AWARD NUMBER: W81XWH-19-1-0681

TITLE: AXL-Targeting Antibody-Drug Conjugate as Novel Therapy for Triple-Negative Breast Cancer

PRINCIPAL INVESTIGATOR: Parkash Gill

CONTRACTING ORGANIZATION: University of Sothern California

REPORT DATE: OCTOBER 2021

TYPE OF REPORT: ANNUAL

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
OCTOBER 2021	Annual	1SEPT2020 - 31AUG2021

4. TITLE AND SUBTITLE

AXL-Targeting Antibody-Drug Conjugate as Novel Therapy for Triple-Negative Breast Cancer

5a. CONTRACT NUMBER
W81XWH-19-1-0681

5b. GRANT NUMBER
BC181309P1

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Parkash Gill

E-Mail: parkashg@usc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

University of Southern California
Department of Contracts and Grants
3720 South Flower Street
Los Angeles, CA 90089-0701

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

We have identified AXL, a receptor protein tyrosine kinase (RTK), being highly expressed and activated (phosphorylated) in Triple Negative Breast Cancer. We have determined that AXL provides survival benefit to the tumor cells. We have also discovered a monoclonal antibody that is highly specific to AXL, and does not bind to other related receptor tyrosine kinases. We have also shown that the antibody internalizes and degrades the AXL receptor. We have humanized the antibody for clinical development. We have established high producer cell line and propagated in chemically defined medium. We thus propose to conduct the following studies using novel target and novel therapeutic.

Specific aims are; Aim 1. To develop a humanized antibody-drug conjugate (ADC) that can effectively target the AXL membrane receptor tyrosine kinase. Aim 2. To test the efficacy of the AXL-targeted ADC in preclinical animal models Aim 3. To develop a mass spectrometry-based method to effectively monitor AXL expression and activation in xenograft tissues and clinical samples.

15. SUBJECT TERMS: NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	10	USAMRDC

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1. Introduction

Triple negative breast cancers (TNBC) represent a continued challenge representing 10-15% of all breast cancers and carries poor outcome. No curative therapy is available in the advanced TNBC. Patients with TNBC are treated with systemic chemotherapy with mostly short-term benefits and associated adverse effects. Immune check point inhibitor antibodies combined with chemotherapy have also shown some efficacy (Kwa M, Adams S Cancer 2018;124:2086-103). In addition, Sacituzumab, a TROP-2 antibody drug conjugate was recently approved for patients who have failed prior chemotherapy. Overall survival of 12.1 months, progression free survival of 5.6 months, overall response rate of 35%, which were substantially longer than chemotherapy. (Bardia A et al, NEJM 2021, 384:1529-41). Sacituzumab has frequent grade 3 and grade 4 toxicity bone marrow suppression, diarrhea among others. Combination therapy with immune check point inhibitors which also can cause gastro-intestinal toxicity requires cautious investigation. There remains a significant need for novel targeted therapies of TNBC.

Mutant oncogenic driver receptor tyrosine kinases (RTKs), or RTKs that are over-expressed and constitutively activated often drive tumor cell survival, or over-expressed tyrosine kinase receptors which cause dependence of tumor cells in cancer initiates a phosphorylation cascade in cells to deliver signals that ultimately leads to inhibition of cell death, increased invasion and tumor metastasis. Identification of novel targets and targeting agents is now used widely for many cancers. Analysis of phosphorylated proteins in TNBC led to the identification of AXL receptor tyrosine kinase (RTK). AXL is overexpressed and activated in highly aggressive TNBC cells (Wu et al. 2015, Wu X et al Cancers (Basel). 2021 Aug 23;13(16):4234. doi: 10.3390/cancers13164234). We have also discovered AXL specific monoclonal antibody that internalizes and degrades the receptor (Liu et al 2010, Brand et al, 2014, Yu et al, 2015, Brand TM et al 2015, Li D, et al, 2014, Liu S, 2014) We have humanized the antibody and established high yield CHO cell line to produce antibody in chemically defined medium. MoAb173 is thus suitable for the proposed aims to investigate a novel humanized anti-AXL monoclonal antibody drug conjugate (ADC) as a novel therapy to treat TNBCs with AXL overexpression.

