Therapeutic Potential of Electrophilic Nitro-Oleic Acid for The Treatment of Triple-Negative Breast Cancer

by

Chen-Shan Woodcock

B.A., Chinese Culture University, 2000

M.S., National Taiwan University, 2003

Submitted to the Graduate Faculty of

The University of Pittsburgh School of Medicine

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Molecular Pharmacology

University of Pittsburgh

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Chen-Shan Woodcock

It was defended on

December 11, 2017

and approved by

Committee Chair: Steffi Oesterreich, PhD, Professor, Pharmacology & Chemical Biology

Nancy Davidson, MD, Professor, Head, Division of Medical Oncology, University of Washington

Yi Huang, MD, PhD, Assistant Professor, Pharmacology & Chemical Biology

Carola Neumann, MD, Associate Professor, Pharmacology & Chemical Biology

Abdolreza Zarnegar, PhD, Professor, Pathology

Dissertation Advisor: Bruce Freeman, PhD, Professor, Chair, Pharmacology & Chemical Biology

Therapeutic Potential of Electrophilic Nitro-Oleic Acid for The Treatment of Triple-Negative Breast Cancer

Chen-Shan Woodcock, PhD

University of Pittsburgh, 2017

Triple negative breast cancer (TNBC) comprises ~20% of all breast cancers and is the most aggressive breast cancer subtype. Devoid of expression of the estrogen and progesterone receptors, along with the receptor tyrosine kinase ERB2 (HER2) that defines most mammary cancers, there are no targeted therapies for patients with TNBC. This, combined with a high metastatic rate and a lower 5-year survival rate than for other breast cancer phenotypes, means there is significant unmet need for new therapeutic strategies.

Herein, the anti-neoplastic effects of the electrophilic fatty acid nitroalkene derivative, 10-nitro-octadec-9-enoic acid (nitro-oleic acid, NO_2-OA), were investigated in multiple preclinical models of TNBC. NO_2-OA reduced TNBC cell growth and viability *in vitro*, attenuated TNFα-induced TNBC cell migration and invasion and inhibited the tumor growth of MDA-MB-231 TNBC cell xenografts in the mammary fat pads of female nude mice. The upregulation of these aggressive tumor cell growth, migration and invasion phenotypes is mediated in part by the constitutive activation of proinflammatory nuclear factor kappa B (NF-κB) signaling in TNBC. NO_2-OA inhibited TNFα-induced NF-κB transcriptional activity in human TNBC cells and suppressed downstream NF-κB target gene expression, including the metastasis-related proteins intercellular adhesion molecule-1 (ICAM-1) and urokinase-type plasminogen activator (uPA).

The mechanisms accounting for NF-κB signaling inhibition by NO_2-OA in TNBC cells were multifaceted, as NO_2-OA a) inhibited the inhibitor of NF-κB subunit kinase β phosphorylation and downstream inhibitor of NF-κB degradation, b) alkylated the NF-κB RelA protein to prevent DNA binding and c) promoted RelA polyubiquitination and proteasomal degradation. Comparisons with nontumorigenic human breast epithelial MCF-10A cells revealed that NO_2-OA more selectively inhibited TNBC function. This was attributed to greater extents of multi-drug resistance protein-1 (MRP1) expression and greater MRP1-mediated efflux of NO_2-OA glutathione conjugates in MCF-10A cells. Blocking MRP1 transporting activity by inhibitor probenecid sensitized MCF-10A cells to NO_2-OA similar to its effect on TNBC cells, indicating that MRP1-mediated efflux can profoundly attenuate the NO_2-OA biological activity in MCF-10A cells. This dissertation study will be an important illustration of how NO_2-OA inhibits TNBC tumor development and its elucidation may have significant impact for the improvement of pharmacological therapies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

Acknowledgments

I would like to thank my advisor, Dr. Bruce Freeman, for his guidance through the years of my training. His mentorship has helped me develop not only as a scientist, but also as a human being. With his positive attitude he leads and demonstrates the importance of humility, honesty, and integrity in both scientific research and life in general.

I would also like to give a special “thank you” to my committee members: Dr. Nancy Davidson, Dr. Steffi Oesterreich, Dr. Yi Huang, and Dr. Carola Neumann, who all welcomed me into their lab groups and were instrumental in cultivating my understanding of the root causes and treatment of breast cancer. You all always helped me understand the broader picture of how my research fits into the wider body of scientific knowledge. Dr. Abdolreza Zarnegar always gave me his valuable advice and insightful discussions that greatly contributed to my research. I would like to sincerely thank Dr. Adrian Lee for his thoughtful suggestions and important feedback for my study. I also would like to thank program director Dr. Patrick Pagano and graduate program coordinator Shannon Granahan for their unending support and assistance through every major milestone in the molecular pharmacology program.

I would like to thank Dr. John Skoko, Dr. Ye Qin, and Dr. Chunyu Cao (WCRC) for kindly helping with the xenograft mice experiments and Dr. Rohit Bhargava (MWRI) for helping with pathology examination. I also like to thank Dr. Eugenia Cifuentes-Pagano and Dr. Yao Li

(VMI) for help with the Amplex Red H_2O_2 measurement assay. I especially would like to thank Dr. Stacy Wendell for mentoring the MRP1 project and Dr. Steven Woodcock for generating all nitro-lipids for my study. I would like to thank Franca Golin-Bisello and Bhupinder Singh for help with western blotting and q-RT-PCR and thank Sonia Salvatore for help with mass spectrometry analysis.

Finally I would like to acknowledge the past and present members of Freeman lab who I have had the pleasure to work with: Dr. Alison Groeger, Dr. Meghan Greenwood, Dr. Tanja Rudolph, Dr. Volker Rudolph, Dr. Gustavo Bonacci, Dr. Francisco Schopfer, Dr. Paul Baker, Dr. Marco Fazzari, Dr. Jeffrey Koenitzer, Dr. Nicholas Khoo, Dr. Dario Vitturi, Dr. Lihua Li, Hajira Hotiana, Gregory Buchan, Soma Jobbagy, and Crystal Uvalle. I thank you all!

To my parents, Yong-Da and Shu-Huei, who taught me to follow my dreams and the work ethic required to achieve them. Further, to my brother King, my husband Steven, and cutest daughter Nora, whose love and support made this possible.

1.0 INTRODUCTION

1.1 INFLAMMATION AND CANCER

The inflammatory response is a complex physiological host-defense system. In order to clear foreign intruders, inflammation is important for the turnover and repair of damaged tissues. However, sometimes it goes awry and becomes a major factor in the pathogenesis of many chronic human diseases, such as cancer.

The role of inflammation in the development of cancer was observed as early as 1863 by Rudolf Virchow, who identified leukocytes in neoplastic tissues and made a connection between inflammation and cancer. He suggested that the " lymphoreticular infiltrate" reflected the origin of cancer at sites of chronic irritation [1]. Today, the link between inflammation and cancer is widely understood. Various types of immune and inflammatory cells are frequently present within tumors. Immune cells affect malignant cells through production of cytokines, chemokines, and growth factors, which mediate signaling pathways such as nuclear factor-κB (NF-κB) to promote cell proliferation, avoid apoptosis, induce proangiogenic formation, activate extracellular matrix-modifying enzymes responsible for epithelial-mesenchymal transition (EMT), increase genome instability, and reprogram energy metabolism evasion [2, 3]. All together, inflammation provides a supportive environment to foster tumor development and progression [4].

1.1.1 Cytokine Tumor Necrosis Factor α (TNFα)

TNFα is a soluble 17 kDa (157 amino acids) that binds as a homotrimer to two distinct homotrimeric receptors on the cell surface: TNFRI (p55 receptor) and TNFRII (p75 receptor) [5]. TNFα is synthesized as a 26 kDa (233 amino acids) membrane-bound propeptide (pro-TNFα) and is secreted after cleavage by TNFα-converting enzyme (TACE) [6, 7]. Pro-TNFα is also functional and binds to TNFRII *via* direct cell-to-cell contact. TNFα is mainly produced by macrophages, but can also be made by various other cells including fibroblasts, astrocytes, smooth muscle cells, keratinocytes, and tumor cells [6].

Evidence for a role of TNFα in human cancer has been provided by several clinical studies [7-10]. To date, TNFα expression has been confirmed in the tumor microenvironment in the following malignancies: breast, ovarian, colorectal, prostate, bladder, renal cell cancer, melanoma, and lymphomas, and leukemia [7].

1.1.2 TNFα Signaling

The TNFα signaling cascade is complex and involves many adaptor proteins, which are recruited after binding of the TNFα ligand to its receptor. Figure 1 summarizes the main elements of the TNFRI signaling pathway; in particular, pathways implicated in inflammation and carcinogenesis

TNFRI is ubiquitously expressed on mammalian cells and regulates various biological functions after TNFα stimulation. Recruitment of TNFα to TNFRI results in dissociation of the

silencer of death domain protein (SODD), whose function is to repress signaling by blocking the binding of adaptor proteins to the death domain of TNFRI. Release of SODD permits the assembly of an activated signaling complex at the death domain of TNFRI, consisting of TNFR-associated death domain (TRADD), TNFR-associated factor 2 (TRAF2), receptor-interacting protein (RIP), and FAS-associated death domain (FADD). These molecules regulate at least four distinct pathways: (1) a proapoptotic pathway that is induced by binding caspase-8 to FADD; (2) an antiapoptotic pathway that is activated by the binding of the cellular inhibitor of apoptosis protein 1 (cIAP-1) to TRAF2; (3) Activator protein 1 (AP1) activation mediated through TRAF2 *via* a c-Jun N-terminal kinases (JNK)-dependent kinase cascade; and (4) NF-κB by RIP [11].

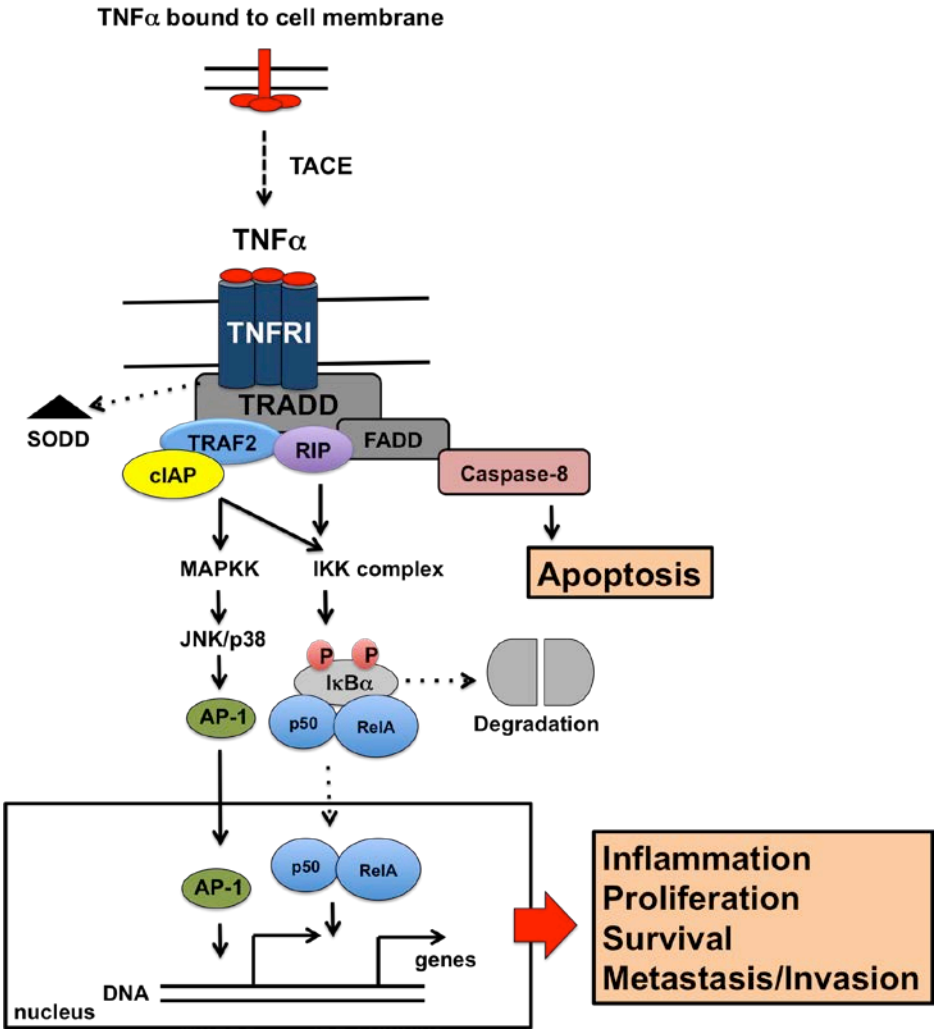


Figure 1. TNFα binds to TNFRI activating several pathways associated with inflammation and tumorigenesis. (Solid arrow: activation; Dash arrow: disassociation)

1.1.3 Role of TNFα Signaling in Cancer Progression

TNFα is a double-edged sword for tumors. There is evidence that it induces tumor necrosis and apoptosis, as its name implies. High concentrations of TNFα can provoke an antitumoral

response in murine and human models of sarcoma [12, 13]. In contrast, low-dose, chronic TNFα production is a feature of many tumor cells, which promotes tumor growth, invasion, and metastasis.Second paragraph.

TNFα acts as growth factor in many tumor types, thereby increasing concentrations of positive cell-cycle regulators and decreasing levels of cyclin-dependent kinase (CDK) inhibitors [6]. In addition, TNFα is a key molecule in the promotion of angiogenesis through the stimulation of endothelial cell proliferation and the enhancement of other pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) [14-16]. TNFα induces the expression of adhesion molecules involved in the invasion of metastatic tumor cells. TNFα promotes further tumor remodeling by stimulating fibroblast and macrophage activity, tumor cell mobility *via* the induction of matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) [17-19]. MMPs and uPA are mutually related to carcinoma progression through adjacent extracellular degradation [20].

A recent study by Hageman and colleagues has shown that non-contact co-culture of ovarian and breast cancer cells with macrophages increases the invasiveness of tumor cells *via* TNFα-dependent activation of JNK and NF-κB pathways [21]. Elevated plasma levels of TNFα have been detected in many malignancies and are often associated with poor prognoses [7-9]. In aggregate, cytokine TNFα plays a crucial role in tumor promotion, and the regulation of NF-κB by TNFα has been shown to be a fundamental signaling pathway of carcinogenesis [22].

1.1.4 Nuclear Factor-κB (NF-κB)

NF-κB is a ubiquitous transcription factor that is regulated by various stimuli, including growth factors, inflammatory mediators (cytokines), cytotoxic agents as chemotherapeutic drugs, and many others. It is a crucial regulator of fundamental cell functions such as proliferation, survival, and mobility [2, 23]. NF-κB is composed of various combinations of the five family members, p50, p52, RelA (p65), RelB, and c-Rel [24]. The most common form of NF-κB is a dimer of RelA and p50, and this dimer is often referred to simply as NF-κB. All Rel proteins share the NH_2-terminal Rel homology domain that mediates dimerization, DNA binding, and nuclear localization and interaction with the inhibitors of NF-κB (IκB). The RelA, c-Rel, and RelB proteins possess COOH-terminal transactivation domains that allow for transcriptional activation of target genes. The binding of Rel proteins with IκBα masks a nuclear translocation signal and effectively sequesters Rel proteins in the cytoplasm where they are inactive [25].

NF-κB activation requires phosphorylation-dependent degradation of IκBα proteins, which retain NF-κB in the cytoplasm. This step is mediated by the IκB kinase (IKK) complex, consisting of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ, also named NEMO), which is activated by RIP. Through this cascade pathway, inflammatory cytokines including TNFα target IκBα for proteasomal ubiquitination, which in turn, stimulates translocation of NF-κB to the nucleus. Within the nucleus, NF-κB directly binds to its promoter-binding element to induce genes encoding pro-survival, pro-proliferative, and metastatic molecules that support cancer development and progression (Figure 1) [26]. Importantly, abnormalities in this cascade have been reported in several malignancies, which suggests that NF-κB plays a key role in cancer development and progression [2, 23].

1.1.5 Amplification of *rel/nfκb* Genes and Aberrant NF-κB Activity in Cancer

A potential role for NF-κB in oncogenesis was evident upon discovery of the retroviral oncogene *v-Rel* as the homolog of the gene encoding c-Rel, one of the NF-κB subunits [27]. The genes encoding c-Rel, NF-κB2 (p100/p52), and Bcl-3 are amplified and/or rearranged in many human cancers. For example, mutations in the gene encoding IκBα have been detected in Hodgkin's lymphoma and are suggested to contribute to the constitutively active NF-κB in Hodgkin's cells [28].

In addition, many human-derived, solid-tumor cell lines have displayed increased nuclear NF-κB (p50-RelA heterodimer) levels and activity [27]. *rela* gene amplification has been detected in squamous carcinomas of the head and neck, and adenocarcinomas of the breast and stomach [29]. An increase in *rela* gene expression was also reported in thyroid carcinoma cell lines [30]. Constitutive activation of NF-κB factors is also emerging as a hallmark of various types of solid tumors, including breast [31-33], ovarian [31, 34], colon [31], pancreatic [35], thyroid [30], bladder [36], and prostate carcinomas [37, 38], as well as in melanomas [39].

NF-κB activity is generally associated with the growth and survival of the tumor cells [2, 27]. Relative to other aspects of oncogenic function, NF-κB is known to activate the expression of genes that contribute to metastasis and invasion. Such genes include MMPs, uPA and cell adhesion molecules such as ICAM-1 [40]. uPA is significantly increased in most breast cancer cell lines that contain constitutively active NF-κB, it is required for intravasation, and is associated with poor prognosis [41]. This observation supports a role for NF-κB in metastasis.

1.1.6 Anti-NF-κB Therapy for Cancer

As described above, NF-κB plays key roles in the inflammatory response, inhibition of apoptosis, cell proliferation, and metastasis. NF-κB is activated in certain cancers and in response to chemotherapy and radiation [26, 40]. Recent findings supported by *in vitro* and xenograft models of cancer implicate NF-κB inhibition as an important approach for the treatment of certain hematological malignancies and as an adjuvant approach in combination with chemotherapy or radiation for a variety of cancers [42]. Potent inhibitors of NF-κB have been studied and developed for cancer treatment.

The molecular cascade of signaling events provides several steps for specific inhibition of NF-κB activity and function [43], and several therapeutic strategies have been developed to target these signaling molecules (Figure 2). One strategy is interference with cytoplasmic IKK activation and IκB degradation using IKK inhibitors and a proteasome inhibitor, respectively. Another is blocking NF-κB translocation to the nucleus or DNA binding capacity by thiol-reactive agents such as 15d-PGJ_2 [44] and dimethyl fumarate (DMF) [45].

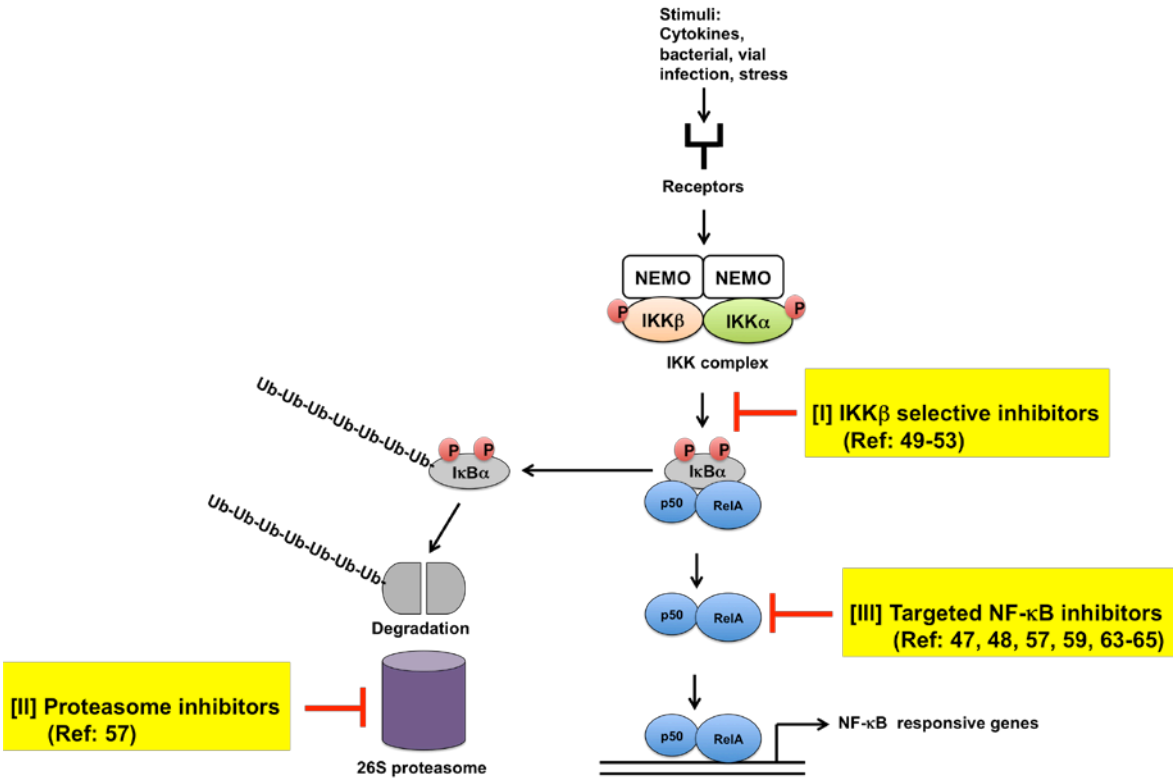


Figure 2. The mechanism of action of inhibitors of the NF-κB signal pathway.

There are three main therapeutic strategies aimed at blocking NF-κB activity. [I] Inhibition of IKK kinase activity. Drugs have the ability to specifically inhibit IKK, thereby preventing phosphorylation of IκBα. [II] Inhibition of protease activity. Drugs such as PS-341 and lactacystin specifically inhibit 26S proteasome complex, thereby preventing IκBα degradation. [III] Inhibition of nuclear translocation and DNA binding. Drugs specifically prevent NF-κB subunits RelA and p50 from entering the nucleus or have the ability to prevent the NF-κB subunit from binding with target genes, therefore inhibiting transcription. The classes of inhibitors that block distinct steps in this pathway are indicated by the red bars and yellow boxes.

1.1.6.1 Selective IKKβ Inhibitors

Three types of IKK inhibitors have been developed: ATP analogs, compounds with an allosteric effect on IKK structure, and compounds that interact with a specific cysteine residue (Cys179) in the activation loop of IKKβ. PS-1145 (Aventis Pharma) was developed from a β-carboline natural product that inhibited several different kinases and has been extensively evaluated in various *in vitro* assays by different groups [46, 47]. Preclinical studies report that PS-1145 inhibits the IKK complex with an IC_{50} of 150 nM, blocking TNFα-induced IκB phosphorylation and degradation in HeLa cells [46]. PS-1145 was also shown to interfere with NF-κB activation, abrogate cytokine production and secretion, and inhibit cell proliferation of multiple myeloma cells [47].

Another well-studied synthetic molecule BMS-345541 (Bristol-Myers Squibb Co.) binds to an allosteric site on both IKKα and IKKβ, but shows an approximately 10-fold greater inhibitory effect on IKKβ [48]. In addition, thiol-reactive compounds such as parthenolide [49] and 15d-PGJ_2 [50] can block IKKβ activity through a direct conjugation to the thiol group of the Cys179 residue. It is likely that interaction of these compounds with Cys179 interferes with phosphorylation-induced activation of IKKβ, as Cys179 is located between Ser177 and Ser181, which are part of the kinase activation loop and required for activation of IKKβ in response to many upstream signals, such as TNFα and LPS [51, 52].

1.1.6.2 Proteasome Inhibitors

The common steps before NF-κB leaves the cytoplasm are the ubiquitination of IκBα by the SCF-β-TrCP ubiquitin ligase complex followed by the rapid degradation of ubiquitinated IκBα by the 26S proteasome [52, 53]. Thus, proteasome inhibitors suppress activation of NF-κB by

stabilizing IκBα. Bortezomib (Velcade; Millenium), has entered clinical development for the treatment of myeloma [54]. However, it is not entirely clear whether its anti-myeloma effects are mediated entirely through inhibition of NF-κB since bortezomib: (a) does not directly target NF-κB and in turn, may affect other cancer-related proteins; and (b) also has NF-κB-independent effects on cancer cell growth [47, 53, 55].

1.1.6.3 Inhibitors Targeting the NF-κB (p50/RelA)

A number of thiol reactive electrophiles/molecules have anti-inflammatory activity and act as inhibitors of NF-κB DNA binding. Cyclopentenone prostaglandin derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is naturally occurring prostaglandin metabolites [56]. This molecule is synthesized during the late phase of the inflammatory response and is a key regulator in the resolution of inflammation [56]. Cysteine residues in the DNA-binding domain of p50 (Cys62) and RelA (Cys38) [57] are targeted by 15d-PGJ_2 [44]. Several studies show that 15d-PGJ_2 has anti-cancer effects on drug-resistant ovarian [58] and breast [59] cancer cells.

Similar to 15d-PGJ_2, the natural products sesquiterpene lactone parthenolide from the plant feverfew and dithiolethione from dietary vegetables can also directly inhibit NF-κB DNA binding primarily through interaction with Cys38 in RelA [60-62]. Moreover, an anti-inflammatory drug dimethyl fumarate (DMF) is already in clinical use for multiple sclerosis. In the preclinical breast cancer model, DMF inhibited mammosphere formation, cell proliferation and xenograft tumor growth of triple-negative breast cancer *via* covalently modifying the RelA protein on Cys38 to block its nuclear translocation and DNA binding activity [45]. Overall, these inhibitors have been primarily characterized as suppressing NF-κB-mediated inflammatory pathways, which may be linked to their anti-cancer therapeutic potential.

1.2 REDOX-DERIVED ELECTROPHILIC FATTY ACIDS

The broad definition of an electrophile is a molecule having one or more electron-withdrawing atoms or groups, which accepts an electron pair from electron-rich donor molecules (nucleophiles) to form a covalent bond *via* Michael addition. These Lewis acids include cations (Hg^{2+} and Zn^{2+}), polarized neutral molecules (iodoacetamide), polarizable neutral molecules (Cl_2 and Br_2), and some oxidizing agents.

In the late 1970s, enzymatic oxygenations of unsaturated fatty acids from autocrine and paracrine signaling mediators were discovered [63]. Soon thereafter, nitric oxide (•NO) was described as a mediator of vascular relaxation *via* heme-iron coordination and the activation of guanylate cyclase (sGC) [64]. It was appreciated that free radical NO also serves as a lipophilic mediator which impacts lipid oxidation *via* the generation of secondary oxidizing and nitrating species [65-69]. The oxidation and nitration of unsaturated fatty acids to electrophilic products has been an emerging area of cell and organ regulation, because these redox-derived electrophilic fatty acids can directly modify protein molecules, leading to modulation of cellular signaling and organism function.

A fundamental characteristic of α,β-unsaturated carbonyl and nitroalkene derivatives (Figure 3) is a soft electrophilic nature, which restricts the breadth of preferred molecular targets in cell and tissue compartments. In a biological context, electrophiles react preferentially and reversibly with biological nucleophiles (i.e. cysteine thiols on proteins) by Michael addition [65]. This reversible reactivity implicates electrophiles as important molecular sensors with signaling capabilities that inform the cell of its redox or stress status.

Figure 3. The β-carbon of an α,β-unsaturated carbonyl and nitroalkene are electrophilic.

The chemical structures of an α,β-unsaturated carbonyl (left) and a nitroalkene (right) are presented above. Asterisk indicates an electrophilic β-carbon.

1.2.1 Introduction to Polyunsaturated Fatty Acids (PUFAs)

Fatty acids (FAs) have a variable length carbon chain with a methyl terminus and a carboxylic acid head group. They can be categorized based on the number and degree of unsaturated carbons. Saturated FAs contain the maximal number of hydrogen atoms, while mono- and poly-unsaturated FAs (PUFAs) have one, two or more double bonds. PUFAs can be further subdivided on the basis of the position of the first double bond relative to the methyl terminus of the chain. For example, n-3 and n-6 FAs are two of the most biologically significant PUFA classes, with their first double bond on either the third or sixth carbon from the methyl chain terminus, respectively. The final carbon in the FA chain is also known as the omega carbon; hence, the common reference to these FAs as ω-3 or ω-6 PUFAs [70].

First paragraph.

1.2.2 Electrophilic Fatty Acids

First paragraph. Two essential FAs, α-linolenic acid (ALA) and linoleic acid (LA), cannot be made by the body and must be obtained through dietary sources. Humans have the capacity to metabolize essential FAs to long chain ω-3 or ω-6 PUFAs [71]. PUFAs can be oxidized or nitrated through enzymatic or non-enzymatic reactions. PUFA oxidation and nitration of at least one of the alkenes (the intrinsic carbon-carbon double bond) results in the formation of an electrophilic fatty acid species. These species are potentially reactive with available biological nucleophiles, such as GSH, protein thiols or imidazolyl (His) residues, *via* the resulting electron-deficient alkene or diene conjugated to a powerful electron-withdrawing group [65, 72].

1.2.3 Redox-derived PUFA Oxidation and Nitration

With multiple unsaturated bonds, PUFAs are susceptible to oxidation, which is categorized into non-enzymatic oxidation and enzymatic oxidation. Non-enzymatic oxidation can be divided into autoxidation (mediated by free radicals) and photooxidation (mediated by ultraviolet light or singlet oxygen). In cells, several types of enzymes including Cyclooxygenases (COXs), Lipoxygenases (LOXs) and Cytochromes (CYPs) are able to specifically oxidize PUFAs to generate various metabolites [73, 74]. The following section will discuss electrophilic PUFA generation *via* autoxidation (Figure 4).

In autoxidation, the reaction is mediated by free radicals (i.e., O_2, O_2^- and $\bullet NO_2$, etc.), which give rise to a lipid hydroperoxide as the primary oxidation product. In many cases, hydroperoxides can be further oxidized to ketones (and ultimately malondialdehyde) [75].

Autoxidation of PUFAs is initiated by hydrogen abstraction from an allylic methylene carbon along the lipid backbone, creating a newly formed radical with an unpaired electron (R•). Specifically, bisallylic hydrogens have a low bond dissociation energy that makes abstraction particularly favorable, and the radical products are resonance stabilized. During the propagation step, molecular oxygen (O_2) adds to a carbon-centered radical to form a peroxyl radical (ROO•). This peroxyl radical (ROO•) can itself be reduced to a hydroperoxide (ROOH) by propagating the reaction by abstracting a hydrogen atom (proton) from an adjacent PUFA; or be prone to oxidation (peroxyl radical abstraction of another hydrogen radical) to yield an electrophilic α,β-unsaturated carbonyl (R=O) [76, 77].

R• and ROO• may also react with other radical species such as •NO_2 to form nitrated fatty acids (NO_2-FA) [78]. Nitration of PUFAs (NO_2 addition) generates double bonds with electrophilic character due to the strong electron-withdrawing activity of the nitro group. The resulting NO_2-FA are themselves strongly electrophilic [79]. Overall, fatty acid-derived electrophiles can be grouped into two categories: α,β-unsaturated carbonyl and NO_2-FA.

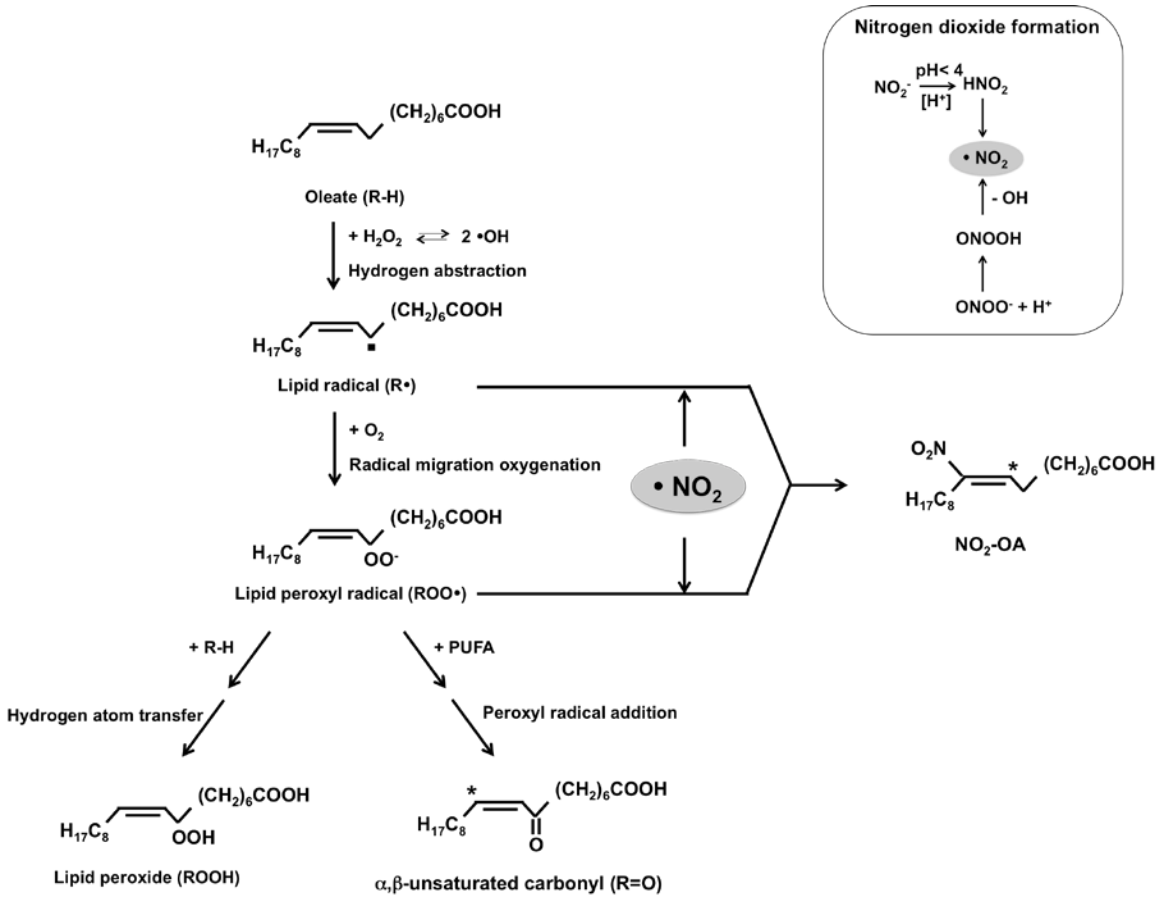


Figure 4. Redox-derived oxidation and nitration of oleic acid.

