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PRINCIPAL INVESTIGATOR: Parkash Gill

CONTRACTING ORGANIZATION: University of Southern California, Los Angeles, CA

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Parkash Gill

E-Mail: parkashg@usc.edu

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University of Southern California
Department of Contracts and Grants
3720 South Flower Street
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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.

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TABLE OF CONTENTS

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 2010j, 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 first synthesized the ADC by conjugating hMAb173 with mertansine, a highly potent microtubule inhibitor. The ADC specifically target AXL overexpressing cells and effectively kill the targeted cells. We have are evaluating the therapeutic potential of this novel ADC in preclinical xenograft models of TNBC.

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 partnering between Dr. Parkash Gill at the University of Southern California Dr. Xinyan Wu at the Mayo Clinic and. This report is based on the progress at the site of University of Southern California.

What were the major goals of the project?

The major goals of the project were as follows:

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

What was accomplished under these goals?

1) Major Activities

a. In year 3, we generated AXL-ADC
b. We established AXL-ADC retains binding to AXL cell surface receptor tyrosine kinase
c. We determined that AXL-ADC retains binding and endocytosed of cell surface AXL
d. We determined that AXL-ADC is cytotoxic to AXL localized to the cell surface

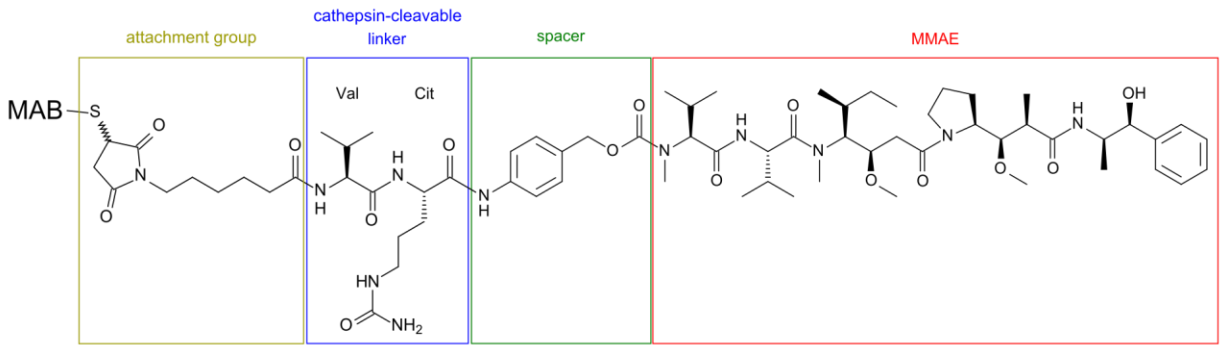
2) Specific Objectives

a. Generate and characterize AXL-ADC
b. To test if the AXL-ADC retains the properties of the naked AXL antibody including endocytosis and receptor degradation
c. To induce cellular toxicity to cells expressing cell surface receptor AXL and determine efficacy in vivo

3) Significant results

We finalized the pay load and protocol for making AXL antibody drug conjugate and verified several features critical for advancing the compound. We established that the AXL ADC retains binding to the cell surface AXL receptor kinase. Second critical attribute is the potency of the compound which is determined by the number of molecules of cytotoxic agent bound to each molecule of the antibody. we have selected the range of cytotoxic molecule bound to each antibody molecule that retains desired function. We have determined that the AXL ADC remains stable.
AXL-ADC retains the functional attributes by AXL degradation as a result of binding, and endocytosis. Lastly, based on the understanding that targeted delivery of the cytotoxic agent leads to cell death, AXL-ADC is cytotoxic only to AXL expressing cells and not AXL knock out isogenic cell lines.
AXL-ADC development was delayed due to CoVID and thus delay to conduct in vivo studies, which will be conducted during the current year under the no cost extension.

AXL was conjugated to monomethyl auristatin E (MMAE) with cleavable linker. MMAE can bind AXL Ab 173.



