AWARD NUMBER: **W81XWH-19-1-0052**

TITLE: **Characterizing Nitro-Fatty Acids as Rad51 Inhibitors and Cotreatment in Triple-Negative Breast Cancer**

PRINCIPAL INVESTIGATOR: **Carola A. Neumann, MD**
COLLABORATING INVESTIGATOR: **Bruce A. Freeman. PhD**

CONTRACTING ORGANIZATION: **University of Pittsburgh**
Pittsburgh, PA 15203

REPORT DATE: **March 2020**

TYPE OF REPORT: **Annual Report**

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2020	Annual Report	01 Feb 2019-31 Jan 2020

4. TITLE AND SUBTITLE
Characterizing Nitro-Fatty Acids as Rad51 Inhibitors and Cotreatment in Triple-Negative Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0052

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Bruce A. Freeman. PhD

E-Mail:freerad@pitt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
Pittsburgh, PA 15203

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This Research Plan is testing a readily-deployed novel drug strategy for treating TNBC, where the inhibition of Rad51-mediated DNA repair by electrophilic nitroalkenes renders TNBC cells more sensitive to PARP inhibition and TNBC cell killing. OA-NO2 and NFA-8 are nitroalkenes, 7-nitro-nonadec-7-enoic acid and 10-nitro-octadec-9-enoic acid, respectively. We have now devised an even more pharmacologically efficacious nitroalkene that shares the same active nitroalkene moiety with OA-NO2 and NFA-8, dimethyl-4-nitro-oct-4-enoiate (CP-1). New data indicates that CP-1 outperforms both OA-NO2 and NFA-8, respectively. CP-1 shows not only enhanced cell killing of TNBC cells as single drugs as well as combination therapy, but also displays cell protective effects in benign breast epithelial cells that are treated with PARP inhibitors or ionizing radiation. Further, preliminary data also show that smaller alkynyl (R-C□CH) terminus on OA-NO2 facilitates secondary "click" reactions with azido-substituted affinity tags that facilitate protein pull-down as well as HPLC-MS/MS-based detection. Preliminary studies support click chemistry-based Rad51 and □-actin pulldown through alkynyl-tagged NFAs from cell lysate.

15. SUBJECT TERMS
Nitroalkenes, triple negative breast cancer, homologous recombination directed DNA repair, PARP, RAD51

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	20	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION:

This Research Plan will test a readily-deployed novel drug strategy for treating TNBC, where the inhibition of Rad51-mediated DNA repair by electrophilic NFA renders TNBC cells more sensitive to PARP inhibition and TNBC cell killing. 15% of all breast cancers are triple negative, being devoid of the three receptors that classify and define treatment strategies for most mammary cancers: estrogen receptor (ER), progesterone receptor (PR) and ERB2 (also known as HER2). Because of this, no targeted therapies are available for TNBC patients. This poses a destitute situation, as TNBC is an aggressive cancer that disproportionally affects younger women and those having African origins. 15-20% of TNBC patients also carry a germline *BRCA1* or *BRCA2* mutation, resulting in defects in homologous-directed DNA repair (HDR). Two open-label Phase 3 clinical trials have now signified the advantage of PARPi monotherapy compared to chemotherapy for germline *BRCA*-mutant TNBC patients. In the OlympIAD trial, progression-free survival was prolonged by 2.8 mo. and in the EMBRACA trial by 3 mo. These findings are promising and significant, as they reinforce the concept that genetic defects in HDR pave a path to PARPi cancer cell killing, by inhibiting single strand DNA repair. This in turn underscores the significance of our Research Plan. We propose to induce HDR-deficiency through Rad51 inhibition, which then in turn will amplify PARPi efficacy. To accomplish this, we have identified a unique reactivity of a novel class of Rad51 inhibitors, nitro alkenes, which are well tolerated and readily testable as new drug candidates in humans: nitro alkene derivatives are endogenously present as nitrated fatty acids in humans and their synthetic homologs have been de-risked by preclinical toxicology and 5 Phase 1 safety evaluations of IV and the oral nitro alkene OA-NO_2 in healthy and obese volunteers (http://www.complexarx.com), as well as ongoing Phase 2 trials in renal and cardiopulmonary disease patients, presenting a promising new opportunity for treating the drug-resistant TNBC breast cancer phenotype.

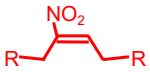

Fig.1 nitro alkene

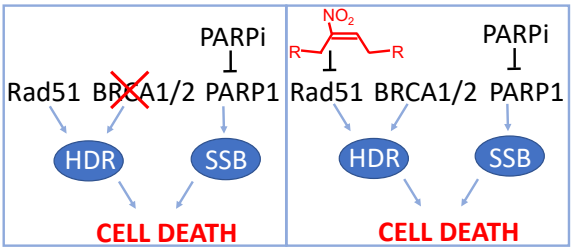

Fig. 2. Chemical induction mimicking loss of BRCA1/2 through targeting Rad51 with OA-NO_2 to induce cell death in TNBC.

2. KEYWORDS:

Triple negative breast cancer, PARP inhibition, DNA-double strand repair, Homologous directed DNA repair deficiency, RAD51, nitro fatty acid, nitroalkene

3. ACCOMPLISHMENTS:

- **MAJOR GOALS:**

Aim 1: Examine if NFA-dependent inhibition of HDR is exclusively mediated by Rad51 Cys319 thiol adduction.

Major Task 1/Neumann: Generation of TNBC CRISPR cell lines expressing Rad51 mutants (MDA-MB-231, BT49 and Hs578T from ATCC TNBC cell line panel). *Completion 65%*

Major Task 2/Freeman: NFA-8 synthesis. *Completion 100%*

Major Task 3/Neumann: Use TNBC CRISPR cell lines and compare responses of OA-NO_2 or NFA-8 plus olaparib. *Completion 0%*

Major Task 4/Neumann: Generate Rad51 recombinant proteins (WT and Cys319Ser) and test effects of NFAs on Rad51 DNA binding, oligomerization, presynaptic filament formation and ATPase activity. *Completion 30%*

Major Task 5/Freeman: Determination of OA-NO_2 protein adduction sites in Rad51 and other HDR proteins. *Completion 50%*

Aim 2: Compare the structure-function relationships of OA-NO_2 with NFA-8 in a panel of 10 TNBC cell lines for HDR-inhibition and lethality by determining combination and drug reduction indices.

Major Task 6/Neumann: Determine proliferation and viability of 10 TNBC cell lines (BT549, BT20, HCC1500, HCC1937, HCC1187, MDA-MB-231, MDA-MB-436, SUM149PT, SUM159PT and HS578T) and 2 immortalized breast epithelial cell lines (MCF-10A, MCF-12A) in response OA-NO_2, NFA-8 and olaparib. *Completion 35%*

Major Task 7/Neumann: Calculation of combination and drug reduction indices (CI and DRI) using CalcuSyn software. *Completion 50%*

Major Task 8/Neumann: Compare OA-NO_2 and NFA-8 responses in TNBC cell lines and benign breast epithelial in DNA DSB repair. *Completion 50%*

Aim 3: Utilize human TNBC patient-derived xenografts (PDXs) and a mutant p53 breast cancer mouse model to evaluate drug synergism of NFAs in combination with PARP inhibition.

Major Task 9/Neumann: Apply for IACUC approval and ACURO approval. *Completion 100%*

Major Task 10/Neumann and Freeman: Human *ex vivo* explants. *Completion 0%*

Major Task 11/Neumann and Freeman: Expand PDXs in Balb/c mice/immunogenic mouse model. *Completion 0%*

- **What was accomplished under these goals?**

To Major Task 1/Neumann: Generation of TNBC CRISPR cell lines expressing Rad51 mutants (MDA-MB-231, BT49 and Hs578T from ATCC TNBC cell line panel).