Asterisk indicates an electrophilic β-carbon. NO_2-OA: nitro-oleic acid (10-nitro-octadec-9-enoic acid)

1.2.4 Interaction of Electrophiles with Nucleophiles

Arthur Michael first reported in 1887 that olefins (also known as alkenes) conjugated with electron-withdrawing groups are susceptible to attack by nucleophiles [80, 81]. This type of reaction, named after Michael, leads to the terms Michael acceptor (electrophiles), Michael

donor (nucleophiles), and Michael addition reaction (general term for electrophile-nucleophile reaction).

According to the hard and soft acid and base (HSAB) concept of Pearson [82], hard electrophiles (e.g., formaldehyde) prefer hard molecule nucleophiles such as amino groups, whereas soft electrophiles (e.g., α,β-unsaturated carbonyl) prefer to react with soft nucleophiles (e.g. thiolate anions, $R\text{-}S^-$) [83, 84]. Soft electrophiles are endogenously generated during inflammation [84]. Soft in this context denotes a soft acid (or base), which is defined as a species having a more diffuse charge density (relying on more ionization and polarization of the outer shell). This perspective focuses on the soft-soft reaction of the electrophilic fatty acid modification of thiol groups by α,β-unsaturated carbonyls and nitroalkenes. Figure 5 is an illustration of a nitrated fatty acid engaging in cell signaling *via* Michael addition reactions with cellular nucleophiles.

The major factor that determines the rate of a Michael addition is the pK_a of the thiol involved. Michael addition is favored by deprotonation of the thiol (R-SH) to the more nucleophilic thiolate anion ($R\text{-}S^-$). In the case of both free cysteines and those cysteines incorporated into proteins, their pK_as are generally around 8.5 [85], indicating a low proportion of reactive thiolates and subsequently mild reactivity at the typical intracellular pH of 7.2. However, the pK_a of cysteine thiols can be significantly lowered by proximity to basic amino acid residues such as lysine or arginine [86].

Protein structure can also affect electrophile association or dissociation with nucleophiles. Some electrophilic fatty acids have structural isomers. For example, nitro-oleic acid (NO_2-OA) has two positional isomers, 9-NO_2-OA and 10-NO_2-OA. 10-NO_2-OA has a

favorable reactivity with nuclear receptor PPARγ on Cys285. This phenomenon may be caused by protein structure [87].

The strength of an electrophile's electron-withdrawing group also partially determines the rate of the Michael addition reaction. As α,β-unsaturated carbonyl-derivatives of PUFAs, 15-deoxy-Δ-^{12,14}-prostaglandin J_2 (15d-PGJ_2) and prostaglandin A_2 (PGA_2) both have a second-order rate constant ~0.7 $M^{-1}s^{-1}$ for reaction with GSH [79]. NO_2-FA react with GSH at a rate constant ~183 $M^{-1}s^{-1}$ [79], which is almost 261 times faster than that of 15d-PGJ_2 or PGA_2. The overall reactivity of these electrophiles with nucleophilic biomolecules, such as cysteine residues of proteins, is thus dictated by these rates. Ultimately, the reaction rate of a particular electrophile will influence its ability to affect cell signaling by formation of adducts with cellular nucleophiles.

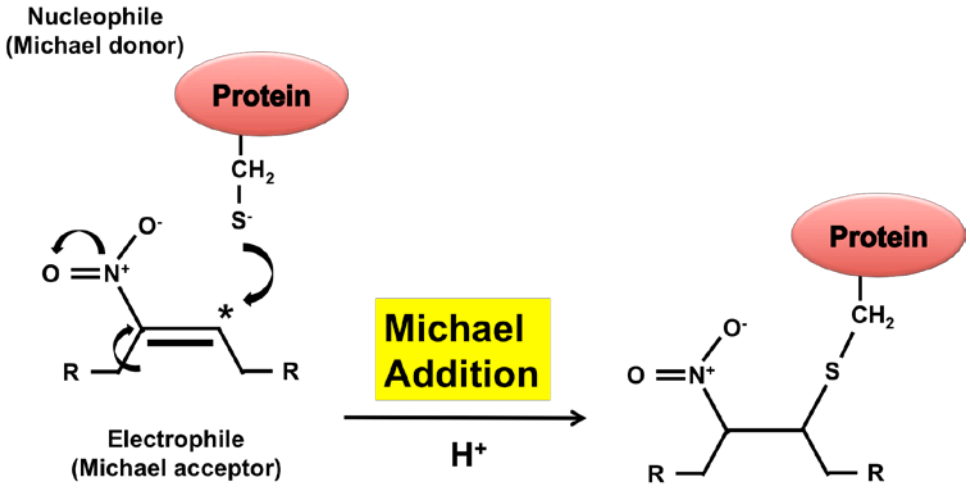


Figure 5. Post-translational modification of a protein by an electrophilic nitrated fatty acid *via* Michael addition.

The electron-withdrawing nitro-group reduced electron density at the carbon indicated by an asterisk. This carbon is subject to attack by a nucleophile (a cysteine thiolate in this example). The resulting Michael addition thus represents a covalent modification.

1.2.5 Electrophilic Fatty Acids as Signaling Mediators

Early appreciation of the occurrence and importance of biomolecule reactions with electrophiles came from studies of chemical carcinogenesis and mutations caused by electrophile adduction to DNA bases [88]. Miller et al. reported that electrophiles which can covalently modify proteins may be carcinogenic [89-91]. Electrophile generation and reactivity comprise an important aspect of cell adaption to environmental stress and metabolic stimuli. The toxicity of electrophiles are ameliorated by conjugation with GSH in the absence and presence of GSH S-transferases (GSTs; phase-II reaction) [92-94] and the resulting conjugates are excreted from cells by phase-III transporters [95] that are regulated by a transcription factor, NF-E2-related factor 2 (Nrf2) [96, 97].

Because electrophilic fatty acids are biological Michael acceptors, they may function by post-translational modification (PTM) of proteins and transcription factors. Multiple classes of lipid-derived electrophilic signaling molecules (PUFAs with α,β-unsaturated carbonyl or nitroalkene) are expected to have unique patterns of downstream signaling. Through the Michael addition reaction, electrophilic fatty acids may adduct susceptible protein cysteine, histidine, or lysine residues to alter protein structure, function, and subcellular distribution [65, 72, 98]. Thus, the potential targets for electrophilic fatty acid modification are diverse; they display pleiotropic effects on an array of key signaling pathways (Figure 6) and provide measureable (and many times beneficial) outcomes in the context of inflammation and other disease processes [66]. The following are key biological targets of electrophilic fatty acids that result in downstream signaling events.

1.2.5.1 Inhibition of NF-κB Activation

As we described in section 1.1.4, NF-κB regulates cell proliferation, survival, and a host of inflammatory responses from cytokines or chemokines [99]. Nitrated fatty acids (NO_2-FA) reduce NF-κB transcriptional activation though inhibition of DNA binding. Nitrolinoleic acid (NO_2-LA, a combination of 9-, 10-, 12-, and 13-nitro-9,12-*cis*-octadecadienoic acid isomers) and nitro-oleic acid (NO_2-OA, 9- and 10-nitro-9-*cis*-octadecenoic acid isomers) block cytokine response *via* PTM of the RelA (also called p65) subunit of NF-κB [100]. *In vivo*, NO_2-OA murine plasma concentrations of 25 nanomolar inhibit leukocyte recruitment to the vascular endothelium and reduce aortic expression of adhesion molecules [101].

15d-PGJ_2 inhibits NF-κB activation and subsequent upregulation of proinflammatory molecules *via* PTM of IKKβ, RelA, and the p50 subunit [44, 50]. More recent findings indicate that induction of RelA glutathionylation by 15d-PGJ_2 is another mechanism of action for 15d-PGJ_2 to inhibit NF-κB signaling [102].

In aggregate, these findings affirm that electrophilic fatty acids are potent NF-κB inhibitors that act at multiple steps within the signaling cascade; such inhibitors show potential for alleviating the proinflammatory response.

1.2.5.2 Activation of PPARγ Transcriptional Activation

Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor that mainly regulates lipid homeostasis and adipocyte differentiation. Importantly, PPARγ regulates the partitioning of lipid stores away from peripheral sites (i.e., liver and muscle) and reduces adipokine expression, thus facilitating increased insulin sensitivity [103].

Both NO_2-FA and α,β-unsaturated carbonyls are PPARγ ligands. In the case of NO_2-FA, NO_2-LA and NO_2-OA directly adduct the Cys285 residue in the PPARγ ligand-binding domain [87]. Direct evidence by a structural analysis showed that the nitro group of NO_2-LA specifically interacts with the Arg288 and Glu343 residues of PPARγ to facilitate ligation [104]. NO_2-OA (EC_{50}= 13 nM) is a more potent agonist for PPARγ than NO_2-LA (EC_{50}= 36 nM) [87]. Moreover, NO_2-OA promotes glucose uptake in adipocytes and also upregulates genes such as PPARγ2, adipocyte protein 1 (aP1), glucose transporter type 4 (Glut4), c-AMP response element binding protein (CREB) binding protein 1 (CBP-1), and PPARγ coactivator-1 (PGC-1), which are involved in adipogenesis and glucose uptake [87]. Unlike full PPARγ agonists such as the thiazolidinedione insulin sensitizer rosiglitazone, NO_2-LA and NO_2-OA act as partial agonists [105] and may reduce the risk of peripheral edema, weight gain, and adverse cardiovascular diseases.

15d-PGJ_2 is also a PPARγ ligand; however, it does not significantly promote activation as does NO_2-LA [106]. Nonetheless, 15d-PGJ_2 directly binds to PPARγ and induces differentiation of C3H10T1/2 fibroblasts to adipocytes [107]. Both 15d-PGJ_2 and NO_2-LA form intracellular conjugates with GSH, which removes 15d-PGJ_2 and NO_2-LA from the cell *via* efflux transporters, such as multidrug resistance-associated protein-1 (MRP1), thus inhibiting PPARγ-dependent transcription [108, 109] and suggesting a cell autonomous limitation mechanism.

1.2.5.3 Activation of Keap1/Nrf2 Pathway

The Keap1/Nrf2 pathway regulates the gene expression of antioxidant proteins, phase II xenobiotic metabolizing enzymes, and phase III transporters [96, 97]. Under basal conditions,

transcription factor Nrf2 undergoes rapid degradation by the ubiquitin-proteasome system, resulting in minimal Nrf2 levels in a variety of cells. During oxidative stress, Nrf2 is released, which is regulated by Kelch ECH associating protein 1 (Keap1), a substrate adaptor protein for a CUL3-based E3 ubiquitin ligase [110, 111]. When oxidants and/or electrophiles invade the cells, the reactive thiols of Keap1 undergo oxidative and covalent modifications, leading to release of Nrf2 [110, 112, 113]. As a result, the released Nrf2 translocates to the nucleus and (together with small Maf) forms a heterodimeric complex that binds to the antioxidant/electrophile responsive element (ARE/EpRE), upregulating its downstream genes (e.g., hemeoxygenase-1 (HO-1), glutamate cysteine ligase (GCL), GSTs, UDP-glucuronosyltransferases (UGTs), and MRPs, etc.) [96, 97, 111].

Keap1 contains a number of highly reactive cysteine residues that have potential to sense reactive electrophiles by forming covalent adducts. Some electrophiles such as sulforaphane [114], HNE [115], 15d-PGJ_2 [112, 116], and NO_2-FAs [117] covalently react with the cysteines in Keap1. Among these cysteine residues, Cys151, Cys273, and Cys288 have been identified as critical cysteines responsible for electrophile-dependent regulation of Keap1 [114, 115, 118]. Cys273 and Cys288 are located in the Keap1 intervening region (IVR; the linker region) and are required for maintaining Nrf2 turnover under basal conditions [114, 115]. By contrast, Cys151 is located in the BTB domain (a conserved protein-protein interaction domain) and is uniquely required for inhibition of Keap1-dependent degradation of Nrf2 by electrophiles and oxidative stress [114]. Because these particular thiols of Keap1 are sensitive to oxidative modification, the Keap1-Nrf2 pathway represents an important detoxification and cell protection mechanism.

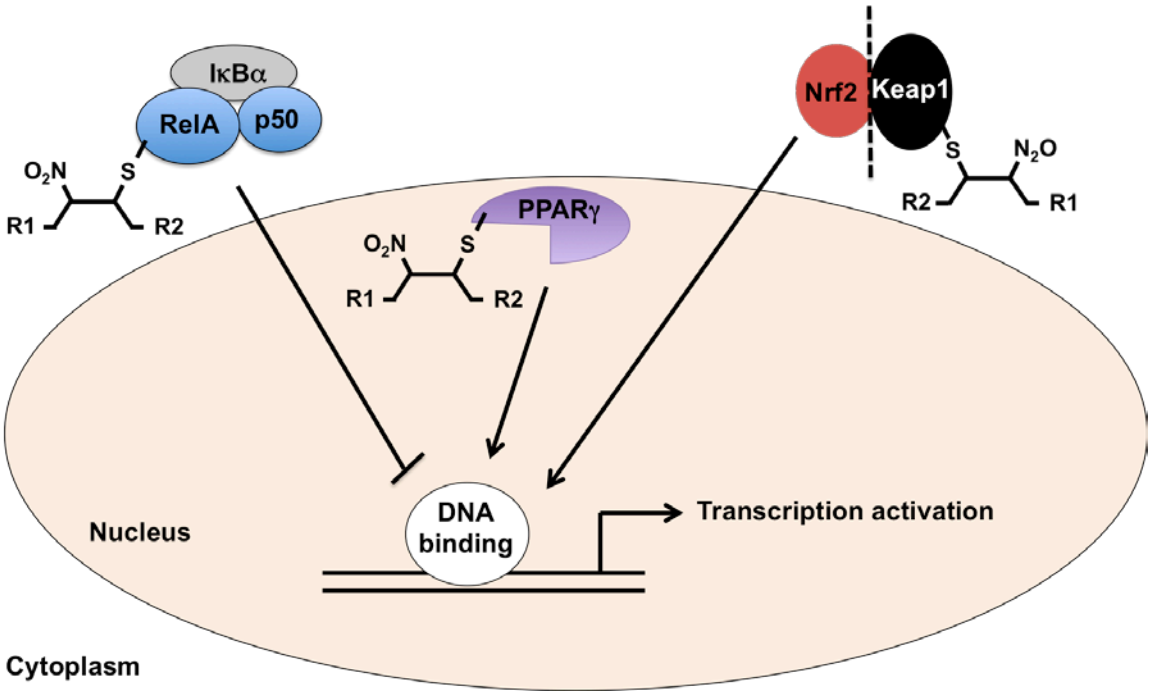


Figure 6. Transcription factors as targets of electrophilic fatty acid (eg. NO_2-FA) modification.

In the cytoplasm, NO_2-FA alkylate the RelA protein of NF-κB, sustaining inhibition by IκBα and blocking p50/RelA-dependent gene transcription. NO_2-FA covalently adduct Keap1, causing dissociation and translocation of Nrf2 to induce ARE gene transcription. In the nucleus, NO_2-FA may also bind and act as partial PPARγ agonists to stimulate gene transcription.

1.2.6 Reversibility of Electrophile-Protein Adducts

In terms of chemical bonding, the carbon-sulfur bond is stable, and thus covalent protein modifications mediated by electrophilies are potentially irreversible. For example, acrylamide is

a soft but weak electrophile of the type 2 alkene chemical class [119] that causes enzyme inhibition and toxicity by irreversibly undergoing Michael addition with Cys151 of GAPDH [120]. Nevertheless, there are several reports on the reversibility of electrophile-protein interactions. GSH conjugates of methyl isocyanate can easily be converted to other thiol conjugates [121], and alkylation of a GAPDH thiol by low concentrations of NO_2-OA is reversible in the presence of GSH [98]. Thus, the reversibility of electrophile-protein interactions may be regulated by transalkylation of GSH since GSH is the most abundant cellular reductant and serves as a cellular redox buffer system [122].

GSH is a cysteine-containing tripeptide (glytamine-cysteine-glycine) that is an evolutionarily conserved molecule employed by plants, animals, fungi and some prokaryotic cells to combat endogenously or exogenously generated oxidative insults [123]. GSH is present in various mammalian tissues at concentrations of more than 1 mM [124, 125], and large numbers of GSH adducts with electrophiles have been identified following exposure to the electrophiles themselves or to xenobiotics capable of forming electrophilic metabolites [93, 126]. GSH can also undergo an exchange reaction with a disulfide. Disulfide exchange reactions are kinetically slow; however, they are accelerated by enzymes such as glutaredoxins, thioredoxin, and sulfiredoxin [86]. The thiol group on the cysteine residue in GSH has a pK_a of 9.2 [127], indicating that GSH exists as a relatively unreactive thiol at physiological pH. However, the reactions of electrophiles with GSH are greatly facilitated by GSTs, which lower its apparent pK_a.

Some electrophiles (including electrophilic fatty acids) permanently and irreversibly modify target proteins, while others induce short half-life and reversible adduction of the target protein [128]. It has been appreciated that reversibly reactive electrophiles display less or no

cytotoxic effects at low concentrations. Moreover, the specific PTM reactions that occur *via* the reversibility of electrophiles may support the notion that these reactions function as a signaling event sensitive to cellular metabolic and redox status [129].

1.2.7 Electrophilic Fatty Acid Metabolism: MRP1-derived Export

It is well established that after xenobiotics are absorbed and distributed in the body, they are extensively biotransformed by cytochrome P450 (CYP) to form hydroxylated metabolites in cells (phase I reaction). Most of the hydroxylated metabolites are detoxification products of the parental substrates. However, some drugs and environmental chemicals undergo metabolic activation to electrophilic species by CYPs or other enzymes that cause tissue injury through covalent modification [130-132]. These hydroxylated and electrophilic metabolites undergo UGT- and GST-mediated conjugation reactions to glucuronides and GSH adducts (phase II reaction) [133]. Finally, these polar metabolites are recognized by ATP-dependent multidrug resistant proteins (MRP) and are excreted (phase III reaction) [134].

Multidrug resistant-associated protein 1 (MRP1/ABCC1) is a member of the ATP-binding cassette (ABC) transporter protein superfamily C [135]. MRP1 is ubiquitously expressed in most normal tissues and protects tissues by reducing the intracellular accumulation of xenobiotics and metabolites [136]. MRP1 is the glutathione S-conjugate (GS-X) pump for multivalent organic anions (such as drug metabolites) conjugated to GSH, glucuronate, or sulfate [137].

A number of lipid-derived signaling molecules are substrates of MRP1. GSH conjugates of the cyclopentenone class--prostaglandins (PGs), prostaglandin A2 (PGA2), and 15d-PGJ_2--are transported by MRP1 [108, 138]. These prostaglandin derivatives contain α,β-unsaturated

carbonyl groups, which enable them to covalently modify target proteins and have anti-proliferative and anti-inflammatory effects. However, both GSH conjugation and subsequent MRP1-mediated efflux of these derivatives also protects cells from the cytotoxic effects of abundant prostaglandin derivatives. 4-Hydroxynonenal (4-HNE) is another relatively stable α,β-unsaturated electrophile produced by the peroxidation of linoleic acid in biological membranes in tissues under oxidative stress [139, 140]. Because it can form adducts with DNA, proteins, and lipids, 4-HNE is genotoxic and cytotoxic, and is causally involved in several human pathologies including cardiac and neurodegenerative diseases as well as cancer [141]. Cellular 4-HNE levels are regulated through their conjugation with GSH. GSH-conjugated 4-HNE is an inhibitor of GST, among other biological activities, and must therefore be exported from cells by MRP1 [142]. In addition, the extracellular transport of GSH-conjugated NO_2-LA is mediated by MRP1, and this reaction mitigates the cellular activation of PPARγ signaling by NO_2-LA [109].

1.2.8 Nitrated Fatty Acids

Nitrated derivatives of unsaturated fatty acids (i.e. NO_2-FA) are formed endogenously upon oxidation of unsaturated fatty acids by oxides of nitrogen (NOx), such as nitrogen dioxide (NO_2), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$) (Figure 4). All NO-derived species can be produced from dietary consumption or oxidation of nitric oxide (NO) during inflammation [143]. Both multiple NO_2-FA and their protein- or GSH-adducts are detected in healthy human plasma and urea. Multiple studies indicate NO_2-FA exhibit anti-inflammatory signaling actions, as well as activate antioxidant responses.

Plasma and urinary NO_2-FA concentrations in healthy humans range from 2-20 nM, with additional pools of NO_2-FA present as a) Michael addition products with the abundant biological

nucleophiles present in tissues and fluids and b) esterified species in complex neutral and polar lipids [144, 145]. Tissue NO_2-FA levels are affected by both dietary lipid and nitrogen oxide concentrations [146, 147]. The unique electrophilic character of nitroalkene substituents promotes kinetically rapid and reversible Michael addition with nucleophilic Cys (and to a lesser extent His) residues of proteins [65, 79]. This reversible protein adduction by NO_2-FA decreases the potential for toxicity stemming from the accumulation of the Schiff's base and Michael addition products characteristic of other lipid electrophiles such as cyclopentanones and α□β-unsaturated oxo- (or keto-) groups [65, 148, 149]. Through transient PTM reactions with hyper reactive protein thiols, NO_2-FA modulate signaling pathways involved in cell proliferation and inflammatory responses. This occurs as a result of the alkylation of functionally-significant Cys residues in transcriptional regulatory proteins, including the Keap1 regulator of Nrf2 signaling, the nuclear lipid receptor PPARγ and NF-κB (Figure 6) [87, 100, 117]. Of relevance to the present study, NO_2-FA inhibit NF-κB-mediated signaling in diverse cell and murine models of metabolic and inflammatory stress in cardiovascular, pulmonary and renal systems [100, 101, 150].

This dissertation study focused on using nitro-oleic acid (NO_2-OA), which was selected for structural simplicity, favorable pharmacokinetics/pharmacodynamics, *in vivo* presence, and an absence of toxicity at an extended therapeutic range.

1.3 BREAST CANCER

Breast cancer is the most commonly diagnosed type of non-skin cancer in women, representing 30% of all new cancer cases in 2017 among women in the United States [151]. It is estimated to develop in one out of every eight women in the United States during their lifetimes [152]. Through a multistage process characterized by the development of a hyperplasia, this disease progresses to an *in situ* carcinoma that ultimately invades surrounding tissue and spreads throughout the body causing distinct metastasis [153]. Advances in surgical techniques, radiation therapy, and novel therapeutics have led to an increase in overall survival rates [154]. However, metastatic breast cancer remains largely an incurable disease with an average 25% five-year survival rate. Like most cancers, this disease is highly variable from patient to patient, but can be classified into four major subtypes based on the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) within a given malignancy (luminal A, luminal B, HER2+, and triple-negative breast cancer), which can guide the use of small molecule or biologic intervention [155].

1.3.1 Molecular Implications of Breast Cancer

The most common breast cancer is referred to as Luminal A, which expresses ER and/or PR with low expression of the proliferation marker Ki-67 [156]. Luminal A breast cancers tend to have the best prognosis and are susceptible to targeted endocrine therapies. Like Luminal A breast cancers, Luminal B breast cancers express ER and/or PR but are characterized by higher proliferation as evident by Ki-67 and are less prevalent. While these cancers tend to have an overall worse prognosis, several targeted treatment options are available to patients. Diseases

exhibiting the worst prognosis are the HER2+ and triple-negative breast cancer (TNBC) subtypes. HER2+ breast cancers account for 7-12% of patients and are diagnosed by either high HER2 staining *via* immunohistochemistry (3+, IHC) or borderline IHC staining (2+) with a demonstrated HER2 gene amplification identified by fluorescence *in situ* hybridization. Patients with this disease will most commonly be treated with a combination of HER2 targeted therapy plus conventional cytotoxic chemotherapy [157]. Finally, TNBC is characterized by the lack of ER, PR, and HER2 and predominantly expresses basal/myoepithelial cytokeratins. Because of the lack of receptors that can be targeted by therapy, these breast cancers are limited to treatment with some combination of cytotoxic chemotherapy. Furthermore, by definition, the term TNBC is exclusionary in nature, meaning that any form of breast cancer that does not express ER, PR, or HER2 is combined into this one subtype. Thus, this group is highly heterogeneous and one report suggests it should be further divided into 6 additional subtypes (basal-like 1, basal-like 2, immunomodulatory, mesenchymal-like, mesenchymal stem-like, and luminal androgen receptor) [158]. Molecular characteristics of the four major subtypes of breast cancer are summarized in Table 1 [159-161].

Table 1. Immunohistochemically defined breast cancer molecular subtypes.

	ER	**PR**	**HER2**
Luminal A	(+)	Usually (+)	(-)
Luminal B	(+)	Often (-)	(-)
Her2-enriched	(-)	(-)	(+)
Triple-Negative	(-)	(-)	(-)

1.3.2 Risk Factors of Triple-Negative Breast Cancer (TNBC)

TNBC accounts for approximately 15% to 20% of all diagnosed breast cancer cases [43] and predominantly presents with aggressive features (high nuclear grade and high mitotic index), poor prognosis, shorter survival, and a high rate of BRCA1-related mutations [155, 162]. TNBC tumors are more frequent in younger patients and black women [163, 164]. The majority of TNBC tumors are “basal-like”, with 5-yr survival rates typically lower than other breast cancer phenotypes (~77% vs. ~93%, respectively) [163]. TNBC patients are at higher risk for relapse within the first 5 years post-treatment, and their recurrent tumors are more aggressive and invasive [165, 166], resulting in life expectancy estimates of only 3 to 22 months [167, 168]. Compared to other types of breast cancers, TNBC patients are four times more likely to develop visceral metastases of the lung, liver and brain within five years of diagnosis [169].

TNBC develops early in life and more often in pre-menopausal women [170, 171]. Individuals with metabolic syndromes such as high blood glucose, triglyceride or low high density lipoprotein (HDL) [172], increased body weight [173] and younger age (<50 years old at diagnosis) [165] are considered as high risk groups of TNBC. In addition, mutations in BRCA1 also correspond with the risk of developing basal-like breast cancer or TNBC [174].

In vitro and clinical studies suggested that development of metastases from TNBC progression may be related to the ability of chemotherapy to trigger an inflammatory response, which causes increased cytokines that are known to favor metastasis development [175, 176]. More specifically, the association between the roles of cytokine TNFα and an increased risk of TNBC progression has been demonstrated [10, 177].

1.3.3 Current Therapies for TNBC

A systemic therapy for the treatment of early breast cancer is given in either the neoadjuvant setting (before primary therapy) or the adjuvant setting (after primary therapy i.e. surgery) [178, 179]. Although TNBC tumors are more often responsive to chemotherapy, tumor resistance and rapid extensive metastatic recurrence remains a major therapeutic obstacle. Thus, it has been a challenge for surgeons and medical oncologists.

Potential targets for TNBC treatment include the surface receptor epidermal growth factor receptor (EGFR), components of the protein kinase B (Akt) pathway, such as PI3K, or inhibition of defective DNA repair such as PARP1 [174].

1.3.4 NF-κB Signaling Pathway Contributing to TNBC

NF-κB signaling pathways are aberrantly activated and involved in tumor development in various cancers, including breast cancer [2, 23, 33]. Hormone-independent breast cancer cells and primary breast tumors exhibit higher NF-κB activity [32, 180-182]. A study by Nakshatri et al. provided the first direct evidence that constitutive NF-κB DNA binding was observed in hormone-independent (ER-) compared to hormone-dependent (ER+) breast cancer cell lines [32]. In another study involving 31 specimens from human breast tumors, predominantly active NF-κB in ER-negative human breast tumor specimens was measured [180]. A more recent study by Ossovskaya et al. demonstrated that NF-κB is one of the key regulators of TNBC and identified highly expressed NF-κB target genes (> 4-fold) in TNBC tumors compared to normal breast tissue [182]. The inhibition of NF-κB activation, induced by over-expression of a non-

degradable IκBα super-repressor (Ser32/36 mutations of IκBα), significantly inhibits the growth of several TNBC cell lines [181].

The cytokine TNFα contributes significantly to the privileged pro-inflammatory microenvironment that promotes tumor progression. TNFα activates tumor metastasis and invasion through NF-κB-mediated up-regulation of extracellular matrix degradation enzymes and adhesion molecule expression [177]. Notably, a meta-analysis revealed that TNBC patients with elevated TNFα expression have an increased risk of tumor metastasis to distant organs [10]. Thus, NF-κB activation and the downstream signaling actions of its pro-inflammatory mediators play a critical role in TNBC malignancy. This motivates the investigation of NF-κB inhibition strategies as a chemotherapeutic approach for countering metastatic TNBC.

1.4 SIGNIFICANCE

Since TNBC tumors do not respond to endocrine therapy or other targeted agents, chemotherapy remains the mainstay of treatment for these cancers; however, it is generally toxic and the lack of effective alternative therapy has been challenging. NO_2-OA is endogenously formed during digestion and inflammation by the reaction of oleic acids with the NO and NO_2^--derived nitrating species. The field of NO_2-OA activity in cancers is still underexplored. This dissertation work describes the first discovery of any potential therapeutic actions of NO_2-OA in human TNBC cells. A number of electrophilic bioactive compounds including isothiocyanates sulforaphane (SFN), 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) have been shown to possess anti-cancer activity (anti-proliferation,

apoptosis, and anti-metastatic) in TNBC cells and tumors. Thus, the present study sought to determine the efficacy of NO_2-OA on proliferative, apoptotic, and metastatic events in TNBC.

Inflammation is intimately involved in the pathogenesis of breast cancer, particularly in TNBC. TNFα increases the risk of distant tumor metastasis in TNBC patients. The current preclinical studies have demonstrated that TNFα activates NF-κB signaling that drives gene expression of the extracellular matrix degradation enzymes and adhesive molecules important for tumor metastasis and invasion [177]. Constitutive NF-κB activation plays a critical role in TNBC malignancy [32, 180] and NF-κB inhibitors are rational therapeutic targets for TNBC. Because NO_2-OA displays anti-inflammatory signaling properties, such as inhibiting inflammatory NF-κB signaling through direct alkylation of the NF-κB RelA subunit *via* Michael addition, consequently blocking DNA binding activity and potently repressing expression of NF-κB-regulated genes. Thus it may be effective as a therapeutic agent for the treatment of metastatic TNBC tumors.

Furthermore, therapeutic specificity is an important issue in cancer treatment. It is likely that the level of MRP1 protein contributes to the biological effects of NO_2-OA between tumors and tissues since MRP1 regulates the transportation of lipid-derived signaling molecules lead to protect cells/tissues from the cytotoxic effects of abundant these molecules. Based on these factors, an initial study was conducted to elucidate differential cellular responses to NO_2-OA in TNBC versus MCF-10A cells. ***We hypothesize that nitro-oleic acid will have therapeutic potential to selectively inhibit proliferation, survival, and metastasis of TNBC.*** This dissertation study will be an important explanation of how NO_2-OA inhibits TNBC tumor development and its elucidation has significant impact for the improvement of pharmacological therapies.

2.0 MATERIALS AND METHODS

2.1 Reagents

The NF-κB inhibitor JSH-23 (4-Methyl-*N*1-(3-phenyl-propyl)-benzene-1,2-diamine), protein synthesis inhibitor Cycloheximide (CHX; 3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-glutarimide), and proteasome inhibitor MG-132 (Z-L-Leu-D-Leu-L-Leu-al) were purchased from Sigma. The IKKβ inhibitor Bay 11-7082 (3-[(4-methylphenyl)sulfonyl]-(2*E*)-propenenitrile) was purchased from Calbiochem. The MRP1 inhibitor Probenecid (4-[(Dipropylamino)sulfonyl]benzoic acid) was purchased from Enzo Life Sciences and dissolved in 1M sodium hydroxide. siRNA directed against human RelA (ON-TARGET plus smart Pool, L-003533-00-0005), human MRP1/ABCC1 (L-007308-00-0005), and non-targeting control siRNA (D-001810-10-05) were purchased from Dharmacon RNAi Technologies. Lipofectamine 2000 or 3000 (Life Technologies) was used for cell transfection.

2.2 Cell Lines

Cell lines were purchased from ATCC. MDA-MB-231, MCF7, and T47D cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 5% fetal bovine serum (Hyclone). MDA-MD-468 and ZR-75-1 cells were cultured in Improved Minimum Essential

medium (Gibco) supplemented with 5% and 10% fetal bovine serum, respectively. SK-BR-3 cells were cultured in McCoy's medium (Gibco) supplemented with 10% fetal bovine serum. MCF-10A cells were cultured in growth medium consisting of DMEM:F12 supplemented with 5% horse serum (Hyclone), 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 20 ng/ml EGF, and 10 μg/ml insulin (Sigma). Cells were incubated at 37°C in a 5% CO_2 atmosphere.

2.3 Synthesis of NO_2-FA and Biotinylated NO_2-FA

NO_2-OA was synthesized as described previously [183]. Nitro-stearic acid (NO_2-SA) was obtained by reduction of the nitroalkene double bond with sodium borohydride (unpublished data). Oleic acid (OA) was purchased from Nu-Check Lipids. N,N'-dicyclohexylcarbodiimide (DCC), 4-dimethylamino pyridine (DMAP) and N-hydroxysuccinimide (NHS) were purchased from TCI-America. Biotinylated NO_2-FA were synthesized from free fatty acids and biotin-(polyethylene glycol)-amine (Pierce, #21346) *via* activation of the carboxylic acid as an NHS-ester. In a typical preparation, NHS (40 mg), DCC (63 mg), and a catalytic amount of DMAP (2-3 mg) were weighed into a 20 mL vial and dissolved in 5 mL CH_2Cl_2, then cooled in an ice bath to 0°C. NO_2-OA (100 mg) was dissolved separately and washed in 1-2 mL CH_2Cl_2. The solution was stirred and allowed to warm to room temperature (RT) overnight. The crude solution was then filtered, purified by flash column chromatography (silica gel, ethyl acetate/hexanes gradient) and the NO_2-OA NHS-ester stored as a solution in DMF (50 mg/mL). Biotin-(polyethylene glycol)-amine (50 mg) was dissolved in DMF and an equivalent molar amount of NO_2-OA NHS-ester (57 mg in 1.14 mL DMF) solution was added to it. The solution was stirred at RT overnight. The next day the solvent was removed by evaporation, after which

the final product was isolated by preparatory thin-layer chromatography (acetic acid/water/methanol/chloroform, 1/4/35/60). The final conjugates were identified by LC/MS (Bt-NO_2-OA calculated mass 684.4, found 684.3 $[M + H]^+$; Bt-NO_2-SA calc 686.5., found 686.5$[M + H]^+$; Bt-OA calc 639.5., found 639.1 $[M + H]^+$) and stored as solutions in DMSO at -20 °C.

