1 μM), and antimycin A (10 μM; C). Caspase 9 activation was assessed in samples harvested from a parallel plate treated identically using Western blot analysis of Procaspase 9 (D). Ethanol (EtOH) was used as a vehicle control. A representative Western blot is shown. Results represent means ± s.e.m., n = 5. ** p < 0.01 compared to EtOH vehicle control. # p < 0.05 compared to 15d-PGJ₂. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society.
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Figure 3-13: Effect of 15d-PGJ$_2$ and mito-15d-PGJ$_2$ on extracellular acidification. MDA-MB231 cells were treated for 30 min with 15d-PGJ$_2$, mito-15d-PGJ$_2$, or mito-PGE$_2$ (10 $\mu$M) in specialized microplates then extracellular acidification rate (ECAR) was assessed using the Seahorse XF24 Analyzer. Ethanol (EtOH) was used as a vehicle control. Results represent means ± s.e.m., n = 5. ** p < 0.01 compared to EtOH vehicle control. # p < 0.05 compared to 15d-PGJ$_2$. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society. http://www.biochemj.org
ing pathways using organelle-selective targeting of the electrophile 15d-PGJ₂. To this end, we synthesized a novel mitochondrially-targeted analog of 15d-PGJ₂ (mito-15d-PGJ₂) and tested its effects on redox cell signaling. Mito-15d-PGJ₂ caused profound defects in mitochondrial bioenergetics and mitochondrial membrane depolarization when compared to 15d-PGJ₂. We also found that mito-15d-PGJ₂ was more potent at initiating intrinsic apoptotic cell death and was less effective than 15d-PGJ₂ at upregulating the expression of heme oxygenase-1 and glutathione. Mito-15d-PGJ₂ and the untargeted analog as modified unique proteomes which may be the basis for the differential effects of these compounds. These data demonstrate the feasibility of modulating the biological effects of electrophiles by using mitochondrial targeting strategies.
CHAPTER 4

REDOX MODULATION OF MAMMARY CANCER MIGRATION BY 15d-PGJ₂

INTRODUCTION

Since 90% of all cancer related deaths are the result of metastasis [1], understanding the regulation of this complex process is important in developing new anti-metastatic treatment strategies. It is becoming clear that a number of steps in the metastatic cascade are regulated by redox signaling. Known downstream effects of modification of redox-sensitive signaling pathways include modulation of matrix metalloproteinase (MMP) expression, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) regulated gene expression, and mitochondrial reactive oxygen species (ROS) generation [62-65]; moreover, activity of these pathways has been shown to be directly linked to metastatic potential in multiple cancer types [62, 109-111]. Taken together, these studies suggest there are redox-sensitive signaling pathways controlling basic processes required for metastasis.

The focus of this work is the redox signaling molecule 15-deoxy-Δ¹²,₁⁴-prostaglandin J₂ (15d-PGJ₂) which modifies primarily cysteine residues through a Michael-type addition [37]. In the context of cancer, 15d-PGJ₂ has garnered much interest because of its ability to inhibit angiogenesis, cause growth arrest, and induce cell death in several cancer cells lines [38-41]. Interestingly, although 15d-PGJ₂ has been shown to be cytotoxic in cancer cells, little is known about its effects on metastasis.
Cellular migration plays an important role in metastasis, and 15d-PGJ2 has been shown to inhibit migration [112, 113]. There is also evidence demonstrating that 15d-PGJ2 alters cytoskeletal structure in multiple cell types including neuroblastoma and mesangial cells; however, these studies reported cytotoxicity associated with cytoskeletal alterations [114, 115]. The cytoskeletal effects of 15d-PGJ2 have been largely attributed to the direct modification of cytoskeletal components such as actin, vimentin, and tubulin [114, 115]. In this chapter, we investigate the effects of 15d-PGJ2 on the filamentous actin (F-actin) cytoskeleton at lower concentrations which do not cause cytotoxicity. The effect of 15d-PGJ2 on the cytoskeleton and migration may have important implications in the inhibition of metastatic processes such as invasion, intravasation, and extravasation.

The goals of this study were to determine the effects of non-toxic, low concentrations of 15d-PGJ2 on the regulation of cytoskeletal organization and its influence on cell migration as well as to determine the mechanism of action of 15d-PGJ2 at these low concentrations. We first investigated the effect of 15d-PGJ2 on cell viability, migration, and focal adhesion disassembly. In addition, we determined the effects of 15d-PGJ2 on F-actin cytoskeletal structure and examined the roles of direct actin adduction, peroxisome proliferator activated receptor gamma (PPARγ) activation, and redox signaling pathways in 15d-PGJ2 mediated cytoskeletal regulation. Our study is the first to demonstrate that 15d-PGJ2 can alter actin organization with minimal direct adduct formation with actin, and that this effect coincides with decreased migration and increased focal adhesion disassembly. These results suggest a role for redox signaling pathways, rather than direct cytoskeletal disruption, in the mechanism of action for 15d-PGJ2 in cancer cells.
RESULTS

15d-PGJ₂ toxicity in JC mouse mammary adenocarcinoma cells

15d-PGJ₂ has been shown to induce apoptotic cell death in a number of cancer cell lines at concentrations ranging from 5-50 μM [30, 102-104]. We also observed cytotoxicity in this concentration range in MDA-MB231 cells in the previous chapter. In order to determine non-toxic concentrations of 15d-PGJ₂, cytotoxicity was assessed using propidium iodide (PI) and Annexin V-FITC co-staining measured by flow cytometry. JC cells were treated with 15d-PGJ₂ (0.01-3 μM) for 16 h. By this method, we are able to distinguish apoptotic and necrotic cell death. As seen in Figure 4-1A, at concentrations ranging from 0.01 to 3 μM, there are no significant changes in viability. Furthermore, there is no indication of apoptotic or necrotic cell death in response to 15d-PGJ₂ treatment, as seen by the lack of cells staining positive for PI (necrosis), Annexin V-FITC (early apoptosis), or both (late apoptosis). In contrast, consistent with reports in the literature [30, 102-104], when JC cells are treated with higher concentrations (20 μM) of 15d-PGJ₂, there is a significant reduction in the number of viable cells as well as an increase in both late apoptotic and necrotic cell populations (Figure 4-1B).

15d-PGJ₂ toxicity was also assessed using the colony formation assay. This assay measures the replicative ability of cells to form colonies after treatment, an important characteristic of cancer cells. Interestingly, when JC cells were treated with 15d-PGJ₂, a marked decrease in colony formation was measured at concentrations as low as 0.01 μM (Figure 4-2). Taken together, these data suggest that treatment with low concentrations of 15d-PGJ₂ (0.01-3 μM) attenuates the clonogenic capacity of JC cells, but does not cause apoptosis or necrosis. Since re-adherence to tissue culture plates and proliferation are two
Figure 4-1: Effect of 15d-PGJ$_2$ on apoptotic and necrotic cell death. The viability of JC cells treated with increasing concentrations of 15d-PGJ$_2$ (0.01 - 3 μM) for 16 h was assessed using PI/Annexin V flow cytometry (A). 15d-PGJ$_2$ (20 μM) is shown as a positive control for cell death (B). Ethanol was used as a vehicle control. Values shown represent means ± s.e.m., n = 6-9. ** p < 0.01 compared to EtOH control, 2-way ANOVA.
Figure 4-2: Effect of 15d-PGJ2 on colony formation. The percentage of JC cells which were able to form colonies after treatment with increasing concentrations of 15d-PGJ2 (0.01 - 3 µM) for 16 h was assessed using the colony formation assay. Representative images of cloning wells (A) and quantification (B) are shown. Ethanol was used as a vehicle control. Values shown represent means ± s.e.m., n = 3-9. ** p < 0.01 compared to EtOH control, 1-way ANOVA.
critical steps for successful colony formation in this assay, these data also suggest that 15d-PGJ₂ may attenuate proliferation and/or adhesion pathways. Further investigation demonstrated that 15d-PGJ₂ does not cause alterations in cell cycle progression in this model; however, re-adherence of cells to tissue culture plastic after treatment with 15d-PGJ₂ and subsequent trypsinization is impaired (data not shown). Therefore, 15d-PGJ₂ does not cause cell death at concentrations at or below 3 μM, and the decreased colony formation caused by 15d-PGJ₂ appears to be due to a decreased ability of cells to re-adhere after plating, rather than decreased cell viability per se.

15d-PGJ₂ attenuates migration

Having established sub-lethal concentrations of 15d-PGJ₂, we next examined the effect of this electrophile on cell motility using a scratch assay. Treatment with 15d-PGJ₂ caused a concentration-dependent decrease in cell migration over 8 h with significant changes seen at concentrations of 15d-PGJ₂ equal or greater than 0.03 μM (Figure 4-3). These results demonstrate that low, non-toxic concentrations of 15d-PGJ₂ attenuate cancer cell migration.

Effects of 15d-PGJ₂ on focal adhesion kinase signaling

Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase whose expression has been shown to be frequently dysregulated in cancer (reviewed in [116]). FAK activation occurs primarily through integrin-mediated signal transduction. Downstream signaling events regulate multiple biological process including cell survival, proliferation, angiogenesis, and of particular interest in the context of this work, migration and
Figure 4-3: Effect of 15d-PGJ2 on cell migration. JC cell migration was assessed using a scratch assay. Cells were treated with 0.03 - 3 μM 15d-PGJ2, and cell migration into cell-free area was assessed after 8 h. Representative images of 0.3 μM 15d-PGJ2 treated wells (A) and quantification of dose response curve are shown (B). Ethanol was used as a vehicle control. Values represent means ± s.e.m., n = 3-12. ** p < 0.01 compared to EtOH control, 1-way ANOVA.
invasion (reviewed in [116]). Chen et al. previously demonstrated that 15d-PGJ2 treatment of thyroid carcinoma cells caused decreased levels of the focal adhesion proteins vinculin, integrin β1, FAK, and paxillin; however, these effects were observed at concentrations of 15d-PGJ2 which also caused cell death [117]. To investigate the potential role of FAK signaling in 15d-PGJ2-induced attenuation of migration, we treated JC cells with 0.3 μM 15d-PGJ2 for 30 min and 4 h, and then total FAK levels were determined. Treatment with this sub-lethal concentration of 15d-PGJ2 did not alter total FAK protein levels (Figure 4-4A).

We also investigated the activity of the FAK signaling pathway by examining levels of phosphorylated FAK (p-FAK) by immunofluorescence. It is well-established that activation of FAK results in the autophosphorylation of Tyr397 which reveals a binding site for Src family kinases and mediates many of the downstream signaling events [118]. FAK phosphorylation is significantly decreased to 45% of control (p ≤ 0.01) in cells treated with 15d-PGJ2 (0.3 μM) for 30 min (Figure 4-4B). Focal adhesions were also quantified after treatment with 15d-PGJ2. No difference in the number of cells scoring positive for focal adhesions was observed after treatment with 15d-PGJ2 for 30 min (data not shown); however, focal adhesion disassembly was evident after treatment for 4 h (Figure 4-5).

It is clear that 15d-PGJ2 can act through multiple mechanisms including redox signaling, PPARγ-dependant pathways, and the G protein-coupled receptor DP2 (reviewed in [119]). Rosiglitazone (ROSI), a PPARγ agonist, has previously been shown to alter focal adhesion signaling and impair migration [117]. Additionally, Powell [120] and Monneret [121, 122] et al. demonstrated that 15d-PGJ2 can bind to and activate the DP2
Figure 4-4: Effect of 15d-PGJ2 on focal adhesion kinase signaling. JC cells were treated with 15d-PGJ2 (0.3 μM) for 30 min or 4 h and total FAK protein levels were determined by Western blot analysis. A representative Western blot image is shown (A). Phosphorylated FAK (p-FAK) was assessed in cells treated with 15d-PGJ2 (0.3 μM, 30 min) using an anti-p-FAK antibody and a fluorophore-conjugated secondary antibody (green channel) using fluorescence confocal microscopy. Cells were co-stained with Alexa Fluor 633® Phalloidin and DAPI to visualize F-actin (red channel) and nuclei (blue channel), respectively. Representative images of merged red, green, and blue channel are shown. Total green fluorescence per field was quantified and normalized to number of cells, and values shown indicate p-FAK/cell (B). Ethanol (EtOH) was used as a vehicle control. Values represent means ± s.e.m., n = 9. ** p < 0.01 compared to vehicle control, 1-way ANOVA.
receptor on eosinophils. Though DP2 receptor expression seems to be limited to Type 2 helper T cells, cytotoxic T cells, eosinophils, and basophils in humans [123] while PPARγ is expressed more ubiquitously [124], we examined the effect of both DP2 and PPARγ agonists, 15(R)-Prostaglandin D₂ (15(R)-PGD₂) and Rosiglitazone, respectively.