CRISPR mutant clones have been generated for MDA-MB-2131 and MCF-10A cells. We are currently expanding the clones, which then will be sequenced to identify successfully target clones.

Major Task 2/Freeman: NFA-8 synthesis:

OA-NO_2 and NFA-8 are nitroalkenes, 10-nitro-octadec-9-enoic acid and 7-nitro-nonadec-7-enoic acid, respectively. We have now devised an even more pharmacologically efficacious nitroalkene that shares the same electrophilic nitroalkene moiety with OA-NO_2 and NFA-8, dimethyl-4-nitro-oct-4-enoiate (CP-1). New data indicates that CP-1 outperforms both OA-NO_2 and NFA-8, respectively (data shown below). 25 gm of CP-1 have been synthesized and is available for both in vitro and in vivo studies.

Major Task 4/Neumann: Generate Rad51 recombinant proteins (Wt and Cys319Ser) and test effects of NFAs on Rad51 DNA binding, oligomerization, presynaptic filament formation and ATPase activity.

We have tested Wt and recombinant Rad51-Cys319Ser reaction with OA-NO_2 and found that OA-NO_2 has the ability to specifically disrupt Rad51 binding from DNA. This was probed by quantifying

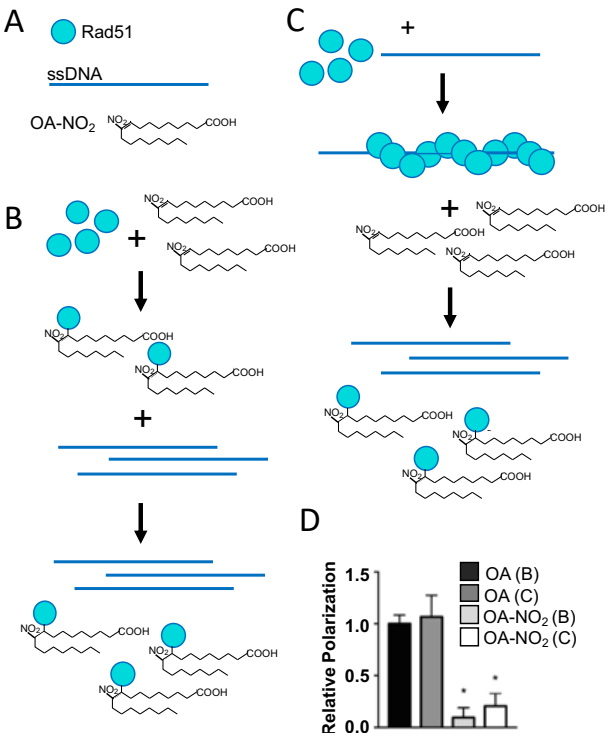

Fig. 3. A. DNA binding was measured using fluorescence polarization (FP) on a Tecan Spark 20M (ex/em 480 nm/535 nm). **B.** Purified Rad51, ATP and 5 μM OA or 5 μM OA-NO_2 was first incubated for one hour before Alexa Fluor 488 conjugated single strand (ss) DNA was added. Fluorescence polarization was quantified and normalized to a control lacking ATP. **C.** Purified Rad51, ATP was incubated for one hour with Alexa Fluor 488 conjugated single strand (ss) DNA. Then, 5 μM OA or 5 μM OA-NO_2 were incubated for two hours Fluorescence polarization was quantified and normalized to a control lacking ATP. **D.** Quantification. Average + SEM, n = 3.

changes in fluorescence polarization of an Alexa Fluor 488 conjugated single-stranded oligonucleotide *in vitro*. In the presence of ATP, Rad51 protein binding to Alexa Fluor 488 conjugated ssDNA will increase the polarization of light emitted by the fluorescent substrate (**Fig. 3A**) (4). OA-NO_2, but not OA, decreased the relative polarization of Rad51 in the presence of ATP and DNA (**Fig. 3D**). This inhibition of polarization increase was observed both a) when OA-NO_2 was pre-incubated with Rad51 and then mixed with DNA (**Fig. 3B**), and b) when Rad51 was already associated with DNA, and then OA-NO_2 or OA was added (**Fig. 3C**). This suggests OA-NO_2 robustly abrogates Rad51-DNA interaction by a) not only preventing Rad51 from DNA binding, but also disrupting already assembled Rad51 oligomers. Control experiments found that OA and OA-NO_2 did not cause non-specific effects such as fluorophore quenching-induced decreases in fluorescence polarization (not shown). This suggests that OA-NO_2 can access C319 of Rad51 monomers in a filament and dissociate Rad51 protein from ssDNA *in vitro*.

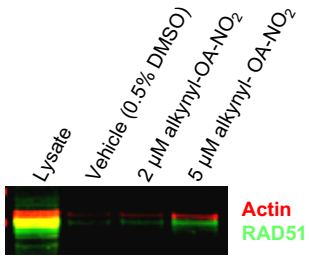

Fig. 4. Click Chemistry-linked affinity capture: In UWB1.289 cells Rad51 and β-actin cysteines were adducted by alkynyl OA-NO_2 and purified via biotin/streptavidin beads.

As proposed, we are currently generating Rad51 mutant proteins (Rad51 Cys319Ser) with our collaborator Dr. Patrick Sung and will soon compare Rad51 WT with mutant protein in the assays listed and compare OA-NO_2 with CP-1 in these assays.

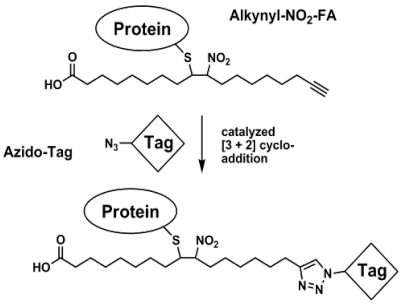

Fig. 5. Click chemistry strategy for detecting protein-adducted NFA. The alkynyl substituent reacts with diazo-biotin and is purified via streptavidin (beads).

Major Task 5/Freeman: Determination of OA-NO_2 protein adduction sites in Rad51 and other HDR proteins.

We have previously used biotinylated electrophilic lipids NFA for affinity-based identification of NFA-protein adduct formation specific proteins, organelles and cells (45, 46). Biotin is bulky (244 g/mol) compared with various NFA species (300-350 g/mol). Thus, integrating a smaller alkynyl (R-C≡CH) terminus on OA-NO_2 facilitates secondary "click" reactions with azido-substituted affinity tags that facilitate protein pull-down as well as HPLC-MS/MS-based detection. Preliminary studies support click chemistry-based Rad51 and β-actin pulldown through alkynyl-tagged NFAs from cell lysate (**Figs. 4 and 5**). This allows specific identification of this and other potential NFA targets in SSA and Alt-EJ-DNA repair pathways. This innovative approach was designed to reveal the degree of specificity of OA-NO_2 Rad51 targeting and lays the foundation for better characterizing new NFA regioisomers and their molecular targets.

Our next step is to identify OA-NO_2 alkynyl-adducted cysteines in cytoplasmic and nuclear cell fractionations using mass spectrometry. U2OS cells (+/-) irradiation will be analyzed in triplicates. We will focus on identifying proteins involved in DNA DSB repair.

Major Task 6/Neumann: Determine proliferation and viability of 10 TNBC cell lines (BT549, BT20, HCC1500, HCC1937, HCC1187, MDA-MB-231, MDA-MB-436, SUM149PT, SUM159PT and HS578T) and 2 immortalized breast epithelial cell lines (MCF-10A, MCF-12A) in response OA-NO_2, NFA-8 and olaparib.