2.4 MTT (Methylthiazolyldiphenyl-tetrazolium bromide) Assay of Cell Viability

Cells at density of 3000-5000 cells per well were plated in 96-well plates and treated with 0 to 15 μM NO_2-OA. At 48 hr, 200 μl of a 1 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (Sigma), diluted in regular culture media, was added to each well. The plates were incubated at 37°C in 5% CO_2 for 4 hr, allowing viable cells to reduce the yellow tetrazolium salt to blue formazan crystals. At 4 hr, the MTT solution was removed and 200 μl of 1:1 (v/v) solution of DMSO: ethanol was added to each well to dissolve the formazan crystals. The absorbance in individual wells was determined at A_{540} nm. The half maximal inhibitory concentration (IC_{50}) of NO_2-OA was determined using CalcuSyn software from Biosoft (Cambridge, UK).

2.5 Hoechst 33258 DAN Stained Assay of Cell Proliferation

Cells were plated at a cell density of 3000-5000 cells per well in 96-well culture dishes. After attachment overnight, the media was replaced and cells were treated with 0 to 20 μM NO_2-

OA or NO_2-SA for 48 hr. In the MRP inhibition study, MCF-10A cells were pre-treated with 0.25 mM probenecid for 1 hr and then co-treated with 0 to 25 μM NO_2-OA indicated above for 48 hr. Cells were counted using the FluoReporter dsDNA quantitation kit (Molecular Probes) according to the manufacturer's instructions. Fluorescence was measured using a SpectraMax M2 plate reader (Molecular Devices). The half maximal inhibitory concentration (IC_{50}) of NO_2-OA was determined by using CalcuSyn software from Biosoft (Cambridge, UK). Three experiments were done in quintuplet.

2.6 Cell Migration Assay

MDA-MB-231 and MDA-MB-468 cells were subjected to cell migration analysis in Boyden chambers. The bottom of a 12-well membrane filter (Falcon) was coated with 10 μg/ml fibronectin (Fisher) for 12 hr before each experiment. Cells were pre-treated with 5 μM NO_2-OA or NO_2-SA for 1 hr and then cultured in the absence or presence of TNFα (20 ng/ml, BD) for an additional 2 hr in medium containing 1% FBS. Cells were trypsinized and washed with migration media (DMEM) containing 0.1% fatty acid-free BSA (Sigma) to remove serum. Cells at a density of 10^5/well were then placed in the upper chamber with migration media containing the same pretreatment condition. The cells were allowed to migrate towards the 5% FBS chemoattractant for 5 hr. Non-migrated cells from the top surface were removed with cotton swabs. Migrated cells were fixed with 4% paraformaldehyde, then stained with 0.5% crystal violet (Sigma) for 15 min. Migrated cell density on the filters was observed by microscopy. The crystal violet on migrated cells was destained with 10% acetic acid, and the absorbance in individual filters was determined at A_{573} nm. The relative cell migration rate in each sample is

defined as the percent increase in migrated cells compared to the migrated cells in the absence of TNFα stimulation (Serum Ctrl).

2.7 Cell Invasion Assay

MDA-MB-468 cells were pre-treated with NO_2-OA (5 μM), NO_2-SA (5 μM), or NF-κB inhibitor JSH-23 (10 μM) for 1 hr and then in the absence or presence of TNFα (20 ng/ml) for an additional 2 hr in culture medium containing 1% FBS. Cells were then suspended in migration media and placed in the top well of invasion chambers (Chemicon ECM554). Chemoattractant (5% FBS) was placed in the lower chamber for 24 hr at 37°C to attract invasive cells. Cells were then harvested and invasion rates were determined according to the manufacturer's protocol.

2.8 NF-κB-luciferase Gene Expression Assay

Luciferase chemiluminescence-based analysis of NF-κB transcriptional activity was performed as previously [100] with minor modification. MDA-MB-231 and MDA-MB-468 cells at about 70% confluence in 12-well plates were transiently transfected with a NF-κB-luciferase reporter plasmid (Stratagene) with Lipofectamine 3000 (Invitrogen). 24 hr after transfection, cells were pretreated with NO_2-OA (5 μM), NO_2-SA (5 μM), OA (5 μM), or JSH-23 (20 μM) for 2 hr, then simultaneously treated with TNFα (20 ng/ml) for an additional 4 hr. Each transfection was performed in triplicate. Luciferase activity was measured using the Dual-luciferase assay kit (Promega). Relative light units (RLU) were measured using a 96-well plate luminometer,

according to the manufacturer's instructions (Victor II, PerkinElmer Life Sciences). Protein concentration was determined using the BCA Assay (Pierce). Data represents the ratio of treated samples to controls in the context of mean RLU/protein content, +/- SD.

2.9 NO_2-FA Protein Alkylation Reactions

To determine whether NO_2-OA binds to RelA (p65) or IKKβ, MDA-MB-231 or MDA-MB-468 cells were treated with 5 μM biotinylated (Bt) lipids Bt-NO_2-OA, Bt-NO_2-SA, or Bt-OA in DMEM containing 5% FBS. After 2 hr, cells were harvested in TGH buffer (1% Triton X, 10% glycerol, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA, 1 mM EGTA) supplemented with a mixture of protease inhibitors and phosphatase inhibitors (Roche Applied Science) [87]. Total cell lysates (0.5-1 mg) were mixed and incubated with streptavidin agarose beads (Sigma) at 4°C overnight. Beads were washed three times using TGH buffer. After SDS-PAGE, western blot analysis was performed using anti-p65 mouse monoclonal antibody (Santa Cruz Biotechnology) or anti-IKKβ rabbit polyclonal antibody (Cell Signaling).

2.10 Fluorescence-Activated Cell Sorting (FACS)

MCF-10A, MDA-MB-231, and MDA-MB-468 cells were plated at a cell density of 2.5 x 10^5 cells in 6-well plates for 24 hr, and then were treated with 0.1% methanol (vehicle), 5 μM NO_2-OA, NO_2-SA, or OA for 24 hr. Adherent and nonadherent cells were collected, centrifuged at 2000 x rpm for 10 min, washed with ice-cold phosphate-buffered saline, fixed with 4.44%

formaldehyde (Electron Microscopy Sciences), and stained with 50μg/mL propidium iodide (Sigma). FACS analysis was performed in the University of Pittsburgh Department of Immunology Unified Flow Core Facility.

2.11 Immunoprecipitation and NO_2-OA-induced RelA Protein Polyubiquitination

To determine the induction level of RelA protein polyubiquitination by NO_2-FA, MDA-MB-231 and MDA-MB-468 cells were treated with 0.1% methanol (vehicle), NO_2-OA (5 μM), or NO_2-SA (5 μM) for 6 hr, then cell lysates were harvested in TGH buffer supplemented with a mixture of protease inhibitors and phosphatase inhibitors (Roche Applied Science). Lysates were clarified by centrifugation at 14,000 x *g* for 10 min. Total protein lysates (1 mg) were incubated with anti-RelA antibody (Santa Cruz Biotechnology) and Protein G/A conjugated agarose beads (EMD Millipore) at 4°C overnight. Immunoprecipitation fractions were obtained by centrifugation at 14,000 x *g* for 1 min at RT and washed with TGH buffer three times. The immunoprecipitated RelA was resolved by 8 % SDS-PAGE gel and transferred to nitrocellulose membrane (Bio-Rad) for probing with an anti-ubiquitin antibody (Santa Cruz Biotechnology). The blot was then stripped and reprobed with an anti-RelA antibody to ensure that an equal amount of RelA protein was immunoprecipitated and loaded.

2.12 Western Blotting

Western blotting was performed as previously described [87]. 20-60 μg of total lysates per lane were loaded on 7%, 10%, 12%, 15%, or gradient SDS-PAGE and transferred onto nitrocellulose or polyvinylidene difluoride membranes (Bio-Rad). The membranes were probed with primary antibodies against ubiquitin, RelA, caspase-3, MRP1, or PARP-1 (Santa Cruz Biotechnology); cyclin D1, p21, caspase-9, MRP4, IKKβ, pIKK, IκBα, or pIκBα (Cell Signaling); or caspase-8 (R&D Systems). Samples were normalized to β-actin (Sigma) or GAPDH (Trevigen, Inc.). Protein bands were visualized and digitized images quantified using Image Lab software (Bio-Rad). Western blots are representative of at least three individual experiments.

2.13 RNA Extraction and qRT-PCR

Total RNA samples of cells or tumors were extracted using TRIZOL reagents according to the manufacturer's instructions (Invitrogen). Total RNA (1 μg) was reverse transcribed using iScript cDNA kit (Bio-Rad) according to manufacturer's recommendation. 25 ng cDNA products were used for each subsequent quantitative real-time PCR (qRT-PCR) reaction. All qRT-PCR was analyzed and performed on the StepOne PLUS PCR system (Life Technologies) by using TaqMan Gene Expression Assays. Fold change was calculated using the ΔΔ*C*t method with 18S ribosomal RNA or human β-actin RNA serving as the internal control.

2.14 Mouse Model of TNBC Tumor Xenografts

All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Approval #15025402). The fourth inguinal mammary fat pad of 8-week-old female BALB c *nu/nu* athymic nude mice was injected with a total of 3 x 10^6 MDA-MB-231 cells (Harlan Bioproducts for Science Inc.). Tumor volumes (0.5 x length [mm] x width [mm] x width [mm]) were measured by caliper every three days, and body weights were measured weekly. Tumors were allowed to grow until volumes reached between 50-100 mm^3; then mice were randomized to treatment with NO_2-OA (7.5 mg/kg in 100 μL), NO_2-SA (7.5 mg/kg in 100 μL) or the vehicle sesame oil (100 μL; Sigma) by oral gavage daily for 4 weeks.

2.15 Measurement of NO_2-FA and Other Metabolites in Xenograft Tumors

To measure the total amount of 10-NO_2-OA (NO_2-18:1) and its metabolites (NO_2-18:0 and NO_2-14:0) in xenograft tumors, tumor homogenates (250 mg/ml) in phosphate buffer (pH 7.4) were incubated with 20 mM mercury (II) chloride ($HgCl_2$) for 30 min at 37°C before lipid extraction. Fatty acids from tumor tissues were spiked with 60 nmol of 10-[^{15}N]O_2-d_4-OA (internal standard) and then were extracted using HPLC grade Hexane/Isopropanol/1M FA (2/1/0.1, v/v/v, Burdick and Jackson). The upper phase (organic phase) was separated and dried under N_2. Samples were reconstituted in methanol. NO_2-FA products were analyzed and quantified by HPLC-ESI-MS/MS using gradient solvent systems consisting of water containing 0.1% acetic acid (solvent A) and acetonitrile containing 0.1% acetic acid (solvent B). Lipid extracts were resolved using a reverse phase HPLC column (5 micron, 2 × 100 mm C18 Luna column;

Phenomenex) at a 0.650 ml/min flow rate. Samples were applied to the column at 30% solvent B (0.3 min) and eluted with a linear increase in solvent B (30–100% solvent B in 9.7 min). 10-NO_2-OA and its metabolites were identified using an API 5000 triple quadrupole mass spectrometer (Applied Biosystems) equipped with an electrospray ionization source. A search for precursors of m/z 46 (in negative ion mode) was used to screen for metabolites containing a NO_2 group. The following parameters for the mass spectrometers were used: electrospray voltage was –4.5 kV, declustering potential -80 eV, CE -35, EP -5, CXP -3, gas1 55 and gas2 60 and the source temperature was set at 600°C. Solvents for mass spectrometric analyses were purchased from Burdick and Jackson.

2.16 LC-MS Analysis of RelA Post-Translational Modifications

The proteomics analysis was performed as described [87] with minor modification. 5 μg of purified human recombinant RelA (residues 1-306, containing a GST tag; Sino Biological) was incubated with NO_2-OA for 1 hr in phosphate buffer (pH 7.4) at 37°C. RelA was then digested using trypsin (Roche Applied Science) at a RelA to trypsin ratio of 50:1 overnight at 37°C. The digested peptides were analyzed by HPLC-MS/MS for post-translational modification. Analyses were performed using a LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with a Dionex nanoLC (Thermo Fisher Scientific). Peptides were eluted from the column at a 0.14 mL/min flow rate using a water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) solvent system. The samples were loaded at 2% solvent B, then the gradient was set to reach 55% solvent B in 50 min, 95% in 1 min and held at 95% solvent B for 3 min, and then re-equilibrated at initial conditions for 10 min. MS analysis was

performed in the positive ion mode with the following source parameters that were optimized for the detection and identification of electrophilic NO_2-OA-modified Cys- and His-containing peptides: source voltage 2.3 kV, capillary temperature 2240 °C, tube lens 95 V, capillary voltage 49 V, and collision energy of 35 V. Searcher was performed using Thermo Proteome Discoverer 1.4.1.14 software (Thermo Fisher Scientific). The covered RelA sequence (>99%) included all peptides containing either Cys or His residues (nucleophilic targets of NO_2-OA), allowing for a comprehensive analysis of putative target residues.

2.17 Preparation of GSH Conjugate (GS-$^{15}NO_2$-d_4-OA) Standard

GS-$^{15}NO_2$-d_4-OA standard was generated by the reaction of 200 mM glutathione with 100 μM $^{15}NO_2$-d_4-OA in 50 mM phosphate buffer (pH 8.0 at 37°C) for 3 hr. The reaction sample was loaded on a C18 SPE column pre-equilibrated with 5% methanol and then eluted with methanol. Mass spectrometric structural analysis of GS-$^{15}NO_2$-d_4-OA was performed in both negative and positive ion mode using accurate mass determinations and their respective MRM transitions.

2.18 Extraction of Glutathione Conjugate of NO_2-OA (NO_2-OA-SG) in Cell Media

One 6-well plate (~$1x10^6$ cells) of MCF-10A, MDA-MB-231, or MDA-MB-468 cells was cultured for 24 hr. Before treatment, cell media were replaced with DMEM containing 5% FBS and 5 μM NO_2-OA was added to the media. Cell treatment was conducted at 37°C for 60 min, and then the cell culture media was collected. GS-NO_2-OA was extracted using a modified

Bligh-Dyer method. Samples were centrifuged at 2,800 rpm for 5 min. The upper layer (aqueous) containing GS-NO_2-OA was extracted using C18 SPE columns (Thermo Scientific). Columns were conditioned with 100% methanol, followed by 2 column volumes of 5% methanol before use. GS-$^{15}NO_2$-d_4-OA was added and used as internal standard (IS). Samples were vortexed and equilibrated at 4°C for 5 min prior to extraction. Samples were loaded into SPE column and washed with two column volumes of 5% methanol and the column was dried under a vacuum for 30 min. Lipids were eluted with 3 ml methanol, solvent was evaporated, and samples were dissolved in methanol for analysis by HPLC-electrospray ionization mass spectrometry (ESI-MS/MS).

2.19 Liquid Chromatography Mass Spectrometry (LC-MS/MS)

NO_2-OA and NO_2-OA-SG were analyzed by high performance liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS) using a Shimadzu/CTC PAL HPLC coupled to a Sciex 5000 triple quadrupole mass spectrometer (Sciex, San Jose, CA). NO_2-OA and NO_2-OA-SG gradient solvent systems consisted of water + 0.1% acetic acid (solvent A) and acetonitrile + 0.1% acetic acid (solvent B). NO_2-OA and its metabolites were resolved using a Luna C18 reversed phase column (2 mm X 100 mm, Phenomenex, Torrence, CA) at a flow rate of 0.65 mL/min. Samples were applied to the column at 30% solvent B and eluted with a linear increase in solvent B (30–100% in 9.7 min). The column was washed at 100% solvent B for 3 min before returning to initial conditions for equilibration (2 min). NO_2-OA-SG conjugates were resolved using a Luna C18 reversed phase column (2 mm × 150 mm, Phenomenex) at a 0.25 mL/min flow rate. Samples were applied to the column at 20% solvent B held for 5 min and

eluted with a linear increase in solvent B (20–98% solvent B in 20 min), followed by a wash step at 98% solvent B for 4.5 min, and switched back to initial conditions for 4 min. MS analyses for NO_2-OA used electrospray ionization in the negative ion mode with the collision gas set at 5 units, curtain gas 40 units, ion source gas #1 55 units and #2 60 units, ion spray voltage −4500V, and temperature 600°C. The declustering potential was −80 eV, entrance potential −5, collision energy −35, and the collision exit potential −3. Multiple reaction monitoring (MRM) was used for the analysis of lipids showing a loss of a nitro group (*m/z* 46) upon collision-induced dissociation (MRM: 326.2/46 and 331/47 for NO_2-OA and $^{15}NO_2$-d_4-OA, respectively) in negative ion mode. The following parameters for the mass spectrometers were used for NO_2-OA-SG conjugates in positive ion mode: gas#1 50 and gas#2 55, ion spray voltage 5000 V, source temperature 550˚C, declustering potential 70 eV, entrance potential 5, collision energy 17 and the collision exit potential 5. MRM transitions 635.2/506.2 and 640.2/511.2 were used for NO_2-OA-SG and $^{15}NO_2$-d_4-OA-SG, respectively.

2.20 Statistical Analysis

Data analyses were conducted using Prism 6 software (GraphPad Software). Results are presented as mean ± SD except tumor volumes except Figure 13, which is presented as mean ±SEM. Statistical analysis was performed using Student's *t*-test, one-way or two-way analysis of variance as appropriate. Statistical significance was achieved with $p < 0.05$.

3.0 IMPACT OF ELECTROPHILIC NITRO-OLEIC ACID ON DEVELOPMENT AND PROGRESSION OF TRIPLE-NEGATIVE BREAST CANCER

3.1 INTRODUCTION

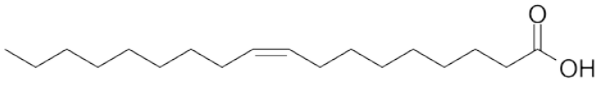
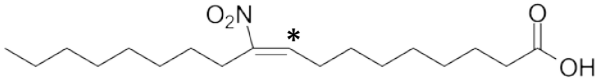
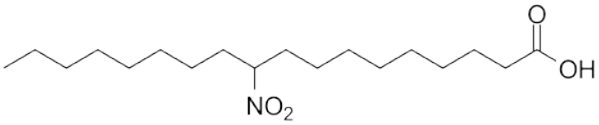
Several electrophilic natural compounds generally derived from plants, vegetables, or endogenous bioactive molecules possess anticancer activity in TNBC cells *via* inhibition of proliferation, induction of apoptosis and suppression of migration. For example, isothiocyanates (sulforaphane, SFN) from broccoli have anti-proliferative and apoptotic effects on TNBC cells [184, 185]. Clinically available dithiolethiones, naturally found in vegetables, inhibit cytokine-induced TNBC cell migration and invasion by directly targeting NF-κB signaling pathway [61]. Phytochemicals from blueberries induce apoptosis in MDA-MB-231 cells *via* modulation of the PI3K/AKT/NFκB pathway [186]. The cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) acts as an NF-κB inhibitor to induce apoptosis by down-regulation of anti-apoptotic protein expression such as cIAPs and Bcl-X_L in both MDA-MB-231 and MDA-MB-468 cells [59]. Lastly, the naturally occurring triterpene oleanolic acid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) induces cell cycle arrest at G2/M phase and tumorsphere formation in TNBC cells [187]. In aggregate, bioactive electrophilic molecules may be considered as potent anticancer agents and provide alternatives of current TNBC therapy.

The anticancer activity of NO_2-FA on cancer development and progression had not been previously examined. Anti-proliferative actions of NO_2-OA on macrophages, vascular smooth muscle cells and fibroblasts are observed in models of chronic vascular and pulmonary disease [188-192]. One recent study reported that NO_2-OA stimulates intrinsic caspase-8 and extrinsic caspase-9 activity in rat aortic smooth muscle cells [193]. Moreover, NO_2-OA inhibits platelet-derived growth factor (PDGF)-stimulated rat aortic smooth muscle cell migration [188]. All together, these data suggest that NO_2-OA may have therapeutic potential for treating cancers, perhaps TNBC.

Here, we first investigated the impact of NO_2-OA on anticancer activity in breast cancer. NO_2-OA treatment was tested against a panel of breast cancer cell lines *in vitro* for cytotoxic and anti-proliferative effects. The breast cancer cell lines that displayed high sensitivities to NO_2-OA compared to non-tumorigeneic epithelial MCF-10A cells were used to examine the therapeutic efficacy of NO_2-OA in a xenograft model. In order to understand the molecular mechanisms of NO_2-OA on proliferation and survival of responsive breast cancer cells, the differentially expressed mRNAs and proteins of key regulators involved in cell cycle and survival pathways were further evaluated. Lastly, to determine whether NO_2-OA affects cytokine-induced cell migration and invasion of TNBC, the Boyden chamber migration/invasion assays were performed.

In this thesis study, the actions of NO_2-OA were compared with native fatty acid OA (oleic acid) and NO_2-SA (the non-electrophilic reduced form of NO_2-OA). Chemical structure and characteristics of these lipids are shown in Table II.

Table 2. Chemical structure and characteristics of lipids

Lipid Name	Lipid Structure	Electrophilicity
Oleic acid (OA)		**NO**
Nitro-oleic acid (NO_2-OA)		**YES**
Nitro-stearic acid (NO_2-SA)		**NO**

Asterisk denotes the electrophilic carbon.

3.2 RESULTS

3.2.1 Effects of NO_2-OA on Breast Cancer Cell Growth

To investigate the effect of NO_2-OA on cell growth of different subtypes of breast cells, Hoechst 33258 cell count assay was accessed in a non-cancerous breast epithelial cell line (MCF-10A), ER^+ breast cancer cell lines (MCF7, T47D, and ZR-75-1), an Her2 amplified breast cancer cell lines (SK-BR-3), and two TNBC cell lines (MDA-MB-231 and MDA-MB-468). Both MDA-MB-231 and MDA-MB-468 cells were sensitive to NO_2-OA compared to non-cancerous MCF-10A cells (Figure 7A-B). The anti-growth response of other subtypes to NO_2-OA treatment was similar to MCF-10A cells (Figure 7C-F).

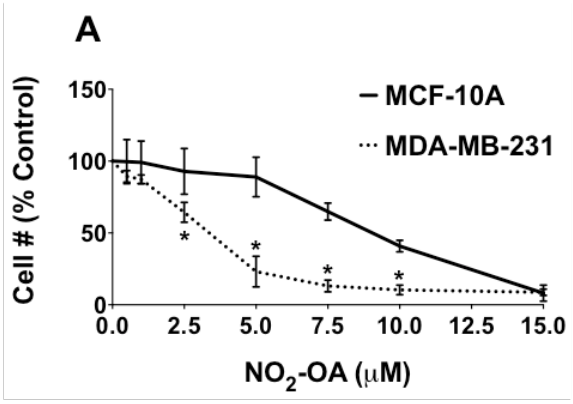
To complement this data, the IC_{50} (the half maximal inhibitory concentration) value of NO_2-OA in these breast cancer cell lines was determined. The IC_{50} value for NO_2-OA was significantly greater for non-cancerous MCF-10A cells (7.7± 1.93 μM), as opposed to MDA-MB-231 (2.7± 0.11 μM) and MDA-MB-468 (1.6± 0.11 μM) cells (Figure 7G). The IC_{50} value for both MCF7 (11.61± 3.59 μM) and T47D (11.69± 4.69 μM) cells was greater than MCF-10A cells (Figure 7G). However, the IC_{50} value of ZR-75-1 and SK-BR-3 cells was unmeasurable since these two cell lines were not responsive to NO_2-OA. These results suggest that NO_2-OA may preferentially inhibit TNBC cell growth.

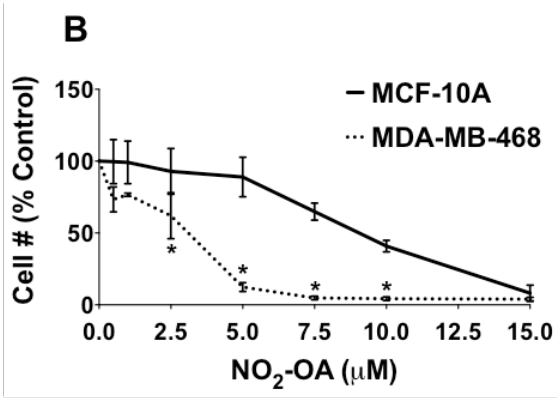
In contrast OA and NO_2-SA, which are structurally very similar to NO_2-OA (Table II), did not affect TNBC cell growth versus untreated control (Figure 8), indicating that NO_2-OA-mediated TNBC cell growth inhibition is associated with the nitroalkene-mediated electrophilicity. Overall, different subtypes of breast cancer cells displayed different responses to NO_2-OA; while TNBC cells were more sensitive to growth inhibition, ER+ and non-tumorigenic breast cancer subtypes were less sensitive. The Her2 subtype may be resistant to NO_2-OA, as there was no growth inhibition in this cell line.

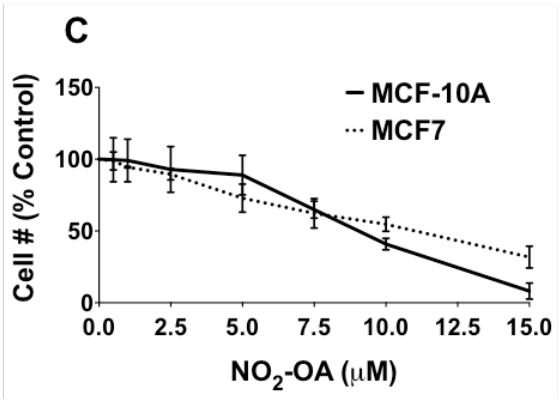
To ensure experimental conditions were consistent in every study, concentrations of 5.0 μM of NO_2-OA were used in most cell culture studies. Methanol was used as vehicle control because NO_2-OA is stored in methanol solution. OA and NO_2-SA were used as negative controls.

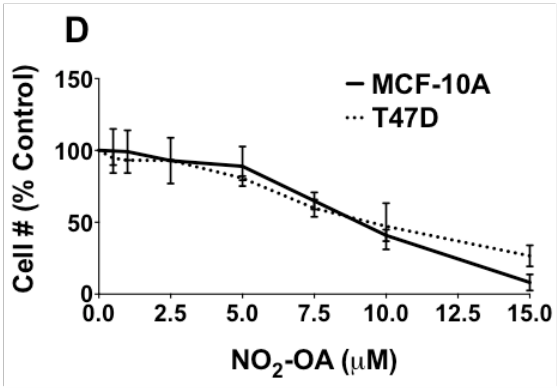
3.2.2 NO_2-OA Inhibits TNBC Cell Viability

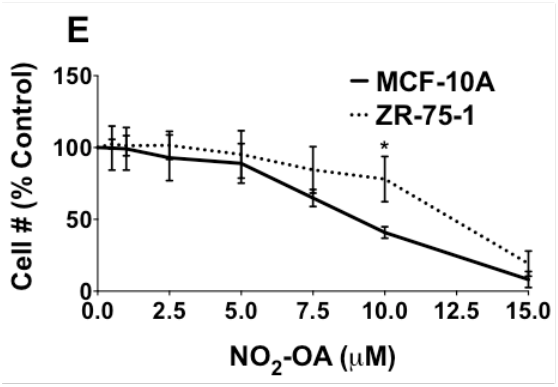
The cytotoxic effects of NO_2-OA on breast cancer cells were evaluated next *via* an MTT assay. The experimental design, cell lines, and treatment conditions were the same as the Hoechst 33258 cell count assay (Figure 7). Results showed the greatest sensitivity to NO_2-OA cytotoxicity in both MDA-MB-231 (Figure 9A) and MDA-MB-468 (Figure 9B) cells among all the tested cell lines with IC_{50} values about 3.5-4.0 μM for a 48 hr treatment (Figure 9E). Again, the IC_{50} values for MCF7 (10.4± 4.38 μM) and SK-BR-3 cells (not determined) were similar to the cell growth assay (Figure 9E), and the results confirmed that NO_2-OA did not inhibit cell growth and viability of ER+ and HER2 subtypes of breast cancer. Altogether, NO_2-OA exhibited significant inhibitory actions against cell growth and viability of TNBC.

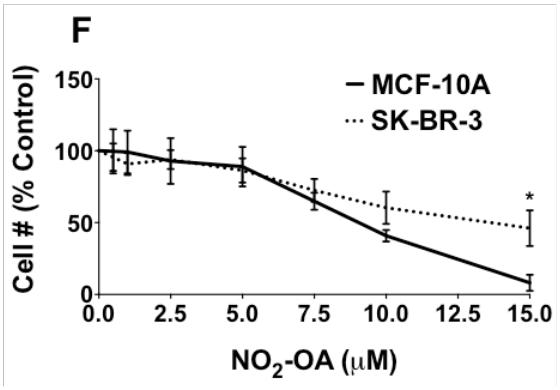

A
Cell # (% Control)
150
100
50
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
NO2-OA (μM)
MCF-10A
MDA-MB-231
*


B
Cell # (% Control)
150
100
50
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
NO2-OA (μM)
MCF-10A
MDA-MB-468
*


C
Cell # (% Control)
150
100
50
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
NO2-OA (μM)
MCF-10A
MCF7


D
Cell # (% Control)
150
100
50
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
NO2-OA (μM)
MCF-10A
T47D


E
Cell # (% Control)
150
100
50
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
NO2-OA (μM)
MCF-10A
ZR-75-1
*


F
Cell # (% Control)
150
100
50
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
NO2-OA (μM)
MCF-10A
SK-BR-3
*

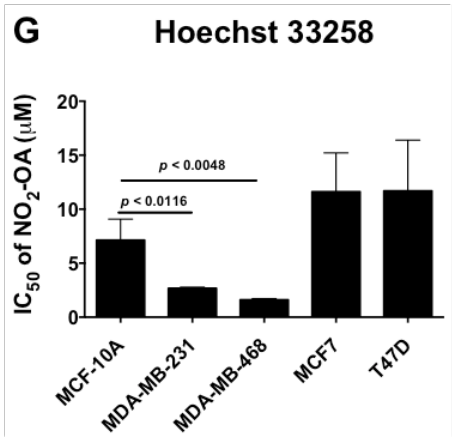

G
Hoechst 33258
IC50 of NO2-OA (μM)
20
15
10
5
0
p < 0.0048
p < 0.0116
MCF-10A
MDA-MB-231
MDA-MB-468
MCF7
T47D

Figure 7. Differential sensitivity of breast cancer cell lines to NO_2-OA.

Non-tumorigenic breast ductal epithelial cell line MCF-10A (A-F) and breast cancer cell lines including MDA-MB-231 (A), MDA-MB-468 (B), MCF7 (C), T47D (D), ZR-75-1 (E), and SK-BR-3 (F) cells were used for the cell growth study. All cells were treated with a range (0-15 μM) of NO_2-OA for 48 hr. Cell numbers were measured using the FluoReporter dsDNA quantitation assay and compared between two cell lines within treatment using two-way analysis of variance followed by Tukey post hoc test. Data are shown as percent of untreated control cells (mean ± SD). P-value < 0.05 was considered significant (*). (G) The IC_{50} values of NO_2-OA in different breast cancer cell lines. Data is shown as mean ± SD. P-value < 0.05 between two cell lines was considered significant and calculated by two-tailed unpaired Student's *t* test.

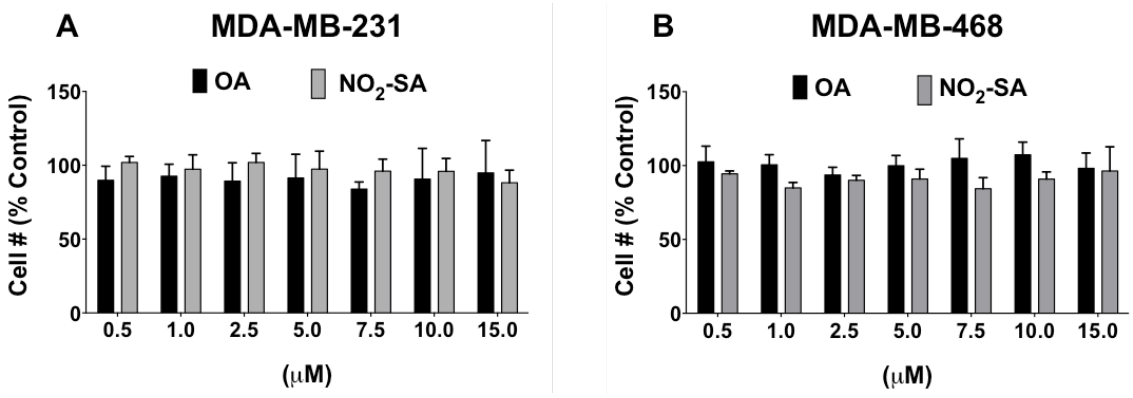


Figure 8. Non-electrophilic OA and NO_2-SA controls do not inhibit TNBC cell growth.

MDA-MD-231 and MDA-MB-468 cells were treated with a range (0-15 μM) of OA or NO_2-SA for 48 hr. Cell numbers were measured using the FluoReporter dsDNA quantitation assay according to the manufacturer's instructions. Data are shown as percent of untreated control cells (mean ± SD).

