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| AWARD NUMBER: | W81XWH-11-1-0211 |
| **TITLE:** | The Role of Novel Substituted Diindolyl Methane Analogues in the Treatment of Triple-Negative and ErbB2-Positive Breast Cancer |
| **PRINCIPAL INVESTIGATOR:** | Mandip S. Sachdeva, PhD |
| CONTRACTING ORGANIZATION: | Florida A&M University<br>Tallahassee, FL 32307 |
| REPORT DATE: | MAY 2016 |
| TYPE OF REPORT | ANNUAL |
| **PREPARED FOR:** | U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012 |
| DISTRIBUTION STATEMENT: | Approved for Public Release; Distribution Unlimited |
| | |

| REPORT DOCUMENTATION PAGE | | *Form Approved*<br>*OMB No. 0704-0188* |
|---|---|---|

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| **1. REPORT DATE**<br>May 2016 | **2. REPORT TYPE**<br>**Annual** | **3. DATES COVERED**<br>1 May 2015 – 30 April 2016. |
|---|---|---|

| | |
|---|---|
| **4. TITLE AND SUBTITLE**<br><br>"The Role of Novel Substituted Diindolyl Methane Analogues in the Treatment of Triple-Negative and ErbB2-Positive Breast Cancer" | **5a. CONTRACT NUMBER**<br><br>**5b. GRANT NUMBER**<br>**W81XWH-11-1-0211**<br>**5c. PROGRAM ELEMENT** |
| **6. AUTHOR(S)**<br><br>**Dr. Mandip S. Sachdeva**<br>email: mandip.sachdeva@famu.edu | **5d. PROJECT NUMBER**<br><br>**5e. TASK NUMBER**<br><br>**5f. WORK UNIT NUMBER** |
| **7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**<br>**Florida A&M University**<br>**Tallahassee, FL 32307** | **8. PERFORMING ORGANIZATION REPORT** |
| **9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**<br><br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012 | **10. SPONSOR/MONITOR'S ACRONYM(S)**<br><br>**11. SPONSOR/MONITOR'S NUMBER(S)** |

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT** The mission of the proposed HBCU/MI Breast Cancer Partnership Training Award at Florida A & M University (FAMU) is to develop a rich intellectual environment that will promote and strengthen the research capabilities of FAMU investigators in the area of breast cancer research. The objectives this proposal are (1) to provide mentorship and training to FAMU researchers in breast cancer research area to enhance the research expertise and competitive ability; (2) to train FAMU investigators through a well defined research project investigating the anticancer potential of C-DIM analogs in treatment of breast cancer; (3) to develop FAMU investigators grantsmanship skills by submitting extramural grants for independent funding; and (4) to create awareness among FAMU researchers and African American Community about breast cancer biology and therapy. The outcome of this proposal will lead to novel oral therapeutic strategies for treatment of triple negative breast cancer (TNBC) and ErbB2-positive breast cancer) EPBC and also result in publications in highly ranked journals. This approach will result in establishment of a successful and independently funded breast cancer research program at FAMU. Our ultimate goal is to become an independent research and training program of excellence for minority investigators

| **15. SUBJECT TERMS**<br>**Triple negative breast cancers** | | | | | |
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| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION** | **18. NUMBER** | **19a. NAME OF RESPONSIBLE PERSON USAMRMC** |
| **a. REPORT**<br>U | **b. ABSTRACT**<br>U | **c. THIS PAGE**<br>U | UU | 11 | **19b. TELEPHONE NUMBER**<br>*(include area code)* |

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## 2. Section I: A brief introduction covering the purpose and scope of the research effort