We have compared OA-NO_2 with the newly identified nitroalkene CP-1 in proliferation and apoptosis assays. MCF10A and MDA-MB-231 cells were respectively infected with IncuCyte® NucLight Red Lentivirus in order to produce red nuclei for the apoptosis IncuCyte assay. Four wells of a 96-well plate were plated with cells at appropriate density the day prior. One well was treated with the reagent with a MOI of 1 and the

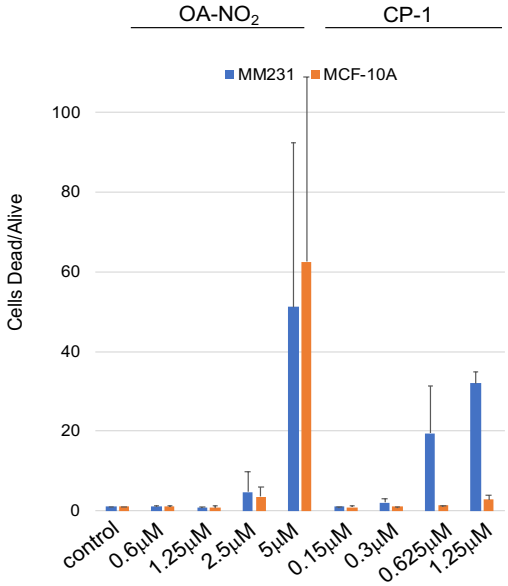

Fig. 6. MDA-MB-231 (MM231, blue) and MCF-10 (red) cells were analyzed for toxicity by OA-NO_2 and CP-1. Cells were treated on day 1 and live as well as dead cells were analyzed on day 3. n=2

other well treated with a reagent with MOI of 3. After the infected cells reached appropriate confluency, they were transferred to a 6-well plate to further proliferate. Cells with red nuclei were selected using fluorescence activated cell sorting (FACS).

Sorted cells (MDA-MB-231 and MCF-10A) were seeded at a density of 2 x 10^3/well on a clear 96-well plate. The next day, cells were incubated with apoptotic markers Cytotox Green (Cat. No. 4633), Annexin V (Cat. No. 4642) and the PARPi olaparib at desired concentrations. Wells were then co-treated with OA-NO_2 or CP1 and then analyzed in the IncuCyte over a time course of 3 days. Nuclei (live cell marker) and apoptotic markers were quantified and the ratio of apoptotic to live cells at the end of day 3 is shown in **Fig. 6.** Notably, OA-NO_2 dosing increased cell death in both, MDA-MB-231 cells and MCF-10A cells, while CP-1 only induced toxicity to the TNBC cell line MDA-MB-231 but not the benign cell line MCF-10A.

Utilizing the same assay, both cell lines were further analyzed for responses to both, OA-NO_2 or CP-1 and olaparib. As show in **Fig. 7** in contrast to the highest dose of OA-NO_2, CP-1 at both 0.625 μM and 1.25μM shows a protective effect in MCF10A cells co-treated with olaparib. These data indicate that CP-1 shows not only higher in vitro tumor cell killing potency compared to OA-NO_2 but also may provide a protective effect towards benign MCF-10A cells when treated with olaparib. Further replicates of these experiment are in progress.

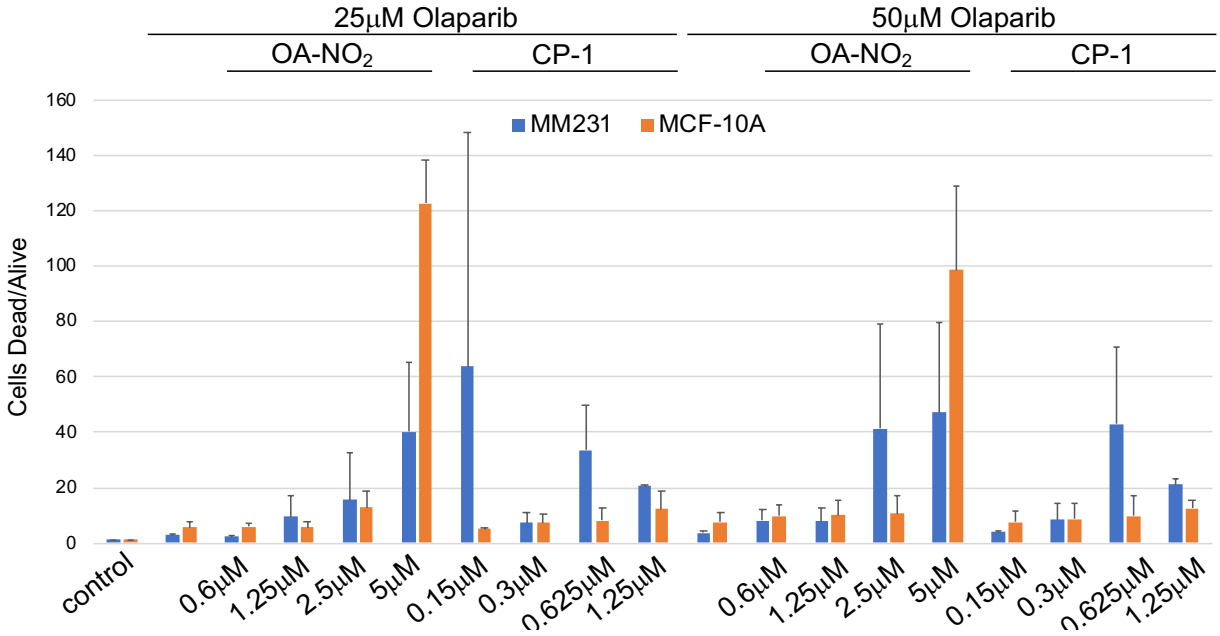

Fig. 7. MDA-MB-231 (MM231) and MCF-10 cells were analyzed for toxicity induced by OA-NO_2 and CP-1 in combination with two different doses of olaparib (ola). Cells were treated on day 1 and live as well as dead cells were analyzed on day 3. n=2

OA-NO_2 and CP-1 potencies were also compared by clonogenic analysis in 6 well plates. 500 cells/well were plated and treated the following days with OA-NO_2 or CP-1. As shown in **Fig. 8**, 0.5μM and 1μM CP-1 significantly decreased clonogenic outgrowth in MDA-MB-231 cells but not in MCF-10A cells.

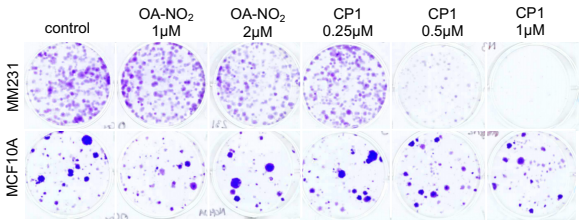

Fig. 8. Comparison of OA-NO_2 and CP-1 for clonogenic survival

To test if CP-1 has a protective effect on MCF-10A cells in response to ionizing radiation (IR), MDA-MB-231 cells as well as MCF-10A cells were examined again for clonogenic survival in 6 well plates. 500 cells/well were plated, then irradiated and subsequently treated with OA-NO_2 or CP-1 12 hr later. Again, CP-1 protects MCF-10A cells from IR toxicity as MCF-10A cells treated with IR only show lower number of colonies than cells treated with IR and CP-1 (**Fig. 9**). This is especially evident in cells treated with 4Gy. In MDA-MB-231 cells, co-treatment of CP-1 with IR shows lower colony numbers compared to MDA-MB-231 cells treated with IR and OA-NO_2. These data emphasize that CP-

1 protects benign cells from DNA damage induced by PARPi or IR and augments both DNA damage and cell death in TNBC cells.