We will first synthesize the ADC by conjugating hMAb173 with mertansine, a highly potent microtubule inhibitor. The ADC will specifically target AXL overexpressing cells, internalize and released cytotoxic payload. We will also develop near-infrared (NIR) labeled AXL antibody to non-invasively monitor the delivery and distribution of the AXL antibody. We will evaluate the therapeutic potential of this novel AXL-ADC in preclinical models using triple negative breast cancer model system.

2. Keywords

Triple negative breast cancer TNBC
Receptor tyrosine kinase RTK
AXL. The word AXL, comes from the Greek word “anexelekto”, means uncontrolled.
Phosphorylation of AXL, pAXL
Antibody drug conjugates (ADC)

3. Accomplishments

The study is partenering between Dr. Parkash Gill at the University of Southern California and Dr. Xinyan Wu at Mayo Clinic.

What were the major goals of the project?

The major goals of the project in year 1 and 2 were as follows:

- To develop a humanized antibody-drug conjugate (ADC) that can effectively target the AXL membrane receptor tyrosine kinase.

- To test the efficacy of the ADC in vitro and in vivo

- To develop Antibody fluorescent conjugate for in vivo monitoring of AXL localization.

- To develop a mass spectrometry-based method to effectively monitor AXL expression and activation in tumor tissues

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

1) Major Activities

During year 2, we developed AXL-ADC and characterized for stable molecule that retains stability and solubility

We tested AXL MDA-MB231 isogenic cell lines with and without AXL knock out for AXL-ADC specificity to binding, degradation of the receptor and cytotoxicity to AXL expressing cells

We produced AXL antibody to produce batch of AXL-ADC to provide Dr. Wu at Mayo clinic for drug distribution and efficacy studies

2) *Specific Objectives*

Develop AXL antibody conjugation with cytotoxic agent

Characterize AXL-ADC

Test specificity of AXL-ADC to retain naked antibody characteristics

Establish assays for receptor degradation in vitro

Establish cytotoxicity in vitro

3) Significant results

Specific AIM 1: To develop a humanized antibody-drug conjugate (ADC) that can effectively target the AXL membrane receptor tyrosine kinase
Subtask

Subtask 1: To synthesize AXL-ADC. Humanized antibody was produced in CHO cells in chemically defined medium. Antibody was purified on protein A column and purity of the antibody was confirmed on HPLC.

Purified antibody binds to recombinant AXL-AP. Purified antibody was conjugated to cytotoxin. AXL-ADC was purified and characterized

Coupling of MC-Val-Cit-PAB-MMAE to hIgG1 (hMoAB #173)

Reagents:

1. hMoAB# 173. Conc: 2mg/mL
2. MC-Val-Cit-PAB-MMAE. The precursor of antibody drug conjugate. The structure includes the following: a) A thio reactive maleimidocaproyl (MC) group; b) A protease-sensitive Val-Cit dipeptide; c) PABC (p-aminobenzyl alcohol p-nitrophenyl carbonate) linker and d) MMAE (Monomethyl auristatin E) payload. MW = 1,317. Catalog: BP-23969
3. DTT; PBS, DMSO, Desalting colum (PD-10)

Protocol:

1. Dialyze hMoAB# 173 (2mg/mL) against 50mM NaPi, pH 7.6 for overnight;
2. Make 10mM solution of MC-Val-Cit-PAB-MMAE in 100% DMSO. For example: disolve 10 mg of MC-Val-Cit-PAB-MMAE in 0.76 mL of DMSO (stored at -80C if necessary;
3. Equilibrate PD-10 column with 50mM NaPi, pH 7.6;
4. Reduce hMoAB# 173 with DTT:
   a) Take 1mL of dialyzed hMoAB# 173 (2mg) and mix it with freshly prepared DTT up to 0.3mM;
   b) Leave on dark at Room temperature for 30 min;
5. Remove DTT from sample by gel filtration on PD-10. Collect 75-80% of loaded protein;
6. Measure the volume of desalted hMoAB# 173. Should be approximately 1.2mL
7. Add of 4 uL of 10mM solution of MC-Val-Cit-PAB-MMAE/DMSO. Mix vigorously;
8. Add other 4 uL of 10mM solution of MC-Val-Cit-PAB-MMAE/DMSO. Mix vigorously;
9. Add final 4 uL of 10mM solution of MC-Val-Cit-PAB-MMAE/DMSO. Mix vigorously; Total amount of MC-Val-Cit-PAB-MMAE/DMSO added: 12uL.
10. Incubate at dark for 1h at room temperature;
11. Dialyze against TBS for O/N
12. Measure concentration and activity.

1. Ligand binding assay

To check the function of MMAE-MoAB #173, we used Ligand binding assay. ProteinA-Agarose beads is loaded with 20ng of either MoAB #173-WT (parental, not labeled antibody) or with the same amount of MMAE-MoAB #173 in 1.5mL eppendorf tubes. 100 ng of antigen fused to alkaline Phosphatase was added into each tube. Samples were incubated in a shaker for 40 min. Beads were washed 3 three times in 1.2 ml TBS and enzymatic reaction was developed by application of substrate p-Nitrophenyl phosphate, pH 10. Experiment was repeated in triplicate. Unrelated human monoclonal IgG1 (HuIgG1) was used as a negative control (Figure 1).

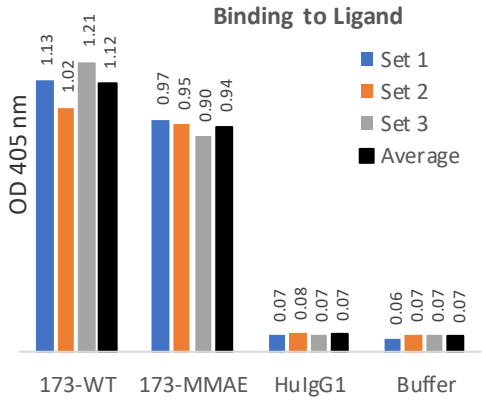

Figure 1. Functional ligand binding assay of MMAE-MoAB 173. WT is a positive control. HuIgG1 is a negative control. No background subtraction.

2. SDS-PAGE. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

SDS-PAGE was run as a quality control. MoAB #173 was reduced with 0.3mM of DTT at room temperature, desalted on PD-10 column and coupled to MC-Val-Cit-PAB-MMAE. 3 samples were loaded: 1) MoAB #173 -WT (Antibody before modification); 2) DTT-Treated and desalted MoAB #173 and 3) MMAE-MoAB #173 Conjugate (Final product). See Figure 2.