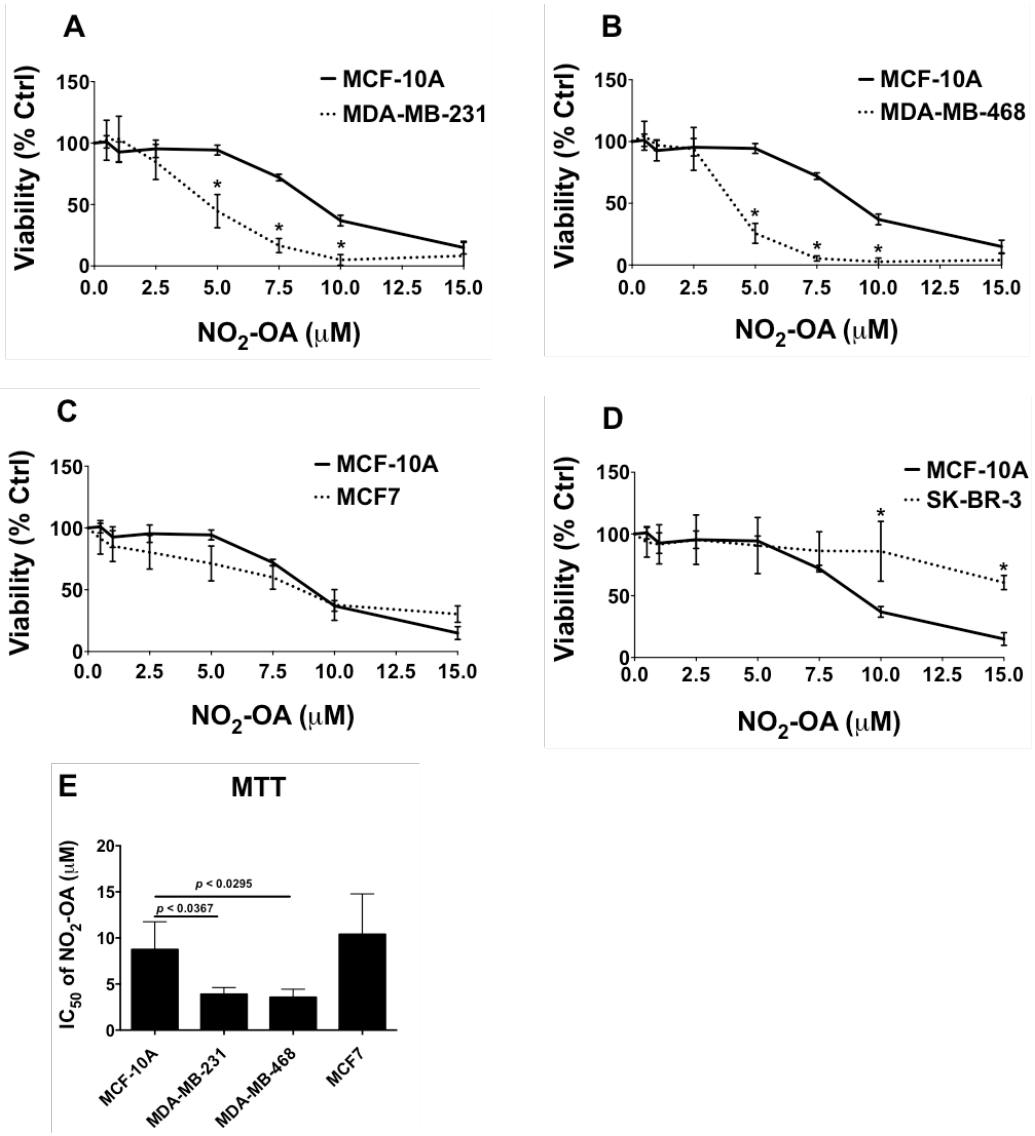


Figure 9. NO_2-OA preferentially inhibits TNBC cell viability.

MCF-10A (A-D), MDA-MB-231 (A), MDA-MB-468 (B), MCF7 (C), and SK-BR-3 (D) cells were treated with a range (0-15 μM) of NO_2-OA for 48 hr. Viable cell numbers were measured by MTT assay normalized to untreated cells (Ctrl). Data shows mean ±SD error bars. Cell viability between two cell lines within treatment was measured by two-way analysis of variance followed by Tukey post hoc test. Data was averaged from 3 independent experiments (n=6). P-value < 0.05 was considered significant (*).

3.2.3 NO_2-OA Arrests Cell Cycle of TNBC

NO_2-OA treatment for 48 hr significantly reduced the cell growth of TNBC (Figure 7A-B). To determine whether the decreased cell number was due to alteration of cell cycle, fluorescence-activated cell sorting analysis was performed. In both MDA-MB-231 and MDA-MB-468 cells, treatment with NO_2-OA for 24 hr significantly increased the percentage of cells at G2-M phase and decreased the percentage of cells at G0-G1 phase (Figure 10A-C). However, all cell populations (G0-G1, S, and G2-M) of MCF-10A were unaltered by NO_2-OA treatment (Figure 10D).

Meanwhile, western blot analysis showed that NO_2-OA down-regulated the protein expression of cyclin D1 and up-regulated the protein expression of p21 in both MDA-MB-231 and MDA-MB-468 cells, but not in MCF-10A or MCF7 cells (Figure 10E). To confirm the western blot results, mRNA levels of cyclin D1 and p21 were measured by quantitative RT-PCR and revealed similar results (Figure 10F-G). Consistent with the cell growth study (Figure 8), NO_2-SA did not affect cell cycle populations (Figure 10B-D) or expression of cell cycle regulator proteins or mRNA in MCF-10A, MDA-MB-231, and MDA-MB-468 cells (Figure 10E-G). These results suggest that NO_2-OA causes cell cycle arrest in TNBC cells.

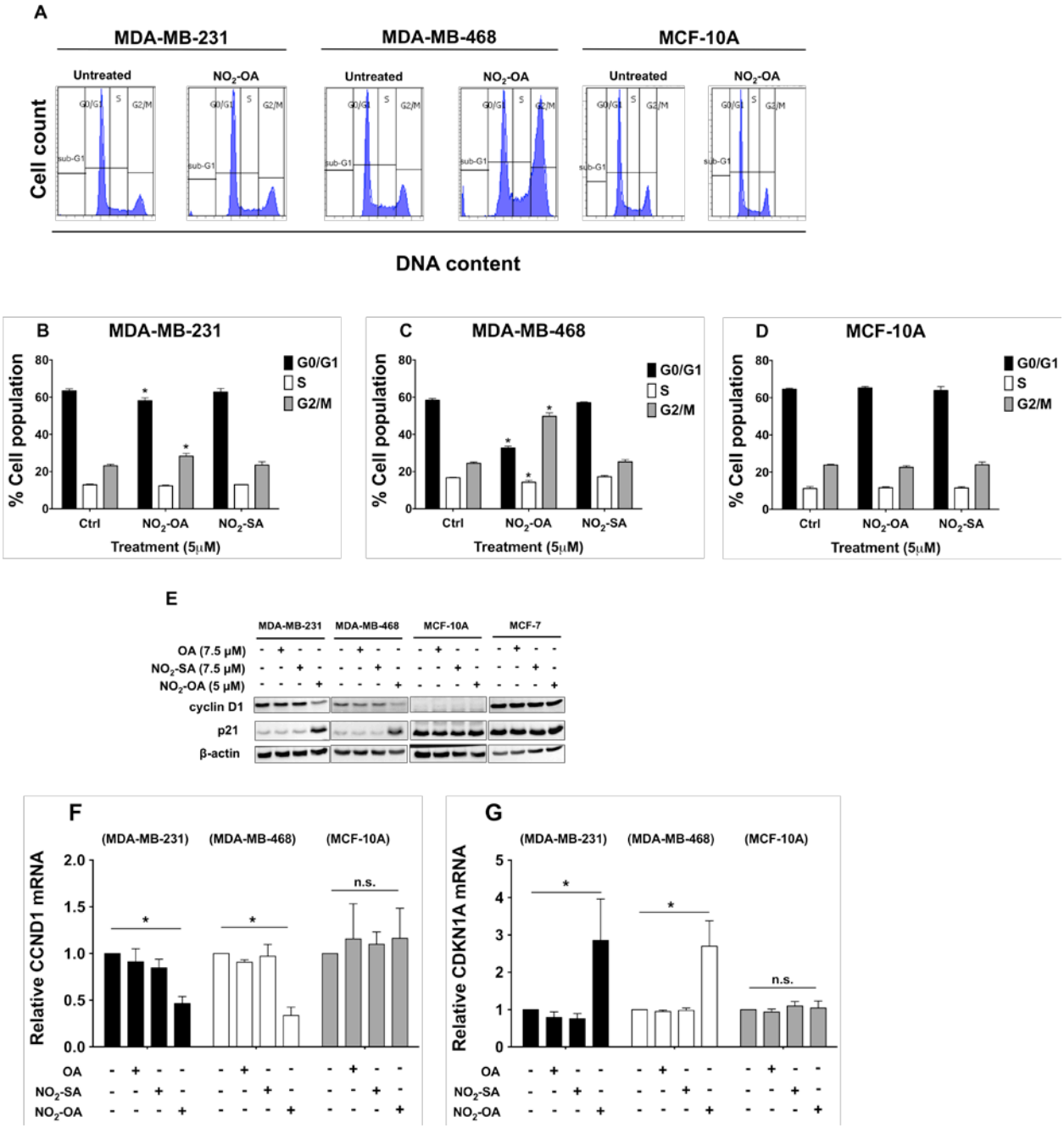


Figure 10. NO_2-OA causes cell cycle arrest in TNBC cells.

(A) Cell cycle distribution (sub-G1, G0/G1, S, and G2/M) was determined by fluorescence-activated cell sorting analysis. MDA-MB-231, MDA-MB-468, and MCF10A cells were treated with or without NO_2-OA (5 μM) for 24 hr. Percentages of cells in each phase of the cell cycle

(G0/G1, S, and G2/M) are presented for MDA-MB-231 (B), MDA-MB-468 (C), and MCF-10A (D) cells. Data shown are mean ± SD, n=3. P-value < 0.05 versus Ctrl was considered significant (*) and was determined by one-way analysis of variance followed by Tukey post hoc test. (E) Western blot analysis of cyclin D1 and p21 protein levels from untreated, OA-treated, NO_2-SA-treated, or NO_2-OA-treated different breast cancer cells: MDA-MB-231, MDA-MB-468, MCF7 and MCF10A. (F and G) Effects of NO_2-OA on expression of *CCND1* (cyclin D1) and *CDKN1A* (p21) mRNA in MCF-10A, MDA-MB-231, and MDA-MB-468 cells. Cells were treated with 5 μM NO_2-OA or controls (OA and NO_2-SA) for 24 hr. CDKN1A and CCND1 mRNA levels were measured by RT-qPCR. 18S rRNA was used as an internal control. Data represent the mean ± SD of three individual experiments. The fold increase relative to untreated control is presented. Statistical significance (*, $p < 0.05$) compared with untreated control was determined by one-way analysis of variance followed by Tukey post hoc test. n.s., not significant.

3.2.4 NO_2-OA Induces Apoptotic Cell Death in TNBC Cells

Since we observed that an increase in sub-G1 cell population was apparent in both MDA-MB-231 and MDA-MB-468 after 24 hr of NO_2-OA treatment (Figure 10A), we sought to determine if the effect of NO_2-OA on sub-G1 cells in TNBC cells was a result of apoptosis. Therefore, cleavage of PARP-1 (a substrate of caspase-3) was examined by western blot analysis. In MDA-MB-231 and MDA-MB-468 cells but not in MCF-10A cells, treatment with NO_2-OA induced cleavage of PARP-1 by caspase-3 (Figure 11A), indicating that NO_2-OA was specifically effective in inducing TNBC apoptosis through caspase-3 activation.

To further investigate the apoptotic pathways activated in TNBC cells, the activation of initiator caspases (caspase-8 for extrinsic pathway and caspase-9 for intrinsic pathway) was

analyzed using antibodies that detect both the pro-caspase and activated (cleaved) forms of the initiator caspases. As shown in Figure 11B, NO_2-OA treatment increased cleavage of both caspase-8 and caspase-9 in both MDA-MB-231 and MDA-MB-468 cells, suggesting that NO_2-OA induced apoptosis through both intrinsic (mitochondrial-dependent) and extrinsic (death receptor-dependent) apoptotic pathways in TNBC cells.

We also examined the potential changes in gene expression levels of BCL2L1 (Bcl-xl; the anti-apoptotic protein) and BIRC5 (also called Survivin; the inhibitor of apoptosis protein) in cells treated with NO_2-OA or OA for 24 hr. Bcl-xl was significantly down-regulated by NO_2-OA treatment in both MDA-MB-231 and MDA-MB-468 cells, but not in MCF-10A cells (Figure 11C). However, BIRC5 gene expression was down-regulated by NO_2-OA treatment in MDA-MB-468 cells, not MDA-MB-231 cells (Figure 11D). In aggregate, these results confirm differential effects of NO_2-OA on cell cycle and survival in MCF-10A versus TNBC cells.

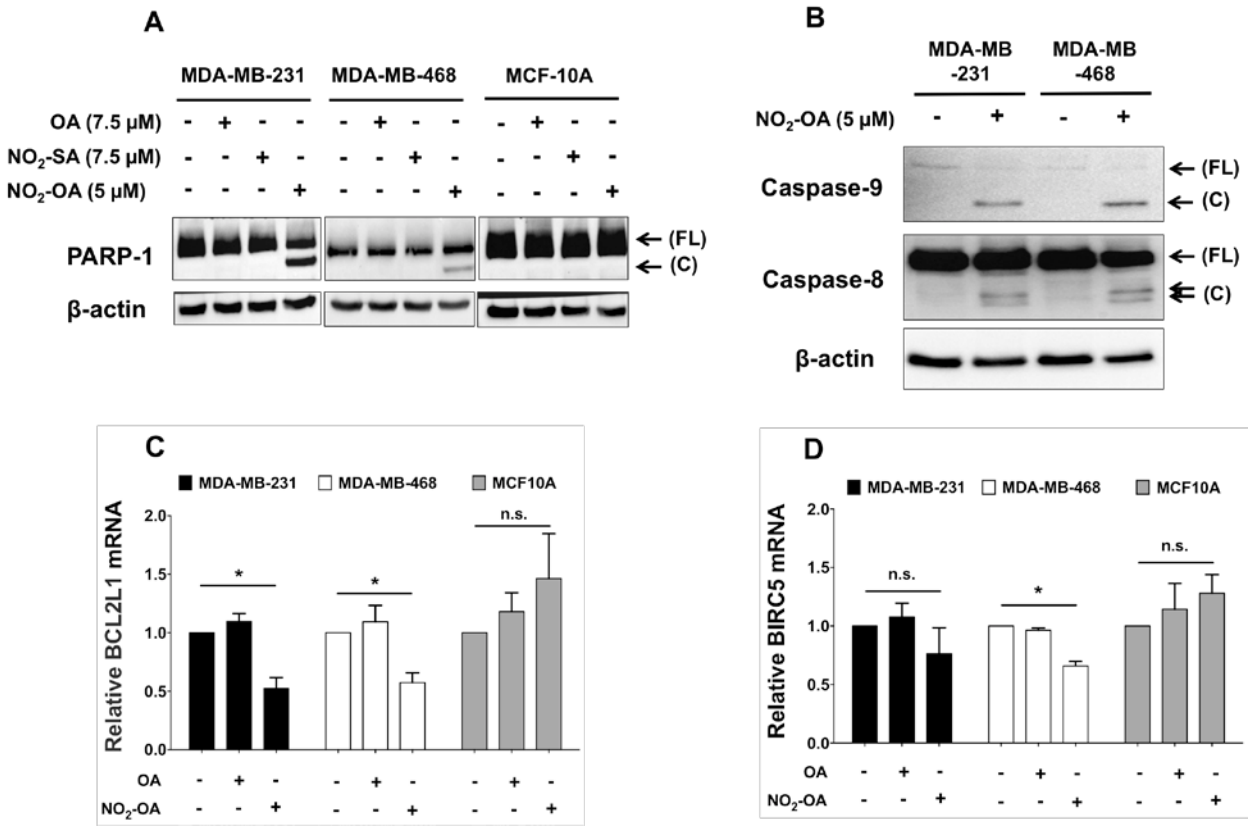


Figure 11. NO_2-OA triggers apoptosis in TNBC cells.

(A) NO_2-OA induces PARP-1 cleavage in TNBC cells. Western blot analysis of PARP-1 cleavage in MCF-10A, MDA-MB-231, and MDA-MB-468 cells, which were treated with OA (7.5 μM), NO_2-SA (7.5 μM) or NO_2-OA (5 μM) for 24 hr. β-actin was used as loading control. Data are representative of 3 independent experiments. (B) NO_2-OA induces caspase-8 and caspase-9 cleavage in TNBC cells. Western blot analysis of caspase-8 and caspase-9 cleavage in MDA-MB-231 and MDA-MB-468 cells treated with or without NO_2-OA (5 μM) for 24 hr. β-actin was used as loading control. Data are representative of 3 independent experiments. (C and D) Effects of NO_2-OA on BCL2L1 and BIRC5 gene expression in breast cancer cell lines. Cells were treated with 5 μM NO_2-OA or OA for 24 hr. BCL2L1 and BIRC5 mRNA levels were measured by RT-qPCR. 18S rRNA was used as an internal control. Data are shown as mean ±

SD (n=3). P-value (*) < 0.05 versus untreated control was considered significant and was determined by one-way analysis of variance followed by Tukey post hoc test.

3.2.5 NO_2-OA Inhibits Cytokine TNFα-induced TNBC Cell Mobility

To determine whether NO_2-OA inhibits TNBC progression promoted by TNFα signaling, we examined the effects on TNBC cell migration and invasion in the presence of TNFα. Boyden chamber migration analyses indicated that TNFα augmented migration in both MDA-MB-231 and MDA-MB-468 cells (Figure 12A, images 3 and 8), compared to basal conditions (Figure 12A, image 2 and 7). NO_2-OA significantly inhibited both MDA-MB-231 and MDA-MB-468 cell migration induced by TNFα (Figure 12A, images 4 and 9; Figure 12B-C). Moreover, NO_2-OA itself reduced the migration of MDA-MB-231 cells (Figure 12B) in basal conditions (Serum Ctrl), but had no effect on MDA-MB-468 cells (Figure 12C).

To evaluate the potential effect of NO_2-OA on TNFα-induced TNBC cell invasion, cells were placed in transwell permeable supports coated with matrigel for invasion assays. TNFα-induced invasion was significantly inhibited by NO_2-OA treatment in MDA-MB-468 cells, whereas the non-electrophilic control fatty acid (NO_2-SA) displayed marginal effects on tumor cell invasion **(**Figure 12D).

The inhibitory actions of NO_2-OA on MDA-MB-468 invasion were further compared to the NF-κB inhibitor JSH-23, which inhibits nuclear translocation of the RelA subunit [194]. Similar to JSH-23, NO_2-OA inhibited invasion induced by TNFα in MDA-MB-468 cells (Figure 12D), suggesting that NO_2-OA may inhibit TNFα-induced breast cancer cell mobility due to its effects on NF-κB signaling.

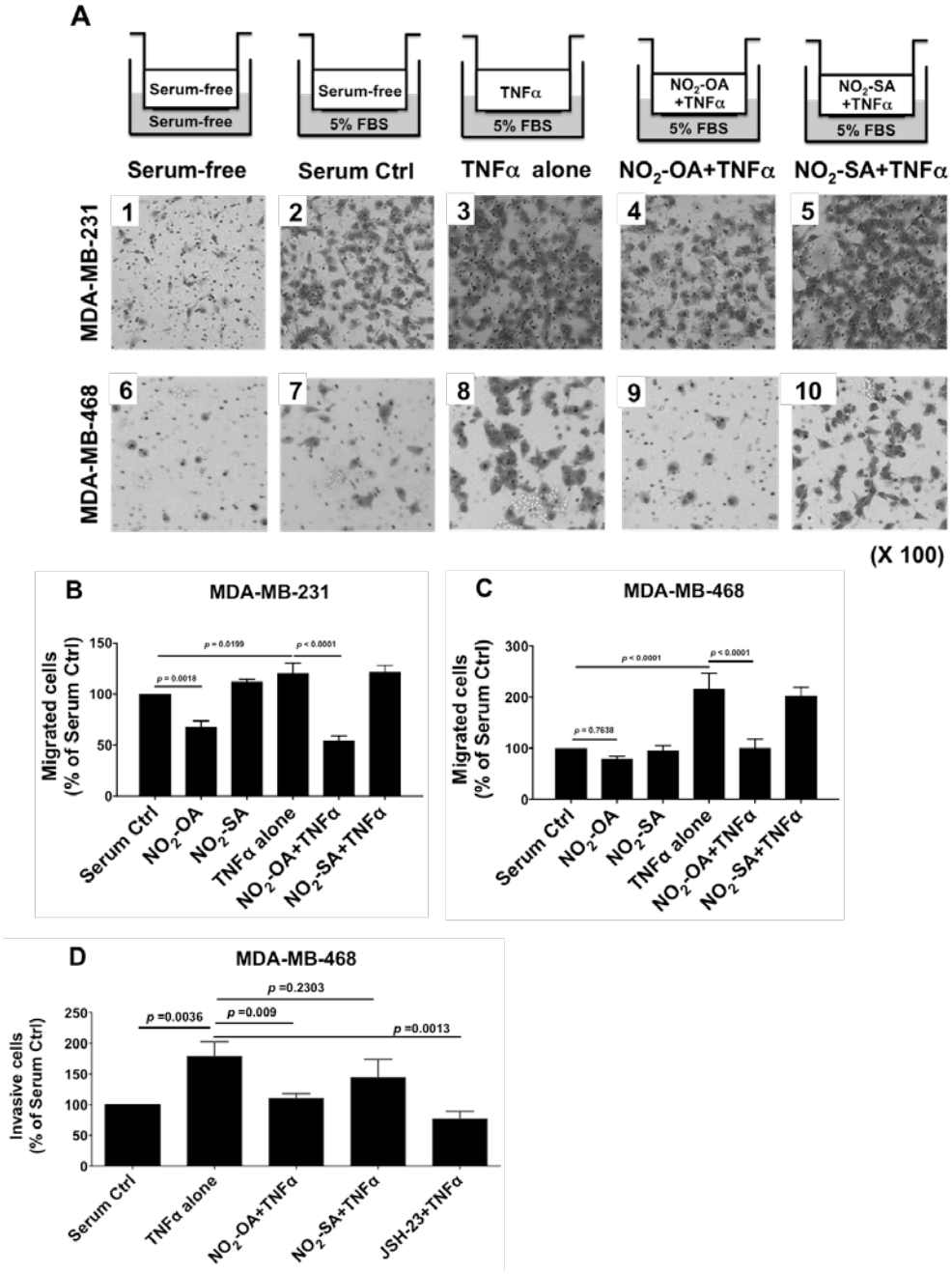


Figure 12. NO_2-OA inhibits TNF*α*-induced TNBC cell mobility.

(A) Diagram and representative images of crystal violet-stained migratory MDA-MB-231 or MDA-MB-468 cells on bottom membrane. 1 x 10^5 pretreated MDA-MB-231 or MDA-MB-468 cells were plated on the top of a porous membrane. NO_2-OA (5 μM) or NO_2-SA (5 μM) in the

presence of TNFα (20 ng/mL) was added in the upper chamber of the wells, and cells were allowed to migrate for 5 hr. Migrated cells were stained with 0.25% crystal violet for 15 mins and then photographed under a light microscope at 100x. (B and C) The migrated cells were quantified by solubilization of crystal violet and spectrophotometric reading at A_{573} nm. The relative cell migration rate in each sample is defined as the percent increase in migrated cells compared to the migrated cells in the absence of TNFα stimulation (Serum Ctrl). (D) MDA-MB-468 cells were incubated in serum-free media containing TNFα combined with NO_2-OA (5 μM), NO_2-SA (5 μM), or JSH-23 (10 μM) and allowed to invade through Matrigel matrix toward 5% FBS chemotractant for 24 hr. Percentage of invading cells in the treatment was compared with Serum Ctrl. All data are shown as mean ± SD of three independent experiments. Statistical $p < 0.05$ was considered significant and was determined by one-way analysis of variance followed by Tukey post hoc test.

3.2.6 Effects of NO_2-OA on the Growth of MDA-MB-231 *in vivo*

Given that TNBC cell growth (Figure 7) and viability (Figure 9) are inhibited by NO_2-OA, the efficacy of NO_2-OA on tumor growth was examined in a murine xenograft model of TNBC. MDA-MB-231 cells have been commonly incorporated into xenograft models of TNBC and shown to be tumorigenic [195]; thus, we used this cell line to primarily examine the efficacy of NO_2-OA on TNBC tumor growth.

MDA-MB-231 (3×10^6/100 μL) cells were injected into the fourth inguinal mammary fat pad of 6-week-old female athymic nude mice. Oral gavage with NO_2-OA (7.5 mg/kg/day), NO_2-SA (7.5 mg/kg/day), or sesame oil (vehicle control; 100 μL/day) was initiated and continued for

4 weeks after the average tumor sizes reached approximately 50–100 mm^3. NO_2-OA significantly reduced MDA-MB-231 xenograft tumor growth compared to vehicle controls (Figure 13). NO_2-SA did not affect the tumor growth, in consistent with the *in vitro* study on cell growth (Figure 8).

During the course of treatment, there was no obvious weight loss observed in NO_2-OA-treated mice (Figure 14). Mice behaved normally and no adverse effects were observed, suggesting that there is a lack of toxicity of NO_2-OA *in vivo*.

3.2.7 Oral Bioavailability of NO_2-OA on MDA-MB-231 Xenograft Tumors

We next sought to test the efficiency of drug delivery and distribution into tumors using oral administration; the bioavailability of NO_2-OA or its metabolites (NO_2-18:0 and NO_2-14:0) was directly quantified from xenograft tumors by mass spectrometry analysis (Figure 15). Total free and protein-bound NO_2-OA was detectable in NO_2-OA-treated tumors, and the range of concentration is around 0.1-1 nM (Figure 15A). Control vehicle- or NO_2-SA-treated tumors were NO_2-OA-free, indicating there was no contamination or treatment mistakes during the treatment.

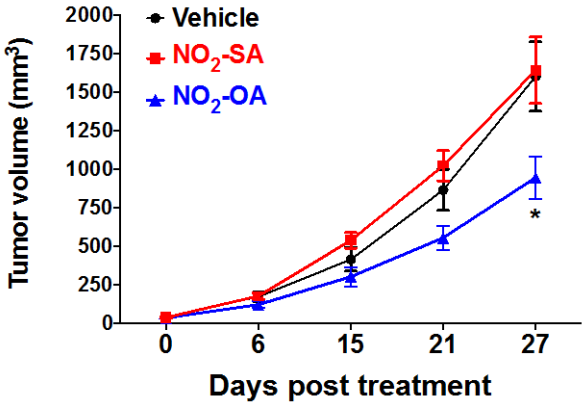


Figure 13. NO_2-OA reduces MDA-MB-231 xenograft tumor growth.

Mammary fat pad MDA-MB-231 tumor xenograft-bearing female athymic nude mice were treated with NO_2-OA (7.5 mg/kg, n=15), NO_2-SA (7.5 mg/kg, n=13), or sesame oil (vehicle control, n=10) by oral gavage every day for 4 weeks. Data are shown as mean ± SEM. Significant difference (*, $p < 0.05$) compared with vehicle group by using two-way analysis of variance followed by Tukey post hoc test.

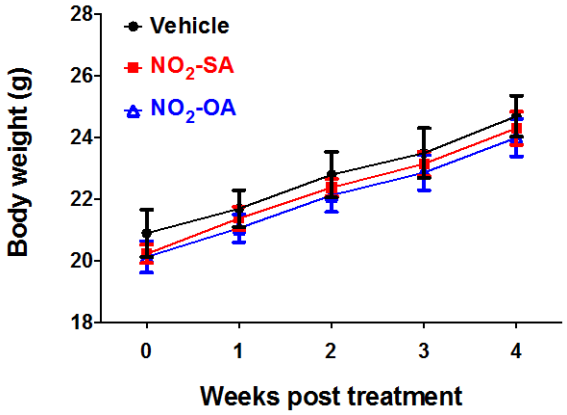


Figure 14. NO_2-OA does not cause mouse body weight loss.

Mammary fat pad MDA-MB-231 tumor xenograft-bearing female athymic nude mice were treated with NO_2-OA (7.5 mg/kg, n=15), NO_2-SA (7.5 mg/kg, n=13), or sesame oil (vehicle control, n=10) by oral gavage everyday for 4 weeks. Body weights were measured weekly. Data are shown as mean ± SD. Statistical analysis was compared with vehicle group by two-way analysis of variance followed by Tukey post hoc test.

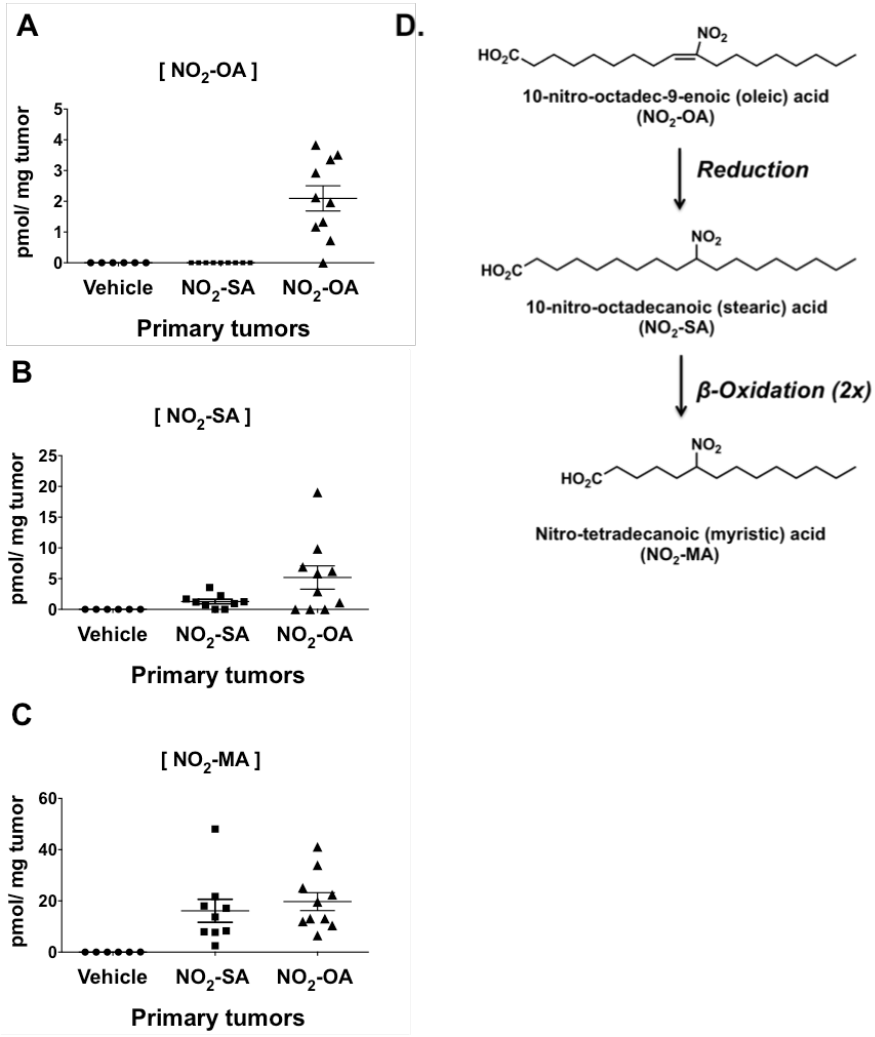


Figure 15. Detection of NO_2-OA and its metabolites in MDA-MB-231 xenograft tumors.

Lipids were extracted from MDA-MB-231 xenograft tumors as described in Materials and Methods. (A) 10-nitro-octadec-9-enoic acid (**NO_2-OA**; NO_2-18:1), (B) 10-nitro-octadecanoic acid (**NO_2-SA**; NO_2-18:0), and (C) Tetranor metabolite 6-nitro-myristic acid (**NO_2-MA**; NO_2-14:0) were quantified by HPLC-MS/MS using [$^{13}C_{18}$]NO_2-OA as internal standard. Quantification of lipids was normalized to tumor weight (mg). Results represent individuals with the mean ± SD. (D) Reduction and β-oxidation reactions of NO_2-OA. First, NO_2-OA is

metabolized *via* reduction to non-electrophilic NO_2-SA. NO_2-SA is then metabolized to chain length 14 carbons (NO_2-MA) through two cycles of β-oxidation.

3.3 DISCUSSION

Compared to other breast cancer phenotypes, TNBC is an aggressive subtype with a poor prognosis [163], with patients four times more likely to show visceral metastases to the lung, liver and brain within five years after diagnosis [169]. This is because TNBC does not respond to endocrine therapy or other more targeted chemotherapeutic agents; thus, DNA damage-inducing strategies such as ionizing radiation, cis-platin and doxorubicin remain mainstay treatments. Adverse systemic responses to DNA-directed chemotherapeutic agents, including cardiac and renal toxicity, limit chemotherapy options because of cytotoxic effects on non-cancerous cells [196-198].

In this study, we demonstrated that NO_2-OA inhibited TNBC cell viability, motility and tumor cell proliferation-related signaling reactions to an extent where *in vivo* tumor growth in MDA-MB-231 xenografted mice was attenuated by oral dosing of NO_2-OA. This observation also motivates more detailed dose-timing and dose-response studies of NO_2-OA effects on tumor growth and metastasis in preclinical animal models.

It has been previously reported that electrophilic sulforaphane induced cell cycle arrest of MDA-MB-231 and MDA-MB-468 cells at G2/M phase [184, 185]. Sulforaphane also induced apoptosis by activation of caspase-8 in MDA-MB-231 cells and activation of caspase-9 in MDA-MB-468 [184]. Our studies showed that treatment with NO_2-OA inhibited the growth of human TNBC cells both *in vitro* and *in vivo*. Other studies have suggested the overexpression of cyclin

D1 is correlated with the aberrant proliferation of human TNBC cells [199]. In one phase II clinical trial study, a significant suppression in cyclin D1 expression was observed when patients with TNBC were treated with carboplatin and eribulin [200]. Our results showed that NO_2-OA significantly inhibited the expression of cyclin D1 mRNA and protein in NDA-MB-231 and MDA-MB-468 cells. p21, the inhibitor of CDK, was also down-regulated by NO_2-OA in TNBC cells. Conversely, the expression of cyclin D1 and p21 in non-cancerous ductal MCF-10A cells were not affected by NO_2-OA. The putative mechanisms of MCF-10A cell protection from NO_2-OA are discussed in Chapter 5 of this dissertation.

In addition, NO_2-OA induced apoptosis by initiation of both mitochondrial (caspase-9 activation) and death receptor (caspase-8 activation) pathways. At this point, more detailed mechanisms of NO_2-OA-induced apoptotic cell death remain to be defined; however, sulforaphane decreases Bcl-2 expression, activates cytochrome c release from the mitochondria, and increases FasL expression in TNBC cells [184]. These actions imply that other electrophilic NO_2-FA derivatives might mediate similar actions in the regulation of apoptosis. In aggregate, these data reveal that NO_2-OA displays pleiotropic anti-cancer properties *via* the inhibition of cell proliferation and induction of apoptosis in TNBC (Figure 16).