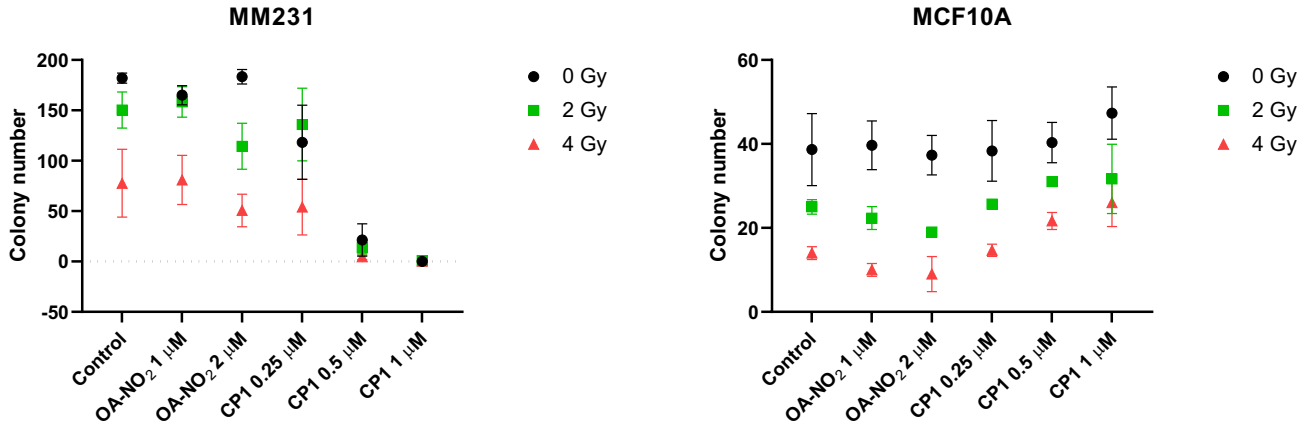

Fig. 9, CP-1 protects the benign breast epithelial cell line MCF-10A from toxic effects of IR while it potentiates IR effects in the TNBC cell line MDA-MB-231; n=3

Major Task 7/Neumann: Calculation of combination and drug reduction indices (CI and DRI) using CalcuSyn software.

MDA-MB-231 cells were analyzed for drug synergism between OA-NO_2 or NFA-8 and the PARP inhibitors olaparib and talazoparib. For this, relative cell numbers were first compared by measuring the luminescent signal generated by ATP using the CellTiter-Glo (Promega) assay. 5 x 10^3 MBA-MD-231 cells/well were plated in a 96-well plate and treated with PARPi at the indicated concentrations for 72 h in the presence or absence of OA-NO_2 or the control oleic acid (OA) and replenished every 24 h. Compusyn software was used to calculate the combination index values and synergy for PARP inhibitors in combination with OA-NO_2. As shown in **Fig. 10** co-treatment of OA-NO_2 with talazoparib, showed higher drug synergism compared to OA-NO and olaparib.

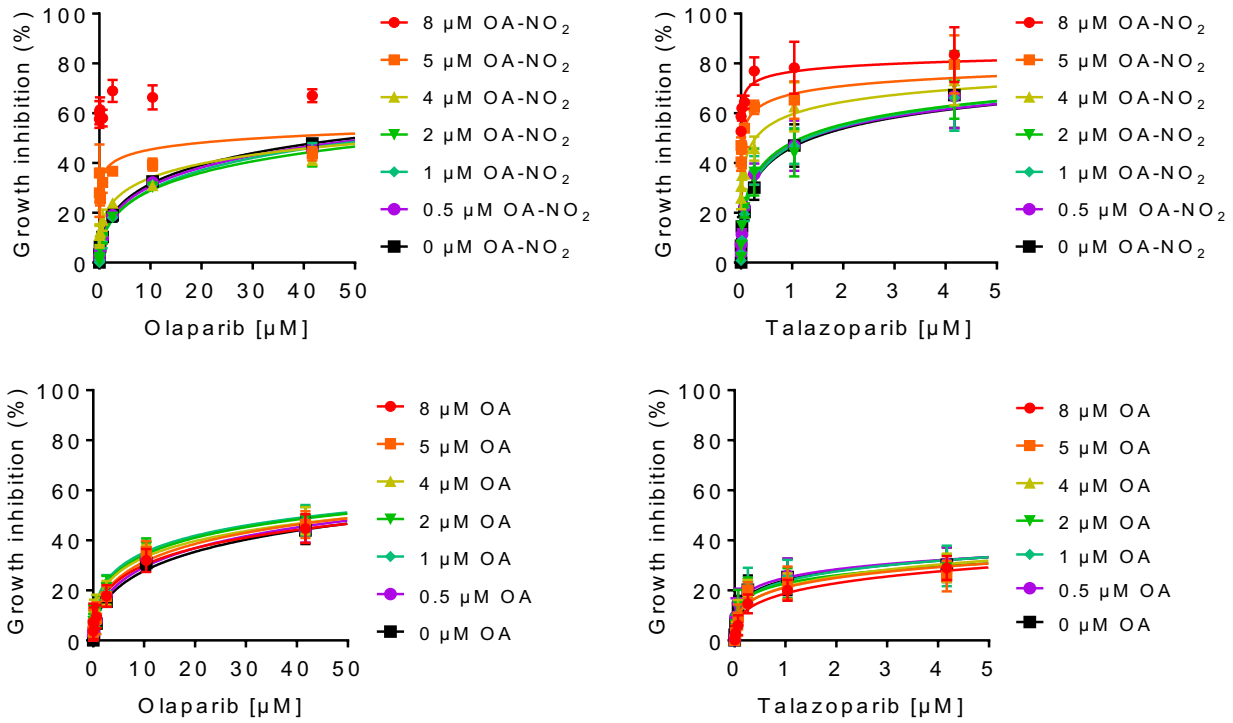

Fig. 10. Co-treatment of MDA-MB-231 cells with OA-NO_2 NFA-8 and the PARP inhibitors olaparib and talazoparib; n=3

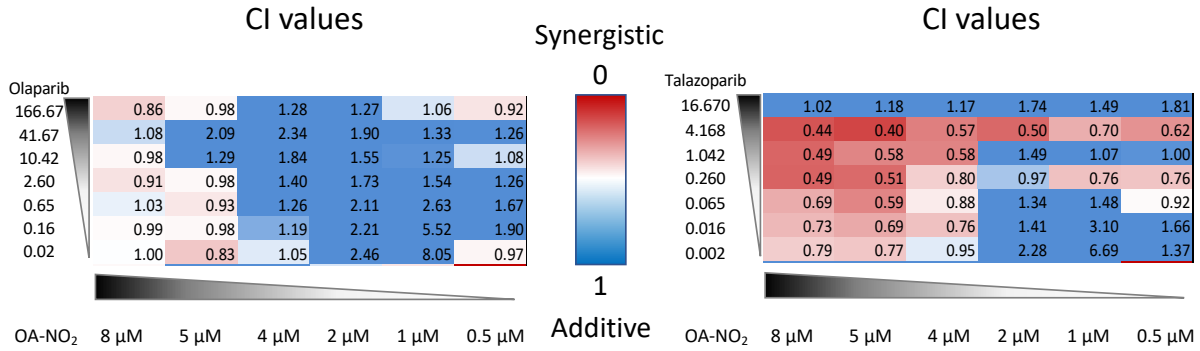

Fig. 11. Drug synergism between OA-NO_2 and the PARP inhibitors olaparib and talazoparib. n=3

X-ray modeling studies of the Rad51-Cys319 region revealed that, as opposed to OA-NO_2 (shown), if one were to synthesize a nitro-fatty acid that has the nitroalkene substituent closer to the carboxylate terminus, there would be an enhancement of hydrogen bonding between the carboxylate and Rad51-Glu322. This also predicted stronger hydrophobic interactions with Pro318, increased Cys319 alkylation and greater inhibition of HDR and tumor cell growth and survival. Thus, 7-NO_2-nonadecenoic acid (NDA-NO_2/NFA-8) was synthesized for comparison with OA-NO_2. Next, utilizing the same assay, OA-NO_2 and NFA-8 were compared and analyzed for drug synergism with talazoparib. As shown in **Fig. 11**, syngergistic growth inhibition is higher in MDA-MB-231 cells co-treated with talazoparib comapred to OA-NO_2. <u>These data suggest that NFA-8 is more efficacious in killing TNBC cells synergistically when combined wirth talazoparib compared to OA-NO_2</u>