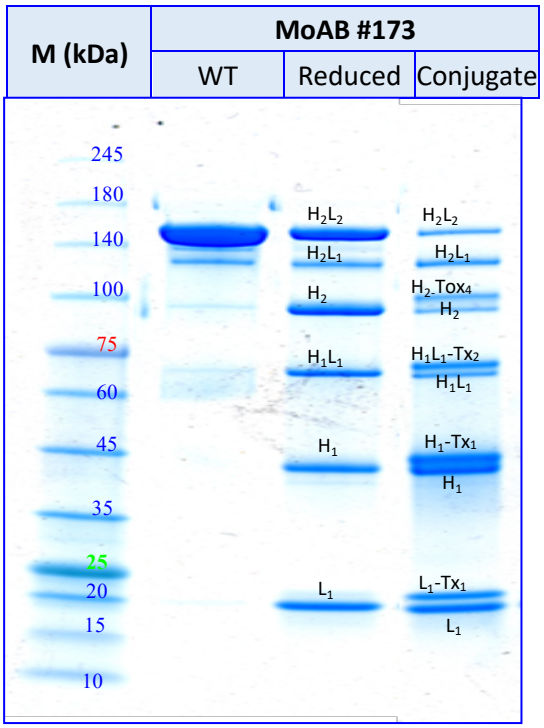

Figure 2. Not-reduced SDS-PAGE of MoAB #173. M: Markers, MWs shown in kDa. WT: MoAB #173 taken into reaction. MoAB #173 – Reduced: Treated with 0.3mM DTT and de-salted on PD-10 column reduced MoAB # 173. Conjugate: Final product. "H_1" - singe heavy chain, "L_1" - singe light chain, "H_1L_1" – disulfide bridged heavy and light chains, "H_1Tx_1" – Heavy chain disulfide linked with single molecule of MMAE and so on.

Subtask 2: To conjugate AXL antibody with 64Cu for in vivo imaging. Dr. Peter Conti is collaborating on the project. Dr. Peter Conti' team leads the effort in generating imaging reagents and conjugate hMAb173 with 64Cu for in vivo imaging. Protocol for the conduct of in vivo studies was approved by the animal care (IACUC) committee. Dr. Peter Conti's co-investigator Dr. Scott Liu, PhD has left the institute. Dr. Kai Chen replaced Dr. Scott Liu to oversee the imaging studies. The USC Cyclotron program no longer has capabilities for Cu-targeted PET tracer development. Thus, we will modify distribution studies, which will be done using direct tissue assessment by Dr. Wu at Mayo.

Subtask 3: AXL-ADC has been characterized. The AXL-ADC retains intact, remains soluble and stable. No aggregation was observed.

3. SE-HPLC. Size exclusion chromatography gives an instant answer on aggerate stage of IgG labeled with MMAE. Figure 3 illustrates this fact with an example of too much reduction (4 mM DTT) and optimal reduction of IgG with 0.3mM DTT. Overreduction of IgG with DTT triggers Intermolecular cross-linking and leads to aggregate formation. In this case tetramerization with a formed aggregate around 600kDa. Optimal reduction (0.3mM DTT) followed by coupling to MMAE shifts the peak slightly on right. This is expected behavior due to increased hydrophobicity of MoAB-MMAE complex. SE-HPLC analysis is important assay, but not sufficient for full characterization of obtained product.