Breast cancer is the second leading cause of cancer-related deaths and approximately 15%-20% of patients are diagnosed with TNBC, which do not express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). It is a particularly lethal subtype of breast cancer with a 5-year survival rate as low as 40%. The life expectancy after detection of visceral metastasis in TNBC patients is estimated as 3 to 22 months. The over-expression of ErbB2 (HER2) occurs in approximately 20–25% of all breast tumors and the outcome of current therapies for ErbB2-positive breast cancers (EPBC) remains unsatisfactory due to short intervals to recurrence, short overall survival, resistance and toxic side effects. African American women are 34% more likely to die from breast cancer than white women. The TNBC and EPBC are high risk breast cancers and the choice of orally available chemotherapeutic agents is limited. Hormonal therapy (ER modulators) and HER antibody based therapy are far safer than cytotoxic drug based regimens. But triple negative breast cancers are not responsive to hormonal or HER targeting therapy. Given the challenge in treating TNBC and EPBC and its inherent poor prognosis, the use of novel orally active C-DIM analogues will have major clinical implications for the treatment of breast cancer. Orally administered Diindolyl methane (DIM) analogue DIM-C-pPhC6H5 alone showed potent anticancer activity and also exhibited additive to synergistic anticancer activity in combination with docetaxel (doc). Based on this success, our ultimate objective is to design, synthesize and evaluate the novel orally effective DIM analogues to treat TNBC and EPBC by establishing the breast cancer research program with the support of proposed HBCU/MI Partnership Training Award. The proposed studies will synthesize C-DIM analogs (DIM10 and DIM 12, 14) and investigate the structure-dependent anticancer activities in TNBC and EPBC using both cell and laboratory animal models. Our hypothesis is that the novel C-DIM analogues will show potent anticancer activity against TNBC and EPBC and the use of C-DIM analogues could be a novel approach for the treatment of breast cancer. Moreover, evaluation of anticancer efficacy of DIM loaded tumor targeted nanoparticles in TNBC patient derived tumorgrafts(PDX) could be helpful in understanding its clinical outcome.

## 3. Section II: Research Accomplishments

Synthesis of various C-DIM analogues, in vitro cytotoxicity, pharmacokinetic in rats and higher animals in vivo anticancer efficacy studies and western blot to analyze various tumor markers were summarized in the previous reports (December 2011, May 2012, April 2013, May 2014, May 2015). This report being an annula report will have some data from the earlier report and some new data. Compounds, C-DIM-10 and C-DIM-14 were found to have superior anticancer activity against TNBC (MDA-MB-468, MDA-MB-231 and MDA-MB-453) and EPBC (BT474 and SKBR3) cells in comparison to other DIM analogs. Both the compounds were founds to have poor bioavailability due to their poor solubility. Therefore, DIM-10 loaded nanostructured lipid carrier (NLC) was developed to improve their oral bioavailability. Pharmacokinetic of free drug and DIM NLC in dog were described in our last report of May 2014 while Pharmacokinetic and anticancer efficacy of DIM loaded nanocarrier in TNBC tumor bearing mice was described in our previous report of 2012 and 2013. In our last report (2014-2015) we have studied the novel mechanism of DIM analogs (DIM-C-pPhCO2Me and DIM-C-pPhCN) in the treatment of TNBC by antagonizing an orphan nuclear receptor 4A1 (NR4A1). Extensive in vitro and in vivo studies were carried out to explore pathways involved in NR4A1 mediated apoptosis and anti-cancer activity.

According to the future plan submitted in our last report, in current period, we have tried to develop TNBC patient derived breast cancer model in our lab. Since, very limited research has been done in the area of PDX so far, there is no well-established protocol available for it. Moreover, unlike human cancer cells xenograft, PDX takes more than 6 months for a complete study. We have initiated preliminary experiments with the help of Dr. Jackson, Director, Animal Facility, Florida A & M University. We received NOD-SCID mice from Charles River laboratories, MS and tumorgrafted with patient derived tumor fragments of TNBC which we obtained from Dr. Michael Lewis, VanderBilt.

**Cytotoxicity of DIM in various TNBC cell lines**

MDA-MB-231, MDA-MB-231/DOX resistant and MDA-MB-231/DTX resistant cells were plated in 96-well tissue culture plate, at a density of $1x10^4$ cells/well and allowed to incubate overnight and were treated with various dilutions of DIM-10 and DIM-12 in DMEM media from stock solution in DMSO. After 48 h of DIM analogues exposure, cells were fixed with 0.5 % v/v gultaraldehyde and viability was assessed by crystal violet assay. The absorbance was measured by a microtiter plate reader (Spectramax 190, Molecular devices, USA) at 540 nm. Our results as depicted in Table 1 showed that DIM10- was superior than DIM-12 for its in vitro cytotoxicity against both Dox and DTX resistant cell lines.

| Cell line | DIM 10 | DIM 12 |
|---|---|---|
| MDA-MB-231 | 4.37±0.95 | 9.75±1.4 |
| MDA-MB-231/DOX | 6.52±1.3 | 13.69±1.6 |
| MDA-MB-231/DTX | 6.64±1.7 | 14.24±1.1 |

Table1: Cytotoxicity of DIM analogs in MDA-MB231 cell lines.