Bifunctional cleavable linker was to attach payload to the antibody. One end of the linker binds to conserved residues in Fab and Fc. The other end of the linker is conjugated to cytotoxic agent. Up to 8 molecules of MMAE can bind to one molecule. Relative narrow range of antibody to toxin ratio was achieved by optimizing the reaction conditions. The protocol is summarized below.

Monoclonal humanized antibody 173 was applied at the concentration of 2 mg/ml. MC-Val-Cit-PAB-MMAE is the precursor payload. The molecule includes 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. DMSO and desalting column PD-10 were applied as described.
Mab173 (2mg/mL) was dialyzed against 50mM NaPi, pH 7.6 for overnight. 10mM solution of MC-Val-Cit-PAB-MMAE in 100% DMSO as prepared by disolve 10 mg of MC-Val-Cit-PAB-MMAE in 0.76 mL of DMSO. This solution was kept at -80C until application. PD-10 column was equilibrated with 50mM NaPi, pH 7.6. Mab173 was reduced with DTT by taking 1mL of dialyzed hMoAB# 173 (2mg) ws mixed with freshly prepared DTT up to final concentration 0.3mM. DTT treated antibody was kept in the dark at room temperature for 30 min. DTT was then removed from the sample by gel filtration on PD-10 and 75-80% of the loaded material was collected. The final volume of the antibody was around 1.2 ml. Payload was added to the antibody, 4 uL of 10mM solution of MC-Val-Cit-PAB-MMAE/DMSO was added and mixed vigorously. Additional 4 uL of 10mM solution of MC-Val-Cit-PAB-MMAE/DMSO and was mixed vigorously. This preocess was repeated again using 4 uL of 10mM solution of MC-Val-Cit-PAB-MMAE/DMSO and mixed vigorously. This in total 12 ul of MC-Val-Cit-PAB-MMAE/DMSO was added. The mixture was Incubate in the dark for 1h at room temperature. Conjugated antibody was dialyzed overnight to remove unconjugated payload. Final antibody concentration was measured and used for analysis.

Ligand binding assay

In order to determine that the mAb173-MMAE retains binding to the target AXL protein, we measured binding to AXL extracellular domain fused to alkaline phosphates, 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 mAb173-MMAE in 1.5mL volume. 100 ng of AXL extracellular domain-AP fusion protein was added into each tube, incubated in a shaker for 40 minutes. The beads were washed three times in 1.2 ml TBS and enzymatic reaction was developed by application of AP substrate p-Nitrophenyl phosphate, pH 10. Experiment was repeated in triplicate.. Control human IgG1 was used as a negative control. Specific binding to AXL-AP fusion protein was observed for both naked AXL antibody mAb173 and mAb173-MMAE. Negative control antibody hIgG1 had no specific binding (Figure 1).

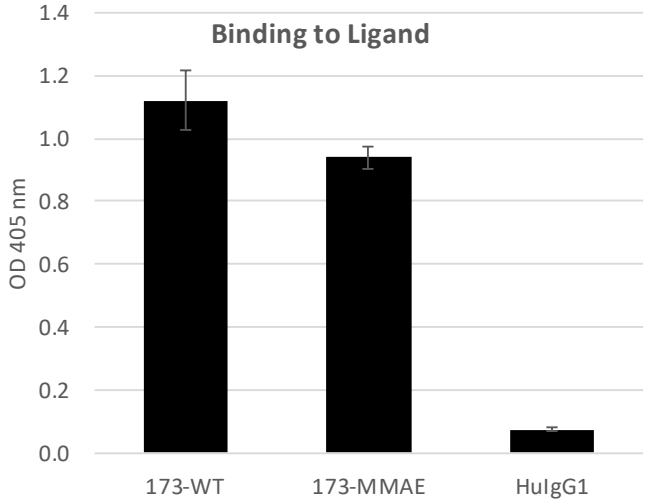


Figure 1. Functional ligand binding assay of MMAE-MoAB 173. WT is a positive control. HuIgG1 is a negative control. No background subtraction.