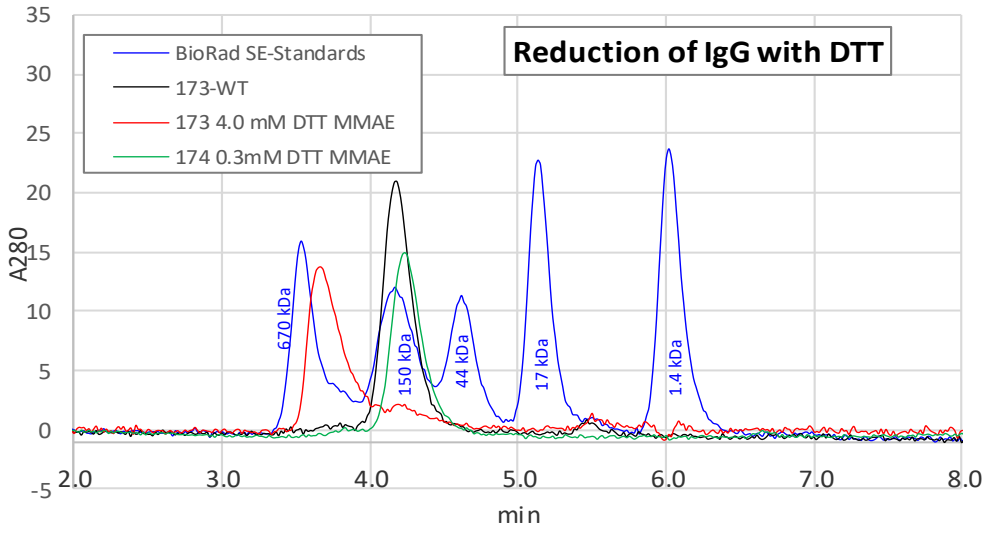

Figure 3: Size exclusion HPLC of MoAB173-MMAE conjugates. Blue Line – SE-HPLC markers from BioRad (MW is shown in kDa); Black line: MoAB-WT (Wilde Type) – Antibody before labeling; Red Line: Reduction of MoAB with 4mM DTT; Green line: Reduction of MoAB with 0.3mM DTT.

Subtask 4: To Axl-ADC conjugation batch production for studies and Mayo Clinic. A batch of the AXL-ADC has been produced

Subtask 5: To generate a batch of AXL- ADC for the in vivo testing in TNBC models.

AXL-ADC in vivo efficacy studies and tissue distribution studies will be done By Dr. Wu at Mayo Clinic.

Major Task2: To test AXL-ADC in vitro in TNBC cell lines

Subtask 1: To test the cell membrane binding and internalization kinetics of the anti-AXL-ADC using confocal microscopy and FACS: Axl-ADC has shown specificity to Axl expressing tumor cell lines and not isogenic Axl knock out cell line.

MDA-MB-231 wild type and AXL knock out cells were plated in six well plates. Cells were treated with AXL-ADC at various concentrations to determine if it retains the characteristics of parent naked antibody. Cells were harvested at 48 hours and analyzed for AXL protein using Western Blot. MDA-MB-231 AXL knock out cell line showed in AXL expression. MDA-MB-231 wild type showed dose dependent decline in the AXL receptor levels. These findings are consistent with the expected function of AXL antibody to internalize and degrade the receptor.

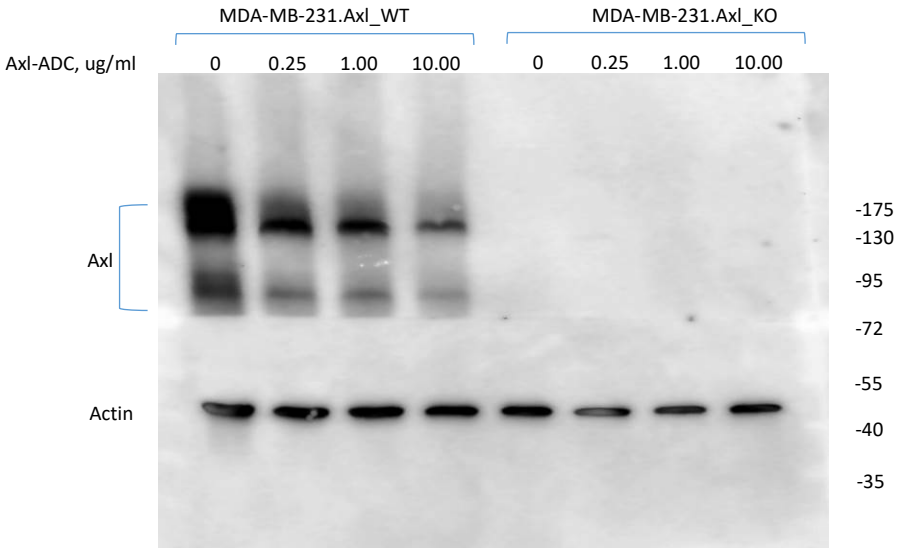

Figure legend: *MDA-MB-231 wild type (left panel) and AXL knock out (right panel), were treated with AXL-ADC and the level of AXL was measured in equal amount of protein loading shown by b-actin levels.*

XL-ADC cytotoxicity Assay: AXL wild type MDA-MB-231 expresses AXL protein and thus is a target of AXL-ADC cytotoxicity. AXL knock out isogenic cell line lacking AXL expression is anticipated to not show cytotoxicity if AXL-ADC has specificity to its target.