***In vitro* migration assay**

In vitro migration (scratch) assay was carried out in MDA-MB-231/DOX resistant cells. MDA-MB-231/DOX cells (10,000 cells per well) were plated 96-well plate in complete media, and cultured overnight. A uniform scratch was made in the center of well with a cell-scraper. Wells

were washed with PBS and treated with 5 µg/ml DIM-10. After 48 h of DIM-10 exposure, cells were fixed with 0.5% glutaraldehyde and stained with crystal violet for 15 min. The images of scratch area were taken using Olympus microscope to calculate the area using ImageJ software. Areas of gap (scratch) before and after treatment were analyzed for calculating the percent bridging of migration area.

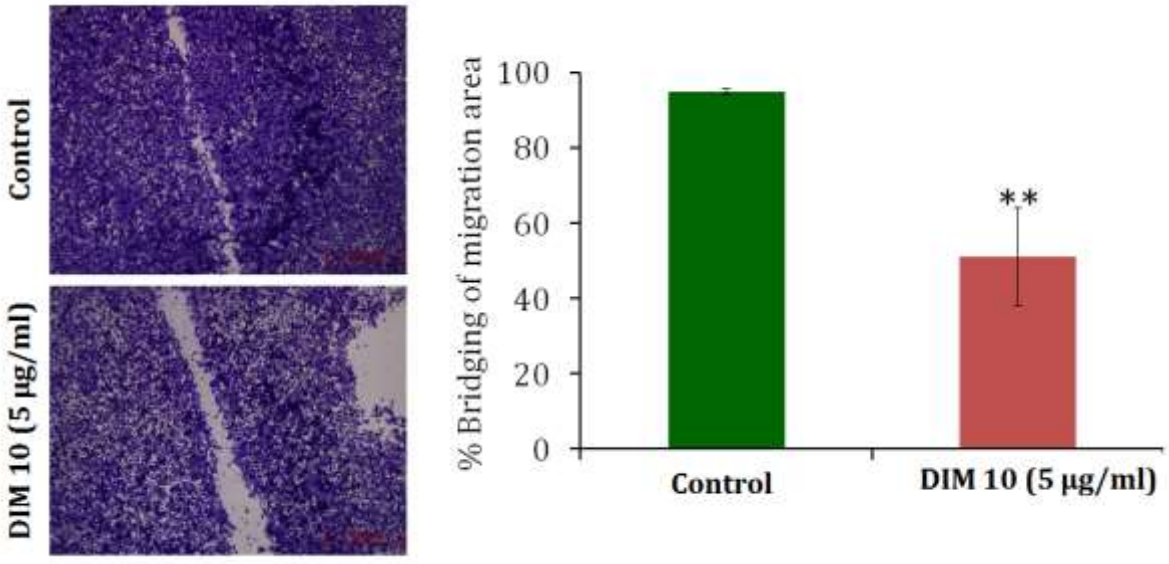


Fig 1. Scratch assay with DIM10 MDA-MB-231/DOX resistant cells.

Our data in Fig.1 shows that there was significant decrease in the bridging with DIM 10 compared to the control in DOX resistant breast tumor cells at doses as low as 5ug/ml.

Formulation of CREKA peptide conjugated DIM-10 liposomes:
We have developed a CREKA (A tumor endothelial cell targeted peptide, cysteine–arginine–glutamic acid–lysine–alanine) coated stealth nanoliposomes of DIM analog (DIM-C-pPhCO2Me, DIM 10). Initial experiments were conducted with DIM-12 but seeing the superior activity of DIM-10, future experiments were only performed with DIM-10. The presence of fibrin and proteins that become cross-linked to fibrin in tumors, but not in normal tissues, are thought to be a result of the leakiness of tumor vessels, which allows plasma proteins to enter from the blood into tumor tissue where fibrinogen is converted to fibrin via tissue pro-coagulant factors. DIM-10 loaded PEGylated liposomes (DPL) and CREKA anchored DIM-10 loaded PEGylated liposomes (CDPL) were prepared using modified hydration method. Briefly, DIM-10:DOGS-NTA-Ni:DOPC:Cholesterol:DSPE-PEG 2000 in 2:5:20:5:2.5 weight ratio were dissolved in chloroform. The solution was drop wise added to mannitol at 45˚C with constant stirring and left overnight for evaporation of chloroform. Resultant powder was dispersed into water and sonicated for 4 min using probe sonicator (Branson probe sonicator, USA). CREKA solution (10 mg/ml) was prepared in water and appropriately added into prepared liposomes to achieve