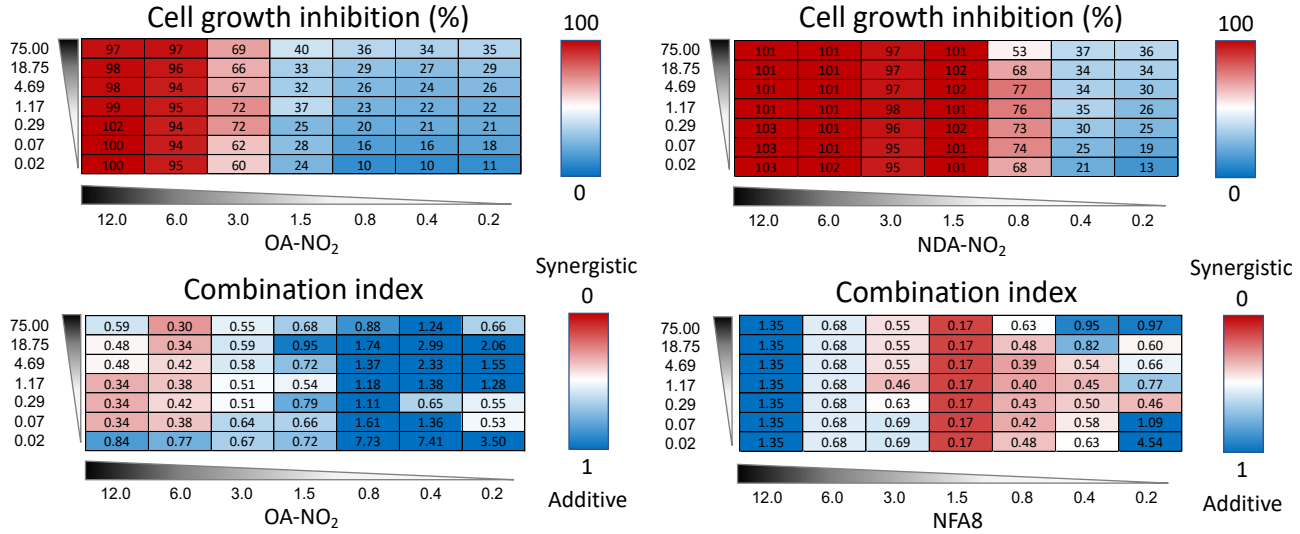

Fig. 12. Comparison of drug synergism between OA-NO_2 , NFA-8 and the PARP inhibitors talazoparib. n=3

Then, OA-NO_2, NFA-8 and CP-1 were compared for effects on cell proliferation by using the CellTiter-Glo assay. These results suggested that diCO_2-O-NO_2 (CP-1) had a 63% lower EC50 compared to OA-NO_2, and 7-NO_2-nonadecenoic acid (NFA-8) an about 30% lower EC50 compared to OA-NO_2. This suggest that CP-1 is the more potent drug candidate out of all nitroalkenes tested (**Fig. 13) and ongoing are drug synergism studies with PARPi and CP-1**.

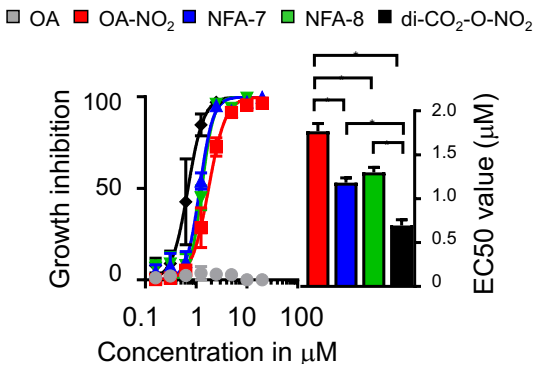

Fig. 13. Comparison of nitroalkenes for EC50 in MDA-MB-231 cells; n=3

Major Task 8/Neumann: Compare OA-NO_2 and NFA-8 responses in TNBC cell lines and benign breast epithelial in DNA DSB repair.

Utilizing the DR-GFP assay for HDR (33), both NFA-8 and CP-1 displayed better inhibition of HDR than OA-NO_2, as predicted, whereas CP-1 displayed the lowest EC50 as well as inhibition of HDR (**Fig. 14**). The nitroalkene diCO_2-O-NO_2 is unique because as the diethylated prodrug it will be readily absorbed and, after de-esterification, is expected to avidly be transported into cells by members of the Organic Anion Transporter Polypeptide (OATP) superfamily. Appreciating that an array of OATPs are highly over-expressed in human tumor cells, including breast, liver, colon, pancreatic and ovarian cancers (57, 58), it is viewed that diCO_2-O-NO_2 will display superior half-life, tumor cell accessibility and Rad51 inhibition.

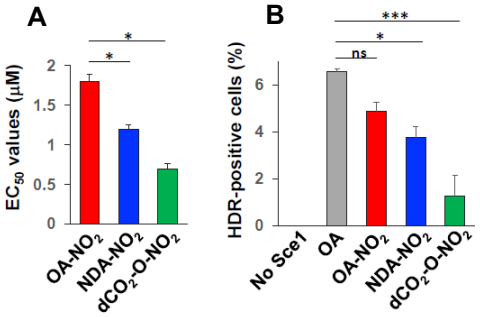

Fig. 14. NFA-8 (7-NDA-NO_2) and CP-1 (diCO_2-O-NO_2) show (A) lower EC_{50} than OA-NO_2 for the inhibition of MDA-MB-231 TNBC cell growth and (B) the greatest extent of inhibition of HDR in U2OS GFP-reporter constructs. Data from 3 independent exps, mean+SEM; n=3.

- **What opportunities for training and professional development has the project provided?**

In the process of conducting experimental endeavors, participating students and fellows have been trained in novel organic chemistry, cell/molecular biology and pharmacokinetics-related experimental methodologies, approaches to statistical analysis and data presentation.

- **How were the results disseminated to communities of interest?**

However, we will communicate our findings to bcRAN (breast cancer research advocacy network) at the coming bcRAN bootcamp in November 2020, where breast cancer survivor will come together in Pittsburgh at the Magee Womens Research Institute, which is affiliated with UPMC. In addition, we will present data at the Great Lakes Breast Cancer Symposium in October 2020 and at a Gordon Research Conference in July, 2020..

- **What do you plan to do during the next reporting period to accomplish the goals?**

To Major Task 1/Neumann: Generation of TNBC CRISPR cell lines expressing Rad51 mutants (MDA-MB-231, BT49 and Hs578T from ATCC TNBC cell line panel). We are currently expanding the clones, which then will be sequenced to identify successfully target clones.

To Major Task 3/Neumann: Once CRISPR clones are established, cell lines will be compared for responses of OA-NO_2, CP-1 plus olaparib.

To Major Task 4/Neumann: Generate Rad51 recombinant proteins (Wt and Cys319Ser) and test effects of NFAs on Rad51 DNA binding, oligomerization, presynaptic filament formation and ATPase activity. As proposed, we are currently generating Rad51 mutant proteins (Rad51 Cys319Ser) with our collaborator Dr. Patrick Sung and will soon compare Rad51 WT with mutant protein in the assays listed. OA-NO2 actions will be compared to CP-1 in these assays.