AXL-ADC mAb 173-MMAE retains binding to AXL receptor in tumor cell line.
We next determined if mAb 173-MMAE retains binding to breast cancer cell line MDA MB 268 cell line that expresses AXL receptor kinase on the cell surface. Various concentrations of Axl mAb 173 or mAb 173-MMAE were added to the tumor cell pellets. Human IgG1 was used as negative control. Excess antibody was washed and the bound antibody was analyzed using AXL extracellular domain Alkaline phosphate fusion protein followed by substrate treatment. Axl mAb 173-MMAE binding to the tumor cell line was similar to that of parent antibody mAb173. Negative control human IgG1 had no binding. Background signal was not subtracted from the data presented below (figure 2).

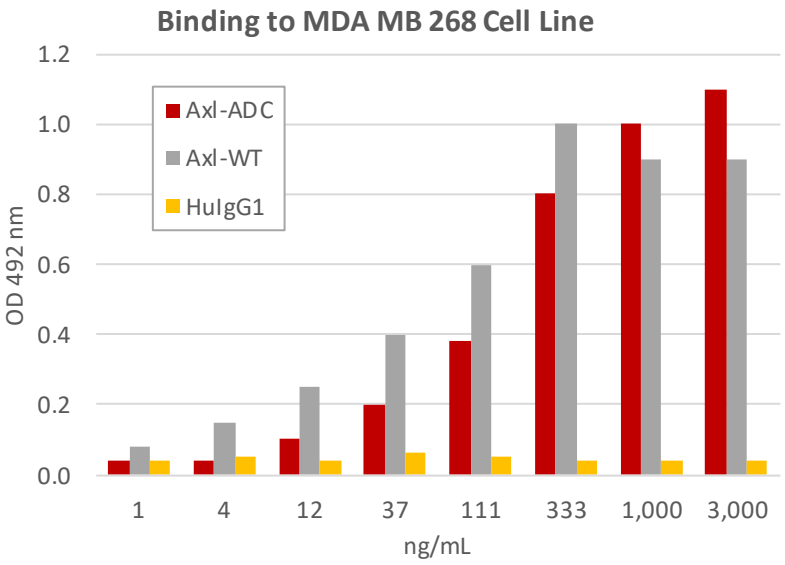
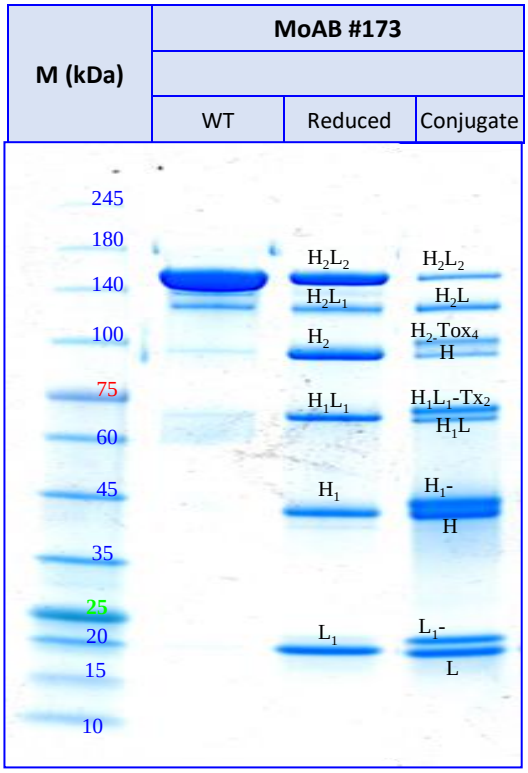


Figure 2. AXL mAb 173_MMAE and parent mAb 173 binding to MDA MB 268 was tested using cell line pellets treated with the antibodies. Human IgG1 was used as a negative control

Characterization of the mAb 173-MMAE using 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 3. mAb173-MMAE is intact and the conjugated MMAE is noted (figure 3).

Figure 3. 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.

Antibody Characterization by SE-HPLC:

Size exclusion chromatography was used to determine antibody aggregation. . mAb173-MMAE, mAb 173 was analyzed after treatment with DTT at 4 mM or 0.3 mM. Figure 4 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.