Isogenic cell lines were plated in 24 well plate and treated with AXL-ADC at various concentrations. Representative images were taken and shown below. Wild type MDA-MB-231 showed marked reduction in the cells and appears non-viable. AXL knock out MDA-MB-244431 showed no change in the cell density. These data are consistent with target specific cytotoxicity. Viable cell count was also assessed as shown below.

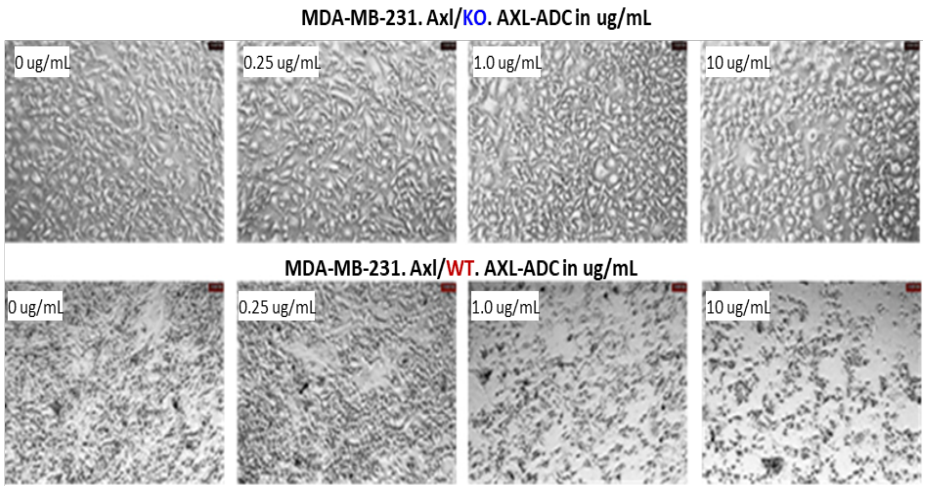

Figure legend: *MDA-MB-231 knock out (top panel) and AXL wild type (bottom panel), were treated with*

AXL-ADC and cell morphology was captured. A dose dependent loss of cell viability and cell density is observed only in wild type cell line consistent with the specificity of the AXL-ADC.

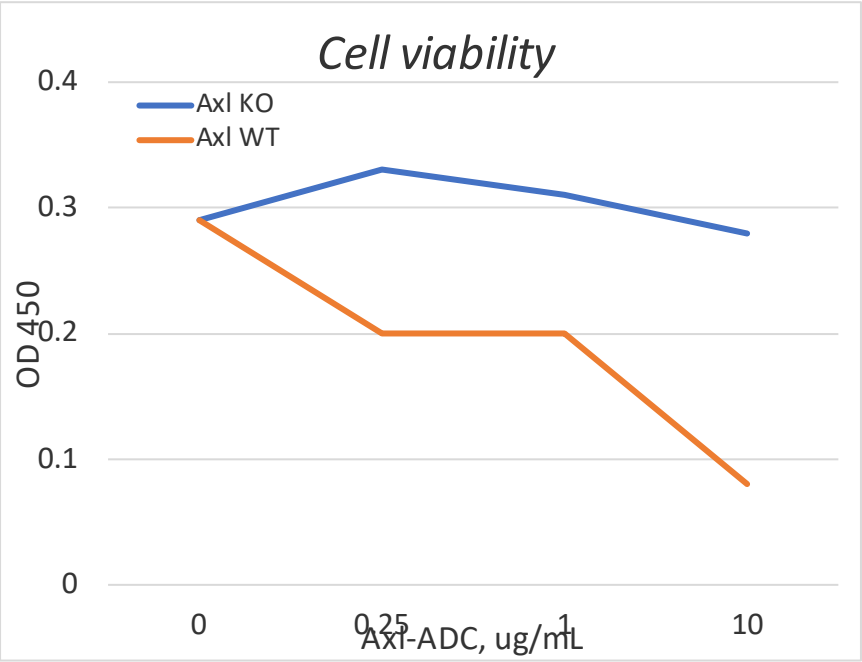

Figure legend: *MDA-MB-231 knock out and AXL wild type, were treated with AXL-ADC at various concentrations. Cell viability was assessed after 72 hour treatment. A dose dependent reduction in cell viability was observed (below)*