Treatment with 15(R)-PGD₂ or Rosiglitazone also resulted in a decrease in the number of cells scoring positive for focal adhesions (Figure 4-5). Taken together, these results indicate that at sub-lethal concentrations, 15d-PGJ₂ can alter FAK mediated signaling not through changes in total FAK levels, but through decreased activation of the FAK signaling pathway. Additionally, 15d-PGJ₂ causes significant focal adhesion disassembly; however, this effect is not specific to 15d-PGJ₂ and can be recapitulated using PPARγ and DP2 agonists. Neither 15(R)-PGD₂ or Rosiglitazone treatment at concentrations 20-fold higher than the reported EC₅₀ for each compound [125, 126] altered cell motility (Figure 4-6).

**Labeling of 15d-PGJ₂ does not alter its biological action**

We have previously reported a series of tagged lipids including biotin tagged 15d-PGJ₂ (bt-15d-PGJ₂) and BODIPY tagged 15d-PGJ₂ (BD-15d-PGJ₂) that can be used in biotin pull-down techniques and fluorescence microscopy localization studies respectively [55, 71, 78]. In order to use these tagged lipids to monitor the subcellular location and protein adduct formation of 15d-PGJ₂, we first determined whether tagging the lipid with either biotin or BODIPY would change its biological action in JC cells. The effect of bt-15d-PGJ₂ and BD-15d-PGJ₂ on migration in the scratch assay was assessed, and as shown in Figure 4-7A, there is no significant difference in the extent of inhibition of migration
Figure 4-5: Effect of 15d-PGJ$_2$ on focal adhesion disassembly. JC cells were treated with 15d-PGJ$_2$ (0.3 μM), 15(R)-PGD$_2$ (0.24 μM), Rosiglitazone (ROSI, 2 μM) or vehicle control for 4 h then fixed in 3% glutaraldehyde. Focal adhesions were quantified using interference reflection microscopy. Values represent the mean percent of cells scored positive for focal adhesions (A). Ethanol and DMSO were used as vehicle controls. Values represent means ± s.e.m., n = 9. ** p < 0.01 compared to vehicle control, 1-way ANOVA.
Figure 4-6: Effects of DP2 and PPARγ agonists on migration. JC cells were scratched with a sterile pipet tip then treated with 15(R)-PGD₂ (0.24 μM, A), ROSI (2 μM, B) or appropriate vehicle control. Cell migration into the cell-free area was assessed after 8 h. Ethanol and DMSO were used as vehicle controls. No significant difference (N.S.) was observed between vehicle control and agonist treated cells, Student’s t-test.
by either of the tagged lipids when compared to the untagged analog. Furthermore, the BODIPY fluorophore and the control non-electrophilic lipid, Prostaglandin E2 (PGE2), had no effect on migration. We also assessed the effect of the BD-15d-PGJ2 on clonogenic survival. BD-15d-PGJ2 (3 μM) decreased clonogenic survival to the same extent as the untagged analog (Figure 4-7B). These data demonstrate that the addition of a biotin or BODIPY tag to 15d-PGJ2 does not alter the biological responses to the electrophile.

15d-PGJ2 changes F-actin morphology

Since focal adhesions are the site at which the actin cytoskeleton is linked to the extracellular matrix [127], we investigated the effect of 15d-PGJ2 on the F-actin cytoskeleton using phalloidin. As seen in Figure 4-8, in untreated and vehicle control treated cells, the F-actin cytoskeleton exhibited a filamentous, elongated morphology. Exposure to BODIPY, the fluorophore conjugated to 15d-PGJ2 for visualization in these studies, did not alter the F-actin cytoskeleton. Interestingly, treatment with 15d-PGJ2 for 30 min caused extensive reorganization of the F-actin cytoskeleton. The effect of PPARγ and DP2 agonists on the F-actin cytoskeleton were also examined. Neither Rosiglitazone nor 15(R)-PGD2 had any effect on the F-actin cytoskeletal morphology (Figure 4-9) suggesting that the reorganization of the F-actin cytoskeleton in response to 15d-PGJ2 occurs through the direct modification of protein cysteine residues, and not through PPARγ or DP2-dependent pathways.
Figure 4-7: Comparison of tagged 15d-PGJ₂ analogs to 15d-PGJ₂. JC cells were scratched with a sterile pipet tip and then treated with 0.3 μM 15d-PGJ₂, bt-15d-PGJ₂, BD-15d-PGJ₂, BODIPY, and PGE₂ or EtOH (vehicle control). Cell migration into the cell-free area was assessed after 8 h (A). JC cells were also treated with 3 μM BODIPY, 15d-PGJ₂, BD-15d-PGJ₂ or EtOH. Clonogenic survival was assessed after a 16 h treatment (B). Ethanol (EtOH) was used as a vehicle control. Values represent means ± s.e.m., n ≥ 3. * p < 0.05, ** p < 0.01 compared to EtOH control, 1-way ANOVA. N.S. = No significant difference was observed between 15d-PGJ₂ and tagged analogs.
Figure 4-8: Effect of 15d-PGJ₂ on the F-actin cytoskeleton. JC cells were treated with BODIPY or BD-15d-PGJ₂ (0.24 μM) for 30 min and then fixed, permeabilized, and stained using Alexa Fluor 633® conjugated Phalloidin to visualize the F-actin cytoskeleton using fluorescence confocal microscopy. Cells were co-stained with DAPI to visualize nuclei. Ethanol (EtOH) was used as a vehicle control. Representative images of merged red (F-actin) and blue (nuclei) channels are shown from samples prepared in triplicate.
Figure 4-9: Effects of DP2 and PPARγ agonists on F-actin cytoskeletal arrangement. JC cells were treated with 15d-PGJ$_2$ (0.3 μM), 15(R)-PGD$_2$ (0.24 μM), Rosiglitazone (ROSI, 2 μM) or vehicle control for 30 min and then fixed, permeabilized and stained with 2 units of Texas Red Phalloidin to visualize F-actin (red channel). Nuclei were visualized with DAPI (blue channel). Ethanol and DMSO were used as vehicle controls. Representative images of red and blue channel merged images are shown from samples prepared in triplicate.
Concentration-dependent effect of bt-15d-PGJ₂ modification of actin and Keap1

It is well accepted that the actin cytoskeleton plays an important role in cellular migration [128]. It was previously shown that 15d-PGJ₂ can form covalent adducts with a number of important cytoskeletal components including actin, tubulin, and vimentin [114, 115]. Moreover, 15d-PGJ₂ can affect cytoskeletal organization in neuroblastoma and mesangial cells [114, 115]. We therefore sought to further characterize the effect of 15d-PGJ₂ on the F-actin cytoskeleton. In order to determine if 15d-PGJ₂ forms a direct adduct with actin at low concentrations, JC cells were treated with 0.3, 3, and 20 μM bt-15d-PGJ₂ for 4 h. Biotin-15d-PGJ₂ adducted proteins were then enriched from cell lysate protein using a neutravidin column. Total and bt-15d-PGJ₂-modified actin was detected by Western blotting. In Figure 4-10A, actin modification can be detected in cell lysates treated with 3 and 20 μM bt-15d-PGJ₂ (approximately 0.4 and 0.8% of total actin respectively), consistent with reports in the literature of a direct modification of actin by 15d-PGJ₂. However, modification of actin was barely detectable at 0.3 μM bt-15d-PGJ₂, the concentration at which alterations in F-actin organization and focal adhesions were observed.

As a positive control, we compared actin adduct formation with another protein which is known to be adducted by 15d-PGJ₂, Kelch-like ECH-associate protein 1 (Keap1). As shown in Figure 4-10B, bt-15d-PGJ₂ directly adducts Keap1 when cells are treated with 3 μM or 20 μM bt-15d-PGJ₂. However, no significant adduct formation on Keap1 was detected at 0.3 μM bt-15d-PGJ₂. The amount (%) of Keap1 and actin which were pulled down from the total cell lysate was assessed by calculating the quantity of each protein from the densitometry of the respective western blot and adjusting for
Figure 4-10: Concentration-dependent effect of bt-15d-PGJ2 on adduct formation with actin and Keap1. JC cells were treated with 0.3, 3, and 20 μM bt-15d-PGJ2 for 4 h and then biotinylated proteins were purified from cell lysates using a neutravidin column. β-actin (A) or Keap1 (B) were each detected in cell lysate or eluent by Western blot analysis. The relative amount of β-actin or Keap1 which was affinity precipitated was determined by comparing the density of lanes containing cell lysate or eluate and correcting for protein loaded (C). Ethanol was used as a vehicle control. Experiments were performed in duplicate. Values represent means ± range. Representative images are shown.
amount of total protein loaded per lane. The amount of bt-15d-PGJ2-modified Keap1 or actin was also measured in the eluate from the western blot by densitometry, and the percentage of each protein which was recovered by neutravidin pull-down from the total cell lysate protein is shown in Figure 4-10C (% modification). Cell lysate and pulled-down samples were analyzed and quantified from the same Western blot membrane in order to minimize variability from blotting development or exposure.

**Activation of EpRE-dependent intracellular antioxidants**

It is known that modification of critical thiols in Keap1 by 15d-PGJ2 results in an increase in activity of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), and subsequent transcription of genes under the control of the Electrophile Response Element (EpRE). Such genes include heme oxygenase-1 (HO-1) and subunits of glutamyl cysteine ligase which controls the production of glutathione (GSH) [55, 105, 129]. To demonstrate that adduct formation with Keap1 correlates with a biological response in JC cells, we monitored Keap1 modification by bt-15d-PGJ2 after 4 h and subsequent increases in HO-1 and GSH levels after 16 h.

These results are consistent with increased expression of HO-1 and GSH levels (Figures 4-11A and 4-11B, respectively), which are not significantly increased at 15d-PGJ2 concentrations below 1 μM. These data demonstrate that adduct formation with Keap1 correlates with activation of EpRE-dependent gene expression in JC cells as observed in other cellular systems, but that this effect occurs at concentrations of 15d-PGJ2 higher than those required to elicit inhibition of migration or reorganization of the F-actin.
Figure 4-11: Induction of Nrf2-dependent intracellular antioxidants. As a read-out of Keap1 modification and subsequent Nrf2 activation, HO-1 protein levels and total glutathione were measured in JC cell lysates which were treated with 15d-PGJ$_2$ (0.1 - 3 μM, 16 h). HO-1 protein levels were assessed by Western blot analysis and quantified (A). Ethanol was used as a vehicle control. Glutathione values were normalized to total lysate protein (B). Values represent the mean ± s.e.m., n=3. ** p < 0.01 compared to EtOH control, 1-way ANOVA.
Effects of p38 inhibition on 15d-PGJ2-induced focal adhesion disassembly and F-actin cytoskeletal changes

Since minimal direct modification of actin was observed by bt-15d-PGJ2 and PPARγ activation and DP2-dependent pathways cannot adequately account for the effects of 15d-PGJ2 on F-actin cytoskeletal structure or migration, we investigated the role of a known redox active MAP kinase signaling pathway. The p38 pathway has been implicated in the regulation of actin dynamics and can be activated downstream of focal adhesion signaling [130-132]. The p38 signaling pathway was also recently shown to be activated by 15d-PGJ2 at low micromolar concentrations in two human endothelial cell models [133, 134]. Figure 4-12A shows a significant decrease (18%, p < 0.01) in the percentage of cells positive for focal adhesions upon treatment with 0.3 μM 15d-PGJ2. The p38 inhibitor SB203580 alone did not significantly alter the percentage of cells positive for focal adhesions, but pretreatment with SB203580 for 30 min prevented the decrease in focal adhesion positive cells observed with 0.3 μM 15d-PGJ2.