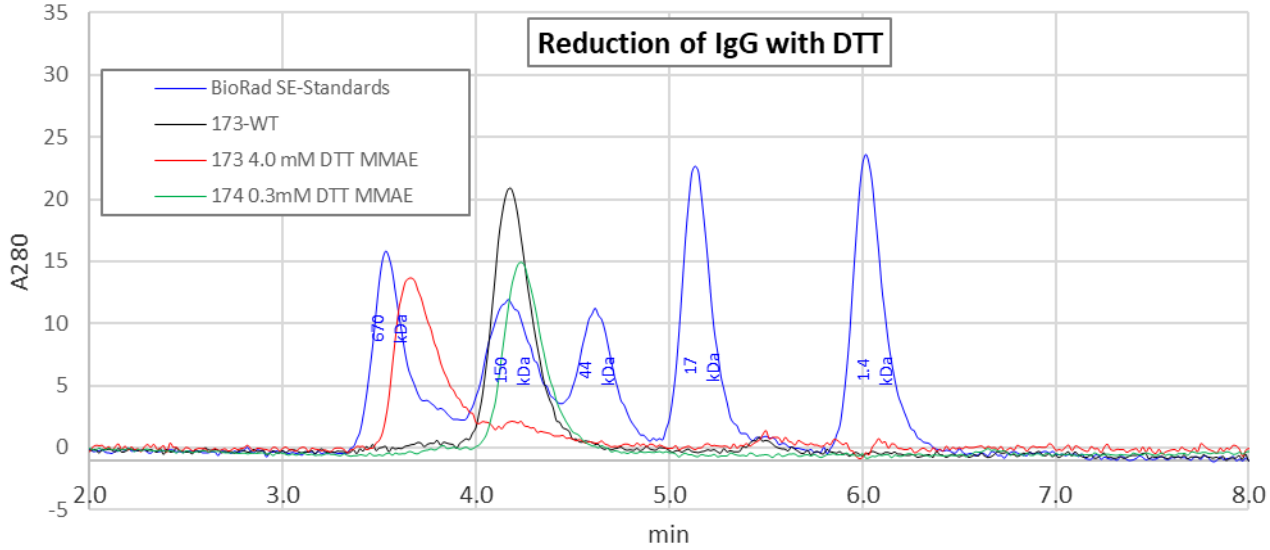
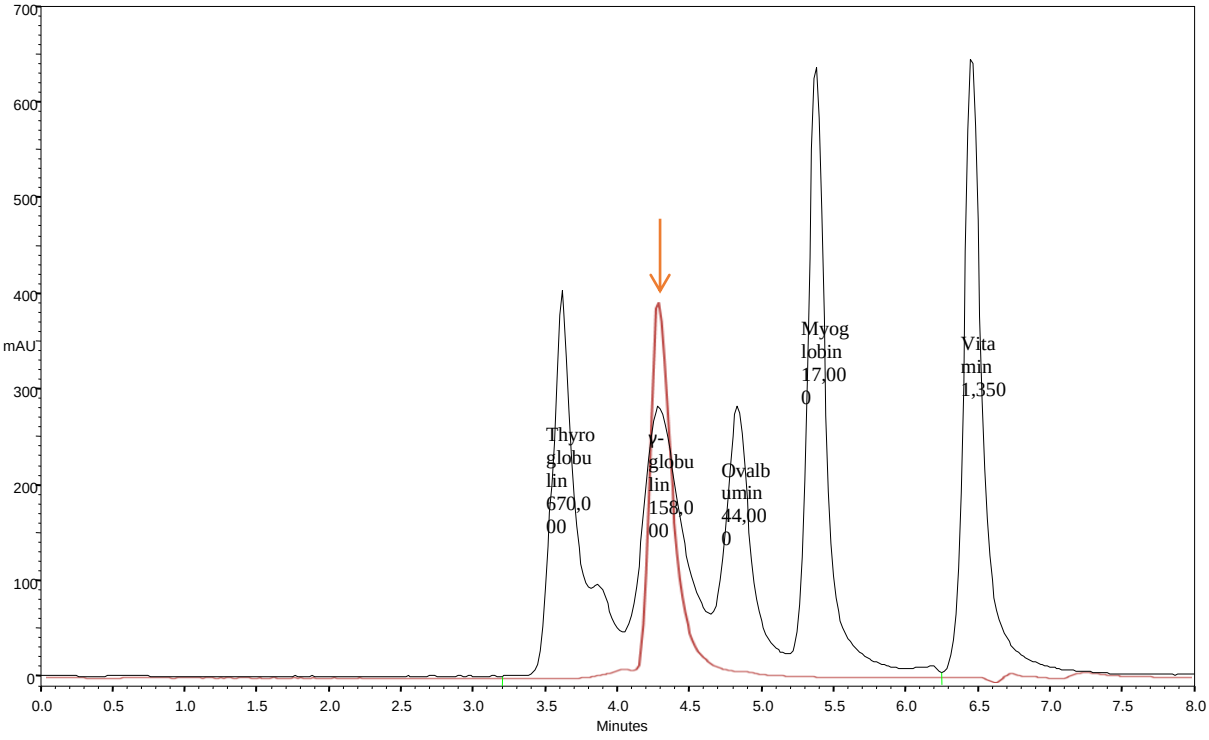