Specific Aim 2: To test the efficacy of the AXL-targeted ADC in preclinical animal model.

Subtask 1: To set up TNBC models (PDX) and TNBC Cell line MDA-MB-231get approval from IACUC and ACURO:

Protocol has been approved by USC IACUC but not ACURO. Due to the loss of CU-targeted PET tracer capabilities at USC, loss of faculty member at USC in Dr. Peter Conti's group, limited time availability of Dr. Kai Chan, we are going to focus on the antibody distribution and efficacy studies at Mayo Clinic partner laboratory.

Subtask 2: To determine bio-distribution and pharmacokinetics of ADC in tumor bearing mice.

Subtask 3: To examine the AXL protein expression in collected TNBC PDX and relate AXL expression to ADC delivery and retention. Dr. Gill will provide the AXL –ADC antibodies for in vivo studies correlate AXL expression in TNBC. In vivo efficacy studies on TNBC models including TNBC patient –derived xenografts (PDX), and MDA-MB-231 implanted in immune deficient mice. Dr. Gill is providing the AXL and AXL-ADC for the ongoing work at Mayo Clinic. Similarly distribution of the antibody in tumor and normal organs is planned and planned through our collaboratoin wtih Dr. Wu at Mayo Dr. Gill has not conducted DOD funded in vivo efficacy studies in TNBC model, however previous in vivo efficacy studies under local approved protocol funded by other sources have provided supporting data

Major Subtask 4: in vivo efficacy studies. Dr. Gill is providing AXL-ADC for in vivo efficacy studies in MDA-MB-231 mouse model. Sytemic toxicity is assessed by histopathology of vial organs, and blood test for hematologic, kidney and liver function.

Dr. Gill has not conducted in vivo efficacy studies with funding from DOD. An efficacy study under a local IACUC approved protocol was done with data supporting the project.

Specific Aim 3: *To establish a mass spectrometry-based method to effectively monitor AXL expression and activation in xenograft tissues and clinical samples.*

Subtask 1. To perform AXL IHC, IP and/or phosphopetides enrichment followed by MS analysis of MDA-MB-231. This work will be done at Mayo Clinic.

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

Nothing to report

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

Nothing to report

Other achievements.

None

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

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

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

We will conduct remaining studies especially in vivo studies as no cost extension to complete the project. Tissue analysis for target degradation, and tumor cell death. We will provide Axl-ADC to our collaborator at Mayo clinical to conduct the studies under approved protocol.

Dr. Peter Conti's group will conduct imaging studies on approved protocols.

4. Impact

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

Nothing to report

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

Nothing to report

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

Nothing to report

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

Nothing to report

5.Changes/Problems

Work was hampered initially due to COVID-19.

We lost one of the faculty – Scott Liu.

We have replaced Dr. Scott Liu with Dr. Kai Chen.

We remain committed to complete the work

6.Products

We generated Antibody-drug conjugates and performed biochemical characterization.

7.Participants & Other Collaborating Organizations

Dr. Binyun Ma post-doctoral fellow has conducted in vitro studies

Dr. Peter Conti collaborated to conduct the imaging studies

Dr. Scott Liu worked with Dr. Peter Conti to lead the imaging studies

Dr. Kai Chen was going to generate imaging reagents after Dr. Liu left USC

Dr. Xinyan Wu at the Mayo clinic works with us on the project.

8. Special Reporting Requirements:

None