JC cells were also pretreated with the p38 inhibitor SB203580, and then the effect of 15d-PGJ2 on F-actin organization was assessed. Figure 4-12B shows the F-actin cytoskeletal structure in JC cells that were pretreated with the p38 inhibitor prior to exposure to 0.24 μM BD-15d-PGJ2. Cells treated with the electrophile exhibited significant F-actin alterations. The p38 inhibitor itself had no apparent effect on the F-actin structure. Interestingly, pretreatment with SB203580 was able to prevent 15d-PGJ2-induced F-actin cytoskeletal rounding. These data suggest a role for the p38 pathway in the F-actin cytoskeletal reorganization and focal adhesion disassembly in response to 15d-PGJ2.
Figure 4-12: Role of p38 in focal adhesion disassembly and cytoskeletal arrangement regulation. JC cells were pretreated with 10 μM SB203580 (SB) for 30 min and then 15d-PGJ₂ (0.3 μM) was added for an additional 4 h. Cells were fixed in 3% glutaraldehyde and focal adhesions were quantified using interference reflection microscopy. Values represent the mean percent of cells scored positive for focal adhesions (A). JC cells were also pretreated with 10 μM SB for 30 min, and then 0.24 μM BD-15d-PGJ₂ was added for an additional 30 min. Cells were then fixed, permeabilized, and stained with 2 units of Alexa Fluor 633® Phalloidin to visualize F-actin (red channel). Nuclei were visualized with DAPI (blue channel). Representative images of red and blue channel merged images are shown (B). Ethanol and DMSO were used as vehicle controls. Values shown represent means ± s.e.m., n ≥ 3. ** p < 0.01 compared to vehicle control, 1-way ANOVA.
Effect of 15d-PGJ2 on GFP-labeled MDA-MB231 viability

It is known that regulation of the F-actin cytoskeleton, focal adhesion disassembly, and migration play an important role in successful metastasis in vivo. Thus far, we have shown that 15d-PGJ2 alters the F-actin cytoskeleton morphology, induces focal adhesion disassembly, and attenuates migration in JC murine mammary adenocarcinoma cells. We next wanted to investigate the effects of 15d-PGJ2 in an experimental metastasis model. In order to do this, we have selected a well-established in vivo model of metastasis used previously in the laboratory of our collaborator, Dr. Danny R. Welch [135, 136]. Lentiviral transfected MDA-MB231 cells which stably over-express green fluorescent protein (GFP; GFP-MB231) are injected into the lateral tail vein of athymic mice and metastasis to the lung is determined 6 weeks post-injection. Prior to investigating the effects of 15d-PGJ2 using this model, we first confirmed that the effects of 15d-PGJ2 were recapitulated in the GFP-MB231 cell line.

To determine cytotoxic concentrations of 15d-PGJ2, GFP-MB231 cells were treated with increasing concentrations of 15d-PGJ2 (0.3-100 μM) for 16 h and cell morphology was assessed. Lactate dehydrogenase (LDH) release was also determined as an indicator of cytotoxicity. Figure 4-13A shows representative light micrographs of cells after lipid exposure. Cells exposed to ≤ 1 μM 15d-PGJ2 show morphology similar to that of vehicle control (EtOH) treated cells. Cells treated with 3 μM 15d-PGJ2 demonstrate cell rounding, and higher concentrations result in cell detachment from the tissue culture plate. As seen in Figure 4-13B, concentration-dependent increases in LDH release occur in response to 15d-PGJ2 exposure. The MTT assay was used as an additional indicator of cell viability, and Figure 4-13C clearly shows that MTT reduction to insoluble formazan
Figure 4-13: Effect of 15d-PGJ₂ on cell viability in GFP-MB231 cells. GFP-MB231 cells were treated with increasing concentrations (0.3-100 μM) of 15d-PGJ₂ for 16 h. Representative light micrographs (10X magnification) after treatment are shown (A). Cell viability was also assessed by LDH release (B) and MTT (C) assays after treatment. Ethanol was used as a vehicle control. Values shown represent means ± s.e.m., n = 4-6. * p < 0.05, ** p < 0.01 compared to EtOH control, 1-way ANOVA.
crystals is significantly impaired in cells treated with 10 μM 15d-PGJ2. The fact that LDH release is not significant at this concentration may be due to the different sensitivities of the assays used. Taken together, these data demonstrate that 15d-PGJ2 elicits significant cytotoxicity in GFP-MB231 cells at concentrations ≥ 10 μM. Moreover, the concentration-dependence of this cytotoxic effect is similar to that which we have previously determined for MDA-MB231 and JC cells (Figures 3-2 and 4-1, respectively). In order to examine the effect of 15d-PGJ2 on experimental metastasis, and not its cytotoxic effects, we have selected 1 μM 15d-PGJ2 for use in all additional experiments in GFP-MB231 since there was no cytotoxicity using any of these assays.

**Effect of 15d-PGJ2 on focal adhesions and migration in GFP-MB231**

We next determined the effect of 15d-PGJ2 on focal adhesion disassembly and migration in GFP-MB231 cells. Cells were treated with 1 μM 15d-PGJ2 for 30 min and the number of cells scoring positive for focal adhesions was determined. Treatment with the lipid resulted in an approximately 50% decrease in the number of cells staining positive for focal adhesions (Figure 4-14A). The effect of 15d-PGJ2 on cell motility was also assessed using a wound healing assay, and 15d-PGJ2 treatment attenuated cell motility by approximately 30% when compared to vehicle control treated cells (Figure 4-14B). Importantly, the extent of the response to 15d-PGJ2 for focal adhesions and cell motility is similar to that which we previously defined for JC cells (Figure 4-5 and 4-3, respectively).
Figure 4-14: Effect of 15d-PGJ$_2$ on focal adhesion disassembly and migration in GFP-MB231 cells. GFP-MB231 cells were treated for 30 min with 15d-PGJ$_2$ (1 μM) and then fixed in 3% glutaraldehyde. Focal adhesions were quantified using interference reflection microscopy. Values represent the mean percent of cells scored positive for focal adhesions (A). GFP-MB231 cell migration was assessed using a scratch assay. Cells were treated with 15d-PGJ$_2$ (1 μM) and cell migration into cell-free area was assessed after 8 h. Ethanol (EtOH) was used as a vehicle control. Values shown represent means ± s.e.m., n = 3-4. ** p < 0.01 compared to EtOH control, Student’s t-test.
Effect of 15d-PGJ$_2$ on experimental metastasis

In order to assess the effect of 15d-PGJ$_2$ on metastasis in vivo, we have utilized an experimental metastasis model whereby GFP-MB231 cells are injected via the tail vein and metastasis to the lung is assessed after 6 weeks. We have selected this model for two reasons: cellular migration plays a critical role in the later steps of the metastatic cascade, and we have observed that 15d-PGJ$_2$ attenuates migration at sub-lethal concentrations. Moreover, this model allows for the pretreatment of the cells ex vivo prior to injection.

Other in vivo models of metastasis, particularly those which result in spontaneous metastasis, would have required treating the mice with 15d-PGJ$_2$ to investigate its effect of metastasis. Instead, we have used the experimental metastasis model for our initial investigation of the effect of 15d-PGJ$_2$ on metastasis with the intention of using spontaneous models to follow-up the initial observations.

To this end, GFP-MB231 cells were treated ex vivo with 15d-PGJ$_2$ (1 µM) or vehicle control (EtOH) for 4 h prior to injection into athymic mice. A second set of cells were treated with 15d-PGJ$_2$ (1 µM, 4 h) followed by replacement of the treatment media with complete media for an additional 12 h (“15d-PGJ$_2$ reversed” group) to allow for reversal of 15d-PGJ$_2$-induced alterations in the F-actin cytoskeleton and focal adhesions (data not shown). After 6 weeks, the number of macroscopic metastatic lesions per lung was quantified. Shown in Figure 4-15, vehicle control treatment had no effect on the number of lesions/lung. Ex vivo treatment with 15d-PGJ$_2$ resulted in a modest, but insignificant decrease in the number of lesions per lung. The 15d-PGJ$_2$ reversed group was similar to control. While these data are very preliminary, they suggest one of two things. Either there is no effect of 15d-PGJ$_2$ on the later steps of the metastatic cascade which
Figure 4-15: Effect of 15d-PGJ₂ on experimental metastasis to lung *in vivo*. GFP-MB231 cells were treated with 15d-PGJ₂ (1 μM) or ethanol (EtOH; vehicle control) for 4 h or treated with 15d-PGJ₂ (4 h) and then allowed to recover in 0.5% FBS medium for an additional 12 h (“15d-PGJ₂ reversed”). Untreated cells were used as a control. After treatment, 500,000 cells were injected into the lateral tail vein of athymic mice and the lungs were analyzed for macroscopic metastases 6 weeks post-injection. Values shown represent means ± s.e.m., n = 6-9 (shown in white on bars).
was modeled in this type of \textit{in vivo} assay, or a single dose of 15d-PGJ$_2$ is not sufficient to significantly impair metastasis. Therefore, additional studies are necessary to further define the effect of 15d-PGJ$_2$ on metastasis.

**SUMMARY**

Recently, a number of steps in the progression of metastatic disease have been shown to be regulated by redox signaling. In this chapter, we investigated the effect of the electrophilic prostaglandin 15d-PGJ$_2$ on the metastatic properties of breast cancer cells. 15d-PGJ$_2$ was shown to decrease migration, stimulate focal adhesion disassembly and cause extensive F-actin reorganization at low concentrations (0.03-0.3 μM). Importantly, the effects on the F-actin cytoskeleton and migration seem to be independent of PPARγ, DP2 receptor activation, and modification of actin or Keap1, which are known protein targets of 15d-PGJ$_2$ at higher concentrations. The p38 inhibitor SB203580 was able to prevent both 15d-PGJ$_2$-induced F-actin reorganization and focal adhesion disassembly. The effects of 15d-PGJ$_2$ on the same parameters were also examined in GFP-MB231 cells both \textit{in vitro} and \textit{in vivo}. Taken together, our results suggest that electrophiles such as 15d-PGJ$_2$ are potential anti-metastatic agents which exhibit specificity for migration and adhesion pathways at low concentrations where there are no observed effects on Keap1 or cytotoxicity. Moreover, these data imply that the concentration-dependence of electrophiles may be exploited to fine-tune biological responses in cancer cells. Further studies examining the pharmacokinetics of 15d-PGJ$_2$ \textit{in vivo} would be necessary to accurately titrate these potentially therapeutic responses in humans.
CHAPTER 5
COMPARISON OF BIOLOGICAL RESPONSES TO ELECTROPHILIC PROSTAGLANDINS IN JC CANCER CELLS

INTRODUCTION

Thus far in this thesis, we have focused our attention on defining the biological responses of cancer cells to a prototypical electrophilic lipid, 15-deoxy-Δ^{12,14}-Prostaglandin J_{2} (15d-PGJ_{2}). We have also examined strategies to fine-tune the biological responses to 15d-PGJ_{2} through intracellular targeting and exploitation of the concentration dependence effects of the lipid. These studies provide important insights into the regulation of redox signaling by lipid electrophiles in cancer.

It is clear that an array of lipid electrophiles can be generated \textit{in vivo} with different reactivities and proteomes which they modify. In this chapter, we will compare the effects of 15d-PGJ_{2} to a second lipid electrophile that has been studied in the context of cancer, Prostaglandin A_{1} (PGA_{1}).