Inhibition of invasive potential is important for the prevention of tumor progression and reoccurrence. Studies have shown that TNFα plays an important role in tumor invasion and metastasis [6, 7] and promotes the metastatic potential of TNBC, with the up-regulation of TNFα expression and activity in TNBC patients strongly linked with tumor metastatic phenotype [10]. *In vitro* experiments showed that treatment with NO_2-OA inhibited cytokine TNFα-induced migration and invasion of TNBC cells. This finding suggests that NO_2-OA might be considered as a potent agent for prevention of inflammatory-mediated in TNBC metastasis. The potential

mechanism of action of NO_2-OA on inhibition of TNFα signaling is proposed in Chapter 4 of this dissertation.

Our results showed that NO_2-OA also reduced migration of MDA-MB-231 cells in the absence of TNFα induction. It is likely that NO_2-OA inhibits cell mobility through multiple different molecular targets along with the TNFα-NF-κB signaling cascade, since the electrophilic cyclopentenone 15d-PGJ_2 also interferes with mammary cancer cell migration *via* inhibition of F-actin reorganization and focal adhesion disassembly [201]. Additional studies are necessary to explore and identify other potential metastasis-related molecules and signaling pathways, which could be targeted by NO_2-OA in TNBC.

In summary, to the best of our knowledge, this is the first evidence demonstrating the inhibitory effects of electrophilic NO_2-OA on the proliferation, survival, and mobility of human TNBC cells and MDA-MB-231 xenograft tumor growth. The concentrations of endogenous NO_2-FAs observed in healthy human plasma and urine are 1-5 nM [143, 202, 203]. Our data revealed that tumor levels of ~ 1 nM *in vivo* were sufficient to result in a pharmacological effect. This preclinical study is built upon a foundation of consistent data and serves as a prelude to a clinical study of electrophilic NO_2-FA as a new therapeutic agent or co-agent that may display selectivity for treating patients with metastatic TNBC, which currently lacks effective treatment options.

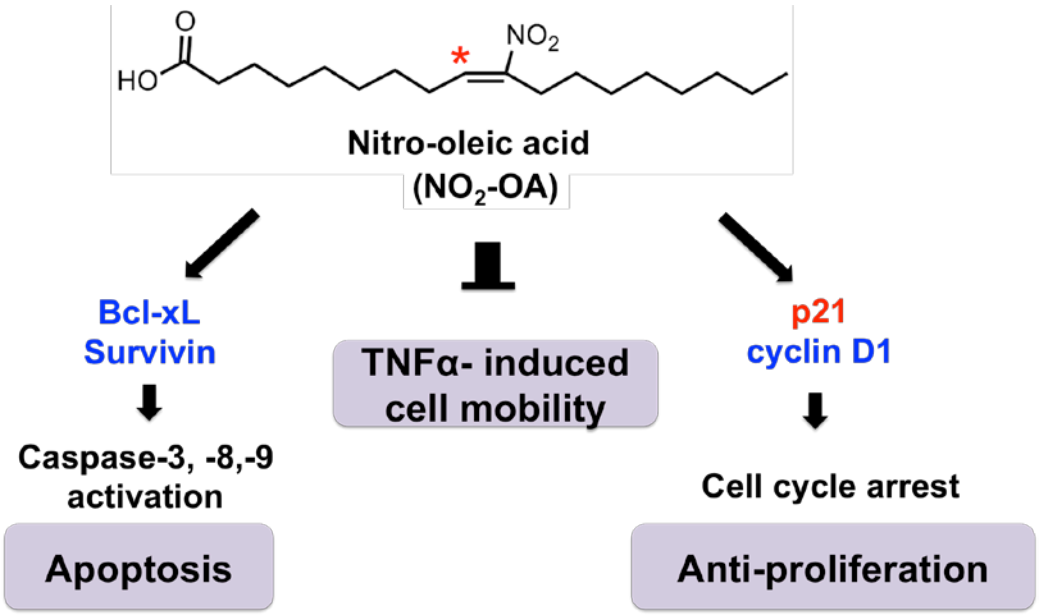


Figure 16. Schematic of NO_2-OA effects on TNBC.

NO_2-OA is an electrophile that plays pleotropic roles in inhibition of TNBC survival, proliferation, and metastasis promoted by TNFα. Red: up-regulation by NO_2-OA; Blue: down-regulation by NO_2-OA. Red asterisk denotes the electrophilic carbon.

4.0 NITRO-OLEIC ACID-MEDIATED INHIBITION OF NF-$_K$B SIGNALING IN TRIPLE NEGATIVE BREAST CANCER

4.1 INTRODUCTION

A meta-analysis reveals that TNBC patients with elevated TNFα expression have an increased risk of distant tumor metastasis [204]. Preclinical studies indicate that TNFα induces NF-κB activation and drives the expression of extracellular matrix degradation enzymes, adhesive molecules, and chemokines, which promote MDA-MB-231 cell migration [61, 205, 206]. Activation of NF-κB has been linked with TNBC development and progression [180, 207, 208], with signaling being constitutively activated in ER-negative breast cancer cell lines and primary tumors [180, 181, 208, 209]. Moreover, available evidence suggests that bioactive molecules or NF-κB-specific inhibitors block NF-κB activity and in turn, inhibited TNBC cell mobility [61, 186, 210]. Therefore, NF-κB inhibition strategies display a rational therapeutic approach for countering metastatic TNBC.

Recent studies reported that NO_2-OA acts as an anti-inflammatory mediator to inhibit LPS-induced NF-κB transcriptional activity in endothelial and immune cells [100, 101]. Because we observed that NO_2-OA attenuated TNBC cell migration and invasion induced by TNFα (Chapter 3, Figure 12), the effect of NO_2-OA on the downstream events of TNFα-induced NF-κB transcriptional activity was explored. In order to identify the potential NF-κB target genes

associated with TNBC cell mobility down-regulated by NO_2-OA, we performed NF-κB target cDNA PCR array on a parallel set of NO_2-OA-treated and NO_2-OA-untreated MDA-MB-468 cells. For those interesting gene candidates, we further examined the impact of TNFα on their gene expression in both MDA-MB-231 and MDA-MB-468 cells for involvement in promoting cell migration.

In addition, we investigated any potential mechanisms of action of NO_2-OA on the TNFα-mediated NF-κB activation cascade in both MDA-MB-231 and MDA-MB-468 cells. Cys179 in the activation loop of IKKβ is known to be a target for several electrophiles, such as the cyclopentenone prostaglandins [211] and the triterpenoid CDDO-Me [212]. A recent study demonstrated that NO_2-OA alkylates Cys38 on the NF-κB subunit RelA in RAW264.7 macrophages [100]. The ability of NO_2-OA to bind IKKβ and RelA was evaluated in TNBC cells by a biotin-streptavidin affinity analysis. In order to determine whether other possible Cys residues of RelA protein (except of Cys38) would be alkylated by NO_2-OA, mass spectrometry analysis was performed.

A recent study reported that NO_2-OA suppresses NF-κB activation induced by LPS *via* dephosphorylation of IKKβ and IκBα in both endothelial cells and macrophages [101]; therefore, the effects of NO_2-OA on phosphorylation of IKKβ and IκBα in TNBC cells were further examined. Proteolytic degradation of NF-κB has been studied as a termination event in NF-κB signaling. An interesting study has revealed that thiol-alkylating and nitrosylating agents induce the degradation of NF-κB subunit p50 *via* the PTM of Cys62 residue in both HT29 and HCT116 tumor cell lines [213]. Based on this concept, the effect of NO_2-OA on proteosomal-dependent degradation machinery of NF-κB RelA protein was investigated.

Finally, in order to determine the impacts of NO_2-OA on NF-κB signaling *in vivo*, primary tumors from the MDA-MB-231 xenograft study (See section 3.2.6) were used to examine the extent of protein expression of IκBα using western blot analysis and mRNA expression of NF-κB target genes using q-RT-PCR analysis in NO_2-OA versus vehicle treatment.

4.2 RESULTS

4.2.1 NO_2-OA Inhibits TNFα-induced NF-κB Transcriptional Activity in TNBC Cells

The inhibition of MDA-MB-468 cell invasion by the NF-κB inhibitor JSH-23 (Chapter 3, Figure 12D) suggests that NO_2-OA may also inhibit TNFα-induced breast cancer cell invasion in a similar manner. To test this concept, the effect of NO_2-OA on TNFα-mediated NF-κB transcriptional activity in TNBC cells was examined. MDA-MB-231 and MDA-MB-468 cells were transiently transfected with a NF-κB luciferase reporter plasmid and pretreated with 5 μM NO_2-OA for 2 hr, followed by stimulation with 20 ng/mL TNFα for additional 4 hr. In addition to NO_2-OA, the non-electrophilic control lipids NO_2-SA (5 μM) and OA (5 μM) were also examined. NO_2-OA significantly inhibited NF-κB-dependent transcription of luciferase compared with TNFα alone in both TNBC cell lines, while NO_2-SA or OA did not alter luciferase production. Moreover, the extent of NO_2-OA inhibition of NF-κB-derived luciferase expression was similar to JSH-23 (Figure 17). These data indicate that NO_2-OA inhibits TNFα-induced NF-κB transcriptional activity in TNBC cells.

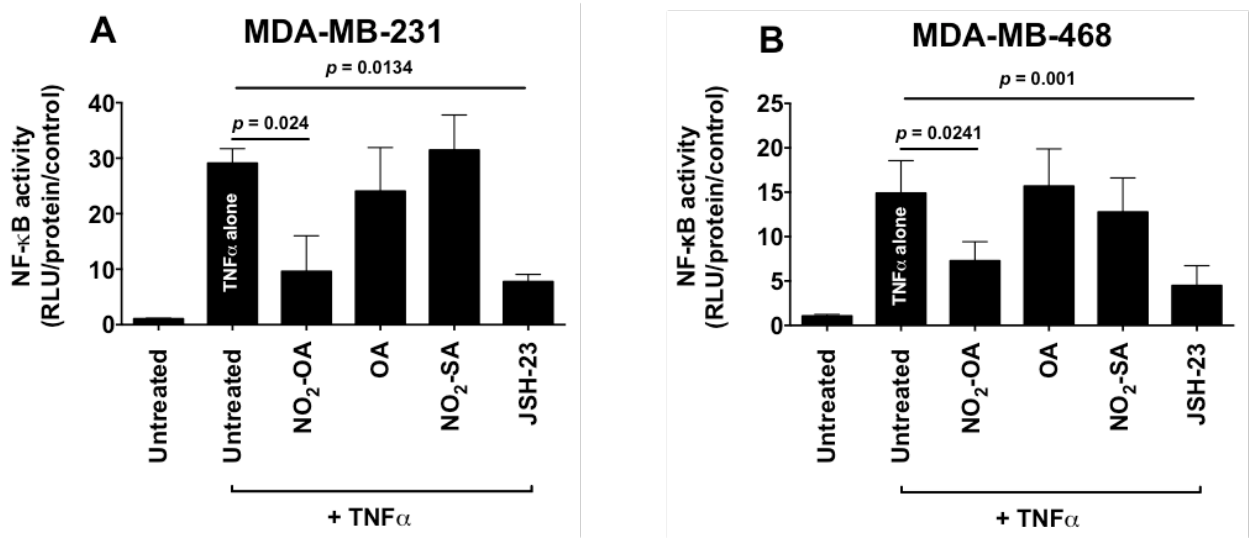


Figure 17. NO_2-OA inhibits TNFα-induced NF-κB transcriptional activity in TNBC cells.

(A) MDA-MB-231 or (B) MDA-MB-468 cells were used in this study. Luciferase activity was assayed and normalized to total protein concentrations. Data (mean RLU ± SD) represent the ratio of treated samples to untreated control (Control) and the fold increase relative to control is presented. P-value < 0.05 was considered significant using Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni corrections for multiple comparisons.

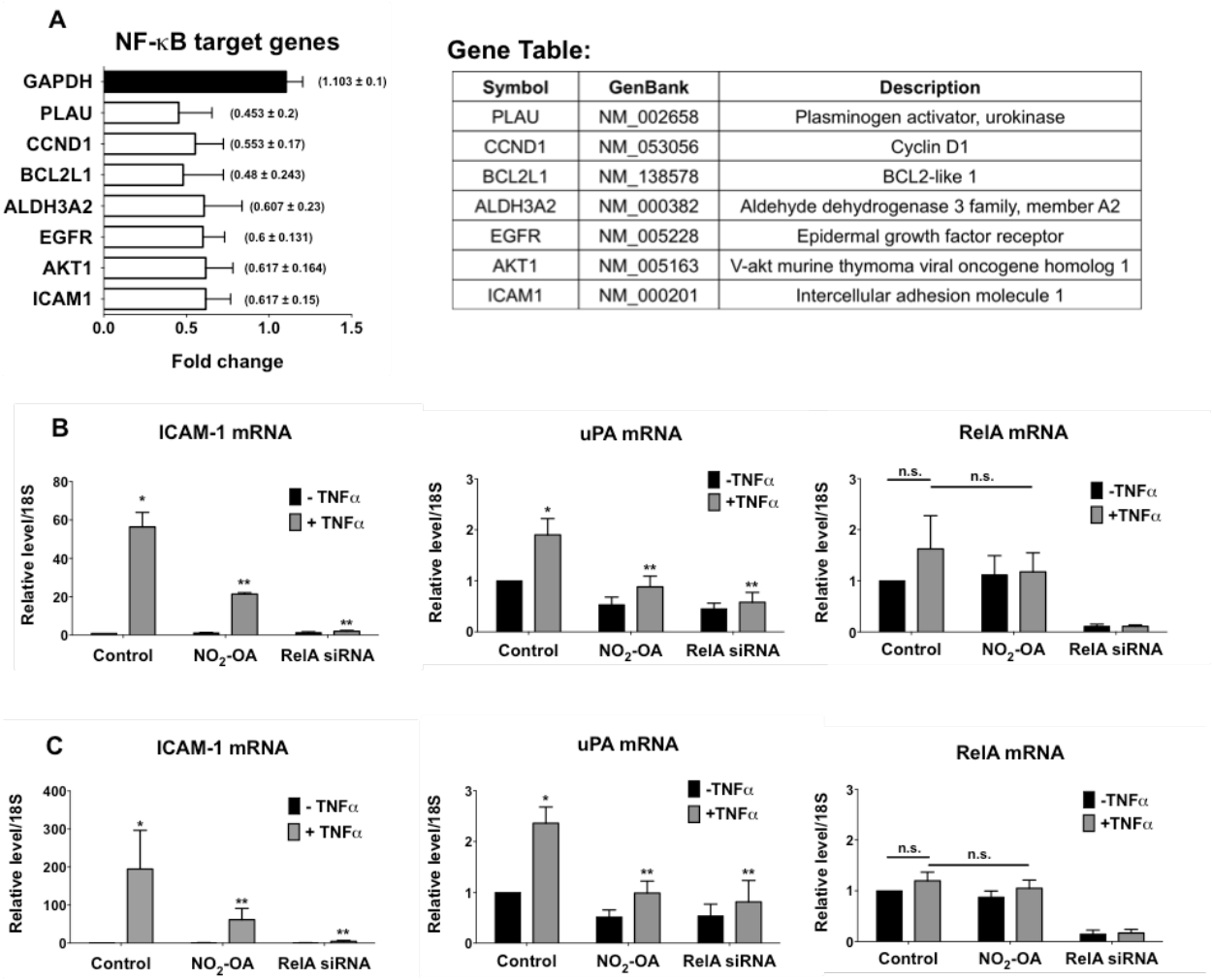
4.2.2 NO_2-OA Inhibits NF-κB-regulated Metastatic Genes in TNBC Cells

Inhibition of NF-κB transcriptional activity by NO_2-OA suggested that the expression of metastasis-related downstream target genes may be decreased. To investigate this, key NF-κB target genes were evaluated in MDA-MB-468 cells treated with NO_2-OA *via* RT^2 profiler PCR array analysis. The expression levels of NF-κB target genes that were regulated by NO_2-OA were compared to MDA-MB-468 untreated control cells. Seven NF-κB target genes were down-regulated with a high degree of change in NO_2-OA treated compared to untreated control cells. These genes play important roles in cell proliferation, survival, and metastasis. ICAM-1 and

uPA, two important regulators of tumor progression and metastasis, were targeted by NO_2-OA (Figure 18A). Treatment with NO_2-OA also decreased the mRNA expression of CCND1 and BCL2L1 genes, in consistent with previous observations (Chapter 3, Figure 10F and Figure 11C).

Previous studies reported that TNFα induces the expression of ICAM-1 and uPA in MDA-MB-231 cells [61, 205]. We sought to determine if electrophilic NO_2-OA suppresses TNFα-induced expression of ICAM-1 and uPA in TNBC cells. MDA-MD-231 or MDA-MD-468 cells were used and pretreated with NO_2-OA before TNFα stimulation. qRT-PCR analysis revealed that TNFα treatment alone resulted in induced expression of ICAM-1 and uPA compared with untreated controls. Simultaneous treatment with NO_2-OA suppressed TNFα-induced expression of ICAM-1 and uPA genes in TNBC cells compared to TNFα treatment alone (Figure 18B-C). However, ICAM-1 and uPA gene expression were abolished by TNFα stimulation in RelA siRNA TNBC cells (Approximately 85-90 percent knockdown efficiency, Figure 18B-C). Together, these data suggest that NO_2-OA inhibited TNFα-induced ICAM-1 and uPA gene expression *via* NF-κB-dependent mechanisms.

In order to determine if TNFα-induced ICAM-1 and uPA gene expression was suppressed by NO_2-OA during cell migration, transcript levels of ICAM-1 and uPA genes were evaluated in MDA-MB-468 cells in Boyden chamber migration assays (Chapter 3, Figure 12C). NO_2-OA significantly inhibited TNFα-induced expression of ICAM-1 and uPA in migrating MDA-MB-468 cells (Figure 19), again supporting that NO_2-OA inhibited expression of NF-κB-regulated genes involved in metastasis.



Symbol	GenBank	Description
PLAU	NM_002658	Plasminogen activator, urokinase
CCND1	NM_053056	Cyclin D1
BCL2L1	NM_138578	BCL2-like 1
ALDH3A2	NM_000382	Aldehyde dehydrogenase 3 family, member A2
EGFR	NM_005228	Epidermal growth factor receptor
AKT1	NM_005163	V-akt murine thymoma viral oncogene homolog 1
ICAM1	NM_000201	Intercellular adhesion molecule 1

Figure 18. NO_2-OA down-regulates NF-κB-regulated metastatic genes in TNBC cells.

(A) Identification of NF-κB target genes down-regulated by NO_2-OA was determined using a human NF-κB target PCR array. MDA-MB-468 cells were treated with or without NO_2-OA (5 μM) for 24 hr. Histograms represent the fraction of mRNA expression in the NO_2-OA-treated cells versus untreated cells. Expression of the housekeeping gene GAPDH was used as an internal control (black bar). The evaluation and effect of NO_2-OA on gene expression of TNFα-induced ICAM-1, uPA, or RelA in MDA-MB-231 (B) and MDA-MB-468 (C) cells. To knock

down the RelA gene, RelA siRNAs were transiently transfected into cells for 24 hrs. Wild-type and RelA siRNA cells were then pretreated with NO_2-OA (5 μM) for 2 hr and then stimulated with TNFα (20 ng/mL) for 6 hr. ICAM-1, uPA, and RelA mRNA levels were measured by RT-qPCR. 18S rRNA was used as an internal control. All data represent the mean ± SD of three individual experiments and the fold increase relative to untreated control is presented. *, $p < 0.05$ versus untreated control, **, $p < 0.05$ versus TNFα alone. n.s., not significant between two groups. Significance was calculated by one-way analysis of variance followed by Tukey post hoc test.

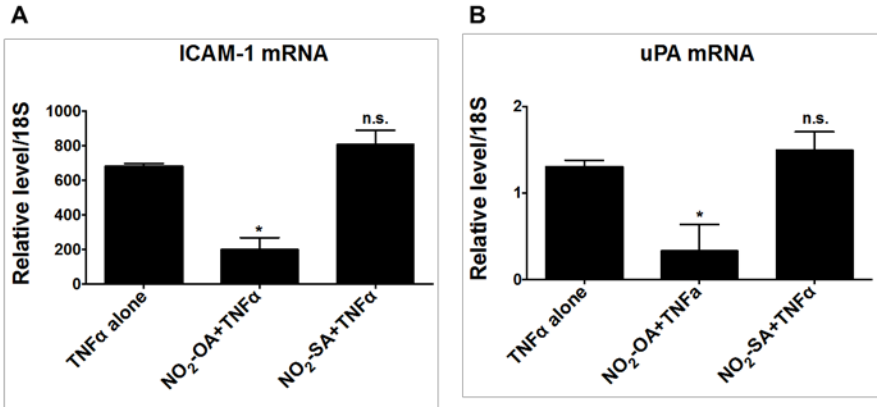


Figure 19. NO_2-OA inhibits ICAM-1 and uPA gene expression induced by TNFα during MDA-MB-468 cell migration.

MDA-MB-468 cells were treated under the same conditions as the cell migration assay (described in Materials and Methods). (A) ICAM-1 and (B) uPA mRNA levels were measured by RT-qPCR. 18S rRNA was used as an internal control. Data represent the mean ± SD and the fold relative to untreated control is presented. *, $p < 0.05$ versus TNFα alone was calculated by one-way analysis of variance followed by Tukey post-hoc test. n.s., not significant.

4.2.3 NO_2-OA Alkylates TNBC IKKβ and RelA.

Because NO_2-OA (but not NO_2-SA) suppresses TNFα-induced NF-κB transcriptional activity (Figure 17) and its targets gene expression (Figure 18B-C) in TNBC cells, the potential for NO_2-OA to directly alkylate NF-κB signaling molecules IKKβ or RelA was investigated. Biotinylated lipids (Bt-NO_2-OA, Bt-NO_2-SA, and Bt-OA) were kindly synthesized and purified by Dr. Steven Woodcock to allow affinity capture-mediated measurement of NO_2-OA and control fatty acid adduction of IKKβ or RelA protein. Followed by streptavidin-biotin-NO_2-OA affinity precipitation from cell lysates, western blot analysis revealed that IKKβ and RelA were pulled down by Bt-NO_2-OA but not control lipids, indicating that NO_2-OA undergoes covalent Michael addition to IKKβ and RelA through its electrophilic nitroalkene moiety (Figure 20). In addition, LC-MS/MS proteomic analysis showed that Cys105 on RelA was also alkylated by NO_2-OA (Figure 21). However, the functional significance of the NO_2-OA alkylation of Cys105 on RelA remains unclear. Together, these results provide mechanistic evidence that NO_2-OA not only directly adducts IKKβ but also RelA, which blocks their activities and leads to inhibition of NF-κB activation.

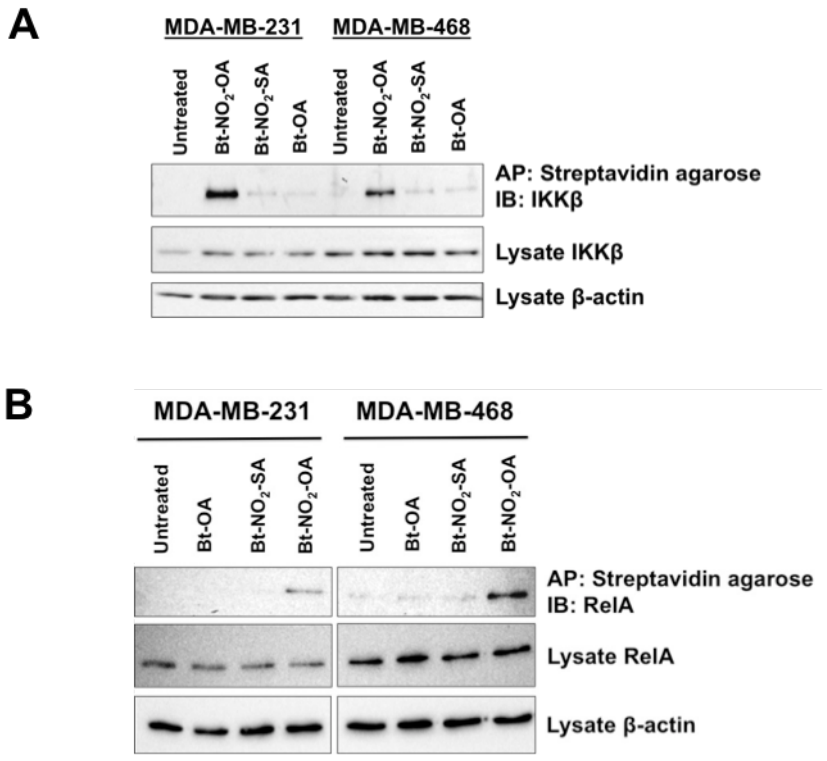


Figure 20. NO_2-OA adducts IKKβ and NF-κB RelA proteins in TNBC cells.

MDA-MB-231 or MDA-MB-468 cells were treated with 5 μM Bt-NO_2-OA Bt-NO_2-SA, or Bt-OA for 2 hr. After cell lysis, biotinylated NO_2-FAs with adducts were affinity purified (AP) by streptavidin agarose beads. Pulled-down IKKβ (A) and RelA (B) proteins were then detected by western blot analysis. IKKβ, RelA, and control β-actin western blots from the same input lysates for AP are shown in the bottom panel. Representative western blot of three individual experiments is presented.

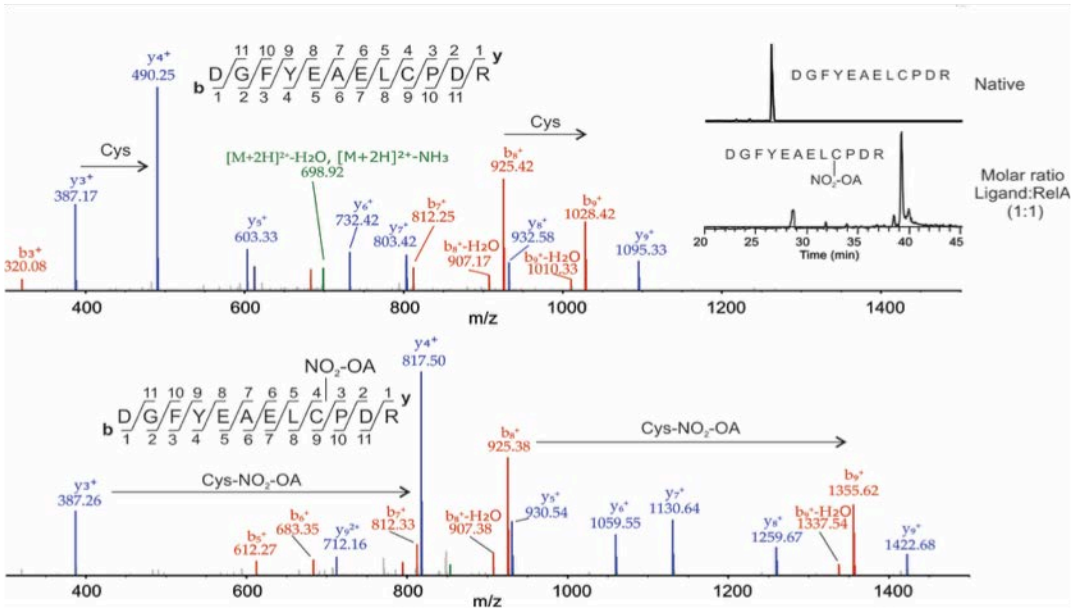


Figure 21. Electrophilic NO_2-OA Alkylates RelA Cys105.

Recombinant RelA (5 μg) protein was incubated for 1 hr in the absence or presence of NO_2-OA at 1:1 mol ratio. The fragmentation pattern of peptide $D^{97}GFYEAELCPDR^{108}$ was obtained from the tryptic digest of untreated- (upper panel) or NO_2-OA-treated RelA (lower panel). Peptides were resolved by reversed phase chromatography and analyzed by electrospray ionization-tandem mass spectrometry. The total ion current (TIC) (insert) shows the differences between the retentions times for both native and NO_2-OA covalently adducts on Cys105 in RelA protein.

4.2.4 NO_2-OA Inhibits TNFα-induced IKKβ Phosphorylation and IκBα Protein Degradation in TNBC Cells

Phosphorylation and degradation of IκBα are critical events in regulation of NF-κB activation. To shed light on the precise role of NO_2-OA inhibition in TNFα-activated NF-κB signaling, the effect of NO_2-OA on IKKβ phosphorylation was first examined. MDA-MB-231 or MDA-MB-468 cells were pretreated with NO_2-OA or the IKK inhibitor BAY11-7082 (positive control) for 2 hr before simultaneous treatment with TNFα for 5 min. Western blot analysis revealed that the basal IKKβ phosphorylation was silent, but TNFα significantly stimulated IKKβ phosphorylation in both MDA-MB-231 and MDA-MB-468 cells. Further, TNFα-induced IKKβ phosphorylation was significantly diminished by either NO_2-OA or BAY11-7082 treatment (Figure 22A).

In order to determine whether NO_2-OA inhibition of IKKβ phosphorylation prevents the degradation of IκBα in response to TNFα stimulation, the abundance of IκBα protein was evaluated after 10 min of TNFα treatment *via* western blot analysis. IκBα protein levels were significantly decreased following TNFα stimulation. Again, both NO_2-OA and BAY11-7082 treatment prevented the degradation of IκBα stimulated by TNFα (Figure 22B). Furthermore, decreased IκBα phosphorylation occurred in cells pretreated with NO_2-OA or BAY11-7082 combined with a proteasome inhibitor MG-132 (Figure 22C). Together, these findings indicate that NO_2-OA but not NO_2-SA suppresses TNFα-induced IKKβ phosphorylation and IκBα degradation, with these actions, in turn, inhibiting downstream NF-κB signaling in TNBC cells.

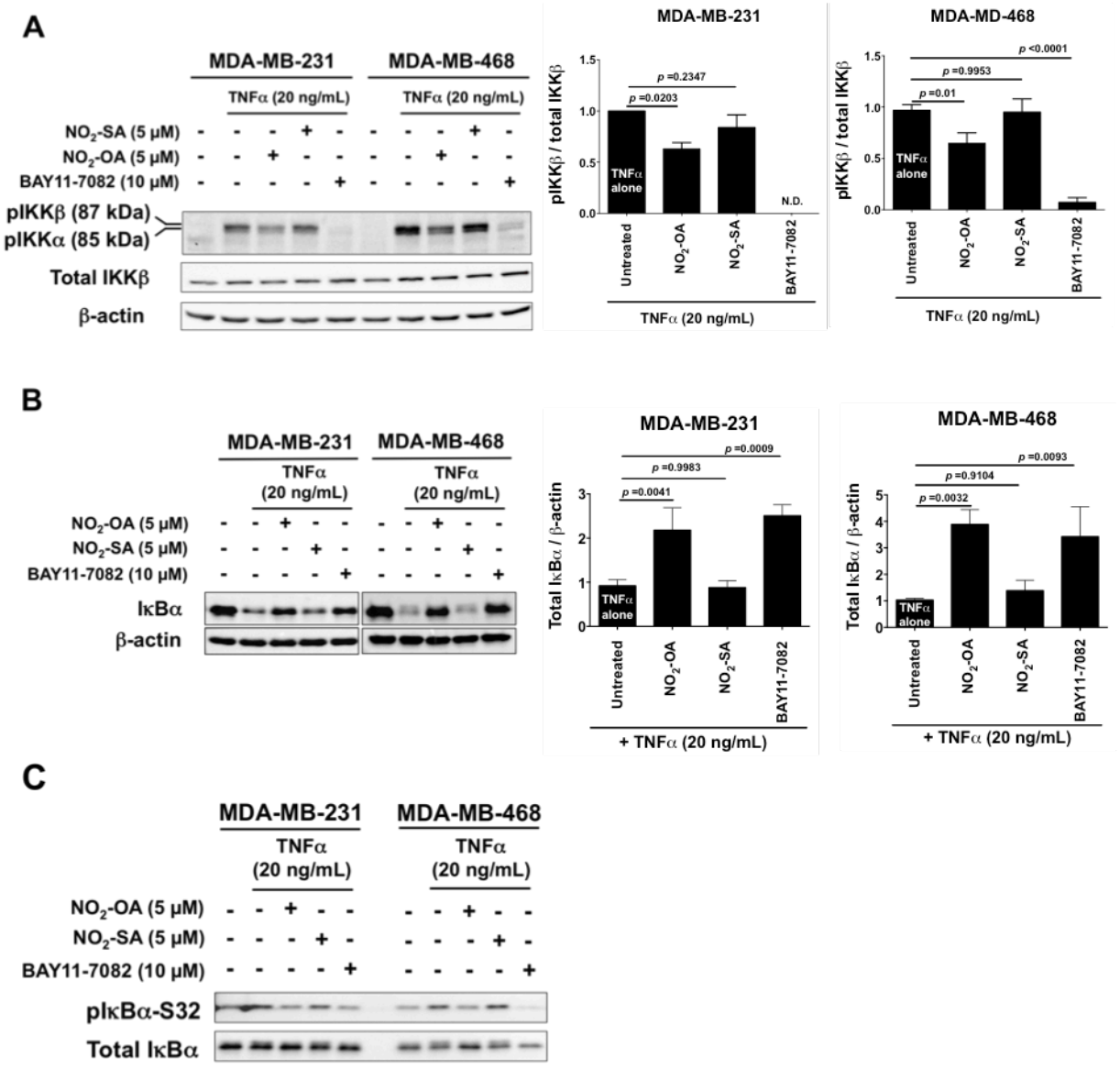


Figure 22. NO_2-OA suppresses TNFα-induced the IKK-IκBα signaling in TNBC cells.

(A) NO_2-OA reduced TNFα-stimulated IKKβ phosphorylation on Ser180 in MDA-MB-231 and MDA-MB-468 cells. Representative western blots of IKKβ (Ser180) phosphorylation and total IKKβ are shown. The relative phosphorylated IKKβ levels (normalized to total IKKβ) from two independent experiments were quantified using Image Lab software (Bio-Rad) and considered statistically significant at $p < 0.05$ when compared with TNFα control using one-way analysis of

variance followed by Tukey post hoc test. N.D. Not detectable. (B) NO_2-OA prevented TNFα-stimulated IκBα degradation in MDA-MB-231 and MDA-MB-468 cells. The levels of IκBα protein were determined by western blot analysis using β-actin as a loading control. The relative total IκBα levels (normalized to total β-actin) from three independent experiments were quantified and considered statistically significant at $p < 0.05$ when compared with TNFα control using one-way analysis of variance followed by Tukey post hoc test. (C) NO_2-OA reduced TNFα-stimulated IκBα phosphorylation on Ser32 in MDA-MB-231 and MDA-MB-468 cells. Cells were pretreated with MG-132 (10 μM) in combination with NO_2-OA (5 μM), NO_2-SA (5 μM), or BAY11-7082 (10 μM) in serum free media for 2 hr before TNFα (20 ng/mL) stimulation for 10 minutes. Representative western blots of IκBα (Ser32) phosphorylation and total IκBα are shown. β-actin was used as a loading control. All graphs are shown as mean ± SD.