Figure 4: Size exclusion HPLC of MoAB173-MMAE conjugates. Blue Line – SE-HPLC markers from BioRad (MW is shown in kDa); Black line: MoAB-WT (Wilde Type) – Antibody before labeling; Red Line: Reduction of MoAB with 4mM DTT; Green line: Reduction of MoAB with 0.3mM DTT.

9

AXL-mAb173-MMAE stability Analysis.

mAb 173-MMAE was characterized in particular to determine if the chemical conjugation leads to antibody aggregate formation. mAb 173-MMAE does not undergo aggregation over 15 days of observation. Furthermore antibody remains stable for 15 days in serum as shown below (figure 5 below).



AXL-173-MMAE retains receptor internalization and receptor degradation:

MDA-MB-231 wild type and AXL knock out cells were plated in six well plates. Cells were treated with AXL-ADC mAb173-MMAE 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.

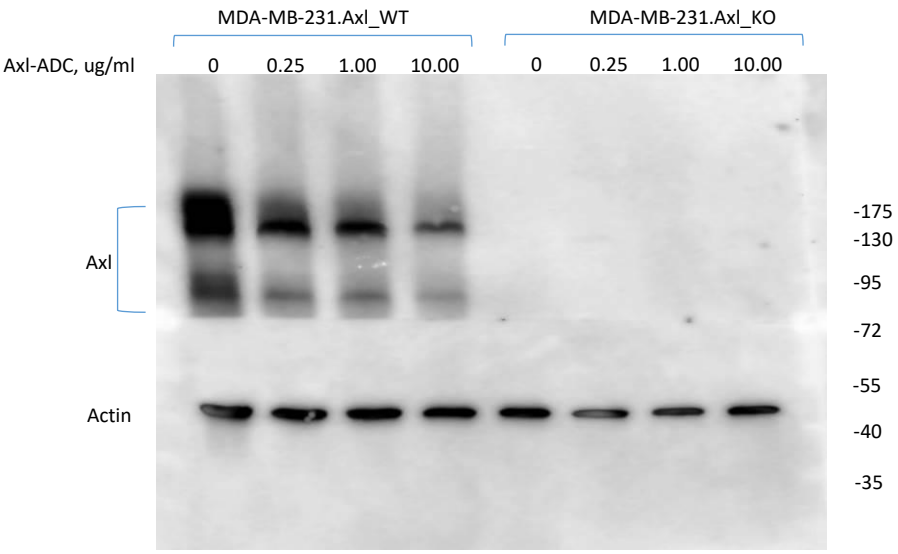


Figure legend: *MDA-MB-231 wild type (left panel) and AXL knock out (right panel), were treated with AXL-ADC and the level of AXL was measured in equal amount of protein loading shown by b-actin levels.*

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.