To Major Task 5/Freeman: Determination of OA-NO_2 protein adduction sites in Rad51 and other HDR proteins. Our next step is to identify OA-NO_2 alkynyl-adducted cysteines in cytoplasmic and nuclear cell fractionations using mass spectrometry. Following acquisition of this information, more selective ion monitoring strategies can be utilized for both the Rad51 Cys319-containing peptide and other OA-NO_2-targeted DNA repair proteins to define CP-1 adduction sites that are anticipated to be similar. U2OS cells (+/-) irradiation will be analyzed in triplicates. We will place primary focus on identifying proteins involved in DNA DSB repair.

Major Task 6/Neumann: Determine proliferation and viability of 10 TNBC cell lines (BT549, BT20, HCC1500, HCC1937, HCC1187, MDA-MB-231, MDA-MB-436, SUM149PT, SUM159PT and HS578T) and 2 immortalized breast epithelial cell lines (MCF-10A, MCF-12A) in response OA-NO_2, NFA-8, CP-1 and olaparib. Expand data and test other cell lines as shown in Figs. 6-13 for proliferation and viability, so that combination indicis can be determined as proposed in Major Task 7.

Major Task 8/Neumann: Compare OA-NO_2 and NFA-8 responses in TNBC cell lines and benign breast epithelial in DNA DSB repair as proposed.

3. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

   - **What was the impact on the development of the principal discipline(s) of the project?**

     We are well along the pathway to discovery of a novel inhibitor of homologous recombination that a) has already been shown from Phase 1 and 2 trials in renal and cardiopulmonary disease patients to be safe in humans, b) we have improved on the potency and pharmacokinetics of NFA-8 and OA-NO_2 (7-nitro-nonadec-7-enoic acid and 10-nitro-octadec-9-enoic acid, respectively) by devising an even more pharmacologically efficacious nitroalkene that shares the same active nitroalkene moiety with OA-NO_2 and NFA-8 (dimethyl-4-nitro-oct-4-enoiate, CP-1).

   - **What was the impact on other disciplines?**

     In addition to the potential discovery of a transformative strategy in cancer therapeutics, we are advancing basic understanding of cell signaling, pharmacology, new drug development and the modulation of genome stability.

   - **What was the impact on technology transfer?**

     We had filed a provisional patent application in November, 2018 entitled “Electrophiles and electrophile prodrugs as Rad51 inhibitors). New data, described in previous sections, that was supported by the DoD award was included in the updating of this new IP in concert with the international PCT filing on November 27, 2019. We have also formally created, with university COI committee approval, a biotechnology company (Creegh Pharmaceuticals) that has as its primary mission the development of novel drugs for the treatment of drug-resistant cancers.

   - **What was the impact on society beyond science and technology?**

     From the previous narrative one can see that we intend to further evolve the present research endeavors to directly limit the morbidity and mortality of breast (and other) cancer patients.

CHANGES/PROBLEMS:

Nothing to report at this time.

PRODUCTS:

Nothing to report at this time.

2. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

   - **What individuals have worked on the project?**

     - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

       ***Example**:*

***Example**:*

Name:	*Bruce A Freeman*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*0.6*
Contribution to Project:	*Oversight of nitroalkene synthesis, analysis, testing*
Funding Support:	*NA*
Name:	*Nicholas Khoo*
Project Role:	*Co-PD/PI*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*1.2*
Contribution to Project:	*Nitroalkene synthesis, analysis, testing*
Funding Support:	*NA*
Name:	*Stacy Wendell*
Project Role:	*Co-PD/PI*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*2.0*
Contribution to Project:	*N itroalkene synthesis, analysis, testing*
Funding Support:	*NA*
Name:	*Lihua Li*
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*12*
Contribution to Project:	*Nitroalkene testing*
Funding Support:	*NA*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Yes, see attached.

- **What other organizations were involved as partners?**

Nothing to report at this time.

Freeman, Bruce

Completed Support

[COMPLETED]
R37HL058115, Bruce Freeman (PI) 7/25/2013 - 5/31/2019 3.0 calendar
Agency: NIH/HL *No-cost Extension until 05/31/19

Title: Redox Transduction of Nitric Oxide Signaling $365,926 Annual Directs
Grant Officer: Ann Marie Brasile Mejac
Email: brasilea@nhlbi.nih.gov
Goals: The major goals of this project are to define redox reactions between nitric oxide and both the lipids and lipophilic antioxidants of vascular cell membranes and serum lipoproteins.

Specific Aims

Specific Aims: 1. Structurally characterize and quantitate the predominant electrophilic fatty acid species present in the inflammatory milieu of obesity, using cell, rodent and clinically-derived specimens. 2. Define the contribution of mitochondrial redox reactions in the formation of electrophilic derivatives of unsaturated fatty acids. 3. Examine the anti-inflammatory actions of electrophilic fatty acids in cell and animal models of obesity-induced diabetes.

Current Support

P01HL103455, Mark Gladwin (PI) 8/1/2016 - 4/30/2021 2.4 calendar
Agency: NIH/HL $118,574 Annual Directs
Title: Vascular Subphenotypes of Lung Disease (Proj. 2)
Grant Officer: Lei Xiao

Email: xiaol@mail.nih.gov

Goals: The overarching translational goal of this program is to define common mechanistic and therapeutic pathways for PAH in the context of major lung and systemic diseases, such as COPD and HIV.

Specific Aims

Specific aims: 1. Define the molecular targets, biochemical responses and physiological actions of a) the dietary NO2-FA precursors (NO2-, NO3-, conjugated linoleic acid) and b) pure NO2-FA in rodent models of obesity-induced pulmonary hypertension. 2. Evaluate the clinical responses of Group 1 pulmonary arterial hypertension patients to the orally administered NO2-FA, 10-nitro-octadec-9-enoic acid (NO2-OA).

R01 HL132550 (Freeman, Bruce) 8/5/2016 - 6/30/2020 3.6 calendar
Agency: NIH/HL $252,550 Annual Directs
Title: Anti-Inflammatory Lipid Mediators in Asthma
Grant Officer: Renee Livshin
Email: livshinr@nhlbi.nih.gov

Goals: We hypothesize that the promotion of nitro-fatty acid signaling alleviates metabolic syndrome-induced hypertension and its pulmonary complications.

Specific Aims

To test this concept, a de-risked drug strategy will be evaluated by pursuing both mechanistically-revealing model system studies and a blinded crossover design Phase 2 clinical study: Aim #1 – Identify the sites of NO2-FA adduction in the lung tissue of obese mice with airway hyperreactivity and define the biochemical and physiological responses to oral NO2-FA administration. Aim #2 – Evaluate the clinical responses of obese asthmatic patients to the orally-administered NO2-FA, 10-nitro-octadeca-9-enoic acid (NO2-OA).

[THIS AWARD]
BC1804671P1 (Freeman, Bruce and Neumann, Carola) 02/01/2019-01/31/2022 0.6 calendar
Agency: DOD $38,316 Annual Directs
Title: Characterizing nitro-fatty acids as Rad51 inhibitors as co-treatment in triple negative breast cancer
Grant Officer: JoAnn Martin
Email: joann.l.martin2.civ@mail.mil
Goals/Aims: This proposal addresses two overarching challenges: 1) Revolutionizing treatment regimens by replacing them with ones that are more effective, less toxic and impact survival and 2) Conquering the problem of overtreatment.
Role: PD\PI

[NEW]
R56CA233817 (Neumann, Carola) 09/01/2019-08/31/2021 0.6 calendar
Agency: NIH/CA $12,105 Annual Directs
Title: Inhibition of DNA Double Strand Break Repair in TNBC by Nitro-Fatty Acids
Grant Officer: John Knowlton
Email: jk339o@nih.gov
Goals: The Research Plan will reveal a novel drug strategy for TNBC therapy, where the inhibition of Rad51--mediated DNA repair by NFAs renders TNBC cells more sensitive to PARP inhibition thus increasing TNBC cell killing.