4.2.5 NO_2-OA Destabilizes NF-κB RelA Protein in TNBC Cells *via* Promotion of Ubiquitination-dependent Degradation

A recent study demonstrated that thiol-alkylating and nitrosylating agents are able to induce the degradation of the NF-κB subunit p50 by the PTM of Cys62 residue in both HT29 and HCT116 tumor cell lines [213]. We observed that NO_2-OA covalently adducted RelA protein in both MDA-MB-231 and MDA-MB-468 cells (Figure 20B). To test our hypothesis that NO_2-OA regulates RelA protein stability by its PTM, we first validated the abundance of endogenous RelA protein expression in response to NO_2-OA by western blot analysis. MDA-MB-231, MDA-MB-468, or MCF-10A cells were treated with 5 μM NO_2-OA or control lipids (OA and NO_2-

SA) for 24 hr. Western blot data revealed that NO_2-OA decreased the abundance of RelA in TNBC cells, while NO_2-SA did not affect RelA protein level. In contrast, RelA protein level in MCF-10A cells was not altered by NO_2-OA (Figure 23A).

To determine whether NO_2-OA affects transcriptional regulation of *RelA* gene in MCF-10A or TNBC cells, *RelA* mRNA level was analyzed by q-RT-PCR after 24 hr NO_2-OA treatment. In all three cell lines, *RelA* mRNA level was also not altered by NO_2-OA treatment (Figure 23B). Taken together, these data suggest that NO_2-OA may regulate RelA protein stability in TNBC cells.

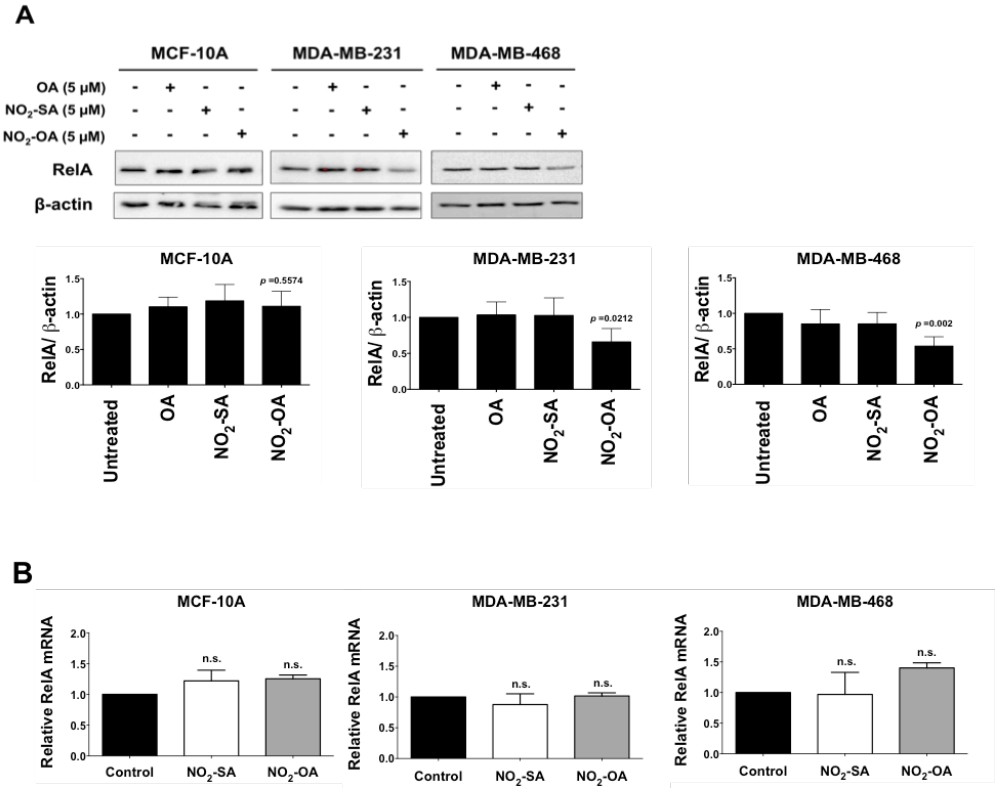


Figure 23. NO_2-OA down regulated RelA protein expression in TNBC cells.

(A) MDA-MB-231, MDA-MB-468, or MCF-10A cells were treated with 5 μM NO_2-OA, NO_2-SA, or OA. Endogenous RelA protein levels were detected by western blot analysis probed with anti-RelA antibody. β-actin was used as a loading control. The relative total RelA levels

(normalized by total β-actin) compared to untreated control were quantified using Image Lab software (Bio-Rad). (B) The effect of NO_2-OA on transcript levels of *RelA* gene in MCF-10A, MDA-MB-231, and MDA-MB-468 cells. Cells were treated with 5 μM NO_2-OA, NO_2-SA, or OA for 24 hr. *RelA* mRNA level was measured by RT-qPCR analysis. 18S rRNA was used as an internal control. All data represent the mean ± SD and the fold relative to untreated control is presented. Statistical analysis was compared with control analyzed by one-way analysis of variance followed by Dunnett post hoc test. n.s., not significant. P-value < 0.05 was considered significant.

4.2.6 NO_2-OA Promotes the Ubiquitination-dependent Degradation of RelA Protein in TNBC Cells

The half-life of RelA protein in colorectal cancer HT29 cells is about 2 hr [214]. In order to explore and confirm the half-life of RelA protein in TNBC cells, MDA-MB-231 and MDA-MB-468 were treated with cycloheximide (CHX) to inhibit protein synthesis compared to non-cancerous MCF-10A cells. The level of RelA protein in TNBC cells was not altered at 6 hr after CHX treatment (Figure 24A). Interestingly, we observed that RelA protein remained at a high level in both MDA-MB-231 (89.5%) and MDA-MB-468 (92%) cells after 20 hr of CHX treatment compared to non-CHX treatment (100%). Conversely, only 60% of RelA protein level remained in non-cancerous MCF-10A cells (Figure 24B). These results suggest that RelA protein is more stable in TNBC cells.

RelA is regulated by ubiquitin- and proteasome-dependent degradation signals that govern NF-κB activation [215-217]. We next sought to determine whether RelA modification by

NO_2-OA induces ubiquitination of endogenous RelA in TNBC cells. The polyubiquitinated level of RelA protein was examined by immunoprecipitation and western blot analysis. MDA-MB-231 or MDA-MB-468 cells were treated with vehicle, NO_2-OA, or NO_2-SA for 6 hr. RelA protein was immunoprecipitated, and polyubiquitinated RelA was detected by an anti-ubiquitin antibody. NO_2-OA, but not NO_2-SA, promoted polyubiquitination of RelA in both TNBC cell lines (Figure 25). All findings indicate that NO_2-OA interacted with RelA and destabilized RelA protein by enhancing its ubiquitination in TNBC cells.

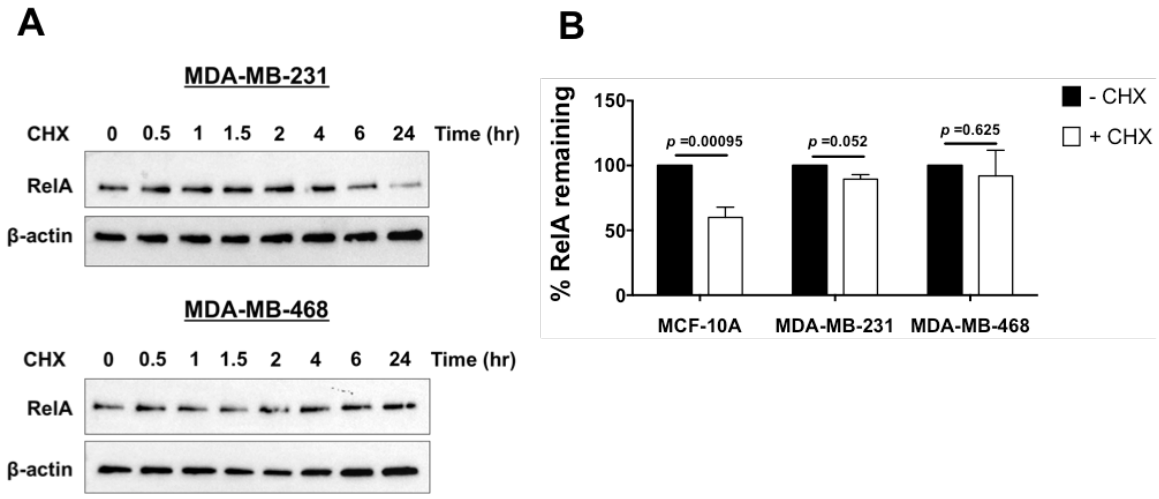


Figure 24. RelA protein stabilizes in TNBC cells.

(A) RelA protein levels in MDA-MB-231 and MDA-MB-468 cells over the time-course (0-24 hr) after treatment with cycloheximide (CHX, 50 μg/mL) was determined by western blot analysis. Western blot representative of one independent experiment. (B) MDA-MB-231, MD-MB-468, or MCF-10A cells were treated with CHX (100 μg/mL) for 20 hr to inhibit *de novo* protein synthesis and harvested for western blot analysis. The levels of RelA with CHX treatment (+CHX) were compared to those without CHX treatment (-CHX). Data show the mean ± SD (n=2). Statistical analysis was compared with -CHX by two-tailed unpaired Student's *t*-test. P-value < 0.05 is considered significant.

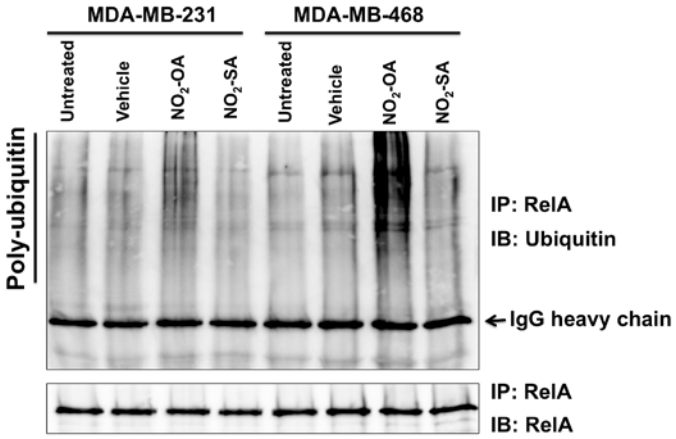


Figure 25. NO_2-OA promoted the ubiquitination of RelA protein in TNBC cells.

MDA-MB-231 or MDA-MB-468 cells were treated with vehicle (methanol), NO_2-OA (5 μM), or NO_2-SA (5 μM) for 6 hr, then cell lysates were harvested and immunoprecipitated (IP) by anti-RelA antibody followed by western blot analysis. The ubiquitin antibody detects covalently linked ubiquitin. Pull-down of RelA proteins is shown in the bottom panel.

4.2.7 The Overall Survival was Better Upon NO_2-OA Treatment During MDA-MB-231 Xenograft Study

Not only did NO_2-OA attenuate MDA-MB-231 xenograft tumor growth (Chapter 3, Figure 13), but it was also noted that various illness phenotypes, such as loss of activity, body temperature, tumor metastasis, or ascites, were observed in vehicle-treated mice about 14 days after treatment. A total 4 of 14 vehicle-treated mice (28.6%) had acute illness phenotypes during the study. Conversely, the illness phenotypes were absent in NO_2-OA-treated mice. Animal survival was

plotted for each group, and data showed that NO_2-OA prolongs survival of TNBC tumor-bearing mice (Figure 26A).

To determine the inhibitory efficacy of NO_2-OA treatment on metastasis formation to other organs, micrometastases in H&E stained tissue sections of lung and liver from all xenograft study mice were evaluated blindly by Dr. Rohit Bhargava (Professor, Dept. of Pathology, University of Pittsburgh). Vehicle treated mice displayed macrometastases in one out of twelve lungs (8.3%) and three out of thirteen livers (23.1%). However, macrometastases were absent in both lung and liver sections of NO_2-OA treated mice (Figure 26B-C).

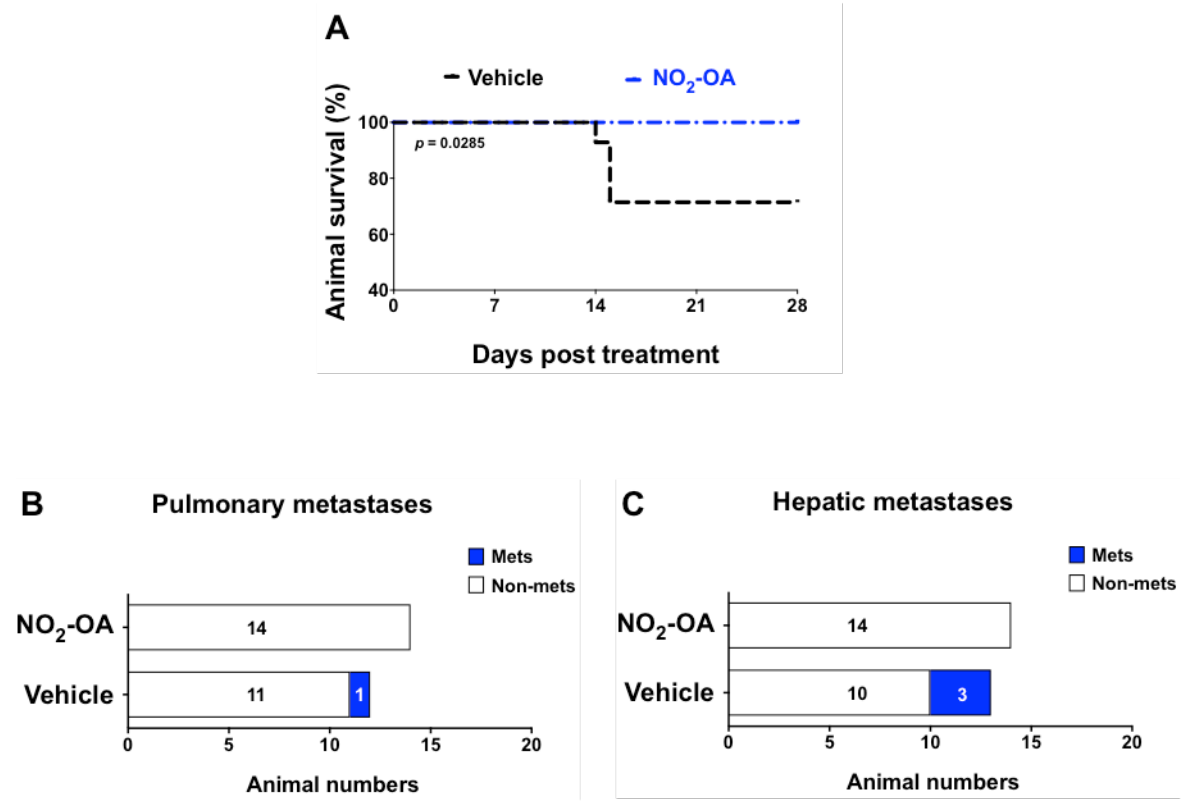


Figure 26. Tumor metastasis was present in vehicle-treated mice, but not in NO_2-OA treated mice.

(A) Comparison of animal survival rate between NO_2-OA-treated and vehicle-treated groups. Animals were monitored up to 28 days after the tumor cell implantation. Survival analysis was plotted on Kaplan-Meier survival curves. Statistical significance (p = 0.0285) between two groups was analyzed using the log-rank test. Evaluation of micrometastases in H&E stained tissue sections of lung (B) and liver (C). The data show numbers of animal diagnosed with lung micrometastases and/or liver micrometastases in vehicle or NO_2-OA treated mice.

4.2.8 NO_2-OA Inhibits Downstream of NF-κB Signaling *in vivo*

Consistent with the *in vitro* experiments, IκBα protein level was higher in NO_2-OA-treated primary tumors (Figure 27A) and the expression level of uPA in tumors was significantly decreased in NO_2-OA-treated mice (Figure 27B). The bioavailable level of NO_2-OA in MDA-MB-231 xenograft primary tumors was around 0.1-1 nM (Chapter 3, Figure 15A). According to recent reports, the concentrations of endogenous NO_2-OA observed in healthy human plasma and urine are typically 1-5 nM [143, 202, 203]. These results suggest that NO_2-OA is orally active and the intake of NO_2-OA may have an inhibitory effect on TNBC tumor metastasis *via* NF-κB signaling.

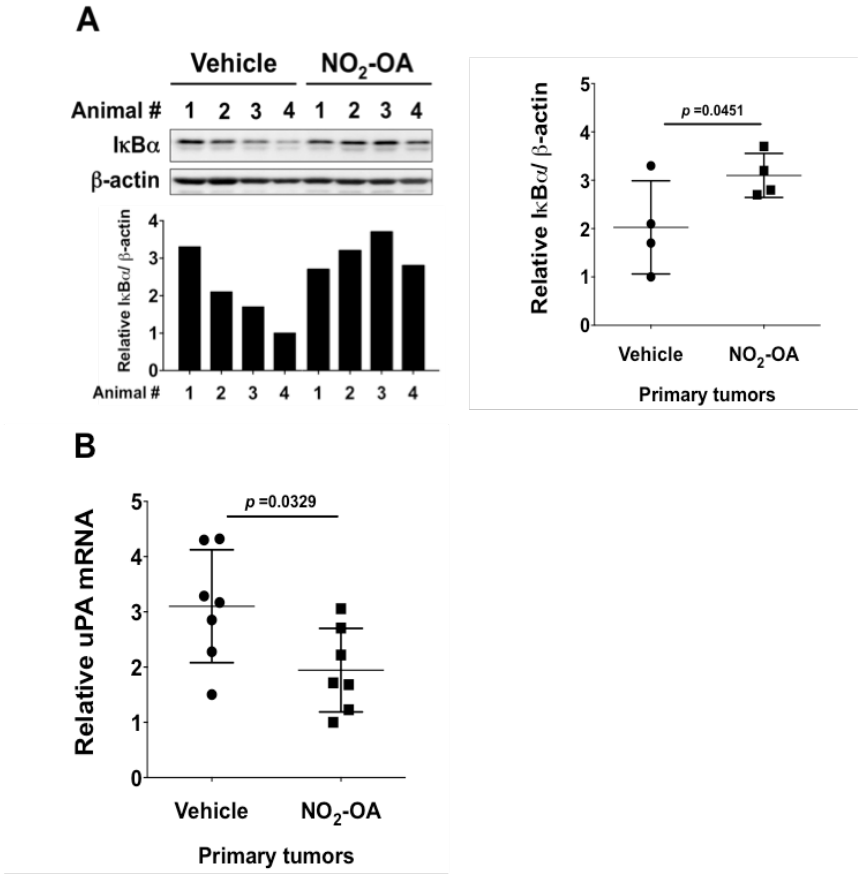


Figure 27. NO_2-OA reduced NF-κB-dependent signaling components in MDA-MB-231 xenograft primary tumors.

(A) Primary tumors from mice treated with vehicle or NO_2-OA were analyzed for total IκBα protein levels by western blot analysis (n=4 each). Bars represent the individual ratio of total IκBα protein normalized to β-actin. Statistical significance (*$p < 0.05$) was calculated by unpaired Student's *t* test (one-tailed). (B) Primary tumor RNAs from mice treated with vehicle or NO_2-OA were isolated, reverse-transcribed, and uPA gene expression was measured by TaqMan PCR assay (n=7 each). Statistical significance (*$p < 0.05$) was calculated by unpaired Student's *t* test (two-tailed).

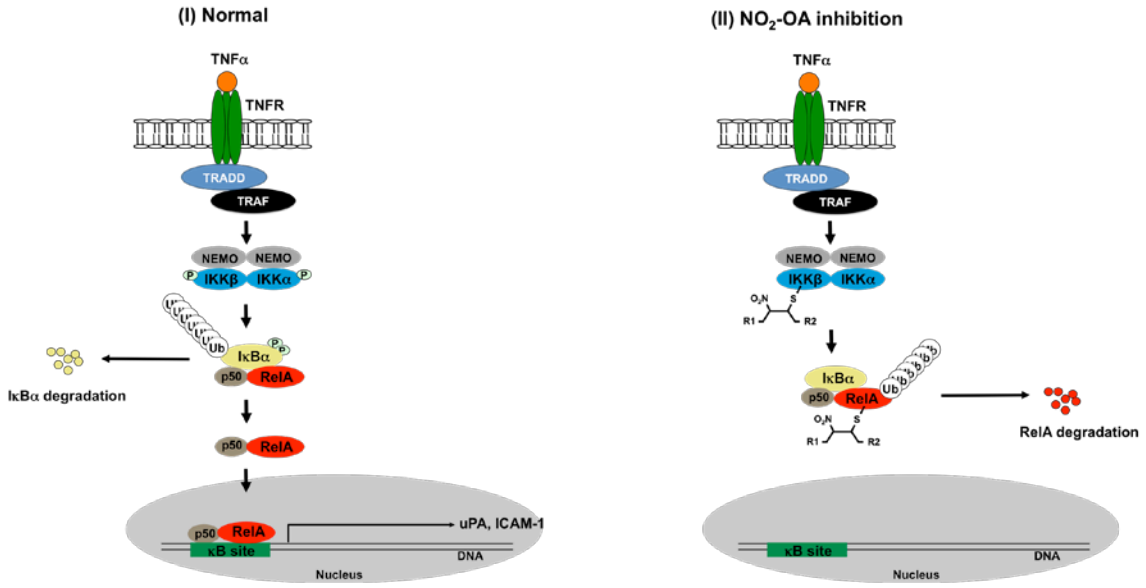


Figure 28. Diagram of NF-κB signaling inhibition by NO_2-OA in TNBC.

(I) TNFα initiates signaling to recruit the adaptors TRADD and TRAF to the membrane where IKKβ is phosphorylated and activated. IKKβ then phosphorylates IκBα to promote its polyubiquitination and subsequent rapid proteasomal degradation. In turn, the RelA/p50 heterodimer is free to translocate to the nucleus and mediate transcription of NF-κB target metastatic genes. (II) Electrophilic NO_2-OA directly binds specific Cys residue in IKKβ and RelA to inhibit IKKβ kinase activity and to induce RelA polyubiquitination, leading to interference with RelA/p50 complex nuclear localization and consequently, the inhibition of transcription of NF-κB-dependent metastatic gene expression (i.e. ICAM-1 and uPA).

4.3 DISCUSSION

Suppression of NF-κB signaling by other small molecules capable of Michael addition can inhibit TNBC cell mobility [45, 61]. We observed that NO_2-OA inhibited TNFα-induced TNBC cell migration (Chapter 3, Figure 12). To test the impact of NO_2-OA on the TNFα-NF-κB signaling cascade in TNBC cells, we examined its inhibitory effect on NF-κB transcriptional activity using a luciferase assay. Our data demonstrate that NO_2-OA inhibited TNFα-induced NF-κB transcriptional activity in both MDA-MB-231 and MDA-MB-468 cells. Furthermore, an array of NF-κB target genes involved in tumor proliferation, survival, and metastasis were down-regulated by NO_2-OA. Those genes included cyclin-D1, Bcl-xl, uPA and ICAM-1. We further demonstrated that both uPA and ICAM-1 genes were induced by TNFα in a NF-κB-dependent manner.

uPA is a serine protease that cleaves the extracellular matrix and activates the conversion of plasminogen to plasmin, which directly degrades matrix proteins such as MMPs [218]. uPA is crucial for tumor metastasis, and its levels are significantly increased in most breast cancer cell lines that contain constitutively active NF-κB [19, 41]. ICAM-1 is a cell surface glycoprotein that acts as an adhesion molecule to promote metastasis and invasion of tumor cells by enhancing the binding of circulating tumor cells and vascular endothelium [219]. ICAM-1 expression is frequently elevated in TNBC cell lines and patient tumors. Blockade of ICAM-1 function by ICAM-1 siRNAs or anti-ICAM-1 antibody reduces human TNBC cell migration and invasion [220, 221]. The results of the present study demonstrate that NO_2-OA significantly reduced uPA and ICAM-1 gene expression during cell migration of TNBC. These findings

implicate a distinguished role of NO_2-OA in inhibition of tumor mobility and support its potential use as an anti-NF-κB agent for treating metastatic TNBC.

Therapeutic targeting of NF-κB activity has studied by inhibiting various players in the pathway [43]. The canonical NF-κB pathway consists of RelA and p50 transcription factors, which are held in the cytoplasm by IκBα. Upon stimulation by inflammatory cytokines, such as TNFα or others, the IKK complex, consisting of IKKα, IKKβ and the scaffolding protein NEMO, is activated. This leads to phosphorylation and proteasomal degradation of IκBα. As a result, RelA/p50 factors are liberated and able to translocate to the nucleus, where they bind to DNA and drive gene transcription [222]. Therefore, inhibitors targeting the proteasome and upstream kinases have been investigated as a new class of anti-inflammatory drugs, but most have failed due to inhibition of non-specific NF-κB signaling molecules and toxic side effects [223].

Inhibition of NF-κB signaling by NO_2-OA *in vivo* and *in vitro* has been reported [100, 101], but the inhibition of NF-κB signaling in TNBC and the discrete mechanisms underlying these actions were previously unknown. In this study, we elucidated two key mechanisms of action by which NO_2-OA inhibits NF-κB activity in TNBC cells. First, NO_2-OA inhibits TNFα-induced phosphorylation of IKKβ and IκBα in TNBC cells, thus limiting IκBα protein degradation. In endothelial cells and macrophages, NO_2-OA also suppresses NF-κB activation induced by LPS *via* dephosphorylation of IKKβ and IκBα [101]. A salient feature of NO_2-FAs is their ability to modulate protein function *via* PTMs induced by reversible Michael addition [65, 98]. We further demonstrated that TNBC cell IKKβ and NF-κB RelA are alkylated by NO_2-OA.

Thus, NO_2-OA not only inhibits NF-κB signaling *via* IKKβ alkylation, but also by directly inhibiting RelA transcriptional function in TNBC cells.

Another innovative aspect of this study is the discovery that NO_2-OA can modulate protein stability upon PTM. Our data demonstrate that NO_2-OA alkylated RelA protein and decreased RelA protein levels in two TNBC cell lines, but not in MCF-10A cells. There was no change in RelA protein levels in TNBC cells upon treatment with NO_2-SA (the non-electrophilic control) in TNBC cells, suggesting nitroalkene electrophilicity is the unique structural feature of NO_2-OA that controls RelA protein stability *via* PTM. Proteolytic degradation of NF-κB subunits leads to termination of NF-κB activation; for example, RelA protein is regulated by ubiquitin- and proteasome-dependent degradation signals that terminate NF-κB [215-217, 224]. Thiol-alkylating and S-nitrosating agents also induce degradation of the NF-κB subunit p50 *via* PTM of the Cys 62 residue in both HT29 and HCT116 tumor cell lines [213]. Intriguingly, alkylation of RelA protein by NO_2-OA increased ubiquitinated RelA protein levels in TNBC cells, a response not observed with NO_2-SA. Thus, the electrophilic nature of the nitroalkene moiety, which is functionally similar to other alkylating agents, is responsible for the covalent modification of NF-κB RelA [213].

The specific Cys of RelA responsible for NO_2-OA alkylation and NO_2-OA-induced ubiquitin-dependent degradation has not been identified at this point; to this question mass spectrometry data shows that NO_2-OA covalently binds Cys105 in addition to redox-sensitive Cys 38 on RelA protein. Future work may determine which specific RelA Cys is critical for NO_2-FA modulation of protein stability.

PPARγ acts as E3 ubiquitin ligase, inducing RelA protein ubiquitination and degradation *via* physically interacting with RelA protein. The PPARγ ligands troglitazone and pioglitazone

increase PPARγ E3 ligase activity by promoting its interaction with RelA protein, in turn, decreasing RelA half-life [225]. Because NO_2-OA is a partial agonist of PPARγ [87], one can speculate that NO_2-OA also activates PPARγ E3 ligase activity, thus further destabilizing RelA protein in TNBC.

We observed high incidence of tumor metastasis during the MDA-MB-231 xenograft study in vehicle-treated mice, but not in NO_2-OA-treated mice. In line with the *in vitro* results, the expression level of uPA in primary tumors was observed to significantly decrease while IκBα protein levels were elevated in NO_2-OA-treated mice. These data indicate that NO_2-OA administration might have the potential to inhibit the expression of a critical metastasis-related NF-κB-dependent target gene *in vivo* and to limit tumor metastasis. However, as we also observed *in vitro* inhibition cell proliferation and viability in MDA-MB-231 by NO_2-OA (Chapter 3, Figure 7A and 9A), the overall anti-metastatic effect of NO_2-OA on MDA-MB-231 xenograft tumors may also be caused by the induction of anti-proliferative and apoptotic activity.

5.0 MODULATION OF NITRO-OLEIC ACID ACTIVITY BY MULTIDRUG RESISTANCE PROTEIN 1

5.1 INTRODUCTION

In the intracellular compartment, GSH and its reactive Cys moiety are more abundant than protein thiols, thus it and other low molecular weight thiols are the primary targets for oxidation and alkylation by free radicals, oxidants and electrophiles [226]. In the case of NO_2-OA, which readily diffuses and gains access to the intracellular compartment and subcellular organelle protein targets [87, 227], GSH conjugates (NO_2-OA-SG) are formed that can be actively transported from cells by the GSH-conjugate efflux pump MRP1 [228]. Up-regulation of MRP1 expression attenuates NO_2-OA-induced PPARγ-dependent transcriptional activity in cells [228]. In the present study, NO_2-OA displayed lower cytotoxic and anti-proliferative effects on non-cancerous breast ductal epithelium (MCF-10A) versus TNBC (Section 3.2.1 and Section 3.2.2). We hypothesized that the variable MRP1 expression observed among different cell types may have a significant influence on the magnitude and efficacy of the chemotherapeutic response to NO_2-OA exposure.

Here, we investigated the role of MRP1 on modulation of NO_2-OA biological availability and activity in breast cancer cell lines. To examine the rate of NO_2-OA-SG conjugate efflux from cells, NO_2-OA-SG was quantified by HPLC-MS/MS analysis in cell media of MCF-10A, MDA-

MB-231, and MDA-MB-468. To determine whether the extent of NO_2-OA-SG efflux associated with the cellular level of MRP1, MRP1 protein was examined and compared between MCF-10A and TNBC cells using western blot analysis. We next examined if decreased MRP1 activity or expression in MCF-10A cells led to accumulate NO_2-OA-SG in cells. MCF-10A cells were treated with MRP1 inhibitor probenecid or MRP1 siRNAs and concentrations of intracellular NO_2-OA-SG were measured by HPLC-MS/MS analysis. Lastly, to determine the impact of MRP1 function in NO_2-OA biological activity, NO_2-OA growth inhibition of MCF-10A cells was examined upon probenecid treatment using Hoechst 33258 dsDNA stain assay. Cell cycle and apoptotic biological markers were examined from cells co-treated with probenecid and NO_2-OA compared to NO_2-OA alone using western blot analysis.

5.2 RESULTS

5.2.1 Extracellular NO_2-OA-SG Efflux is Linked with MRP1 Expression

To determine whether the abundance of NO_2-OA-SG exported outside the cells was different between non-cancerous and TNBC cell lines, we measured concentrations of extracellular NO_2-OA-SG in the media of MCF-10A, MDA-MB-231, and MDA-MB-468 cells treated with 5 μM NO_2-OA for 1 hr using HPLC-MS/MS analysis. In comparison to NO_2-OA-SG levels in MCF-10A culture media, there were significantly lower levels in both MDA-MB-231 and MDA-MB-468 cell culture media (Figure 29).

GSH conjugates NO_2-OA-SG are actively transported out of cells by MRP1 [228]. This 4- to 5-fold difference in extracellular NO_2-OA-SG levels produced by MCF-10A and TNBC

cells prompted comparing the relative expression of MRP1 protein. Western blot analysis detected MRP1 protein expression in MCF-10A cells, but MRP1 was undetectable in both TNBC cell lines (Figure 30).

An additional ABCC (ATP binding cassette subfamily C) family member MRP4 transporter might also mediate GSH conjugate efflux [229]. Therefore, we also examined the level of MRP4 transporter, and its protein expression was similar in three cell lines (Figure 30). Together, these results indicate that a higher extracellular level of NO_2-OA-SG in MCF-10A is associated with MRP1 protein expression.

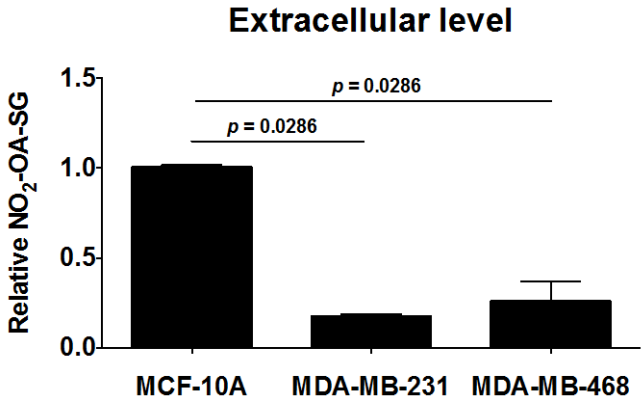


Figure 29. TNBC cells secreted less NO_2-OA conjugated GSH adducts.

NO_2-OA-SG was measured from MCF-10A, MDA-MB-231, and MDA-MB-468 cell media. Cells were treated with NO_2-OA (5 μM) for 1 hr. Cell media was collected, and the extracellular material was extracted. The abundance of NO_2-OA-SG was determined from these extracts using HPLC-MS/MS analysis. Relative amount was calculated as the abundant ratio of NO_2-OA-SG to $^{15}NO_2$-d_4-OA-SG standard from each NO_2-OA-treated cell media divided by the abundance of MCF-10A treated cell media. Data are shown as mean ± SD (n=4). P-value < 0.05 was considered significant using Mann-Whitney U test.