various DOGS-NTA-Ni:CREKA molar ratio. Mixture was incubated for 30 mins. Effect of CREKA concentration on particle size and zeta potential was evaluated. CREKA binding was evaluated by BCA protein estimation kit (Thermoscientific, USA). Briefly, 500 µl of CREKA coated liposomes were filled into Amicon Ultra centrifugal filters (30 kDa) (Millipore, Ireland) and free CREKA was separated by centrifuging the samples at 10,000 rpm for 10 mins. Concentration of free CREKA was analyzed using BCA protein estimation kit.

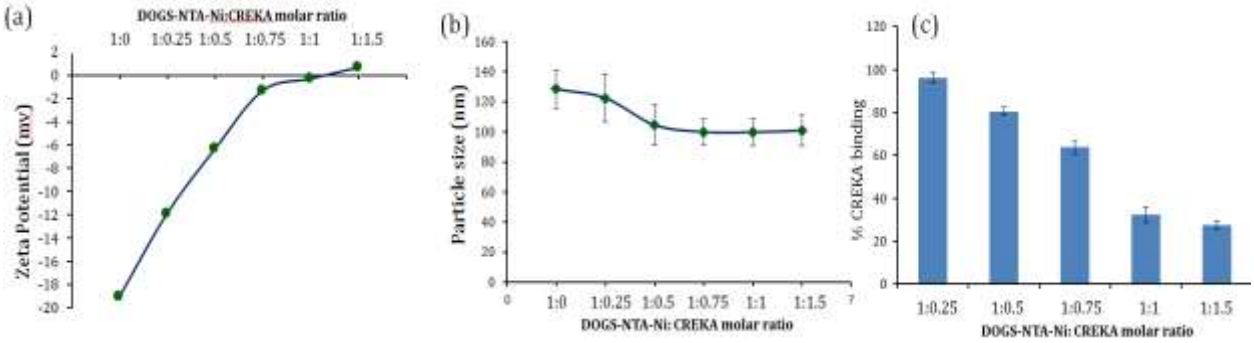


**Figure 2.** Effect of CREKA concentration on (a) zeta potential, (b) particle size and (c) percentage of CREKA binding on DIM-12 loaded DOGS-NTA-Ni containing PEGylated liposomes (DPL). Data were given as mean±SD (n=5)

Immediately after ultra-sonication of DPL, it was incubated with various amount of CREKA. Addition of CREKA peptide significantly affected the zeta potential while marginal effect on particle size was observed (Fig. 2 a&b). Zeta potential was drastically reduced from -23.22 mV to -2.64 mV on addition of 1:0.5 molar DOGS-NTA-Ni:CREKA. Surface complexation of CREKA increased the zeta potential of liposomes in concentration dependent manner. Thereafter, there was a slight decrease in zeta potential at higher ratios e.g. 1:1 and 1:1.5. Hydrodynamic diameter of DPL after separation of mannitol was 189.3 ± 5.7 nm. However, particle size was nearly similar at all the DOGS-NTA-Ni:CREKA ratios. There was a slight decline in particle size from 189.3 nm to around 152 nm after incubation with CREKA (Fig. 1b). Hexahistidine chain of CREKA peptide was expected to form complex with Ni present on liposomal surface. Percentage CREKA binding consistently decreased with increasing the DOGS-NTA-Ni: CREKA ratio. Highest percentage of CREKA binding - 95.14 % was observed at lowest (1:0.25) molar ratio while 74.38 percent CREKA was bound at 1:0.5 molar ratio, which suggested that the free Ni site on liposomal surface was saturated (Fig. 2c). Further addition of CREKA over 1:0.5 molar ratio led to poor percent CREKA binding of around 45 percent. CREKA anchored nanoliposomes with further evaluated for in vitro cytotoxicity, cellular uptake and in vivo anticancer efficacy in TNBC patient derived tumorgraft.