The anti-tumor effects of PGA_{1} have been recognized for over 20 years. \textit{In vitro} and \textit{in vivo} studies have shown potent anti-proliferative effects of PGA_{1}. It has been shown to inhibit growth in multiple cancer cells lines [137], and a more stable derivative of PGA_{1}, Δ^{7}-PGA_{1}, has been shown to extend the life of Ehrlich ascites tumor-bearing mice [138]. These findings lead to the development of stable derivatives more suitable for \textit{in vivo} delivery [47, 49].
Recently, PGA1 has been recognized along with other A series prostaglandins to be a potent anti-viral agent. It has been shown to significantly inhibit viral replication of several viruses including influenza A, human immunodeficiency virus (HIV), and viruses which cause vesicular stomatitis [139]. It was subsequently determined that the anti-viral activity of PGA1 was due to its ability to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling at multiple points in the signaling cascade and the induction of heat shock responses in cells.

It is clear that 15d-PGJ2 and PGA1 both possess similar anti-inflammatory activity through their ability to inhibit NF-κB signaling [140]. Both 15d-PGJ2 and PGA1 inhibit the IκB kinase (IKK). In the case of 15d-PGJ2, IKK inhibition was shown to occur through direct modification of Cys179 and a similar mechanism is thought to govern IKK inhibition by PGA1 [140]. Interestingly, other common targets have been reported for 15d-PGJ2 and PGA1 including cytoskeletal components [114, 141, 142], rat sarcoma viral oncogene homolog (Ras) isoforms [16], and thiol-metabolizing enzymes like thioredoxin and thioredoxin reductase (TrxR) [74, 143, 144]. Common targets of 15d-PGJ2 and PGA1 are shown in Table 5-1.

Table 5-1: Common targets of 15d-PGJ2 and PGA1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Targeted Cysteine(s)</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>15d-PGJ2</td>
<td>C179</td>
<td>Inhibition of IκB kinase</td>
<td>[101, 140]</td>
</tr>
<tr>
<td>PGA1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-Ras</td>
<td>C181, C184, C118</td>
<td>Activation of H-Ras signaling</td>
<td>[16]</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>C35, C69</td>
<td>Inhibition of reducing activity</td>
<td>[143, 144]</td>
</tr>
<tr>
<td>TrxR</td>
<td></td>
<td>Inhibition of TrxR</td>
<td>[74, 144]</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>Cytoskeletal derangement</td>
<td>[114, 141, 142]</td>
</tr>
<tr>
<td>Tubulin</td>
<td></td>
<td>Cytoskeletal derangement</td>
<td>[114, 142]</td>
</tr>
<tr>
<td>Vimentin</td>
<td>C328</td>
<td>Cytoskeletal derangement</td>
<td>[114, 142]</td>
</tr>
</tbody>
</table>

*Only demonstration of site-selective modification was used to report targeted cysteines.*
Though a number of common protein targets have been identified for 15d-PGJ$_2$ and PGA$_1$, it is clear that the proteomes modified by these compounds differ substantially. Not only do the concentrations of the compounds required to elicit a similar biological response differ by up to an order of magnitude [145, 146], but Gayarre et al. demonstrated using 2D-IEF-SDS-PAGE techniques with biotin-tagged analogs of the two compounds, that many of the protein targets are distinct [145].

Based on these findings, we sought to determine if the effects of 15d-PGJ$_2$ on filamentous actin (F-actin), focal adhesions, and migration could be recapitulated using PGA$_1$. We anticipated that if the effects of 15d-PGJ$_2$ and PGA$_1$ were similar in this context, this information would aid in identifying the protein targets which mediate these effects. If the effects on F-actin, focal adhesions, and migration were specific for 15d-PGJ$_2$, that would suggest that the protein target(s) were distinct to 15d-PGJ$_2$.

**RESULTS**

**Cytotoxicity of 15d-PGJ$_2$ and PGA$_1$ in JC cells**

The anti-inflammatory and anti-tumor activity of several cyclopentenone prostaglandins has been examined extensively [137, 138, 140]; however, the potential anti-metastatic activity of these compounds has not been well characterized. In order to determine non-toxic concentrations of PGA$_1$, we first defined the cytotoxic activity of PGA$_1$ using a lactate dehydrogenase (LDH) release assay and compared it to 15d-PGJ$_2$. JC cells were treated with increasing concentrations of 15d-PGJ$_2$ or PGA$_1$ (3-30 μM) for 16 h, and LDH release was assayed as an indicator of cytotoxicity. Both lipids caused toxicity at high concentrations; however, the concentration-dependence of this effect dif-
ferred. 15d-PGJ2 caused significant cytotoxicity at concentrations lower than those which were observed with PGA1 (Figure 5-1). For additional experiments comparing the effects of 15d-PGJ2 and PGA1 in JC cells, we have selected 3 μM as a non-toxic concentration for both lipids.

**Induction of EpRE-dependent intracellular antioxidants by 15d-PGJ2 and PGA1**

We have previously shown that both 15d-PGJ2 and PGA1 cause increases in intracellular glutathione pools in endothelial cells presumably through a Keap1/Nrf2 (Kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2)-dependent mechanism [147]. To assess whether PGA1 caused upregulation of Electrophile Response Element (EpRE)-dependent proteins, we assessed levels of heme oxygenase-1 (HO-1) protein and glutathione after exposure to PGA1 or 15d-PGJ2 in JC cells. Cells were exposed to increasing concentrations of the electrophiles (0.3-3 μM, 16 h) and then intracellular antioxidants were measured. 15d-PGJ2 caused significant induction of HO-1 at 3 μM when compared to PGA1 (Figure 5-2A). Moreover, 15d-PGJ2 treatment caused a greater induction of glutathione levels when compared to PGA1 (Figure 5-2B).

Since 15d-PGJ2 caused significant toxicity at concentrations higher than 3 μM, HO-1 and glutathione levels could not be assessed under those exposure conditions; however, we were able to measure HO-1 and glutathione levels in response to treatment with 10 μM PGA1. Exposure to 10 μM PGA1 resulted in significant induction of both HO-1 and glutathione (Figure 5-3). Taken together, these results demonstrate that both 15d-PGJ2 and PGA1 stimulate induction of intracellular antioxidants; however, high concentrations of PGA1 are required to elicit comparable effects when compared to 15d-PGJ2.
Figure 5-1: Assessment of cytotoxicity in response to PGA₁ or 15d-PGJ₂. JC cells were treated with increasing concentrations of PGA₁ or 15d-PGJ₂ (3-30 μM) for 16 h and then cytotoxicity was assessed using the LDH release assay. Values are expressed as the percentage of LDH released to the medium of total LDH. Ethanol (EtOH) was used as a vehicle control. Values shown represent means ± s.e.m, n = 3. ** p < 0.01 compared to EtOH control, # p < 0.05 compared to 15d-PGJ₂, 1-way ANOVA.
Figure 5-2: Induction of intracellular antioxidants by PGA₁. JC cells were treated with increasing concentrations (0.3-3 µM) of PGA₁ and 15d-PGJ₂ for 16 h and then cell lysates were probed for HO-1 protein levels and glutathione. A representative Western blot and quantification is shown for HO-1 (A). Glutathione values were normalized to total lysate protein (B). Ethanol (EtOH) was used as a vehicle control. Values shown represent means ± s.e.m, n = 3. * p < 0.05, ** p < 0.01 compared to EtOH control. # p < 0.05 compared to 15d-PGJ₂, 1-way ANOVA.
Figure 5-3: Induction of intracellular antioxidants using a higher concentration of PGA₁. JC cells were treated with PGA₁ (10 μM) for 16 h and then cell lysates were probed for HO-1 protein levels and glutathione. A representative Western blot and quantification is shown for HO-1 (A). Glutathione values were normalized to total lysate protein (B). Ethanol (EtOH) was used as a vehicle control. Values shown represent means ± s.e.m, n = 3. ** p < 0.01 compared to EtOH control, 1-way ANOVA.
Comparison of the effect of 15d-PGJ2 and PGA1 on focal adhesion disassembly

We have previously shown that 15d-PGJ2 can cause focal adhesion disassembly even though these effects do not seem to be mediated via the same mechanisms required for the migration and F-actin effects. Nonetheless, the effect of PGA1 on focal adhesion disassembly and cellular migration was examined. JC cells were treated with PGA1 (3 μM) for 30 min, and then the number of cells staining positive for focal adhesions was assessed using interference reflection microscopy. 15d-PGJ2 was used as a positive control. Both 15d-PGJ2 and PGA1 caused a decrease in the number of cells staining positive for focal adhesions (Figure 5-4, 54% and 40% decrease respectively); however, 15d-PGJ2 was more potent at initiating focal adhesion disassembly than PGA1.

Effect of 15d-PGJ2 and PGA1 on cell motility

Cell motility with treatment of PGA1 and 15d-PGJ2 was also assessed using a scratch assay. JC cells were scratched with a sterile pipet tip to create a cell-free area, and then treated with PGA1 or 15d-PGJ2 (3 μM). Cell migration into the cell-free area was assessed after 8 h. Consistent with previous data (Figure 4-3), 15d-PGJ2 attenuated migration; however, unlike 15d-PGJ2, PGA1 was unable to significantly inhibit migration (Figure 5-5).

Differential effects of 15d-PGJ2 and PGA1 on F-actin cytoskeletal organization

We previously demonstrated that 15d-PGJ2 causes rapid and robust reorganization of the F-actin cytoskeleton in JC cells. We next investigated the effect of PGA1 on F-
Figure 5-4: Effect of PGA₁ on focal adhesion disassembly in JC cells. JC cells were treated with 3 μM PGA₁ or EtOH (vehicle control) for 30 min and then fixed in 3% glutaraldehyde. Focal adhesions were quantified using interference reflection microscopy. 15d-PGJ₂ (3 μM) was used as a positive control for focal adhesion disassembly. Values represent the mean percent of cells scored positive for focal adhesions. Ethanol (EtOH) was used as a vehicle control. Values represent means ± s.e.m., n = 3. ** p < 0.01 compared to EtOH control, # p < 0.05 compared to 15d-PGJ₂, 1-way ANOVA.
Figure 5-5: Effect of PGA₁ on migration in JC cells. JC cell migration was assessed using a scratch assay. Cells were treated with 3 μM PGA₁ and cell migration into cell-free area was assessed after 8 h. 15d-PGJ₂ (3 μM) was used as a positive control for attenuation of migration. Representative images of treated wells (A) and quantification (B) are shown. Ethanol (EtOH) was used as a vehicle control. Values represent means ± s.e.m., n = 6. ** p < 0.01 compared to EtOH control, # p < 0.05 compared to 15d-PGJ₂, 1-way ANOVA.
actin morphology. JC cells were treated with either 15d-PGJ2 or PGA1 (3 μM) for 30 min and then the F-actin was visualized using fluorescence confocal microscopy. 15d-PGJ2 caused extensive reorganization and rounding of the F-actin cytoskeleton while cells exposed to PGA1 resembled control cells (Figure 5-6). Taken together, these data demonstrate that the structurally related lipid electrophiles, 15d-PGJ2 and PGA1, have differential effects on focal adhesion disassembly, migration, and the F-actin cytoskeletal morphology. This suggests that these proteins are differentially targeted by these electrophiles.