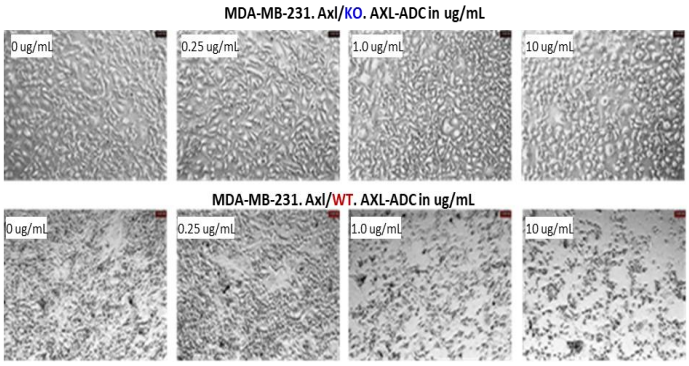


Figure legend: *MDA-MB-231 knock out (top panel) and AXL wild type (bottom panel), were treated with AXL-ADC and cell morphology was captured. A dose dependent loss of cell viability and cell density is observed only in wild type cell line consistent with the specificity of the AXL-ADC.*

AXL-MMAE retains cytotoxicity to AXL expressing MDA MB268 cells.

Antibody drug conjugate was studied for cytotoxicity to cells expressing AXL receptor tyrosine kinase or isogenic cell line with AXL knock out. Specificity of mAb 173-MMAE cytotoxicity to AXL expressing cells is confirmed. AXL negative cell line does not display significant cell toxicity. (figure 6)

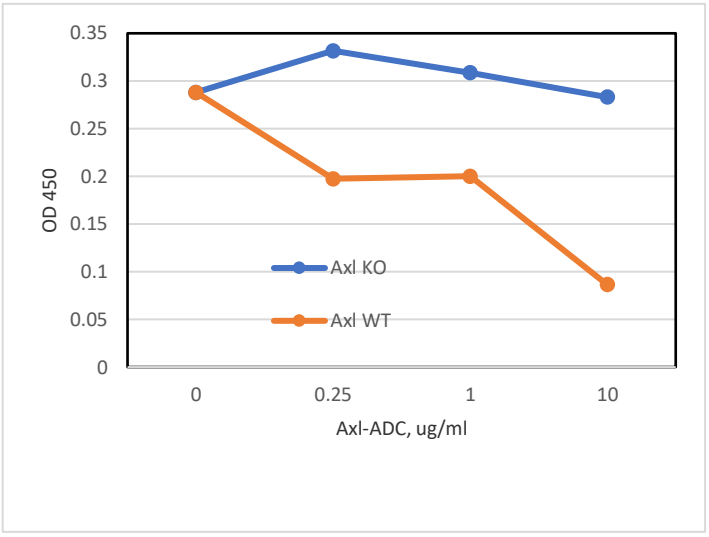


Figure 6: Cells were plated in 24 well plates, and treated with increasing concentrations of the AXL-ADC. Three days after treatment cell viability assay was performed with XTT (tetrazolium) /PMS (phenazine methosulfate). Absorbance was read at 450nm. AXL-ADC has potent cellular toxicity below 100 nM concentration.

How were the results disseminated to communities of interest?
Nothing to report

4) 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 generate larger batch of the AXL ADC. We will provide the mAb173-MMAE, unconjugated mAb-173 to Dr. We at Mayo clinic to conduct studies on organoids and in vivo efficacy studies.
We will conduct efficacy studies in organoids and in vivo studies for safety and efficacy.

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

Changes/Problems
The outbreak of COVID-19 severely hampered our progress. We will make best effort to accomplish the aims in a timely manner in the next year work plan.

Products
We generated Antibody-drug conjugates and characterized the purity.

Participants & Other Collaborating Organizations
We were closely with Dr. Xinyan Wu at the Mayo clinic

What individuals have worked on the project?

Name:	Parkash Gill
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-8083-9639
Nearest person month worked:	2.4
Contribution to Project:	Designed, and overseen the experiments
Funding Support:	None

Special Reporting Requirements
Nothing to report