**Collaboration and training:**

a) Dr. Sachdeva and Dr. Stephen Safe are in touch on a weekly basis to plan the experiments. Further several discussions have led to understanding novel mechanism of action of C-DIM compounds and explore it for other cancers. Further, Dr. Musa who was involved in the

synthesis of compounds has prepared some compounds which have been further evaluated.

b) Ms. Valerie Marcellus and Ms. Jane Hong, undergraduate students have been trained in various aspects of invitro assays.

c) Current post-doc in this project, Dr. Ketan Patel has been actively involved in the exploring DIM analogs for their pharmacokinetic evaluation and in formulating nanoparticles to enhance their bioavailability.

d) PI has been conducting several journal clubs in his lab to discuss the latest developments in the field of cancer chemotherapy and nanotechnology. The aim of these journal clubs is to stimulate discussions and new ideas in breast cancer.

**Section III: Problem Areas**

The problem area here could be the development of the patient derived model for triple negative breast cancer which may take more time and experimentation. We are currently developing the doxorubicin resistant MDA-MB231 tumor model and also simultaneously developing the PDX model. Since DIM-10 has shown significant activity against DOX resistant MDA-MB231, it seems logical to evaluate the efficacy of the DIM-10 formulations. For the PDX model, There is support available from Dr. Michael Lewis from Vanderbilt University. Dr. Stephen Safe is also a collaborator with Dr. Lewis, hence we have dual support from two investigators in the development of this model.

**Section IV: A description of work to be performed during the next reporting period.**
**For the next one year, the following experiments and activities are planned:**

a. To conduct experiments with transgenic mice and also patient derived tumor models (PDX models). These experiments will demonstrate the translational outcome of this research.
b. CREKA anchored nanoliposomes (CDPL) will be evaluated in MDA-MB231 Dox resistant tumors and also patient derived tumorgraft.
c. To submit R01 proposal in the area of breast cancer

**Section V: Administrative Comments (Optional) - Description of proposed site visits**

**and participation in technical meetings, journal manuscripts in preparation,**

**coordination with other organizations conducting related work, etc.**

**Presentations:**

1. Jaganmohan Somagoni, Chandraiah Godugu, Ravi Doddapaneni, Stephen H Safe and **Mandip Singh**. Treatment of pulmonary arterial hypertension by the use of a novel PPAR gamma agonist, diindolylmethane in a nanoparticle formulation. American Association of Pharmaceutical Science (AAPS) Annual Meeting & Exposition 2014, San Diego, CA.

2. Ketan Patel, Stephen Safe and Mandip Singh. 'Fibrin binding peptide anchored, Diindolylmethane derivative loaded liposomes for overcoming doxorubicin resistance in triple negative breast cancer. To be presented at the AAPS meeting in Denver, Colorado, 2016.

**Publications:**

**1.** Erick Hedrick, Syng-Lee, Ravi Doddapeneni, Mandip Singh and Stephen Safe. Nuclear receptor 4A1 as a drug target for breast cancer chemotherapy. Endocr Relat Cancer. 2015 Oct;22(5):831-40. doi: 10.1530/ERC-15-0063. Epub 2015 Jul 30. **Both Mandip Singh and Stephen Safe are corresponding authors in this paper.**

2. Safe S[1], Jin UH, Morpurgo B, Abudayyeh A, **Singh M**, Tjalkens RB. Nuclear receptor 4A (NR4A) family - orphans no more. J Steroid Biochem Mol Biol. 2015 Apr 23. pii: S0960-0760(15)00113-2. doi: 10.1016/j.jsbmb.2015.04.016. [Epub ahead of print].

3. Reeder, A, **Singh M**, Patel K, Doddapeni R, Hedric E, Safe S. Nuclear receptor 4A1 (NR4A1) as a drug target for treating rhabdomyosarcoma (RMS). Oncotarget. 2016 Apr 29. doi: 10.18632/oncotarget.9112. [Epub ahead of print]

4. Hedrick E, Lee SO, Doddapaneni R, Singh M, Safe S. NR4A1 Antagonists Inhibit β1-Integrin-Dependent Breast Cancer Cell Migration. Mol Cell Biol. 2016 Apr 15;36(9):1383-94. doi: 10.1128/MCB.00912-15. Print 2016 May 1.

**Submitted:**

1. Chandu Godugu, Ravi Doddapeneni and **Mandip Singh**. Novel Diindolylmethane derivatives based NLC formulations to improve the oral Bioavailability and anticancer effects in Triple Negative Breast Cancer. Submitted to European Journal of Pharmaceutics, May 2016.

**1. References**

**2. Appendices**