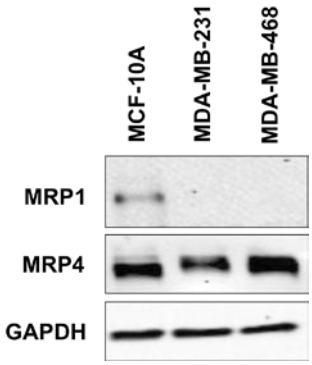


Figure 30. High protein level of MRP1, but not MRP4 was expressed in human epithethal MCF-10A cells.

Representative western blot of endogenous MRP1 and MRP4 in MCF-10A, MDA-MB-231, and MDA-MB-468 cells. A total of 25 μg of protein lysates was loaded in each lane. Endogenous MRP1 and MRP4 protein levels were detected by western blot analysis probed with anti-MRP1 (ABCC1) and anti-MRP4 (ABCC4) antibodies using GAPDH as a loading control. Western blot representative of three independent experiments.

5.2.2 MRP1 Regulates NO_2-OA-SG Trafficking in MCF-10A Cells

To further test if MRP1 is critical in regulating NO_2-OA-SG efflux in mammary epithelial cells, we inhibited MRP1 drug efflux activity in MCF-10A cells using probenecid organic anion transport inhibitor in combination with NO_2-OA. Co-treatment with probenecid resulted in a significant increase in intracellular level of NO_2-OA-SG versus without probenecid treatment (Figure 31A). Consistent with the inhibitor study, down-regulation of MRP1 using siRNA

knockdown accumulated more intracellular NO_2-OA-SG in MCF-10A cells compared to non-specific siRNA control (Figure 31B). In parallel treatment, additional scramble or MRP1 siRNA-treated MCF-10A cells were used for examining the siRNA knockdown efficiency. RT-qPCR data showed about 70 percent of MRP1 mRNA was knocked down by human specific MRP1 siRNAs (Figure 31C).

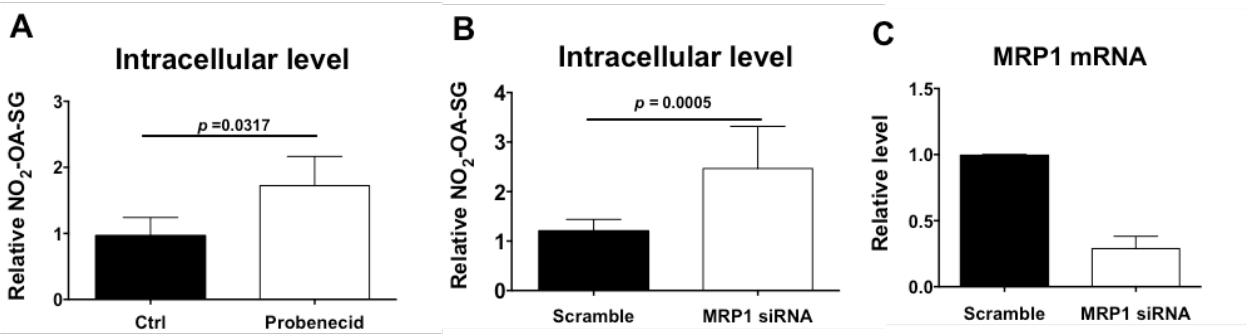


Figure 31. Suppression of MRP1 activity and expression increases intracellular level of NO_2-OA-SG adducts in MCF-10A cells.

(A-B) The relative amount represents the relative abundance of NO_2-OA-SG to $^{15}NO_2$-d_4-OA-SG standard, normalized to protein concentrations from each NO_2-OA-treated sample divided by the abundance of Ctrl or scramble sample. $p < 0.05$ versus Ctrl (n=6) or scramble (n=9) was considered significant using Mann-Whitney U test. (C) The knockdown efficiency of MRP1 siRNA. MCF-10A cells were transiently transfected with MRP1 or non-target (Scramble) siRNAs for 48 hr. MRP1 mRNA level was measured by RT-qPCR. 18S rRNA was used as an internal control. Data represent the mean ± SD, n=4.

5.2.3 Inhibition of MRP1 Influences NO_2-OA Bioactivity in MCF-10A

We observed that NO_2-OA effectively induced cytotoxic and anti-proliferative effects on MDA-MB-231 and MDA-MB-468 cells versus non-cancerous MCF-10A cells (Chapter 3, Figure 7A-B and Figure 9A-B). One possible explanation for MCF-10A cell toleration of NO_2-OA is that MRP1 promotes NO_2-OA-SG transport from cells in order to attenuate NO_2-OA bioactivity. To investigate the role of MRP1 in cellular responses to NO_2-OA, the effect of probenecid on NO_2-OA growth inhibition of MCF-10A cells was performed using Hoechst 33258 dsDNA stain assay. Results showed that co-treatment with probenecid significantly enhanced MCF-10A cell growth inhibition by NO_2-OA at 5 μM, compared to NO_2-OA treatment alone (Figure 32A). The IC_{50} of NO_2-OA (7.23 ± 0.15 μM) was displayed to decrease 2-fold in MCF-10A cells pretreated with probenecid versus NO_2-OA treatment alone (14.23 ± 1.05 μM; Figure 32B).

Moreover, probenecid increased the extent of NO_2-OA-induced cell cycle arrest of MCF-10A cells, as reflected by increased p21 levels and a concomitant decrease in cyclin D1 expression (Figure 32C). Probenecid also enhanced NO_2-OA-induced apoptosis in MCF-10A cells in the context of increased caspase-3 activation and PARP-1 cleavage (Figure 32D). These observations are consistent with both the intracellular concentration and the cell growth/ survival-related signaling actions of NO_2-OA being modulated by subsequent MRP1 export of NO_2-OA-SG. In aggregate, our findings indicate that MRP1-mediated efflux can profoundly attenuate the ability of NO_2-OA to inhibit proliferation and survival in MCF-10A cells (Figure 33). Altogether, down-regulation of MRP1 transporting activity sensitized MCF-10A cells to NO_2-OA similar to its effect on TNBC cells.

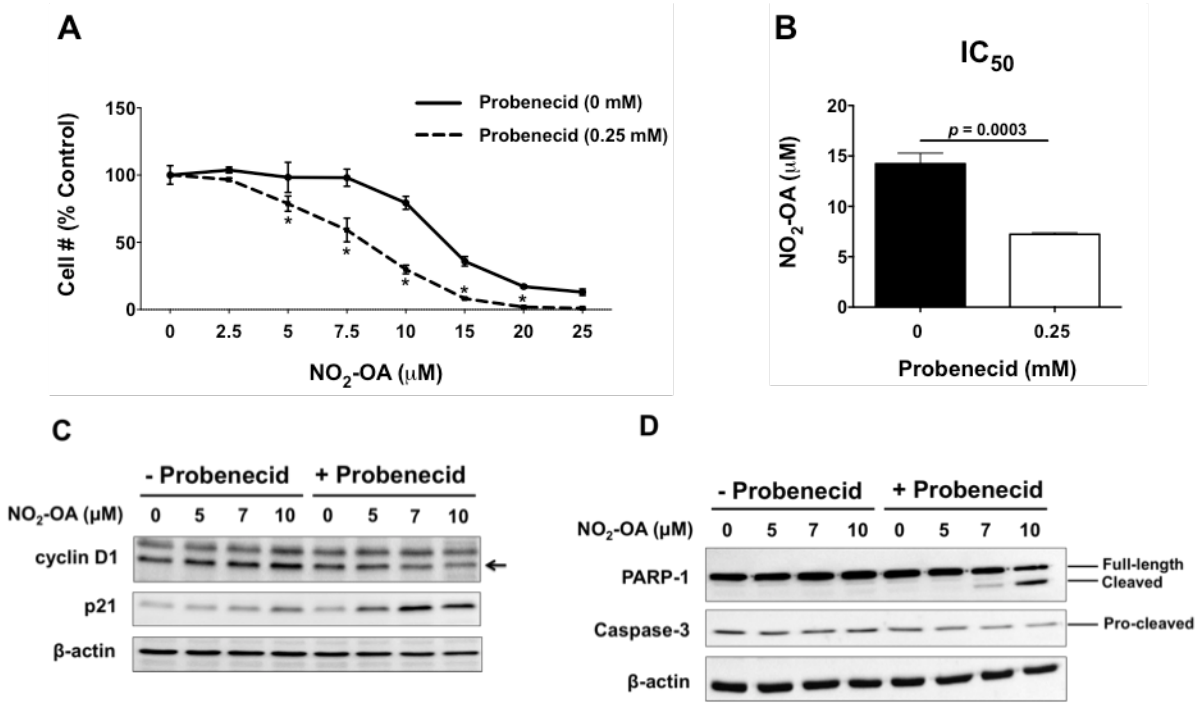


Figure 32. Inhibition of MRP1 activity sensitizes MCF-10A cells to NO_2-OA.

(A) The effect of probenecid on NO_2-OA-induced growth inhibition of MCF-10A cells. Cells were pretreated with or without 0.25 mM probenecid for 1 hr, and then combined with 0, 2.5, 5, 7.5, 10, 15, 20, or 25 μM NO_2-OA for 48 hr. FluoReporter dsDNA stain assay was performed to measure cell numbers. Data are shown as percent of untreated control cells (mean ± SD). * $p <$ 0.05 indicates significant difference between without (solid line) and with probenecid (dashed line) within each dose of NO_2-OA treatment using two-way analysis of variance followed by Tukey post hoc test. Three independent experiments were performed in quintuplet. (B) The average IC_{50} values of NO_2-OA in MCF-10A cell treated with or without probenecid. Data are shown as mean ± SD. $p < 0.05$ was considered significant using two-tailed unpaired Student's *t* test. (C) The effect of probenecid on expression of cyclin D1 and p21 proteins in NO_2-OA-treated MCF-10A cells. Cells were treated with NO_2-OA (5 μM) in the presence or absence of

probenecid (1 mM) for 24 hr. cyclin D1 and p21 protein levels were determined by western blot analysis using β-actin as a loading control. (D) The effect of probenecid on PARP-1 cleavage and pro-caspase-3 protein in NO_2-OA-treated MCF-10A cells. Cells were treated with NO_2-OA (5 μM) in the presence or absence of probenecid (1 mM) for 24 hr. The full-length and cleaved forms of PARP-1 and pro-caspase-3 protein level were determined by western blot analysis using β-actin as a loading control. Western blots are representative of three independent experiments.

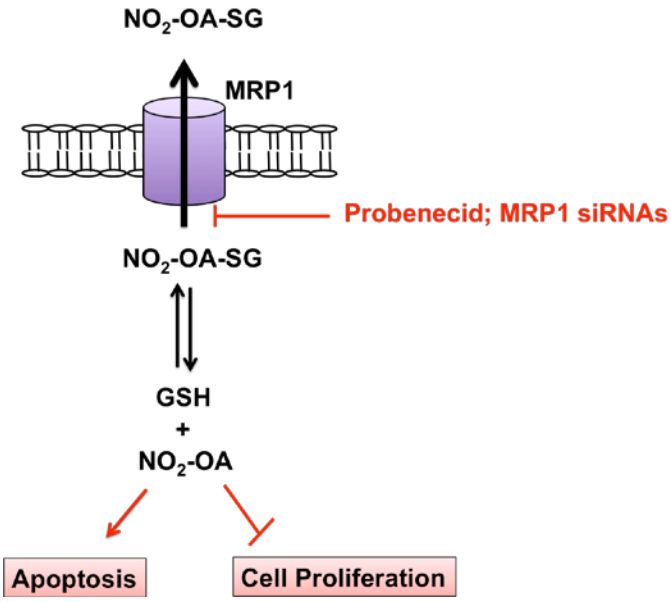


Figure 33. Proposed scheme illustrating the role of MRP1 in intracellular NO_2-OA activity and metabolism in human epithelial cells.

The formation of the GSH conjugate of NO_2-OA (NO_2-OA-SG) is mediated in a non-enzymatic manner. MRP1 transporter pumps out the NO_2-OA-SG conjugate from cells (Black line). Inhibition of the MRP1 transport activity by the organic acid probenecid or MRP1 siRNAs prolongs the half-life of intracellular NO_2-OA and enhances NO_2-OA biological activity in anti-proliferation and apoptosis of cells (Red line).

5.3 DISCUSSION

Adverse effects associated with chemotherapeutic agents limit their use and are commonly associated with cytotoxic effects on non-cancerous cells. An ideal chemotherapeutic agent should be maximally toxic to malignant cells with minimal toxicity in normal cells. However, there are currently limited agents with this capacity available for clinical use. Therefore, the development of selective chemotherapeutic agents that specifically target cancer cells (but not normal cells) are essential to achieving this goal. Our results showed that tumorigenic TNBC cells are more sensitive to NO_2-OA than non-tumorigenic epithelial breast MCF-10A cells, suggesting that this agent exhibits selective cytotoxicity (Section 3.2.1 and Section 3.2.2). Hence, an important goal of this study was to understand the mechanisms that regulate cellular responses to NO_2-OA.

MRP1/ABCC1 is a pump for GSH conjugates that protects normal tissues by reducing the intracellular accumulation of xenobiotics and metabolites [136]. A number of lipid-derived signaling molecules such as leukotriene C4, cyclopentenone 15d-PGJ_2, and NO_2-FA are transported by MRP1 through conjugation with GSH [109, 228, 230, 231]. We observed that MRP1 protein is relatively highly expressed in MCF-10A cell versus TNBC cells. Consistent with MRP1 expression, MCF-10A cells form and export 4-5-fold greater amounts of NO_2-OA-SG adducts into the extracellular compartment compared with TNBC cells, implicating that MRP1 may play a role in cellular responses to NO_2-OA in breast cancer.

When the MRP1 transport activity of MCF-10A cells is inhibited by the organic acid probenecid [232], a more TNBC-like phenotype is conferred in regards to the sensitivity to NO_2-OA. The impact of NO_2-OA on cell growth arrest and killing, cell cycle arrest (cyclin D1, p21) and apoptosis-regulating mediators (PARP-1, caspase-3) all support the concept that signaling

actions are enhanced in MRP1-depleted cells because of enhanced NO_2-OA availability in the intracellular compartment. Altogether, these results affirm that the rates of MRP1-mediated efflux of GSH-adducted NO_2-OA from cells will contribute significantly to tissue and breast tumor responses to NO_2-OA.

Although MRP1 overexpression is associated with drug resistance in prostate, lung, and breast cancer [233], NO_2-OA does not significantly induce MRP1 mRNA expression after 24 hr treatment in MCF-10A, MDA-MB-231, or MDA-MB-468 cells (data not shown). But, it would be necessary to investigate whether there is any potential drug resistance caused by up-regulation of MRP1 induced *via* NO_2-OA after long-term treatment in TNBC cell lines or tumor xenografts.

We have observed that basal GSH levels in MCF-10A cells (19.3 ± 1.9 nmol/10^6 cells) were >2-fold that of MDA-MB-231 (8.3 ± 0.8 nmol/10^6 cells) and ~1.5-fold greater than MDA-MB-468 cells (12.9 ± 0.5 nmol/10^6 cells). Interestingly, MCF-10A cells maintained the GSH level over the first 6 hr after NO_2-OA treatment, whereas the GSH level rapidly decreased at the first hr of NO_2-OA treatment in both MDA-MB-231 and MDA-MB-468 cells (Data not shown). The GSH synthesis pathway promotes cancer initiation and progression, and targeting this pathway by inhibiting xCT (the cysteine/glutamate exchanger) or GCL (glutamate cysteine ligase) has shown some promise in inhibiting tumor growth in combination with chemotherapy in mouse models of breast cancer [234, 235]. The xCT inhibitor sulfasalazine reduces tumor growth and distant metastasis in xenograft models of breast cancer [235], while TNBC (basal-type) cells are sensitive to sulfasalazine that blocks cysteine uptake [235]. A more recent study revealed that cysteine-addition TNBC cells and tumors show a strong activation of TNFα and MEKK4-p38-Noxa pathways that render them susceptible to cysteine deprivation-induced necrosis [236]. More interestingly, a number of studies reported that TNBC is sensitive to anti-

GSH therapy using sulfasalazine and γGCS inhibitor buthionine sulphoximine (BSO), which robustly inhibited tumor proliferation and depleted GSH content [237, 238]. Overall, these studies support the potential of targeting GSH biosysthesis as a therapeutic strategy in TNBC. The effect of NO_2-OA on GSH depletion in TNBC cells may partially mediate inhibition of cell proliferation or survival. Future studies are needed to investigate in more detail the GSH biosynthesis that influences the role of NO_2-OA in TNBC cell survival and proliferation.

6.0 CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

Treatment options for breast cancer vary depending on the subtype. Luminal A and luminal B breast cancer have multiple treatment options, including endocrine and conventional cytotoxic chemotherapy. Her2+ breast cancers may respond well to anti-HER2 therapy in combination with chemotherapy [239]. However, TNBC represent the vast majority of basal and claudin-low subtypes and are largely restricted and resistant to most current treatment [240]. Because TNBC has a highly aggressive nature, it accounts for a disproportionate number of metastatic disease cases and deaths [241]. Unfortunately, TNBC patients have a high risk of relapse and a sharp decrease in survival in the first 3-5 years after treatment [165, 169]. Thus, there remains an urgent unmet need for novel therapeutic agents for TNBC. Throughout this dissertation, the anticancer activity of NO_2-OA in human TNBC cells has been investigated.

In Chapter 3 of this dissertation, NO_2-OA was demonstrated to preferentially inhibit the cell growth and viability of TNBC following a comprehensive evaluation of the efficacy of treatment in the major subtypes of breast cancer (ER+, HER2+, and TNBC) compared to non-cancerous mammary epithelial MCF-10A cells. NO_2-OA is capable of inhibiting two TNBC cell lines (MDA-MB-231 and MDA-MB-468) at concentrations of >3-fold lower IC_{50} than MCF-10A cells. In an orthotopic mouse xenograft model, NO_2-OA is capable of reducing the growth

of MDA-MB-231 xenografted tumors by oral administration (7.5 mg/kg daily for 4 weeks). Data from *in vitro* studies suggest that NO_2-OA causes cell cycle arrest and induces apoptosis in TNBC cells, but not in MCF-10A cells, by modulation of multiple targets important for cell proliferation and death. For example, increased p21 and decreased cyclin D1 expression are observed in TNBC cells with NO_2-OA treatment. Two distinct pathways of apoptotic signaling are engaged by NO_2-OA in TNBC cells, initiated through both mitochondrial (caspase-9 activation) and death receptor (caspase-8 activation) regulated mechanisms. Furthermore, NO_2-OA significantly attenuates TNFα-induced TNBC cell migration and invasion. In aggregate, these data reveal that NO_2-OA displays pleiotropic anti-cancer properties *via* the inhibition of cell proliferation, induction of apoptosis, and blockade of TNFα-mediated cell mobility in TNBC.

The beneficial effect of NO_2-OA on metastatic TNBC is confirmed *via* inhibition of NF-κB signaling. In Chapter 4 of this dissertation, we demonstrate that inhibition of NF-κB signaling by NO_2-OA is evident by blockade of its transcriptional activity following TNFα stimulation. We identified two specific NF-κB target genes, ICAM-1 and uPA (two critical mediators of tumor progression and metastasis), were suppressed by NO_2-OA during TNBC cell migration. When TNBC cells were exposed to TNFα, NO_2-OA inhibited gene expression of uPA and ICAM-1, providing evidence that this compound attenuates inflammation induced by TNFα, which is known to be upregulated in TNBC metastasis. The mechanisms accounting for NF-κB signaling inhibition by NO_2-OA in TNBC cells are multifaceted, as NO_2-OA i) blocks IKKβ phosphorylation and IκBα degradation, ii) alkylates the NF-κB RelA protein to prevent DNA binding and iii) promotes RelA polyubiquitination and proteasomal degradation. *In vivo*, tumor metastasis was not found in NO_2-OA treated animals and NF-κB signaling was repressed in

NO_2-OA treated primary tumors compared to vehicle tumors. Taken together, these data provide *in vitro* and *in vivo* evidence that NO_2-OA possesses the potential to prevent TNBC metastasis *via* suppression of NF-κB signaling.

Treatment with NO_2-OA was observed to be more effective on growth and viability in TNBC cells than in MCF-10A cells. These findings provide evidence for exploring insights into the mechanism of NO_2-OA metabolism that could translate into new therapeutic strategies for selectively treating breast cancer. The rates of MRP1-mediated efflux of GSH-adducted electrophiles from cells have contributed significantly to the net intracellular concentration, half-life, target protein reactions, and tissue responses to lipid electrophiles [109, 231, 242]. In Chapter 5 of this dissertation, we examined the role of MRP1 in NO_2-OA biological activity between MCF-10A and TNBC cell lines. MRP1 protein was highly expressed in MCF-10A cells compared to TNBC cells. Consistent with relative MRP1 expression, MCF-10A cells formed and exported 4-5-fold greater amounts of NO_2-OA-SG adducts into the extracellular compartment than TNBC cells. In contrast, intracellular NO_2-OA-SG is accumulated in MCF-10A cells once MRP1 inhibitor probenecid and MRP1 siRNAs were present. Further, blocking MRP1 activity by probenecid potentiated NO_2-OA-mediated growth inhibition in MCF-10A cells and also enhanced NO_2-OA-induced cell cycle arrest and apoptosis of MCF-10A cells. This affirms that MRP1 activity and levels will influence tumor responses to NO_2-OA.

In summary, data presented in this dissertation suggest NO_2-OA may be considered a potent agent for treatment of TNBC. This preclinical study provides biochemical support for the design of a clinical study that tests whether electrophilic NO_2-OA represents a new therapeutic agent or co-agent that may display selectivity for treating patients with TNBC, a cancer which currently lacks effective treatment options.

6.2 FUTURE DIRECTIONS

In vitro studies

Although NO_2-OA was demonstrated to preferentially inhibit the cell growth and viability of two TNBC cell lines (MDA-MB-231 and MDA-MB-468), two TNBC cell lines are not sufficient to conclude that NO_2-OA displays therapeutic potential for the specific treatment of TNBC subtype. A necessary further step would be to confirm and examine the anti-proliferative and cytotoxic effects of NO_2-OA in more TNBC cell lines, but also identify the specific sub-phenotypes of TNBC which would be most influenced by NO_2-OA.

In vivo studies

Our findings suggest that NO_2-OA was effective to reduce tumor growth in an orthotopic xenograft mice model; however, this model system is limited due to its reduced intra-tumoural heterogeneity and poor record of predicting clinically effective therapies [243]. Future studies for the therapeutic efficacy of NO_2-OA will be verified using other study models, such as patient-derived xenografts (PDX) or genetically engineering mouse models (GEMMs), which is more relevant to clinical outcomes. For example, PDX models represent many relevant features of the primary human tumor, including growth kinetics, histological features, metastatic capacity, and response to therapy [243]. In GEMM models, spontaneous tumor initiation occurs in mammary gland tissue by mouse mammary tumor virus (MMTV) that has been attempted by targeting a variety of oncogenic drivers to the different mammary lineages. Approximately 50% of the mammary cancers that develop in the *Brca1*$^{f11/f11/p53+/-/MMTV-Cre}$ mice (two floxed exon 11 alleles were combined with the MMTV-Cre transgene to effect mammary epithelial cell targeted

BRCA1 loss) demonstrate TNBC or basal-type cancers [244]. This strain may be suitable for exploring the general effects of NO_2-OA on TNBC development and progression.

Future *in vivo* study end-points for efficacy evaluation would be to determine treatment ability to: (a) reduce primary tumor volume and/or metastasis, (b) display lower Ki67 (proliferative marker) in primary tumors by IHC, (c) increase active form of caspase-3 by IHC or DNA fragmentation by TUNEL assay, and/or (d) suppress NF-κB signaling in primary tumor by NO_2-OA.

BIBLIOGRAPHY

1. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* The Lancet, 2001. **357**(9255): p. 539-545.
2. Karin, M., *Nuclear factor-[kappa]B in cancer development and progression.* Nature, 2006. **441**(7092): p. 431-436.
3. Hanahan, D. and Robert A. Weinberg, *Hallmarks of cancer: The next generation.* Cell, 2011. **144**(5): p. 646-674.
4. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-899.
5. MacEwan, D.J., *TNF receptor subtype signalling: Differences and cellular consequences.* Cellular Signalling, 2002. **14**(6): p. 477-492.
6. Szlosarek, P., K.A. Charles, and F.R. Balkwill, *Tumour necrosis factor-α as a tumour promoter.* European Journal of Cancer, 2006. **42**(6): p. 745-750.
7. Szlosarek, P.W. and F.R. Balkwill, *Tumour necrosis factor α: a potential target for the therapy of solid tumours.* The Lancet Oncology, 2003. **4**(9): p. 565-573.
8. Warzocha, K., et al., *Tumor necrosis factor ligand-receptor system can predict treatment outcome in lymphoma patients.* Journal of Clinical Oncology, 1997. **15**(2): p. 499-508.
9. Nakashima, J., et al., *Association between tumor necrosis factor in serum and cachexia in patients with prostate cancer.* Clinical Cancer Research, 1998. **4**(7): p. 1743-1748.
10. Li, H.-H., et al., *Tumour necrosis factor-α gene polymorphism is associated with metastasis in patients with triple negative breast cancer.* Scientific Reports, 2015. **10244**.
11. Chen, G. and D.V. Goeddel, *TNF-R1 signaling: A beautiful pathway.* Science, 2002. **296**(5573): p. 1634-1635.
12. Havell, E.A., W. Fiers, and R.J. North, *The antitumor function of tumor necrosis factor (TNF), I. Therapeutic action of TNF against an established murine sarcoma is indirect, immunologically dependent, and limited by severe toxicity.* The Journal of Experimental Medicine, 1988. **167**(3): p. 1067-1085.
13. Wiemann, B. and C.O. Starnes, *Coley's toxins, tumor necrosis factor and cancer research: A historical perspective.* Pharmacology & Therapeutics, 1994. **64**(3): p. 529-564.
14. Leibovich, S.J., et al., *Macrophage-induced angiogenesis is mediated by tumour necrosis factor-[alpha]*. Nature, 1987. **329**(6140): p. 630-632.
15. Leek, R., et al., *Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma.* British Journal of Cancer, 1998. **77**: p. 2246-2251.

16. Yoshida, S., et al., *Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis.* Molecular and Cellular Biology, 1997. **17**(7): p. 4015-23.
17. Battegay, E.J., et al., *TNF-alpha stimulation of fibroblast proliferation. Dependence on platelet-derived growth factor (PDGF) secretion and alteration of PDGF receptor expression.* The Journal of Immunology, 1995. **154**(11): p. 6040-6047.
18. Leber, T.M. and F.R. Balkwill, *Regulation of monocyte MMP-9 production by TNF-alpha and a tumour-derived soluble factor (MMPSF).* British Journal of Cancer, 1998. **78**(6): p. 724-732.
19. Sliva, D., M.T. Rizzo, and D. English, *Phosphatidylinositol 3-kinase and NF-κB regulate motility of invasive MDA-MB-231 human breast cancer cells by the secretion of urokinase-type plasminogen activator.* Journal of Biological Chemistry, 2002. **277**(5): p. 3150-3157.
20. Brinckerhoff, C.E. and L.M. Matrisian, *Matrix metalloproteinases: a tail of a frog that became a prince.* Nature Reviews Molecular Cell Biology, 2002. **3**: p. 207.
21. Hagemann, T., et al., *Macrophages Induce Invasiveness of Epithelial Cancer Cells Via NF-κB and JNK.* The Journal of Immunology, 2005. **175**(2): p. 1197-1205.
22. Baldwin, A.S., *Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB.* The Journal of Clinical Investigation, 2001. **107**(3): p. 241-246.
23. Inoue, J.-i., et al., *NF-κB activation in development and progression of cancer.* Cancer Science, 2007. **98**: p. 268-274.
24. Ghosh, S. and M. Karin, *Missing pieces in the NF-κB puzzle.* Cell, 2002. **109**(2): p. S81-S96.
25. Gilmore, T.D., *Introduction to NF-[kappa]B: players, pathways, perspectives.* Oncogene, 2006. **25**(51): p. 6680-6684.
26. Ben-Neriah, Y. and M. Karin, *Inflammation meets cancer, with NF-[kappa]B as the matchmaker.* Nat Immunol, 2011. **12**(8): p. 715-723.
27. Rayet, B. and C. Gelinas, *Aberrant rel/nfκb genes and activity in human cancer.* Oncogene, 1999. **18**: p. 6938-6947.
28. Cabannes, E., et al., *Mutations in the IkBa gene in Hodgkin's disease suggest a tumour suppressor role for IkappaBalpha.* Oncogene, 1999. **18**: p. 3063-3070.
29. Mathew, S., et al., *Chromosomal localization of genes encoding the transcription factors, c-rel, NF-kappa Bp50, NF-kappa Bp65, and lyt-10 by fluorescence in situ hybridization.* Oncogene, 1993. **8**: p. 191-193.
30. Visconti, R., et al., *Expression of the neoplastic phenotype by human thyroid carcinoma cell lines requires NFkappaB p65 protein expression.* Oncogene, 1997. **15**: p. 1987-1994.
31. Dejardin, E., et al., *Regulation of NF-kappaB activity by I kappaB-related proteins in adenocarcinoma cells.* Oncogene 1999. **18**: p. 2567-2577.
32. Nakshatri, H., et al., *Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth.* Molecular and Cellular Biology, 1997. **17**(7): p. 3629-39.
33. Sovak, M.A., et al., *Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer.* The Journal of Clinical Investigation, 1997. **100**(12): p. 2952-2960.

34. Bours, V., et al., *The NF-κB transcription factor and cancer: high expression of NF-κB- and IκB-related proteins in tumor cell lines.* Biochemical Pharmacology, 1994. **47**(1): p. 145-149.
35. Wang, W., et al., *The nuclear factor-κB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells.* Clinical Cancer Research, 1999. **5**(1): p. 119-127.
36. Sumitomo, M., et al., *Induction of apoptosis of cytokine-producing bladder cancer cells by adenovirus-mediated I kappa B alpha overexpression.* Human Gene Therapy, 1999. **10**: p. 37-48.
37. Herrmann, J.L., et al., *Bcl-2 suppresses apoptosis resulting from disruption of the NF-κB survival pathway.* Experimental Cell Research, 1997. **237**(1): p. 101-109.
38. Sumitomo, M., et al., *An essental role for nuclear factor kappa B in preventing TNF-alpha-Iinduced cell death in prostate cancer cells* The Journal of Urology, 1999. **161**(2): p. 674-679.
39. Devalaraja, M.N., et al., *Elevated constitutive IκB kinase activity and IκB-α phosphorylation in Hs294T melanoma cells lead to increased basal MGSA/GRO-α transcription.* Cancer Research, 1999. **59**(6): p. 1372-1377.
40. Pahl, H.L., *Activators and target genes of Rel/NF-kappaB transcription factors.* Oncogene, 1999. **18**: p. 6853-6866.
41. Newton, T.R., et al., *Negative regulation of transactivation function but not DNA binding of NF-κB and AP-1 by IκBβ1 in breast cancer cells.* Journal of Biological Chemistry, 1999. **274**(26): p. 18827-18835.
42. Orlowski, R.Z. and A.S. Baldwin, Jr., *NF-κB as a therapeutic target in cancer.* Trends in Molecular Medicine, 2002. **8**(8): p. 385-389.
43. Gilmore, T.D. and M. Herscovitch, *Inhibitors of NF-[kappa]B signaling: 785 and counting.* Oncogene, 2006. **25**(51): p. 6887-6899.
44. Straus, D.S., et al., *15-Deoxy-Δ(12,14)-prostaglandin J(2) inhibits multiple steps in the NF-κB signaling pathway.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(9): p. 4844-4849.
45. Kastrati, I., et al., *Dimethyl fumarate inhibits the nuclear factor κB pathway in breast cancer cells by covalent modification of p65.* Journal of Biological Chemistry, 2016.
46. Castro, A.C., et al., *Novel IKK inhibitors: β-carbolines.* Bioorganic & Medicinal Chemistry Letters, 2003. **13**(14): p. 2419-2422.
47. Hideshima, T., et al., *NF-κB as a therapeutic target in multiple myeloma.* Journal of Biological Chemistry, 2002. **277**(19): p. 16639-16647.
48. Burke, J.R., et al., *BMS-345541 Is a highly selective inhibitor of IκB kinase that binds at an allosteric site of the enzyme and blocks NF-κB-dependent transcription in mice.* Journal of Biological Chemistry, 2003. **278**(3): p. 1450-1456.
49. Kwok, B.H.B., et al., *The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IκB kinase.* Chemistry & Biology, 2001. **8**(8): p. 759-766.
50. Rossi, A., et al., *Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase.* Nature, 2000. **403**(6765): p. 103-18.

51. Perkins, N.D., *Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway.* Oncogene, 2006. **25**(51): p. 6717-6730.
52. Scheidereit, C., *I[kappa]B kinase complexes: gateways to NF-[kappa]B activation and transcription.* Oncogene, 2006. **25**(51): p. 6685-6705.
53. Adams, J., *The development of proteasome inhibitors as anticancer drugs.* Cancer Cell, 2004. **5**(5): p. 417-421.
54. Lenz, H.-J., *Clinical update: proteasome inhibitors in solid tumors.* Cancer Treatment Reviews, 2003. **29**: p. 41-48.
55. TAKIGAWA, N., et al., *Proteasome Inhibition with Bortezomib Enhances Activity of Topoisomerase I-targeting Drugs by NF-κB-independent Mechanisms.* Anticancer Research, 2006. **26**(3A): p. 1869-1876.
56. Gilroy, D.W., et al., *Inducible cyclooxygenase may have anti-inflammatory properties.* Nat Med, 1999. **5**(6): p. 698-701.
57. Chen, F.E., et al., *Crystal structure of p50/p65 heterodimer of transcription factor NF-[kappa]B bound to DNA.* Nature, 1998. **391**(6665): p. 410-413.
58. de Jong, E., et al., *Anticancer effects of 15d-Prostaglandin-J2 in wild-type and doxorubicin-resistant ovarian cancer cells: Novel actions on SIRT1 and HDAC.* PLOS ONE, 2011. **6**(9): p. e25192.
59. Ciucci, A., et al., *Induction of apoptosis in estrogen receptor-negative breast cancer cells by natural and synthetic cyclopentenones: role of the IκB kinase/nuclear factor-κB pathway.* Molecular Pharmacology, 2006. **70**(5): p. 1812-1821.
60. Wagner, S., et al., *Development of a structural model for NF-κB inhibition of sesquiterpene lactones using self-organizing neural networks.* Journal of Medicinal Chemistry, 2006. **49**(7): p. 2241-2252.
61. Switzer, C.H., et al., *Dithiolethiones inhibit NF-κB activity via covalent modification in human estrogen receptor–negative breast cancer.* Cancer Research, 2012. **72**(9): p. 2394-2404.
62. García-Piñeres, A.J., et al., *Cysteine 38 in p65/NF-κB plays a crucial role in DNA Binding inhibition by sesquiterpene lactones.* Journal of Biological Chemistry, 2001. **276**(43): p. 39713-39720.
63. Borgeat, P., M. Hamberg, and B. Samuelsson, *Transformation of arachidonic acid and homo-gamma-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases.* Journal of Biological Chemistry, 1976. **251**(24): p. 7816-7820.
64. Busse, R., A. Lackhoff, and E. Bassenge, *Endothelium-derived relaxant factor inhibits platelet activation.* Naunyn-Schmiedeberg's Archives of Pharmacology, 1987. **336**(5): p. 566-571.
65. Schopfer, F.J., C. Cipollina, and B.A. Freeman, *Formation and signaling actions of electrophilic lipids.* Chemical Reviews, 2011. **111**(10): p. 5997-6021.
66. Delmastro-Greenwood, M., B.A. Freeman, and S.G. Wendell, *Redox-dependent anti-inflammatory signaling actions of unsaturated fatty acids.* Annual Review of Physiology, 2014. **76**(1): p. 79-105.
67. Beckman, J.S., et al., *Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide.* Proceedings of the National Academy of Sciences, 1990. **87**(4): p. 1620-1624.