Specific Aims
Aim 1: Define the mechanisms underlying the effects of nitro-fatty acids on HDR and downstream lethality towards TNBC cells.
Aim 2: Utilize patient-derived TNBC xenografts and a p53-mutant breast cancer mouse model to evaluate synthetic lethality responses of NFAs administered in combination with PARP inhibition.

Overlap
NONE

Khoo, Nicholas

Completed Support

[COMPLETED}
Corporate Research Agreement (Khoo, Nicholas) 8/31/2018-8/30/2019 3.4 calendar
Agency: Complexa, Inc. $83,262 Annual Directs
Title: (1) Assessment of 8,9-alkene and 10-nitrostearate effects on NF-kB signaling pathway and (2) Evaluation of Nrf2 activation by 10-nitrostearate and 8,9-alkene
Grant Officer: Diane K Jorkasky MD, FACP
Email: diane.jorkasky@complexarx.com
Goals: Scope 1: Determine the Nrf2 activation properties of the 10-nitro-octadec-9-enoic acid (CXA-10) metabolites, the double bond reduction product 10-nitro-octadecanoic acid (nitro-stearic acid) and the double bond isomerization product 10-nitro-octadec-8-enoic acid (8,9-alkene) using a murine macrophage cell line
Scope 2: Determine the inhibitory activity of the two CXA-10 metabolites, the double bond isomerization and reduction products 8,9-alkene and 10-nitro-stearate, on NF-κB signaling by assessing the effect on lipopolysaccharide-induced expression MCP-1
Specific Aims
Determine whether the metabolites alter the transactivation of NF-kB and Nrf2 promoter activity using luciferase-based assays from stable cell line reporters. The metabolite responses will then be compared with CXA-10 and marketplace competitors Tecfidera and Bardoxolone
Determine whether pharmacological concentrations of the 8,9-alkene metabolite and nitro-stearic acid induce Nrf2-dependent gene expression of heme oxygenase 1 (HO1) and NAD(P)H: quinone oxidoreductase 1 (NQO1) in murine macrophages
Determine whether 8,9-alkene and nitro-stearic acid inhibits MCP-1, a canonical downstream effector of NF-κB, steady-state mRNA expression, protein synthesis and activity.

Current Support

P01HL103455, Mark Gladwin (PI) 8/1/2016 - 4/30/2021 9.5 calendar
Agency: NIH/HL $80,745 Annual Directs
Title: Vascular Subphenotypes of Lung Disease (Proj. 2)
Grant Officer: Lei Xiao
Email: xiaol@mail.nih.gov
Goals: The overarching translational goal of this program is to define common mechanistic and therapeutic pathways for PAH in the context of major lung and systemic diseases, such as COPD and HIV.
Specific Aims
Specific aims: 1. Define the molecular targets, biochemical responses and physiological actions of a) the dietary NO2-FA precursors (NO2-, NO3-, conjugated linoleic acid) and b) pure NO2-FA in rodent models of obesity-induced pulmonary hypertension. 2. Evaluate the clinical responses of Group 1 pulmonary arterial hypertension patients to the orally administered NO2-FA, 10-nitro-octadec-9-enoic acid (NO2-OA).

R01 HL132550 (Freeman, Bruce) 8/5/2016 - 6/30/2020 1.2 calendar
Agency: NIH/HL $12,207 Annual Directs
Title: Anti-Inflammatory Lipid Mediators in Asthma
Grant Officer: Renee Livshin
Email: livshinr@nhlbi.nih.gov
Goals: We hypothesize that the promotion of nitro-fatty acid signaling alleviates metabolic syndrome-induced hypertension and its pulmonary complications.
Specific Aims

To test this concept, a de-risked drug strategy will be evaluated by pursuing both mechanistically-revealing model system studies and a blinded crossover design Phase 2 clinical study: Aim #1 – Identify the sites of NO2-FA adduction in the lung tissue of obese mice with airway hyperreactivity and define the biochemical and physiological responses to oral NO2-FA administration. Aim #2 – Evaluate the clinical responses of obese asthmatic patients to the orally-administered NO2-FA, 10-nitro-octadeca-9-enoic acid (NO2-OA).

[THIS AWARD]
BC1804671P1 (Freeman, Bruce and Neumann, Carola) 02/01/2019-01/31/2022 1.2 calendar
Agency: DOD $39,703 Annual Directs
Title: Characterizing nitro-fatty acids as Rad51 inhibitors as co-treatment in triple negative breast cancer
Grant Officer: JoAnn Martin
Email: joann.l.martin2.civ@mail.mil
Goals/Aims: This proposal addresses two overarching challenges: 1) Revolutionizing treatment regimens by replacing them with ones that are more effective, less toxic and impact survival and 2) Conquering the problem of overtreatment.
Role: Co-PD\PI

Overlap
None

Wendell, Stacy

Completed Support

[COMPLETED]
S10OD023402, Wendell, Stacy (PI) 5/18/2018 - 5/17/2019
Agency: NIH/HL $593,679 Annual Directs
Title: Bringing Untargeted Metablomics to Pitt *Equipment Grant
Grant Officer: Sudha Veeraraghavan
Email: sudha.veeraraghavan@nih.gov
Goals: If awarded, this grant will fill an unmet need at the University of Pittsburgh and accelerate research activity leading to more publications and grant submissions.

Specific Aims

The requested instrumentation in this proposal is a high resolution mass spectrometer coupled to an ultra high performance liquid chromatograph (UHPLC) system that will be used for untargeted metabolomics and lipidomics experiments.

Current Support

P01HL103455 Gladwin, Mark (PI) 8/1/2016-4/30/2021 1.2 calendar
Agency: NIH/HL $17,520 Annual Directs
Title: Vascular Subphenotypes of Lung Disease (Core C)
Grant Officer: Lei Xiao
Email: xiaol@mail.nih.gov
Goals: The overarching goal of this TPPG is to elucidate common pathogenic mechanisms of pulmonary arterial hypertension (PAH) and target these pathways with novel drug strategies.
Specific Aims
1) The chemiluminescence core will utilize reductive chemistry in conjunction with chemiluminescence NO detection to measure NO and its metabolites in biological specimens and assess bacterial nitrite/nitrate reductase activity in the microbiome. 2) The EPR component will utilize cutting edge EPR technology with spin trapping to directly measure radicals in biological samples. Additionally, EPR technology will enable the differentiation of endogenous and 15-N labeled NO species in biological samples. 3) The Mass Spectroscopy component will enable the measurement of NO and oxo-modified lipid and protein biomolecules.

[NEW]
P01HL114453 (Ray, Prabir) 5/1/2019 - 4/30/2024 1.2 calendar
NIH/HL $8,709 Annual Directs

Title: Immunosuppression in Acute Lung Injury

Goal: The broad, long-term objective of this Project is to better understand the underlying mechanisms of immunosuppression following infection in the lungs by examining distinct host-pathogen interplay.

Specific Aims
Aim 1. Determine immune cell dynamics and gene signatures in PBMCs from critically ill patients and the role of mTOR in immune suppression. Aim 2. Determine the role of PPARγ and PON2 in persistent anti-inflammatory cytokine gene expression in the lungs of mice subjected to the 2-hit model.

U54DK112079, Zhou Wang (PI) 9/22/2016 - 7/31/2021 0.6 calendar
Agency: NIH/DK $8,829 Annual Directs
Title: Impact of Cox-2 on estrogen receptor beta action in prostate epithelial cells (Proj. 3)
Grant Officer: Christopher Mullins
E-mail: mullinsc@niddk.nih.gov
Goals: This project examines the molecular basis for the lack of efficacy of NSAIDs for treatment of BPH and will reveal new molecular and metabolic biomarkers that could both predict response to NSAIDs and lead to development of new agents that enhance anti-inflammatory drug action in BPH patients.
Specific Aims
Three Aims are proposed to test this hypothesis: Aim 1 will determine the impact of Cox-2 on ERß ligand production and action in prostate epithelial cell cultures. Aim 2 will identify the impact of Cox-2 and ERß on targets relevant to polarized epithelial cell function in 3- dimensional cultures of prostate epithelial cells. Finally, Aim 3 will determine whether Cox-2 expression and/or activity influences steroidogenic and cell junction pathways in BPH patients.