**Effect of kinase inhibitors on 15d-PGJ2-induced changes in the F-actin cytoskeleton**

We have previously shown that a p38 inhibitor was able to prevent 15d-PGJ2-induced alterations in the F-actin cytoskeleton and focal adhesion disassembly (Figure 4-12). In an effort to further elucidate the pathways affected by the protein targets of 15d-PGJ2, we next examined the effects of other kinase inhibitors on 15d-PGJ2-induced changes in F-actin morphology. The effect of the PI3K inhibitor LY294002 and the ERK inhibitor FR180202 was tested. JC cells were pretreated for 30 min with the inhibitors (10 μM) then challenged with BODIPY conjugated 15d-PGJ2 (BD-15d-PGJ2; 0.24 μM) for an additional 30 min in the presence of inhibitor. As shown in Figure 5-7, neither the PI3K nor ERK inhibitor alone caused appreciable changes in the F-actin cytoskeleton. As expected, exposure to BD-15d-PGJ2 caused extensive reorganization and rounding of the F-actin cytoskeleton. Interestingly, pretreatment with either the PI3K or ERK inhibitor blocked the 15d-PGJ2-induced changes in F-actin organization such that inhibitor and BD-15d-PGJ2 treated cells resembled control cells (Figure 5-7).
Figure 5-6: Effect of PGA₁ on cytoskeletal arrangement in JC cells. JC cells were treated with 3 μM PGA₁ for 30 min and then fixed, permeabilized and stained with 2 units of Texas Red Phalloidin. 15d-PGJ₂ (3 μM) was used as a positive control for actin reorganization. Nuclei were visualized with DAPI: F-actin (red) and nuclei (blue). Ethanol (EtOH) was used as a vehicle control. Representative red and blue channel merged images are shown from samples prepared in triplicate.
Figure 5-7: Effect of kinase inhibitors on 15d-PGJ₂-induced cytoskeletal reorganization in JC cells. JC cells were pretreated with 10 μM LY294002, an Akt inhibitor, or FR180202, an ERK inhibitor, for 30 min, followed by treatment with or without 0.24 μM BD-15d-PGJ₂ for an additional 30 min. Cells were then fixed, permeabilized and stained with 2 units of Texas Red Phalloidin. Nuclei were visualized with DAPI: F-actin (red), nuclei (blue), BD-15d-PGJ₂ localization (green). Ethanol and DMSO were used as vehicle controls. Representative images of red, green, and blue channel merged images are shown from samples prepared in triplicate.
The fact that the p38, PI3K, and ERK inhibitors were all able to block the F-actin reorganization in response to 15d-PGJ₂ suggests that the target(s) of 15d-PGJ₂ which mediate the aforementioned effects on F-actin, focal adhesions, and migration may be involved in pathways which converge on kinase signaling. Moreover, since the effects of 15d-PGJ₂ on F-actin and migration are not recapitulated using PGA₁, these data suggest that the protein target(s) are which mediate these effects are unique to 15d-PGJ₂ and may not be targets of PGA₁.

**Candidate protein approach for identification of mediators of 15d-PGJ₂ effects**

Recently, Renedo et al. demonstrated that there is a site-selective modification of H-Ras by 15d-PGJ₂ which does not occur with PGA₁ [16]. Both 15d-PGJ₂ and PGA₁ modify H-Ras; however, 15d-PGJ₂, and not PGA₁, modifies Cys184, a cysteine residue unique to H-Ras when compared to other Ras isoforms. Cys118 which is present in all three Ras isoforms (H-Ras, N-Ras, and K-Ras) is modified by PGA₁. In this study, they go on to show that modification of Cys184 by 15d-PGJ₂ results in activation of H-Ras, but not other isoforms and impacts on cell proliferation.

Because we identified differential effects of 15d-PGJ₂ and PGA₁ with regards to F-actin reorganization and migration, and H-Ras is known to regulate multiple kinase signaling pathways, we investigated H-Ras as a candidate protein target of 15d-PGJ₂ which mediates the effects observed here.
**In vitro modification of H-Ras by bt-15d-PGJ₂**

We first wanted to determine if H-Ras can be modified by 15d-PGJ₂ *in vitro*. This work was conducted in the laboratory of our collaborator, Dr. Richard Cohen. To do this, purified 6x-His-tagged H-Ras protein was incubated with bt-15d-PGJ₂ (50 μM) at room temperature for increasing amounts of time. After incubation, H-Ras protein was resolved using SDS-PAGE, and then total H-Ras protein and modified H-Ras were detected using Western blot analysis. Protein lipid adducts were detected using Streptavidin conjugated to horseradish peroxidase (HRP). As seen in Figure 5-8, after 30 min incubation, maximal modification of H-Ras by bt-15d-PGJ₂ can be detected. This demonstrates that bt-15d-PGJ₂ is capable of modifying H-Ras; however, it does not address whether this modification occurs in the cellular context.

**siRNA knockdown of H-Ras protein**

Because direct modification of specific, low abundance proteins by electrophiles can be difficult to assess using currently available techniques, we first wanted to examine whether loss of our candidate protein, H-Ras, can block the biological effects of 15d-PGJ₂. The strategy we employed to examine H-Ras as a candidate protein target was to knockdown the target protein using a siRNA approach and then investigate the effect of 15d-PGJ₂ on F-actin and migration. siRNA-mediated knockdown of H-Ras was confirmed both at the level of mRNA and protein. H-Ras siRNA construct #3 resulted in approximately a 75% decrease in total H-Ras protein (Figure 5-9). The H-Ras siRNA construct #4 caused no appreciable loss of H-Ras protein. For this reason, all additional stu-
Figure 5-8: *In vitro* modification of H-Ras by bt-15d-PGJ₂. Purified 6x-His-tagged H-Ras was incubated with 50 μM bt-15d-PGJ₂ at room temperature for indicated times. Protein was then resolved using SDS-PAGE and probed for lipid-protein adducts using Streptavidin conjugated to HRP and total H-Ras protein using an antibody specific for the 6x-His tag. Representative Western blots are shown. Courtesy of Dr. Richard Cohen.
Figure 5-9: siRNA knockdown of H-Ras in JC cells. JC cells were treated with siRNA constructs directed against murine H-Ras or a murine non-silencing control construct (nsRNA) complexed with Lipofectamine 2000 at a final concentration of 20 μM. Cells were transfected for 8 h, and then transfection media was removed and replaced with complete RPMI media for an additional 40 h. H-Ras knockdown was confirmed by Western blot analysis for H-Ras protein. Values represent means ± s.e.m., n = 3. ** p < 0.01 compared to control, 1-way ANOVA.
dies utilized construct #3. Using this approach, we were able to obtain approximately 75% knockdown of H-Ras protein levels compared to untreated control cells.

**Impact of H-Ras knockdown on 15d-PGJ₂-induced effects on F-actin morphology**

The effect of H-Ras knockdown on 15d-PGJ₂-induced alterations in the F-actin cytoskeleton was examined. Forty eight hours after transfecting with H-Ras siRNA construct #3 or nsRNA, JC cells were treated with 2.4 μM BD-15d-PGJ₂ for 30 min and then fixed, permeabilized, and stained with 2 units of Texas Red Phalloidin to visualize the F-actin cytoskeleton. Transfection with either the nsRNA or H-Ras siRNA constructs did not cause any marked changes in the F-actin cytoskeleton when compared to the control treatment (Figure 5-10). As expected, in the control, untransfected cells, BD-15d-PGJ₂ caused robust rounding of the F-actin cytoskeleton similar to the effects observed previously (Figure 4-8). It appears that there may be a slight difference between the F-actin cytoskeletal arrangement in H-Ras siRNA treated cells compared to control cells; however, knockdown of H-Ras protein levels did not substantially alter the response to BD-15d-PGJ₂.

**Effect of H-Ras knockdown on 15d-PGJ₂-induced attenuation of migration**

We next investigated the effect of H-Ras knockdown on 15d-PGJ₂-induced attenuation of cell motility. To do this, JC cells were transfected with H-Ras siRNA construct #3 or nsRNA, and then 48 h post-transfection, the effect of 15d-PGJ₂ (3 μM) was assessed using the scratch assay. Transfection with either the nsRNA or H-Ras siRNA construct #3 did not cause any marked relative motility when compared to the control treat-
Figure 5-10: Effect of H-Ras knockdown on 15d-PGJ₂-induced changes in cytoskeletal arrangement. 48 h after transfecting with H-Ras siRNA or nsRNA, JC cells were treated with 2.4 µM BD-15d-PGJ, for 30 min and then fixed, permeabilized and stained with 2 units of Texas Red Phalloidin. Nuclei were visualized with DAPI: F-actin (red), BD-15d-PGJ₂ (green), and nuclei (blue). Ethanol (EtOH) was used as a vehicle control. Representative red, green, and blue channel merged images are shown from samples prepared in triplicate.
ment (Figure 5-11). As expected, exposure to 15d-PGJ₂ attenuated cell migration over 8 h; however, siRNA transfection with either the nsRNA or H-Ras siRNA attenuated the 15d-PGJ₂ effect on migration. Since the effect of 15d-PGJ₂ was completely ablated in the transfected control cells (nsRNA), we can not interpret the data which examine the effect of 15d-PGJ₂ in the H-Ras siRNA treated cells. Although the effect of 15d-PGJ₂ on migration is blocked in this group, the result can be attributed to either an effect of 15d-PGJ₂ or the transfection itself.

Taken together, while these data do not conclusively define a role for H-Ras in mediating the 15d-PGJ₂ effects on the F-actin cytoskeleton and cell motility, they also do not eliminate H-Ras as a potential mediator of these effects. Perhaps the fact that only a 75% decrease in H-Ras protein levels could be attained using this method leaves sufficient H-Ras protein available for modification by 15d-PGJ₂. Additional studies using new experimental approaches are necessary to further elucidate the role of H-Ras signaling in this context.

SUMMARY

In this chapter, we examined the concept that structurally related lipid electrophiles cause differential biological responses through the modification of unique protein targets. To do this, we compared the effects of 15d-PGJ₂ and PGA₁ on the F-actin cytoskeleton, focal adhesion disassembly, and migration, and show differential effects on the F-actin cytoskeleton and attenuation of migration. We also demonstrated that multiple kinase inhibitors are capable of blocking the 15d-PGJ₂-induced alterations in the F-actin cytoskeleton. Finally, we examined the potential role of H-Ras as a protein target of 15d-
Figure 5-11: Effect of H-Ras knockdown on 15d-PGJ$_2$-induced attenuation of migration. 48 h after transfecting with H-Ras siRNA or nsRNA, JC cell migration was assessed using a scratch assay. Cells were treated with 3 μM 15d-PGJ$_2$ and cell migration into cell-free area was quantified after 8 h. Values represent means ± s.e.m., n = 3.
PGJ$_2$ to mediate these effects. A siRNA approach to decrease the protein levels of H-Ras was employed; however, an unexpected attenuation of the activity of 15d-PGJ$_2$ resulted from the transfection of cells, and consequently, the data neither conclusively define nor eliminate H-Ras as a potential mediator of these effects.
CHAPTER 6

DISCUSSION

INTRODUCTION

In this thesis, we have examined the biological responses to lipid electrophiles in breast cancer. Using multiple approaches, the effects of a model electrophilic lipid, 15-deoxy-\(\Delta^{12,14}\)-Prostaglandin J\(_2\) (15d-PGJ\(_2\)), were characterized with respect to cell death, induction of intracellular antioxidants, and the ability to regulate processes important for metastasis both \textit{in vitro} and \textit{in vivo}. We have also developed new strategies to fine-tune the biological responses to lipid electrophiles and thereby control breast cancer cell function. To this end, we investigated how cell function can be regulated by organelle-specific targeting of electrophiles (Chapter 3), exploiting the concentration dependent effects of electrophiles (Chapter 4), and activation of redox signaling pathways by modification of specific members of the electrophile responsive proteome (Chapter 5). We have also determined a candidate protein target, H-Ras, may be mediator of the anti-metastatic effects of 15d-PGJ\(_2\). Our work now provides a better understanding of the role of redox signaling by lipid electrophiles in the regulation of breast cancer metastasis.

This chapter will discuss the conclusions and implications of these studies in depth. We will also provide insight into how these studies integrate with current knowledge regarding the role of electrophiles in breast cancer metastasis and discuss the potential impact of other types of electrophiles. In specific, a brief review of studies examining
the anti-metastatic effects of electrophiles derived from the diet such as curcumin and sulforaphane will be put forth, and we will also discuss additional ideas regarding the therapeutic potential of modulating levels of endogenously-derived electrophiles in vivo. Taken together, this discussion will provide important insight into the role of electrophiles in breast cancer metastasis and how redox signaling regulates important aspects of metastasis.