68. Eiserich, J.P., et al., *Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils.* Nature, 1998. **391**(6665): p. 393-397.
69. Castro, L. and B.A. Freeman, *Reactive oxygen species in human health and disease.* Nutrition, 2001. **17**(2): p. 161-165.
70. Nelson, D.L. and M.M. Cox, *Lehninger principles of biochemistry 6th edition.* 2012.
71. Abedi, E. and M.A. Sahari, *Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties.* Food Science & Nutrition, 2014. **2**(5): p. 443-463.
72. Groeger, A.L. and B.A. Freeman, *Signaling actions of electrophiles: Anti-inflammatory therapeutic candidates.* Molecular Interventions, 2010. **10**(1): p. 39-50.
73. Murphy, R.C. and S. Zarini, *Glutathione adducts of oxyeicosanoids.* Prostaglandins & Other Lipid Mediators, 2002. **68-69**: p. 471.
74. Ceaser, E.K., et al., *Mechanisms of signal transduction mediated by oxidized lipids: the role of the electrophile-responsive proteome.* Biochemical Society Transactions, 2004. **32**(1): p. 151-155.
75. Pryor, W.A. and J.P. Stanley, *A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation.* . The Journal of Organic Chemistry, 1975. **40**(24): p. 3615-3617.
76. Halliwell, B. and S. Chirico, *Lipid peroxidation: its mechanism, measurement, and significance.* The American Journal of Clinical Nutrition, 1993. **57**(5): p. 715S-724S.
77. Yin, H., L. Xu, and N.A. Porter, *Free radical lipid peroxidation: mechanisms and analysis.* Chemical Reviews, 2011. **111**(10): p. 5944-5972.
78. O'Donnell, V.B. and B.A. Freeman, *Interactions between nitric oxide and lipid oxidation pathways.* Implications for Vascular Disease, 2001. **88**(1): p. 12-21.
79. Baker, L.M.S., et al., *Nitro-fatty acid reaction with glutathione and cysteine: Kinetic analysis of thiol alkylation by a Michael addition reaction.* Journal of Biological Chemistry, 2007. **282**(42): p. 31085-31093.
80. Tokoroyama, T., *Discovery of the Michael reaction.* European Journal of Organic Chemistry, 2010. **2010**(10): p. 2009-2016.
81. Michael, A., *Ueber die Addition von Natriumacetessig- und Natriummalonsäureäthern zu den Aethern ungesättigter Säuren.* Journal für Praktische Chemie, 1887. **35**(1): p. 349-356.
82. Pearson, R.G., *Hard and soft acids and bases.* Journal of the American Chemical Society, 1963. **85**(22): p. 3533-3539.
83. LoPachin, R.M., et al., *Application of the hard and soft, acids and bases (HSAB) theory to toxicant-target interactions* Chemical Research in Toxicology, 2012. **25**(2): p. 239-251.
84. LoPachin, R.M., D.S. Barber, and T. Gavin, *Molecular mechanisms of the conjugated α, β-unsaturated carbonyl derivatives: relevance to neurotoxicity and neurodegenerative diseases.* Toxicological Sciences, 2008. **104**(2): p. 235-249.
85. Benesch, R.E., H.A. Lardy, and R. Benesch, *The sulfhydryl groups of crystalline proteins: I. some albumins, enzymes, and hemoglobins* Journal of Biological Chemistry, 1955. **216**(2): p. 663-676.

86. Netto, L.E.S., et al., *Reactive cysteine in proteins: Protein folding, antioxidant defense, redox signaling and more.* Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 2007. **146**(1): p. 180-193.
87. Schopfer, F.J., et al., *Covalent peroxisome proliferator-activated receptor γ adduction by nitro-fatty acids: Selective ligand activity and anti-diabetic signaling actions.* Journal of Biological Chemistry, 2010. **285**(16): p. 12321-12333.
88. Lawley, P.D. and P. Brookes, *The action of alkylating agents on deoxyribonucleic acid in relation to biological effects of the alkylating agents* Exp. Cell Res., 1963. **9**(suppl. 1): p. 512-520.
89. Miller, J.A. and E.C. Miller, *The metabolism and carcinogenicity of p-dimethylaminoazobenzene and related compounds in the rat.* Cancer Research, 1947. **7**: p. 39-41.
90. Miller, J.A. and E.C. Miller, *The carcinogenicity of certain derivatives of p-dimethylaminozobenz in the rat.* J. Exp. Med., 1948. **87**: p. 139-156.
91. Miller, J.A., R.W. Sapp, and E.C. Miller, *The absorption spectra of certain carcinogenic aminoazo dyes and the protein-bound derivatives formed from these dyes in vivo.* J. Am. Chem. Soc., 1948. **70**: p. 3458-3463.
92. Coles, B., et al., *The spontaneous and enzymatic reaction of N-acetyl-p-benzoquinonimine with glutathione: A stopped-flow kinetic study.* Archives of Biochemistry and Biophysics, 1988. **264**(1): p. 253-260.
93. Ketterer, B., *Protective role of glutathione and glutathione transferases in mutagenesis and carcinogenesis.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1988. **202**(2): p. 343-361.
94. Hayes, J.D. and D.J. Pulford, *The glutathione S-transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance part I.* Critical Reviews in Biochemistry and Molecular Biology, 1995. **30**(6): p. 445-520.
95. Kaplowitz, N., et al., *GSH transporters: molecular characterization and role in GSH homeostasis.* Biol Chem Hoppe Seyler., 1996. **377**(5): p. 267-273.
96. Itoh, K., et al., *An Nrf2/Small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements.* Biochemical and Biophysical Research Communications, 1997. **236**(2): p. 313-322.
97. Itoh, K., K.I. Tong, and M. Yamamoto, *Molecular mechanism activating nrf2–keap1 pathway in regulation of adaptive response to electrophiles.* Free Radical Biology and Medicine, 2004. **36**(10): p. 1208-1213.
98. Batthyany, C., et al., *Reversible post-translational modification of proteins by nitrated fatty acids in vivo.* Journal of Biological Chemistry, 2006. **281**(29): p. 20450-20463.
99. Tak, P.P. and G.S. Firestein, *NF-κB: a key role in inflammatory diseases.* Journal of Clinical Investigation, 2001. **107**(1): p. 7-11.
100. Cui, T., et al., *Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators.* Journal of Biological Chemistry, 2006. **281**(47): p. 35686-35698.
101. Villacorta, L., et al., *Electrophilic nitro-fatty acids inhibit vascular inflammation by disrupting LPS-dependent TLR4 signalling in lipid rafts.* Cardiovascular Research, 2013. **98**(1): p. 116-124.

102. Lin, Y.-C., et al., *The glutathionylation of p65 modulates NF-κB activity in 15-deoxy-Δ 12,14-prostaglandin J2-treated endothelial cells.* Free Radical Biology and Medicine, 2012. **52**(9): p. 1844-1853.
103. Tontonoz, P. and B.M. Spiegelman, *Fat and beyond: The diverse biology of PPARγ*. Annual Review of Biochemistry, 2008. **77**(1): p. 289-312.
104. Li, Y., et al., *Molecular recognition of nitrated fatty acids by PPARγ*. Nature structural & molecular biology, 2008. **15**(8): p. 865-867.
105. Baker, P.R.S., et al., *Fatty acid transduction of nitric oxide signaling: Multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands.* Journal of Biological Chemistry, 2005. **280**(51): p. 42464-42475.
106. Schopfer, F.J., et al., *Nitrolinoleic acid: An endogenous peroxisome proliferator-activated receptor γ ligand.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(7): p. 2340-2345.
107. Kliewer, S.A., et al., *A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation.* Cell, 1995. **83**: p. 813-819.
108. Paumi, C.M., et al., *Multidrug resistance protein (MRP) 1 and MRP3 attenuate cytotoxic and transactivating effects of the cyclopentenone prostaglandin, 15-Deoxy-Δ 12,14prostaglandin J2 in MCF7 breast cancer cells.* Biochemistry, 2003. **42**(18): p. 5429-5437.
109. Alexander, R.L., et al., *Modulation of nitrated lipid signaling by multidrug resistance protein 1 (MRP1): Glutathione conjugation and MRP1-mediated efflux inhibit nitrolinoleic acid-induced, PPARγ-dependent transcription activation.* Biochemistry, 2006. **45**(25): p. 7889-7896.
110. Itoh, K., et al., *Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain.* Genes & Development, 1999. **13**(1): p. 76-86.
111. Kobayashi, M. and M. Yamamoto, *Nrf2–Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species.* Advances in Enzyme Regulation, 2006. **46**(1): p. 113-140.
112. Kobayashi, M., et al., *The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds.* Molecular and Cellular Biology, 2009. **29**(2): p. 493-502.
113. Sekhar, K.R., G. Rachakonda, and M.L. Freeman, *Cysteine-based Regulation of the CUL3 Adaptor Protein Keap1*. Toxicology and applied pharmacology, 2010. **244**(1): p. 21-26.
114. Zhang, D.D. and M. Hannink, *Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress.* Molecular and Cellular Biology, 2003. **23**(22): p. 8137-8151.
115. McMahon, M., et al., *Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals.* Proceedings of the National Academy of Sciences, 2010. **107**(44): p. 18838-18843.
116. Itoh, K., et al., *Transcription Factor Nrf2 Regulates Inflammation by Mediating the Effect of 15-Deoxy-Δ12,14-Prostaglandin J2.* Molecular and Cellular Biology, 2004. **24**(1): p. 36-45.

117. Kansanen, E., et al., *Electrophilic nitro-fatty acids activate Nrf2 by a Keap1 cysteine 151-independent mechanism.* Journal of Biological Chemistry, 2011. **286**(16): p. 14019-14027.
118. Eggler, A.L., et al., *Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(29): p. 10070-10075.
119. LoPachin, R.M., et al., *Structure-toxicity analysis of type-2 alkenes: In vitro neurotoxicity.* Toxicological Sciences, 2007. **95**(1): p. 136-146.
120. Martyniuk, C.J., et al., *Molecular mechanism of glyceraldehyde-3-phosphate dehydrogenase inactivation by α,β-unsaturated carbonyl derivatives.* Chemical Research in Toxicology, 2011. **24**(12): p. 2302-2311.
121. Pearson, P.G., et al., *S-(N-Methylcarbamoyl)glutathione: A reactive S-linked metabolite of methyl isocyanate.* Biochemical and Biophysical Research Communications, 1990. **166**(1): p. 245-250.
122. Winterbourn, C.C., *Reconciling the chemistry and biology of reactive oxygen species.* Nature Chemical Biology, 2008. **4**: p. 278-286.
123. Meister, A., *Glutathione metabolism and its selective modification.* Journal of Biological Chemistry, 1988. **263**(33): p. 17205-8.
124. Smith, C.V., et al., *Compartmentation of glutathione: Implications for the study of toxicity and disease.* Toxicology and Applied Pharmacology, 1996. **140**(1): p. 1-12.
125. Hwang, C., H.F. Lodish, and A.J. Sinskey, *[18] Measurement of glutathione redox state in cytosol and secretory pathway of cultured cells*, in *Methods in Enzymology*. 1995, Academic Press. p. 212-221.
126. Madhu, C., Z. Gregus, and C.D. Klaassen, *Biliary excretion of acetaminophen-glutathione as an index of toxic activation of acetaminophen: effect of chemicals that alter acetaminophen hepatotoxicity.* Journal of Pharmacology and Experimental Therapeutics, 1989. **248**(3): p. 1069-1077.
127. Jung, G., E. Breitmaier, and W. Voelter, *Dissoziationsgleichgewichte von Glutathion.* European Journal of Biochemistry, 1972. **24**(3): p. 438-445.
128. Liebler, D.C., *Protein damage by reactive electrophiles: targets and consequences.* Chemical Research in Toxicology, 2008. **21**(1): p. 117-128.
129. Rudolph, T.K. and B.A. Freeman, *Transduction of redox signaling by electrophile-protein reactions.* Science Signaling, 2009. **2**(90).
130. Smithgall, T.E., R.G. Harvey, and T.M. Penning, *Spectroscopic identification of ortho-quinones as the products of polycyclic aromatic trans-dihydrodiol oxidation catalyzed by dihydrodiol dehydrogenase. A potential route of proximate carcinogen metabolism.* Journal of Biological Chemistry, 1988. **263**(4): p. 1814-20.
131. Brodie, B.B., et al., *Possible mechanism of liver necrosis caused by aromatic organic compounds.* Proceedings of the National Academy of Sciences of the United States of America, 1971. **68**(1): p. 160-164.
132. Guengerich, F.P., *Metabolic activation of carcinogens.* Pharmacology & Therapeutics, 1992. **54**(1): p. 17-61.
133. Schrenk, D., et al., *Phase II metabolism of benzene.* Environmental Health Perspectives, 1996. **104**(Suppl 6): p. 1183-1188.

134. The International Transporter, C., *Membrane transporters in drug development.* Nature Reviews. Drug Discovery, 2010. **9**(3): p. 215-236.
135. Cole, S., et al., *Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line.* Science, 1992. **258**(5088): p. 1650-1654.
136. Leslie, E.M., R.G. Deeley, and S.P.C. Cole, *Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense.* Toxicology and Applied Pharmacology, 2005. **204**(3): p. 216-237.
137. Jedlitschky, G., et al., *Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump.* Cancer Research, 1996. **56**(5): p. 988-994.
138. Evers, R., et al., *Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1.* FEBS Letters, 1997. **419**(1): p. 112-116.
139. Uchida, K., et al., *Immunochemical detection of 4-hydroxynonenal protein adducts in oxidized hepatocytes.* Proceedings of the National Academy of Sciences, 1993. **90**(18): p. 8742-8746.
140. Girotti, A.W., *Lipid hydroperoxide generation, turnover, and effector action in biological systems.* Journal of Lipid Research, 1998. **39**(8): p. 1529-1542.
141. Dalleau, S., et al., *Cell death and diseases related to oxidative stress:4-hydroxynonenal (HNE) in the balance.* Cell Death and Differentiation, 2013. **20**(12): p. 1615-1630.
142. RENES, J., et al., *Multidrug resistance protein MRP1 protects against the toxicity of the major lipid peroxidation product 4-hydroxynonenal.* Biochemical Journal, 2000. **350**(2): p. 555-561.
143. Bonacci, G., et al., *Conjugated linoleic acid is a preferential substrate for fatty acid nitration.* Journal of Biological Chemistry, 2012. **287**(53): p. 44071-44082.
144. Fazzari, M., et al., *Nitro-fatty acid pharmacokinetics in the adipose tissue compartment.* The Journal of Lipid Research, 2016. **58**: p. 375-385.
145. Salvatore, S.R., et al., *Characterization and quantification of endogenous fatty acid nitroalkene metabolites in human urine.* The Journal of Lipid Research, 2013. **54**(7): p. 1998-2009.
146. Delmastro-Greenwood, M., et al., *Nitrite and nitrate-dependent generation of anti-inflammatory fatty acid nitroalkenes.* Free Radical Biology & Medicine, 2015. **89**: p. 333-341.
147. Hughan, K.S., et al., *Conjugated linoleic acid modulates clinical responses to oral nitrite and nitrate.* Hypertension, 2017. **70**(3): p. 634-644.
148. Codreanu, S.G., et al., *Alkylation damage by lipid electrophiles targets functional protein systems.* Molecular and Cellular Proteomics, 2014. **13**(3): p. 849-859.
149. Levonen, A.L., et al., *Redox regulation of antioxidants, autophagy, and the response to stress: implications for electrophile therapeutics.* Free Radical Biology & Medicine, 2014. **71**: p. 196-207.
150. Snyder, N.W., et al., *15-Oxoeicosatetraenoic acid is a 15-hydroxyprostaglandin dehydrogenase-derived electrophilic mediator of inflammatory signaling pathways.* Chemico-Biological Interactions, 2015. **234**: p. 144-153.
151. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2017.* CA: A Cancer Journal for Clinicians, 2017. **67**: p. 7-30.
152. DeSantis, C., et al., *Breast cancer statistics, 2013.* CA: A Cancer Journal for Clinicians, 2014. **64**(1): p. 52-62.

153. Bombonati, A. and D.C. Sgroi, *The molecular pathology of breast cancer progression.* The Journal of Pathology, 2011. **223**(2): p. 308-318.
154. Edwards, B.K., et al., *Annual report to the nation on the status of cancer, 1975-2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast or prostate cancer.* Cancer, 2014. **120**(9): p. 1290-1314.
155. Sørlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proceedings of the National Academy of Sciences, 2001. **98**(19): p. 10869-10874.
156. Sims, A.H., et al., *Origins of breast cancer subtypes and therapeutic implications.* Nat Clin Prac Oncol, 2007. **4**(9): p. 516-525.
157. Slamon , D.J., et al., *Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.* New England Journal of Medicine, 2001. **344**(11): p. 783-792.
158. Lehmann, B.D., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies.* The Journal of Clinical Investigation, 2011. **121**(7): p. 2750-2767.
159. Perou, C.M., et al., *Distinctive gene expression patterns in human mammary epithelial cells and breast cancers.* Proceedings of the National Academy of Sciences, 1999. **96**(16): p. 9212-9217.
160. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-752.
161. Neve, R.M., et al., *A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.* Cancer Cell, 2006. **10**(6): p. 515-527.
162. Foulkes, W.D., et al., *Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer.* JNCI: Journal of the National Cancer Institute, 2003. **95**(19): p. 1482-1485.
163. Bauer, K.R., et al., *Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype.* Cancer, 2007. **109**(9): p. 1721-1728.
164. Millikan, R.C., et al., *Epidemiology of basal-like breast cancer.* Breast Cancer Research and Treatment, 2008. **109**(1): p. 123-139.
165. Dent, R., et al., *Triple-negative breast cancer: Clinical features and patterns of recurrence.* Clinical Cancer Research, 2007. **13**(15): p. 4429-4434.
166. Kwan, M.L., et al., *Epidemiology of breast cancer subtypes in two prospective cohort studies of breast cancer survivors.* Breast Cancer Research, 2009. **11**(3): p. R31.
167. Heitz, F., et al., *Triple-negative and HER2-overexpressing breast cancers exhibit an elevated risk and an earlier occurrence of cerebral metastases.* European Journal of Cancer, 2009. **45**(16): p. 2792-2798.
168. Dawood, S., et al., *Survival among women with triple receptor-negative breast cancer and brain metastases.* Annals of Oncology, 2009. **20**(4): p. 621-627.
169. Dent, R., et al., *Pattern of metastatic spread in triple-negative breast cancer.* Breast Cancer Research and Treatment, 2009. **115**(2): p. 423-428.
170. Carey, L.A., et al., *Race, breast cancer subtypes, and survival in the carolina breast cancer study.* JAMA, 2006. **295**(21): p. 2492-2502.
171. Rhee, J., et al., *The clinicopathologic characteristics and prognostic significance of triple-negativity in node-negative breast cancer.* BMC Cancer, 2008. **8**: p. 307-307.

172. Maiti, B., et al., *The association of metabolic syndrome with triple-negative breast cancer.* Breast Cancer Research and Treatment, 2010. **121**(2): p. 479-483.
173. Trivers, K.F., et al., *The epidemiology of triple-negative breast cancer, including race.* Cancer causes & control : CCC, 2009. **20**(7): p. 1071-1082.
174. Cleator, S., W. Heller, and R.C. Coombes, *Triple-negative breast cancer: therapeutic options.* The Lancet Oncology, 2007. **8**(3): p. 235-244.
175. Vyas, D., et al., *Rapid extensive recurrence of triple negative breast cancer: Are both therapy and cancer biology the culprit?* J Clin Med Res., 2016. **8**(2): p. 162-167.
176. Panayotopoulou, E.G., et al., *Targeting of apoptotic pathways by SMAC or BH3 mimetics distinctly sensitizes paclitaxel-resistant triple negative breast cancer cells.* Oncotarget, 2017. **8**(28): p. 45088-45104.
177. Qiao, Y., et al., *AP-1 is a key regulator of proinflammatory cytokine TNFα-mediated triple-negative breast cancer progression.* Journal of Biological Chemistry, 2016.
178. Joensuu, H. and J. Gligorov, *Adjuvant treatments for triple-negative breast cancers.* Annals of Oncology, 2012. **23**(suppl_6): p. vi40-vi45.
179. von Minckwitz, G. and M. Martin, *Neoadjuvant treatments for triple-negative breast cancer (TNBC).* Annals of Oncology, 2012. **23**(suppl_6): p. vi35-vi39.
180. Biswas, D.K., et al., *NF-κB activation in human breast cancer specimens and its role in cell proliferation and apoptosis.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(27): p. 10137-10142.
181. Yamaguchi, N., et al., *Constitutive activation of nuclear factor-κB is preferentially involved in the proliferation of basal-like subtype breast cancer cell lines.* Cancer Science, 2009. **100**(9): p. 1668-1674.
182. Ossovskaya, V., et al., *Exploring molecular pathways of triple-negative breast cancer.* Genes & Cancer, 2011. **2**(9): p. 870-879.
183. Woodcock, S.R., et al., *Nitrated fatty acids: synthesis and measurement.* Free Radical Biology and Medicine, 2013. **59**: p. 14-26.
184. Pledgie-Tracy, A., M.D. Sobolewski, and N.E. Davidson, *Sulforaphane induces cell type–specific apoptosis in human breast cancer cell lines.* Molecular Cancer Therapeutics, 2007. **6**(3): p. 1013-1021.
185. Yang, M., et al., *Sulforaphene inhibits triple negative breast cancer through activating tumor suppressor Egr1.* Breast Cancer Research and Treatment, 2016. **158**(2): p. 277-286.
186. Adams, L.S., et al., *Blueberry Phytochemicals Inhibit Growth and Metastatic Potential of MDA-MB-231 Breast Cancer Cells through Modulation of the Phosphatidylinositol 3-Kinase Pathway.* Cancer Research, 2010. **70**(9): p. 3594-3605.
187. So, J.Y., et al., *A synthetic triterpenoid CDDO-Im inhibits tumorsphere formation by regulating stem cell signaling pathways in triple-negative breast cancer.* PLOS ONE, 2014. **9**(9): p. e107616.
188. Cole, M.P., et al., *Nitro-fatty acid inhibition of neointima formation after endoluminal vessel injury.* Circulation Research, 2009. **105**(10): p. 965-972.
189. Klinke, A., et al., *Protective Effects of 10-nitro-oleic Acid in a Hypoxia-Induced Murine Model of Pulmonary Hypertension.* American Journal of Respiratory Cell and Molecular Biology, 2014. **51**(1): p. 155-162.

190. Ambrozova, G., et al., *Nitro-oleic acid modulates classical and regulatory activation of macrophages and their involvement in pro-fibrotic responses.* Free radical biology & medicine, 2016. **90**: p. 252-260.
191. Rudolph, T.K., et al., *Nitrated fatty acids suppress angiotensin II-mediated fibrotic remodelling and atrial fibrillation.* Cardiovascular Research, 2016. **109**(1): p. 174-184.
192. Verescakova, H., et al., *Nitro-oleic acid regulates growth factor-induced differentiation of bone marrow-derived macrophages.* Free Radical Biology and Medicine, 2017. **104**: p. 10-19.
193. Tang, X., et al., *Nitroalkenes induce rat aortic smooth muscle cell apoptosis via activation of caspase-dependent pathways.* Biochemical and Biophysical Research Communications, 2010. **397**(2): p. 239-244.
194. Shina, H.-M., et al., *Inhinitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-κB without affecting IκB degradation.* FEBS Letters, 2004. **571**: p. 50-54.
195. Holliday, D. and V. Speirs, *Choosing the right cell line for breast cancer research.* Breast Cancer Research, 2011. **13**(4): p. 215.
196. Wang, W., C. Li, and T. Yang, *Protection of nitro-fatty acid against kidney diseases.* American Journal of Physiology Renal Physiology, 2015. **310**(8): p. F697-F704.
197. Wang, H., et al., *Nitrooleic acid protects against cisplatin nephropathy: role of COX-2/mPGES-1/PGE2 cascade.* Mediators of Inflammation, 2015. **2015**: p. 293474.
198. Liu, S., et al., *Nitro-oleic acid protects against adriamycin-induced nephropathy in mice.* American Journal of Physiology Renal Physiology, 2013. **305**(11): p. F1533-F1541.
199. Mylona, E., et al., *Cyclin D1 in invasive breast carcinoma: favourable prognostic significance in unselected patients and within subgroups with an aggressive phenotype.* Histopathology, 2013. **62**(3): p. 472-480.
200. Kaklamani, V.G., et al., *Phase II neoadjuvant clinical trial of carboplatin and eribulin in women with triple negative early-stage breast cancer (NCT01372579).* Breast Cancer Research and Treatment, 2015. **151**(3): p. 629-638.
201. Diers, Anne R., et al., *Modulation of mammary cancer cell migration by 15-deoxy-Δ12,14-prostaglandin J2: implications for anti-metastatic therapy.* Biochemical Journal, 2010. **430**(1): p. 69-78.
202. Delmastro-Greenwood, M., et al., *Nitrite and nitrate-dependent generation of anti-inflammatory fatty acid nitroalkenes.* Free Radical Biology and Medicine, 2015. **89**: p. 333-341.
203. Salvatore, S.R., et al., *Characterization and quantification of endogenous fatty acid nitroalkene metabolites in human urine.* Journal of Lipid Research, 2013. **54**(7): p. 1998-2009.
204. Kalimutho, M., et al., *Targeted therapies for triple-negative breast cancer: Combating a stubborn disease.* Trends in Pharmacological Sciences, 2015. **36**(12): p. 822-846.
205. Evani, S.J., et al., *Monocytes mediate metastatic breast tumor cell adhesion to endothelium under flow.* The FASEB Journal, 2013. **27**(8): p. 3017-3029.
206. Qiao, Y., et al., *AP-1 is a key regulator of proinflammatory cytokine TNFα-mediated triple-negative breast cancer progression.* Journal of Biological Chemistry, 2016. **291**(10): p. 5069-5079.

207. Huber, M.A., et al., *NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression.* The Journal of Clinical Investigation, 2004. **114**(4): p. 569-581.
208. Nakshatri, H., et al., *Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth.* Molecular and Cellular Biology, 1997. **17**(7): p. 3629-39.
209. Sovak, M.A., et al., *Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer.* The Journal of Clinical Investigation, 1997. **100**(12): p. 2952-2960.
210. Smith, S.M., Y.L. Lyu, and L. Cai, *NF-κB Affects Proliferation and Invasiveness of Breast Cancer Cells by Regulating CD44 Expression.* PLoS ONE, 2014. **9**(9): p. e106966.
211. Rossi, A., et al., *Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of I[kappa]B kinase.* Nature, 2000. **403**(6765): p. 103-18.
212. Ahmad, R., et al., *Triterpenoid CDDO-Me blocks the NF-κB pathway by direct inhibition of IKKβ on Cys-179.* Journal of Biological Chemistry, 2006. **281**(47): p. 35764-35769.
213. Paranjpe, A. and K.S. Srivenugopal, *Degradation of NF-κB, p53 and other regulatory redox-sensitive proteins by thiol-conjugating and -nitrosylating drugs in human tumor cells.* Carcinogenesis, 2013. **34**(5): p. 990-1000.
214. Hou, Y., F. Moreau, and K. Chadee, *PPARγ is an E3 ligase that induces the degradation of NFκB/p65.* Nat Commun, 2012. **3**: p. 1300.
215. Natoli, G. and S. Chiocca, *Nuclear ubiquitin ligases, NF-κB degradation, and the control of inflammation.* Science Signaling, 2008. **1**(1): p. pe1-pe1.
216. Tanaka, T., M.J. Grusby, and T. Kaisho, *PDLIM2-mediated termination of transcription factor NF-κB activation by intranuclear sequestration and degradation of the p65 subunit.* Nat Immunol, 2007. **8**(6): p. 584-591.
217. Ryo, A., et al., *Regulation of NF-κB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA.* Molecular Cell, 2003. **12**(6): p. 1413-1426.
218. Besser, D., et al., *Signal transduction and the u-PA/u-PAR system.* Fibrinolysis, 1996. **10**(4): p. 215-237.
219. Reymond, N., B.B. d'Agua, and A.J. Ridley, *Crossing the endothelial barrier during metastasis.* Nat Rev Cancer, 2013. **13**(12): p. 858-870.
220. Rosette, C., et al., *Role of ICAM1 in invasion of human breast cancer cells.* Carcinogenesis, 2005. **26**(5): p. 943-950.
221. Guo, P., et al., *ICAM-1 as a molecular target for triple negative breast cancer.* Proceedings of the National Academy of Sciences, 2014. **111**(41): p. 14710-14715.
222. Hayden, M.S. and S. Ghosh, *Shared principles in NF-kappaB signaling.* Cell, 2008. **132**(3): p. 344-362.
223. Garber, K., *The second wave in kinase cancer drugs.* Nat Biotech, 2006. **24**(2): p. 127-130.
224. Maine, G.N., et al., *COMMD1 promotes the ubiquitination of NF-κB subunits through a cullin-containing ubiquitin ligase.* The EMBO Journal, 2007. **26**(2): p. 436-447.

225. Hou, Y., F. Moreau, and K. Chadee, *PPAR γ is an E3 ligase that induces the degradation of NF-κB/p65.* Nature Communications, 2012. **3**: p. 1300.
226. Lin, D., S. Saleh, and D.C. Liebler, *Reversibility of covalent electrophile-protein adducts and chemical toxicity.* Chemical Research in Toxicology, 2008. **21**(12): p. 2361-2369.
227. Koenitzer, J.R., et al., *Fatty acid nitroalkenes induce resistance to ischemic cardiac injury by modulating mitochondrial respiration at complex II.* Redox Biology, 2016. **8**: p. 1-10.
228. Bates, D.J.P., et al., *Noncatalytic Interactions between Glutathione S-Transferases and Nitroalkene Fatty Acids Modulate Nitroalkene-Mediated Activation of Peroxisomal Proliferator-Activated Receptor γ.* Biochemistry, 2009. **48**(19): p. 4159-4169.
229. Bai, J., et al., *Multidrug resistance protein 4 (MRP4/ABCC4) mediates efflux of bimane-glutathione.* The International Journal of Biochemistry & Cell Biology, 2004. **36**(2): p. 247-257.
230. Cole, S.P.C., *Multidrug resistance protein 1 (MRP1, ABCC1), a "multitasking" ATP-binding cassette (ABC) transporter.* Journal of Biological Chemistry, 2014. **289**(45): p. 30880-30888.
231. Song, N.-Y., et al., *Multidrug Resistance-Associated Protein 1 Mediates 15-Deoxy-Δ 12,14-prostaglandin J2-Induced Expression of Glutamate Cysteine Ligase Expression via Nrf2 Signaling in Human Breast Cancer Cells.* Chemical Research in Toxicology, 2011. **24**(8): p. 1231-1241.
232. Hagos, F.T., et al., *Probenecid, an organic anion transporter 1 and 3 inhibitor, increases plasma and brain exposure of N-acetylcysteine.* Xenobiotica, 2017. **47**(4): p. 346-353.
233. Munoz, M., et al., *Role of the MRP1/ABCC1 multidrug transporter protein in cancer.* IUBMB Life, 2007. **59**(12): p. 752-757.
234. Harris, Isaac S., et al., *Glutathione and Thioredoxin Antioxidant Pathways Synergize to Drive Cancer Initiation and Progression.* Cancer Cell. **27**(2): p. 211-222.
235. Timmerman, Luika A., et al., *Glutamine Sensitivity Analysis Identifies the xCT Antiporter as a Common Triple-Negative Breast Tumor Therapeutic Target.* Cancer Cell, 2013. **24**(4): p. 450-465.
236. Tang, X., et al., *Cystine addiction of triple-negative breast cancer associated with EMT augmented death signaling.* Oncogene, 2017. **36**(30): p. 4235-4242.
237. Beatty, A., et al., *Metabolite profiling reveals the glutathione biosynthetic pathway as a therapeutic target in triple negative breast cancer.* Molecular Cancer Therapeutics, 2017.
238. Gross, M.I., et al., *Antitumor Activity of the Glutaminase Inhibitor CB-839 in Triple-Negative Breast Cancer.* Molecular Cancer Therapeutics, 2014. **13**(4): p. 890-901.
239. Cardoso, F., et al., *Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up †.* Annals of Oncology, 2012. **23**(suppl_7): p. vii11-vii19.
240. André, F. and C.C. Zielinski, *Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents.* Annals of Oncology, 2012. **23**(suppl_6): p. vi46-vi51.
241. Cleere, D.W., *Triple-negative breast cancer: a clinical update.* Community Oncology, 2010. **7**(5): p. 203-211.
242. Sibhatu, M.B., et al., *Expression of MRP1 and GSTP1-1 modulate the acute cellular response to treatment with the chemopreventive isothiocyanate, sulforaphane.* Carcinogenesis, 2008. **29**(4): p. 807-815.

243. Holen, I., et al., *In vivo models in breast cancer research: progress, challenges and future directions.* Disease Models & Mechanisms, 2017. **10**(4): p. 359-371.
244. Herschkowitz, J.I., et al., *Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors.* Genome Biology, 2007. **8**(5): p. R76-R76.