R01 DK112854, Schopfer, Francisco (PI) 4/1/2018 - 3/31/2022 0.9 calendar
Agency: NIH/DK $17,087 Annual Directs
Title: Predominant protective role in hepatic steatosis and obesity by fish oil-derived furans
Grant Officer: Raul Rojas
E-mail: rojasr@mail.nih.gov
Goals: A successful completion of this project may greatly impact our current understanding of fish oil effect. Moreover, it will fill critical gaps in knowledge in omega-3 FA therapeutics and can potentially re-define current paradigms related to the impact of omega-3 FA supplementation on lipogenesis, lipolysis, and glycolysis.

Specific Aims

Specific Aims Aim 1: Define FuFA levels in Lovaza and FO, dose, exposure and pharmacokinetics.
Aim 2: Establish the role of FuFA on Lovaza-dependent TG reduction and NAFLD protection.
Aim 3: Define hepatic protein targets, metabolic effects and role of ACC on FuFA protective effects.

R01HL140963 Alison Morris
(PI) 8/1/2018 - 5/31/2022 0.6 calendar
Agency: NIH/HL $29,532 Annual Directs

Title: Systems Biology of Diffusion Impairment in HIV

Goal: This project will leverage existing resources to identify complex associations and causal relationships in DLco impairment, identify novel therapeutic targets and biomarkers, and improve care of HIV+ individuals.

Aim 1: To identify key causal molecular pathways of DLco impairment by integrating clinical features and –omics data from the lung in HIV+ individuals. We will utilize high-throughput RNA sequencing and mass spectrometry to quantify miRNAs, mRNAs, the microbiome, and metabolites in bronchoalveolar fluid and lung epithelial cells in HIV+ individuals with detailed pulmonary function, radiographic, and echocardiographic measurements to construct probabilistic network models of DLco. Key pathways will be validated. Aim 2: To identify predictive signatures of DLco decline from clinical features, transcriptomic, microbiome, and metabolite data in easily accessible clinical specimens. We will build predictive models to identify individuals at risk of developing DLco impairment or having significant decline based on –omics data collected from easily accessible tissues (miRNA and metabolic profiles from serum and PBMCs; microbiome of the oral cavity), coupled with detailed clinical and phenotypic data. Aim 3. To investigate the systems-wide relationship between HIV-induced miRNAs and lung epithelial and endothelial gene reprogramming in HIV+ individuals.

R01 HL132550, Freeman, Bruce (PI) 08/05/2016-06/30/2020 0.6 calendar
Agency: NIH/HL $35,228 Annual Directs
Title: Anti-Inflammatory Lipid Mediators in Asthma
Grant Officer: Patricia Noel
Email: noelp@nhlbi.nih.gov
Goals: We hypothesize that the promotion of nitro-fatty acid signaling alleviates metabolic syndrome-induced hypertension and its pulmonary complications.
Specific Aims
To test this concept, a de-risked drug strategy will be evaluated by pursuing both mechanistically-revealing model system studies and a blinded crossover design Phase 2 clinical study: Aim #1 – Identify the sites of NO2-FA adduction in the lung tissue of obese mice with airway hyperreactivity and define the biochemical and physiological responses to oral NO2-FA administration. Aim #2 – Evaluate the clinical responses of obese asthmatic patients to the orally-administered NO2-FA, 10-nitro-octadeca-9-enoic acid (NO2-OA).

[NEW]

R01HL142589 (Finkel, Toren) 2/1/2019 - 1/31/2023 0.6 calendar
NIH/HL $8,475 Annual Directs

Title: The role of calcium entry through the mitochondrial uniporter in regulating cardiac metabolism and physiology

Goal: We propose to analyze the role of the MCUC in basal and stress-induced cardiovascular physiology.
Specific Aims
Specific Aim 1: To define how mitochondrial calcium uptake through the MCUC regulates necrotic cell death in the setting of cardiac I/R injury.
Specific Aim 2: To determine the metabolic and transcriptional adaptations that occur following genetic manipulation of mitochondrial calcium levels.
Specific Aim 3: To determine the role of MCUC-dependent mitochondrial calcium entry in the agedependent decline in myocardial function.

[THIS AWARD]
BC1804671P1 (Freeman, Bruce and Neumann, Carola) 2/01/2019-1/31/2022 1.2 calendar
Agency: DOD $34,755 Annual Directs
Title: Characterizing nitro-fatty acids as Rad51 inhibitors as co-treatment in triple negative breast cancer
Grant Officer: JoAnn Martin
Email: joann.l.martin2.civ@mail.mil
Goals/Aims: This proposal addresses two overarching challenges: 1) Revolutionizing treatment regimens by replacing them with ones that are more effective, less toxic and impact survival and 2) Conquering the problem of overtreatment.
Role: Co-PD\PI

[NEW]
R56CA233817 (Neumann, Carola) 09/01/2019-08/31/2021 0.3 calendar
Agency: NIH/CA $4,490 Annual Directs
Title: Inhibition of DNA Double Strand Break Repair in TNBC by Nitro-Fatty Acids
Grant Officer: John Knowlton
Email: jk339o@nih.gov
Goals: The Research Plan will reveal a novel drug strategy for TNBC therapy, where the inhibition of Rad51--mediated DNA repair by NFAs renders TNBC cells more sensitive to PARP inhibition thus increasing TNBC cell killing.
Specific Aims
Aim 1: Define the mechanisms underlying the effects of nitro-fatty acids on HDR and downstream lethality towards TNBC cells.
Aim 2: Utilize patient-derived TNBC xenografts and a p53-mutant breast cancer mouse model to evaluate synthetic lethality responses of NFAs administered in combination with PARP inhibition.

[NEW]
Research Grant (Wendell, Stacy; Methe, Barbara) 12/1/2019 - 11/30/2020 2.4 calendar
UPMC Immune Transplant and Therapy Center (ITTC) $145,725 Annual Directs
Title: An integrated platform for investigating metformin-derived alterations of the microbial metabolome to improve healthy aging

Goal: The main goal of this project is to develop the proper analytical tools to understand how metformin (or any therapy) affects the microbiome profile, microbial function and in turn the microbial and host metabolomes. These effects can in turn be correlated to numerous other indices including health records, inflammatory markers, etc.
Specific Aims
Aim 1: To develop a model to study the effects of metformin on the microbiome and changes to the host metabolome in aging
Aim 2: To develop an analytics platform to analyze microbiome and metabolome data sets for which we can focus on the microbial changes that take place in the presence of standard therapies.

[NEW]

R01DK120986 (Kevin Mollen) 1/1/2020 - 12/31/2024 0.6 calendar
NIH/DK $9,070 Annual Directs

Title: Mitonuclear Communication During the Pathogenesis of Inflammatory Bowel Disease

Goal: Successful completion of these experiments will frame the causes and consequences of mitochondrial dysfunction during IBD, offering the opportunity to improve disease outcomes by mitigating ongoing inflammation.
Specific Aims
Aim 1: To evaluate the cause of mitochondrial dysfunction during IBD. Aim 2: To characterize the physiologic response to mitochondrial dysfunction during IBD. Aim 3: To target mitochondrial dysfunction therapeutically in murine colitis and characterize the metabolic effects of mitochondrial dysfunction in human IBD.

Director, Health Sciences Metabolomics and Lipidomics Core (HSMLC) 7/1/2017—Present 1.8 calendar
Directs Health Sciences mass spectrometry facility $33,210/year