**STRATEGIES TO REGULATE BIOLOGICAL RESPONSES TO LIPID ELECTROPHILES IN BREAST CANCER**

**Intracellular targeting of lipid electrophiles**

The control of redox signaling in cells is now emerging as a key area for our understanding of both the etiology of disease and the development of novel therapeutics. It is well recognized that redox signaling offers potential therapeutic targets in cancer; however, a major limitation in this field has been the inability to selectively activate or inhibit a specific signaling pathway [9, 148]. To our knowledge, the work presented in Chapter 3 of this thesis is the first demonstration of selective targeting of a redox signaling pathway by modifying an electrophilic lipid to target the mitochondrion. We selected the compound 15d-PGJ2 for these studies because of its recognized potential as a therapeutic agent in cancer [30, 102, 149] and its well established ability to modify proteins and activate cell signaling in both the cytosol [16, 69] and mitochondrion [54, 78]. We have demonstrated that by conjugating 15d-PGJ2 to the delocalized cation triphenylphosphonium (TPP⁺), we are able to enhance its cytotoxicity by promoting apoptosis over necrosis when compared to the parent compound in MDA-MB231 cells (Figure 3-2). It is likely that the mechanism through which this occurs is the covalent modification of proteins
since the non-electrophilic lipid analog of mito-15d-PGJ2, mito-PGE2, has no effect on cell death or caspase 9 activation. There are multiple potential mechanisms by which mito-15d-PGJ2 could enhance apoptosis in cells. It is unlikely that mito-15d-PGJ2 activates PPARγ-dependent cell death since PPARγ is localized primarily in the cytosol and blocking the COOH functional group on the lipid leads to loss of its PPARγ-dependent properties [150, 151].

Since mito-15d-PGJ2 is added to the outside to the cell, it will accumulate first in the cytosol based upon the membrane potential across the plasma membrane and only then will cross the inner mitochondrial membrane to accumulate in the mitochondrion [79, 152]. Consistent with this model, we found that the protein adducts with mito-15d-PGJ2 presented a pattern distinct from untargeted 15d-PGJ2 when separated by 2D-IEF-SDS-PAGE. As expected, the proteome for mito-15d-PGJ2 shares some common targets with 15d-PGJ2, but a far greater number of proteins (53 for bt-15d-PGJ2 and 22 for mito-15d-PGJ2) are distinct (Figure 3-9). The fact that mito-15d-PGJ2 has substantially fewer protein targets is consistent with our hypothesis that the sub-proteomes modified by reactive electrophiles are cell domain specific. There are, however, some limitations to this analysis of the electrophilic proteome. For example, not all of the proteins which are modified are capable of entering the second dimension of the 2D-IEF-SDS-PAGE due to precipitation in the isoelectric focusing strip (result not shown). Nevertheless, it is clear from those proteins that are represented that the lipid-protein adduct patterns are distinct. Moreover, as expected, modified proteins were present in both the cytosolic and mitochondrial fractions of cells exposed to either mito-15d-PGJ2 or the untargeted analog (Figure 3-10). Fractionation of the cells after exposure to the electrophiles demonstrated a
greater mitochondrial enrichment of adducted proteins relative to the unfractionated sample for mito-15d-PGJ2 compared to 15d-PGJ2. In further support of a selective impact on mitochondrial function by the addition of the TPP+ group to 15d-PGJ2, we found that mito-15d-PGJ2 caused a profound mitochondrial defect in oxidative phosphorylation at concentrations where 15d-PGJ2 had no discernable effect (Figure 3-12).

There are several potential mechanisms for the increased toxicity of mito-15d-PGJ2. First, mito-15d-PGJ2 may more extensively modify mitochondrial targets of the parental compound, 15d-PGJ2. We have previously reported that 15d-PGJ2 modifies components of the mitochondrial permeability transition pore including the adenine nucleotide translocator (ANT) and promotes permeability transition in isolated mitochondria [71]. Second, there is also the potential that mito-15d-PGJ2 enhances cell death by limiting the cytoprotective response of cells to the electrophilic stress they experience. Nrf2-dependent gene transcription results in an increase in the overall antioxidant capacity of cells and has recently been demonstrated to also initiate anti-apoptotic responses [56]. A final potential mechanism for increased cytotoxicity of mito-15d-PGJ2 may be through the modification of new mitochondrial protein targets as a result of targeting 15d-PGJ2 to the mitochondrion. Our data suggest that the loss of Nrf2-dependent gene transcription does not explain the enhanced cytotoxicity of mito-15d-PGJ2 (Figure 3-5 and 3-6). Coincubation of a Nrf2-activating concentration of 15d-PGJ2 does not protect against mito-15d-PGJ2 induced cell death. Moreover, wild-type and Nrf2-/- murine embryonic fibroblasts have similar cytotoxicity profiles in response to either 15d-PGJ2 or mito-15d-PGJ2 (Figure 3-6) pointing to the fact that Nrf2 does not prevent cell death phenotype on acute exposure to an electrophile. However, it is important to note that induction of antioxidant
defenses with low, non-toxic concentrations of electrophiles has been shown to be essential for the protection against a subsequent oxidative stress [153].

The fact that the loss of Nrf2 signaling does not appear to be a mediator of the enhanced cell death effects of mito-15d-PGJ2 does not preclude the role of mitochondrial thiol modification as a mechanism. We have demonstrated that both mito-15d-PGJ2 and 15d-PGJ2 localize to the mitochondrion and using a high resolution proteomic format, we have identified a number of common protein targets of the two compounds. The cell death target is likely to be one or more of these common target proteins (Figure 3-9 and 3-10). Interestingly, we also show that mito-15d-PGJ2 has profound effects on mitochondrial bioenergetics that are not observed with untargeted 15d-PGJ2 (Figure 3-12). These data suggest that both the extent of modification of common targets and a gain-of-function activity of mito-15d-PGJ2 through the modification of unique mitochondrial protein targets may be important for these diverse cellular effects observed here. Consistent with this idea, our data also demonstrate that concentrations at which mitochondrial function is impaired are also those at which apoptosis is observed.

We reasoned that if mito-15d-PGJ2 was accumulated in the mitochondrion it would be less effective at activating cytosolic signaling pathways. To test this, we chose to measure the initiation of EpRE-dependant genes such as HO-1 and the proteins controlling GSH synthesis by the electrophilic lipids [106]. We found that mito-15d-PGJ2 upregulates HO-1 to a lesser a extent and GSH induction is essentially abolished when compared to 15d-PGJ2 (Figure 3-4).

In Chapter 3, we have demonstrated the feasibility of modulating specific cell responses by targeting a subcellular proteome (the mitochondrion). Using this strategy we
successfully selected for one cellular response (cell death) and selected against another (antioxidant upregulation). It is important to note that the addition of the TPP⁺ moiety to 15d-PGJ₂ may impact on the electrophile responsive proteome through mechanisms distinct from mitochondrial targeting. For example, it likely changes the lipophilicity of the parent electrophile and may also provide additional steric factors which change the reactivity to target proteins. Taken together these data indicate that both the reactivity of the thiol proteome and the physical-chemical properties of the electrophile will determine the specific electrophile responsive sub-proteomes which are modified and thus the biological responses. This has important implications for both the understanding of the basic mechanisms through which electrophiles mediate redox cell signaling and the potential to refine the protein targets of electrophiles through intracellular targeting strategies.

**Concentration-dependent effects of 15d-PGJ₂**

Breast cancer metastasis is a major cause of mortality and morbidity in patients, and therefore agents which can inhibit this process, particularly with minimal toxicity to the patient, are desirable therapeutic options. In the studies presented in Chapter 4, we demonstrate that 15d-PGJ₂ at low concentrations (< 1 μM), which do not cause cytotoxicity, stimulates focal adhesion disassembly, causes F-actin reorganization, and attenuates migration of JC mouse mammary adenocarcinoma cells. Since these processes are required for successful metastasis, our data point to a potential anti-metastatic activity of 15d-PGJ₂. Importantly, the effects on F-actin and migration appear to be independent of activation of PPARγ or the Prostaglandin D₂ receptor (DP2) and direct modification of actin, but instead can be attributed to modulation of one or more redox signaling path-
ways through the covalent modification of yet undetermined protein targets of 15d-PGJ2. Interestingly, the effects observed on focal adhesion disassembly appear to be mediated by distinct a mechanism since these effects can be recapitulated by PPARγ and DP2 agonists.

There have been a number of previous studies demonstrating the ability of 15d-PGJ2 to cause cancer cell death, and this is thought to occur primarily through PPARγ mediated activation of cell death pathways [44, 117]. However, studies by our group and others have also shown that 15d-PGJ2 causes apoptosis in a number of cell types, including endothelial cells, through the direct modification of protein thiols in mitochondrial proteins [71]. The modification of these protein leads to permeability transition and activation of apoptotic cell death [71]. These results have been further confirmed by the data shown in Chapter 3 of this thesis. This has raised the concern that 15d-PGJ2 might have toxic side effects when used therapeutically at doses which kill cancer cells [149]. For this reason, we chose to investigate the possibility of targeting metastatic properties of cancer cells at concentrations of 15d-PGJ2 which are not lethal. Our results demonstrate that cell processes which promote metastasis including migration, focal adhesion disassembly, and F-actin reorganization can be effectively modulated by low, sublethal concentrations of 15d-PGJ2.

Our observation that 15d-PGJ2 causes profound reorganization of the F-actin cytoskeleton (Figure 4-8) is consistent with previous reports in neuroblastoma cells [115]. In their study, Aldini et al. attributed the F-actin changes to direct modification of actin by 15d-PGJ2 through formation of covalent adducts [115]. In our study, we were able to recapitulate this result in that direct protein adduct formation of 15d-PGJ2 with actin was
significant at 3 and 20 μM bt-15d-PGJ$_2$ (Figure 4-10). Interestingly, bt-15d-PGJ$_2$ did not appreciably form protein adducts with actin at 0.3 μM, though there was still a profound effect on the F-actin cytoskeleton at this concentration. These results indicate that whereas 15d-PGJ$_2$ forms protein adducts with actin at higher concentrations, this adduct formation does not adequately explain the extensive effect on F-actin reorganization observed at low concentrations of 15d-PGJ$_2$ (<1 μM).

Instead, we have focused on the p38 signaling pathway which is known to be redox regulated and has been implicated in actin structural dynamics in a number of cancer model systems [154]. Multiple stimuli that regulate the actin cytoskeleton, focal adhesion disassembly, cell motility, and invasion converge on the p38 signaling pathway. For example, in neuroblastoma cells, the WASP/WAVE family member WAVE3 has been shown to regulate actin polymerization and cytoskeletal organization through p38-dependent signaling [155]. Orr et al. also showed that focal adhesion disassembly in response to thrombospondin is regulated by p38 [156]. Furthermore, activation of Hsp27 by p38 is well established as an important regulator of actin polymerization and depolymerization [157]. Phorbol 12-myristate 13-acetate (PMA) induced migration of glioblastoma cells has been shown to occur through the p38/Hsp27 signaling axis [158]. More recently, it was shown that the motility of glioma cells is inhibited by flavonid silibinin by a mechanism involving ROS generation and p38 activation [159]. Together, these reports demonstrate the integral role p38 plays in modulating cytoskeleton organization, focal adhesion disassembly, motility, and invasion initiated by diverse stimuli. While our experiments implicate p38 in the mechanism of 15d-PGJ$_2$-mediated actin reorganization and
focal adhesion disassembly (Figure 4-12), further studies are necessary to determine the role of other potentially important redox signaling pathways on this effect.

The mechanism by which 15d-PGJ2 causes focal adhesion disassembly appears to be distinct from those responsible for migration and F-actin reorganization. Focal adhesions have been shown to be modulated by a number of pathways including FAK, extracellular matrix components, and integrin signaling (reviewed in [116]). FAK signaling is altered in response to 15d-PGJ2 not through changes in total FAK levels as previously described [117], but through activation of FAK signaling (Figure 4-4). Since PPARγ and DP2 agonists were able to decrease the number of cells which stain positive for focal adhesions to a similar extent as 15d-PGJ2, it is likely that focal adhesion signaling is regulated by multiple mechanisms. Future studies will examine the role of signaling downstream of PPARγ and DP2 in the regulation of focal adhesion disassembly to determine if 15d-PGJ2 activates common pathways.

In addition, the effects of 15d-PGJ2 on focal adhesions and migration do not appear to be cell line specific. In characterizing the effects of 15d-PGJ2 on GFP-MB231 cells, we noted very similar responses to 15d-PGJ2 for migration and focal adhesion disassembly when compared to the JC cells. This is important because of the extensive heterogeneity found in human cancers. We have also attempted to examine the effect of 15d-PGJ2 on metastasis in vivo; however, no significant effect of 15d-PGJ2 on metastasis was observed using the treatment protocol and in vivo metastasis model we selected. This may be due to the fact that we used only a single dose of 15d-PGJ2 prior to injecting the cells into mice instead of treating tumor-bearing mice with the electrophile. Further studies are necessary to define the effects of 15d-PGJ2 in vivo.
In summary, we have shown that 15d-PGJ2 attenuates mammary cancer cell motility at sublethal concentrations. This effect is preceded by extensive alterations in the F-actin cytoskeletal organization resulting in the rounding of the F-actin cytoskeleton and significant focal adhesion disassembly. Moreover, the effects on the F-actin cytoskeleton appear to be independent of PPARγ activation, signaling downstream of DP2 receptors, or the direct modification of actin by the electrophile. Our data indicate that the p38 signaling pathway plays an integral role in mediating the 15d-PGJ2-induced alterations in the aforementioned parameters. While further studies are required to identify the redox-sensitive protein target or targets of 15d-PGJ2 responsible for these changes, it is clear that modulation of redox signaling pathways by electrophiles may be important anti-metastatic therapeutic avenues in the future. Finally, exploitation of the concentration-dependence of biological responses to electrophiles may allow for targeting of metastatic processes while avoiding undesirable toxicity to normal cells.

**Differential effects of structurally-related lipid electrophiles**

It is increasingly evident that electrophilic lipids are emerging as important mediators of redox cell signaling [10, 37, 45, 160]. We have shown that the electrophilic lipid 15d-PGJ2 regulates important redox-sensitive aspects of metastasis including cytoskeletal regulation and migration. In Chapter 5, we compared the effects of 15d-PGJ2 to a structurally related electrophilic lipid, PGA1. In order to examine the effects of these electrophilic lipids, we first defined sub-lethal concentrations of these compounds to use in further studies. We found that treatment with both 15d-PGJ2 and PGA1 resulted in concentration dependent increases in cytotoxicity (Figure 5-1). Interestingly, PGA1 was
somewhat less toxic and higher concentrations were required to elicit cell death than those required with 15d-PGJ2. This is consistent with previous reports that showed that higher concentrations of PGA1 are required to get quantitatively similar levels of lipid-protein adducts in NIH-3T3 cells [145].

Similarly, the concentration dependence of the induction of intracellular antioxidants by 15d-PGJ2 and PGA1 differed (Figures 5-2 and 5-3) and required higher concentrations of PGA1 for maximal induction of HO-1 and glutathione. Of note is the fact that exposure to PGA1 (10 μM) caused a greater induction of both HO-1 and glutathione than 15d-PGJ2 (3 μM). More careful titration of these responses is necessary to confirm the maximal response to each electrophile; however, these data imply that PGA1 is equally or more potent than 15d-PGJ2 at inducing maximal intracellular antioxidant expression and may provide important insight into the unique proteomes modified by these compounds. Moreover, these data also provide a further example of how the concentration dependence of biological responses to electrophiles can be exploited to regulate cell behavior.

The specificity of 15d-PGJ2 for causing alterations in cytoskeletal structure, focal adhesion disassembly, and cell motility was also addressed by comparing its effects to those of PGA1. Both 15d-PGJ2 and PGA1 caused significant focal adhesion disassembly; however, only 15d-PGJ2 exposure altered the F-actin cytoskeletal morphology and attenuated migration. Based on these data and the data presented in Chapter 4, it appears as though the ability of a compound to initiate rounding of the F-actin cytoskeleton correlates better with inhibition of motility than does changes in focal adhesions. This is true for PPARγ and DP2 agonists as well as PGA1.
In the study presented in Chapter 5, only a single concentration of each electrophile was examined. Understanding the concentration dependence of these effects for both 15d-PGJ2 and PGA1 will allow for the determination of maximal effects of electrophiles on the parameters examined. Furthermore, these studies will provide important insight into the regulation of redox-sensitive metastatic processes by electrophiles.

The fact that we observed differences in F-actin cytoskeletal alterations with 15d-PGJ2 and PGA1 led us to hypothesize that the protein(s) responsible for this may by uniquely modified by 15d-PGJ2. Since it has been previously shown that 15d-PGJ2 and PGA1 participate in site-selective modification of H-Ras [16], one of the only well-described differentially-modified targets of these electrophiles, we examined it as a potential target of 15d-PGJ2 in our model. In vitro, H-Ras is modified by biotin tagged 15d-PGJ2 (Figure 5-8), confirming previous reports that it can be modified by 15d-PGJ2 [16, 75].

We examined the potential role of H-Ras in the cellular context using siRNA-mediated knockdown of H-Ras protein. Though we observed an effect of H-Ras siRNA on 15d-PGJ2-induced attenuation of migration, we also observed as effect of the nsRNA as well thereby complicating the interpretation of the results. Since there appears to be an unexpected interaction between the transfection of the cells and the biological activity of 15d-PGJ2 further studies are needed to conclusively define a role for H-Ras. Complementary H-Ras knockdown strategies that result in more substantial knockdown of total H-Ras protein or proteomic approaches to identify H-Ras-lipid adducts from treated JC cells will address this in the future.
The signaling pathways involved in the regulation of the F-actin cytoskeletal changes in response to 15d-PGJ2 were also investigated. Our results show that multiple kinase inhibitors are capable of blocking 15d-PGJ2-induced changes in the F-actin cytoskeleton (Figure 5-7). These include inhibitors of p38, Akt, and ERK signaling. Interestingly, all of these pathways have been described to be activated downstream of Ras signaling cascades [161, 162]. This is shown in a very simplified schematic in Figure 6-1. However, p38, Akt, and ERK are not the only downstream effectors of Ras activation (reviewed in [162]) and substantial cross-talk occurs between these pathways. If Ras is in fact a target of 15d-PGJ2 in our model, it will be necessary to further characterize the signaling events which occur downstream of Ras including possible effects on the protein kinase C, JNK, and Rho signaling pathways.

Moreover, Ras activation is classically thought be oncogenic in nature, and as a consequence, if 15d-PGJ2 indeed modifies H-Ras, this modification seems to be initiating non-canonical, anti-metastatic signaling and biological responses. The comparison of the effects of 15d-PGJ2 and PGA1 characterized in Chapter 5 demonstrate the level of specificity that can be attained using electrophiles even though these compounds act through innately pleiotropic mechanisms. This is also further evidence for the ability to fine-tune the biological responses to electrophiles using electrophiles capable of modifying different sub-proteomes.

**LIMITATIONS AND POTENTIAL PITFALLS**

Though these findings provide important insight into the role of redox signaling by electrophiles in cancer, it is important to address the potential limitations and pitfalls
Figure 6-1: Downstream effectors of Ras signaling. This simplified schematic shows signaling cascades which are activated downstream of Ras activation. Data shown in this chapter demonstrate that H-Ras can be directly adducted by bt-15d-PGJ$_2$ in vitro and certain biological responses to 15d-PGJ$_2$ can be inhibited by blocking multiple signaling pathways downstream of Ras (starbursts indicate pathways examined).
of the work presented here. In this section, we will address both the limitations of our studies as well as provide potential strategies to overcome some of these limitations in future studies.

**Specificity of effects for cancer versus normal cells**

The data presented in Chapter 3 demonstrate the feasibility of activating specific redox cell signaling pathways using organelle selective targeting. This is an exciting concept since a major limitation of using redox signaling pathways as therapeutic targets is the inability to selectively target specific pathways. Our data demonstrate that by targeting the electrophile 15d-PGJ₂ to the mitochondrion, its ability to initiate apoptotic cell death is significantly enhanced. However, in assessing the toxicity of mito-15d-PGJ₂ in immortalized, non-tumorigenic mammary epithelial cells (MCF10A), no differential cytotoxicity was observed between normal and cancerous cells (Figure 6-2).

Achieving both organelle-specific and tumor-specific targeting of therapeutic electrophiles may present a challenge. Our collaborator, Dr. B. Kalayanaraman, has some encouraging data that suggest that the non-specific cytotoxicity of mito-15d-PGJ₂ in vitro may not present a problem in the whole animal setting. Using tumor-bearing rats, Lopez et al. showed there is preferential uptake of a triphenylphosphonium conjugated, mitochondrially-targeted MRI contrast agent in mammary tumors after intravenous administration of the agent [163]. These data suggest that the biological distribution of mitochondrial targeted compounds may exhibit some preferential uptake in tumors. The mechanism for the preferential uptake in tumors has not yet been defined, and additional studies are necessary to further understand this effect.
Figure 6-2: Effect of mito-15d-PGJ$_2$ on cell viability in non-tumorigenic versus tumorigenic mammary cells. Viability of MCF10A and MDA-MB231 cells exposed to increasing concentrations (3-30 μM) of mito-15d-PGJ$_2$ for 16 h was determined by LDH release. Results represent means ± s.e.m., n = 3, ** p < 0.01 compared to EtOH vehicle control. Reproduced with permission from Diers AR, Higdon AN, Ricart KC, Johnson MS, Agarwal A, Kalyanaraman B, Landar A, Darley-Usmar VM, 2010, Biochem J, 425, doi:10.1042/BJ20091293, © the Biochemical Society.

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**Challenges of electrophile delivery in vivo**

Because electrophiles are reactive with proteins, a major challenge in their utilization clinically may be bioavailability and delivery of therapeutic electrophiles. In *in vitro* studies, we and others have previously shown that 15d-PGJ₂ readily reacts with the available thiol on albumin, a major protein component in blood [164, 165]. Adduction to proteins also underlies the reason that levels of free 15d-PGJ₂ detected in biological samples have been difficult to assess *in vivo* [166] and have only been reported in the high picomolar to low nanomolar range [167, 168].

A number of attempts have been made to generate more bioavailable formulations of electrophiles for use *in vivo*. In the case of the lipid electrophile PGA₁, more biologically stable analogs have been synthesized (e.g. Δ⁷-PGA₁ and Δ⁷-PGA₁ methyl ester) [47] and lipid microspheres have been generated to enhance delivery *in vivo* [49]. These types of strategies may aid in the development of electrophile therapies in the clinical setting.

It is encouraging to note that lipid electrophiles have been used in a number of preclinical animal models with success. Studies of the effects of 15d-PGJ₂ in rodent models have demonstrated efficacy of the electrophile using multiple routes of delivery including intravenous [169], intraperitoneal [170], subcutaneous [171], and topical administration [172].

Finally, in elegant work conducted by Oh et al., it was shown that 15d-PGJ₂ lipid-protein adducts accumulate over time at low, biologically-relevant concentrations, and this characteristic of accumulation of protein adducts over time increases the potency of 15d-PGJ₂ as a cell signaling molecule [164]. These data imply that lipid electrophiles
may have therapeutic efficacy even if only very low, steady-state concentrations of these compounds can be achieved in vivo.

Together, these results suggest that lipid electrophiles may have sufficient bioavailability to be used therapeutically by multiple routes of delivery. Moreover, additional pharmacokinetic and pharmacodynamic studies are warranted to further elucidate the effects and fates of lipid electrophiles in vivo.

**Identification of protein targets of 15d-PGJ2**

The data presented in this thesis demonstrate that 15d-PGJ2 may possess important anti-metastatic activity. Moreover, these data suggest that most of the anti-metastatic activity of 15d-PGJ2 is mediated through redox signaling processes and not through other pathways activated by 15d-PGJ2 (e.g. PPARγ and DP2 receptor mediated effects). The identification of the protein target or targets which are responsible for the effects of 15d-PGJ2 described in this thesis would substantially strengthen these data. Identification of the protein target(s) of 15d-PGJ2 would also allow for further fine-tuning of biological responses to electrophiles using strategies similar to those described in this thesis such as use of electrophiles with differing reactivities and intracellular targeting techniques.

We have presented data examining candidate protein targets of 15d-PGJ2 (e.g. H-Ras); however, a candidate protein approach has several limitations, and our results were largely inconclusive. Since 15d-PGJ2 modifies multiple protein targets concomitantly, it is unlikely that a single protein target is responsible for the effects observed with this lipid. A candidate protein approach only examines a single protein and cannot account for effects which are mediated by the simultaneous modification of multiple proteins. Addi-
tionally, candidate protein approaches are limited to examination of known protein targets of 15d-PGJ2. It is clear that many of the protein targets of 15d-PGJ2 are yet to be identified; therefore, this approach inevitably misses potentially important, yet still unknown targets of 15d-PGJ2.

Broad proteomic approaches to identify protein targets of lipid electrophiles have had limited success to date because of the technically challenging characteristics of lipid-proteins adducts and the difficulty of identifying adducts using traditional mass spectrometry techniques. Our laboratory is continuing to develop new proteomic techniques for the identification of protein targets of 15d-PGJ2 which will hopefully yield important insight into the biological responses of lipid electrophiles in the future.

**ROLE OF ELECTROPHILES IN CANCER AND METASTASIS**

**Dietary electrophiles in cancer and metastasis**

The data presented herein demonstrate that electrophilic lipids can regulate processes important in metastasis. Our work has focused on the effects of the endogenously-generated prostaglandin 15d-PGJ2; however, the findings described here may be applicable to other electrophilic compounds in the context of metastasis. Electrophilic compounds with similar reactivity to 15d-PGJ2 can be derived from the diet. Sulforaphane (SFN) and curcumin are dietary electrophiles which are found in cruciferous vegetables and the Indian spice turmeric, respectively. The structures of these compounds are shown in Figure 6-3. Curcumin contains 2 electrophilic carbon centers which are due to the $\alpha,\beta$-unsaturated carbonyl moieties. SFN is also electrophilic in nature due to the elec-
tron withdrawing properties of the nitrogen and sulfur atoms through the conjugated pi electron system.

In the context of cancer, both SFN and curcumin possess potent chemopreventative activity. Treatment with either SFN or curcumin protects against tumor formation in a number of carcinogen-induced models of cancer [173-177]. SFN is thought to work primarily through the induction of phase II detoxification enzymes like glutathione transferases, glucuronosyltransferases, and NAD(P)H:quinone reductase [178, 179]. An emerging literature also suggests that SFN alters gene transcription through the regulation of histone deacetylase activity [180]. Much more varied biological responses to curcumin have been described. Although, it appears as though its chemopreventative action is mediated at least in part through its anti-inflammatory effects [181-183]. Beyond a role in chemoprevention, more recent data also suggest SFN and curcumin may be effective chemotherapeutic agents [184-187].

A few studies have also examined the effects of SFN or curcumin in the context of metastasis. In *in vivo* models of metastatic melanoma [188, 189], hepatocellular carcinoma [190], and breast cancer [191, 192], experimental metastasis can be suppressed by treatment with these dietary electrophiles. For example, in a spontaneous breast cancer metastasis model, curcumin fed in the diet (2% w/w) resulted in an approximately 40% reduction in the number of mice with metastases to the lung when compared to control-fed animals [192]. Another study demonstrated that treatment of mice with SFN (500 µg/kg) either during or after the establishment of metastatic melanoma lesions in the lung decreased the total number of macroscopic metastases by 82-95% compared to control animals [188].
Figure 6-3: Dietary electrophiles. The structures of the dietary electrophiles curcumin and sulforaphane are shown. Electrophilic carbons are denoted with asterisks.
Interestingly, there are a number of common biological responses to 15d-PGJ2, SFN and curcumin have been reported. These include anti-inflammatory action through inhibition of NFκB [193, 194], cytoprotective responses through the induction of intracellular antioxidants [195, 196], and regulation of kinase signaling pathways [197, 198]. Future comparative studies integrating the effects of both endogenously- and dietary-derived electrophiles on metastatic processes are likely to yield additional insight into the common mechanisms which underlie these effects as well as the protein-electrophile interactions that initiate the biological responses. To date, little epidemiological data exists investigating the relationship between dietary electrophile consumption and cancer metastasis. This type of data would be particularly interesting and may provide a basis for dietary electrophiles as adjuvant therapy in the treatment of cancer.

**Modulating levels of endogenous electrophiles**

Our data suggest that modulation of the levels of endogenous electrophiles may also have therapeutic benefits. Multiple lines of evidence indicate that endogenous levels of electrophilic COX-2 metabolites are regulated in cancer. Since relative levels of both electrophilic and non-electrophilic COX-2 metabolites are enzymatically controlled, the enzymes in these pathways may be important therapeutic targets.

Notably, direct pharmacologic inhibition of COX-2 has shown efficacy in the treatment of colon [199] and breast cancers [200]. COX-2 catalyzes the first and rate-limiting step of the metabolism of arachidonic acid which generates a number of lipid mediators including the prostaglandin family (synthesis pathways are reviewed in [28]). While some of these prostaglandins are thought to drive tumorigenesis (e.g. Prostaglan-
Prostaglandin E2 (PGE2), substantial evidence demonstrates that more minor products have anti-tumor effects [39, 40]. This is shown schematically in Figure 6-4. Prostaglandin E2 is formed enzymatically from the precursor prostaglandin, Prostaglandin H2 (PGH2) through the activity of PGE synthase. PGE2 levels can also be regulated through its removal by the detoxification enzyme of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) to an inactive derivative, 15-keto-PGE2.

Prostaglandin D2 (PGD2) is also formed from the precursor PGH2 by PGD synthase. Relative to PGE2 levels, PGD2 represents a much smaller proportion of the total prostaglandin pool than PGE2. For example, in macrophages, PGE2 constitutes 63% of total prostaglandins produced whereas PGD2 represents only 6% [201]. Important aspects of sleep regulation, platelet aggregation, inflammation, smooth muscle contraction, and bronchoconstriction are mediated by PGD2 [202, 203]. PGD2 is non-electrophilic in nature. Moreover, it is relatively unstable under physiologic conditions and can be non-enzymatically converted to the electrophilic lipid 15d-PGJ2 [27].

COX inhibitors have been shown to have clinical efficacy in the treatment of cancer [204]; however, recent concerns regarding the adverse side effects of this class of drugs including increased stroke and myocardial infarction in patients with cardiovascular risk factors has limited their use [205, 206]. New therapies are under development to modulate PGE2 levels and biological action downstream of COX-2. This may be achieved by inhibiting PGE synthase activity, stimulating 15-PGDH activity, or inhibiting binding of PGE2 to the E-prostanoid receptors EP1-4 (reviewed in [207]).

Interestingly, in multiple cancers, prostaglandin metabolism has been shown to be shunted towards increasing PGE2 levels. Mehrotra et al. determined that PGE synthase
Multiple enzymatic pathways modulate the levels of electrophilic prostaglandins. These pathways may also be potential targets for therapeutic modulation of levels of endogenous electrophiles (denoted as starbursts). Inhibition of cyclo-oxygenase 2 (COX-2; \(a\)) has been shown to be efficacious in cancer treatment; however, significant cardiovascular side effects have been reported. Inhibition of PGE synthase (\(b\)), blockade of PGE\(_2\) receptors (EP-14, \(c\)), and stimulation of detoxification of PGE\(_2\) through the enzyme 15-hydroxyprostaglandin dehydrogenase may inhibit the pro-tumorigenic effects of PGE\(_2\). Stimulation of PGD synthase (\(e\)) may increase the levels of the endogenous electrophile 15d-PGJ\(_2\). Some evidence also indicates that 15d-PGJ\(_2\) can stimulate the its own production through increased COX-2 activity which does not result in increases in PGE\(_2\) levels (\(f\)).
was upregulated in nearly 80% of breast cancer cell lines assessed [208]. It has also been reported that the PGE\textsubscript{2} detoxification enzyme, 15-PGDH, is epigenetically silenced through a mechanism involving an interaction of histone deacetylases with the 15-PGDH promoter in colorectal cancer cell lines and patient tissues. HDAC inhibitors were effective in stimulating re-expression of 15-PGDH \textit{in vitro} [31]. Finally, EP4 antagonists are commercially available and have been shown to block some aspects of PGE\textsubscript{2} signaling in macrophages [209]. Taken together, these results suggest that compounds directed towards enzymes which modulate PGE\textsubscript{2} levels may be relevant therapeutic targets. Moreover, these types of strategies may also limit adverse vascular effects since PGE synthase expression is not detectable in endothelial cells [208].

Evidence also exists which suggests that redirecting prostaglandin synthesis towards PGD\textsubscript{2} may have anti-tumorigenic effects. Loss of PGD synthase has been shown to be a key molecular event in the transition of low-grade astrocytoma to higher grade anaplastic tumors [210]. Moreover, PGD synthase expression from infiltrating macrophages or stromal cells suppresses tumor formation in intestinal adenoma [32] and prostate cancer models [211] respectively. Finally, 15d-PGJ\textsubscript{2} production downstream of PGD synthase inhibits PGE\textsubscript{2} synthesis [212] and plays a decisive role in chemotherapeutic-induced apoptosis [30].

Summarily, based on these reports, the balance of electrophilic and non-electrophilic prostaglandins seems to be tightly regulated. Shunting of prostaglandin synthesis towards increasing PGE\textsubscript{2} levels by increasing synthesis, decreasing detoxification, and limiting diversion of prostaglandin precursors towards other pathways appears to play an important role in promoting tumorigenic progression. Synthesis of prostaglandins
the D- and J-series is also actively inhibited during tumorigenesis. This implies that redirecting prostaglandin synthesis towards PGD synthase metabolites may provide additional means to modulate endogenous electrophile levels for therapeutic benefit.

CONCLUSIONS

In this thesis, we have been able to demonstrate multiple mechanisms by which lipid electrophiles regulate redox signaling in breast cancer. We have developed new strategies to regulate cancer cell behavior in response to lipid electrophiles, and using multiple approaches, we have defined the effects of a model electrophilic lipid, 15d-PGJ2, on cell death, induction of intracellular antioxidants, and the ability to regulate processes important for metastasis both *in vitro* and *in vivo*. We have shown that intracellular targeting of an electrophile enhances its anti-apoptotic activity and that the concentration-dependence of responses to electrophiles can be exploited to inhibit metastasis while limiting undesirable toxicity. Examination of the concentration-dependence of the responses to electrophiles has also allowed us to further define the relative sensitivity of targets of 15d-PGJ2 (shown schematically in Figure 6-5). Finally, by comparing the biological responses to 15d-PGJ2 to a structurally related lipid electrophile, PGA1, we have defined responses which are relatively specific for 15d-PGJ2. In addition, we propose that further examination of the role of electrophiles derived either endogenously or through the diet may also have therapeutic benefit in the treatment of breast cancer metastasis. Taken together, our work now provides a basis for strategies to fine-tune the biological response to electrophiles and better understanding of the role of redox signaling by lipid electrophiles in the regulation of breast cancer metastasis.
Figure 6-5: Defining the electrophile responsive proteome. Examination of the concentration-dependent effects of 15d-PGJ$_2$ in this thesis along with integration of known responses to 15d-PGJ$_2$ described in the literature, the relative sensitivity to modification is shown schematically on the bull’s-eye. The most sensitive protein targets are shown in the center and the less sensitive targets are shown on the outside of the bull’s-eye. The concentration dependence of the biological responses is also shown (italicized text).
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