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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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TECHNICAL REPORT

1. Introduction

Triple negative breast cancers (TNBC) represent 10-15% of all breast cancers. Patients with TNBC are treated with systemic chemotherapy or immune check point inhibitors combined with chemotherapy. The advancement in therapy has been significant but still remains limited to minority of the patients. There remains a significant need for novel targeted therapies for TNBC.

Identification of highly expressed cell surface proteins with or without driver mutations provide opportunity to define novel targets that promote cell replication and survival as novel targets. Our recent analysis for such proteins has led to the discovery of AXL receptor tyrosine kinase (RTK). AXL is overexpressed and activated in highly aggressive TNBC cells (Wu et al. 2015). We have 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 plan to generate antibody drug conjugate (ADC) by conjugating hMAb173 with cytotoxic agents such as mertansine, a highly potent microtubule inhibitor. We will also develop near-infrared (NIR) labeled Antibody conjugate to non-invasively monitor the ADC delivery and distribution *in vivo*. We will evaluate the therapeutic potential of this novel ADC in preclinical models in vitro and in vivo.

2. Keywords

Triple negative breast cancer TNBC
Receptor tyrosine kinase RTK
AXL
Phosphorylation of AXL pAXL
Antibody drug conjugates (ADC)

3. Accomplishments

◦**What were the major goals of the project?**

The major goals of the project in year 1 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

- 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 xenograft tissues and clinical samples.

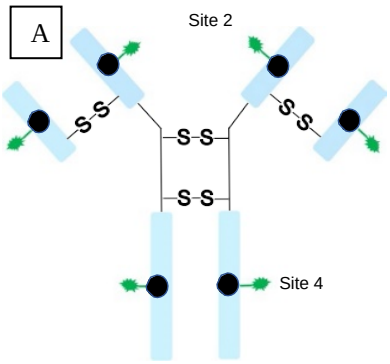
◦**What was accomplished under these goals?**

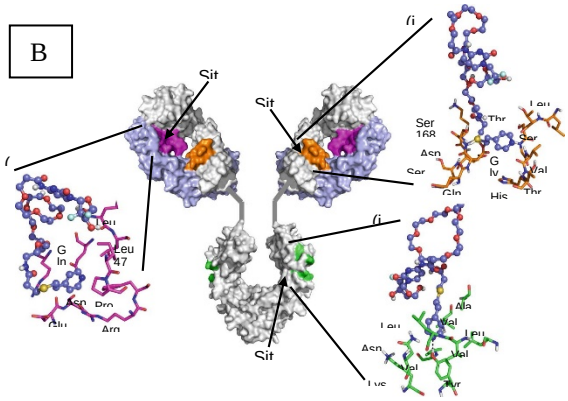
Specific aim 1: To develop humanized antibody-drug conjugate (ADC) that can effectively target the AXL membrane receptor tyrosine kinase

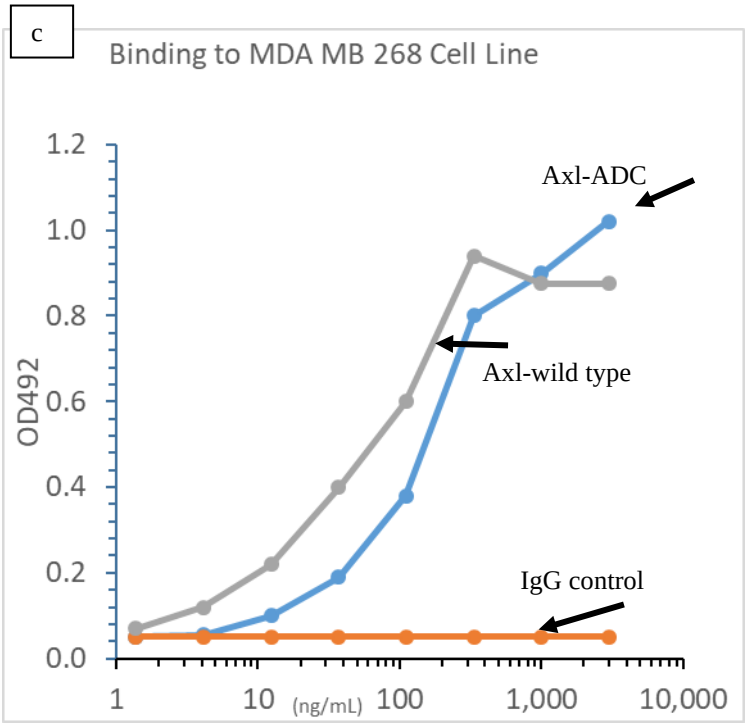
Major Task 1: To synthesize, characterize, and optimize novel ADCs for specificity, affinity, potency, solubility and stability

Subtask 1: To synthesize ADC using hMAb 173 antibodies containing microtubule inhibitor mertansine and bifunctional chemical linker.

We have developed antibody drug conjugate using bifunctional linker. One end of the linker binds to conserved residues in Fab and Fc. The other end of the linker is conjugated to cytotoxic agent, mertansine. Linker binding sites to the antibody are shown in Figure 1. There are six binding sites, four in Fab and 2 in Fc. Linker is an optimized derivative of mercaptoethylpyridine. The conjugate retains binding to the target tumor cell line (MDA MB 268)

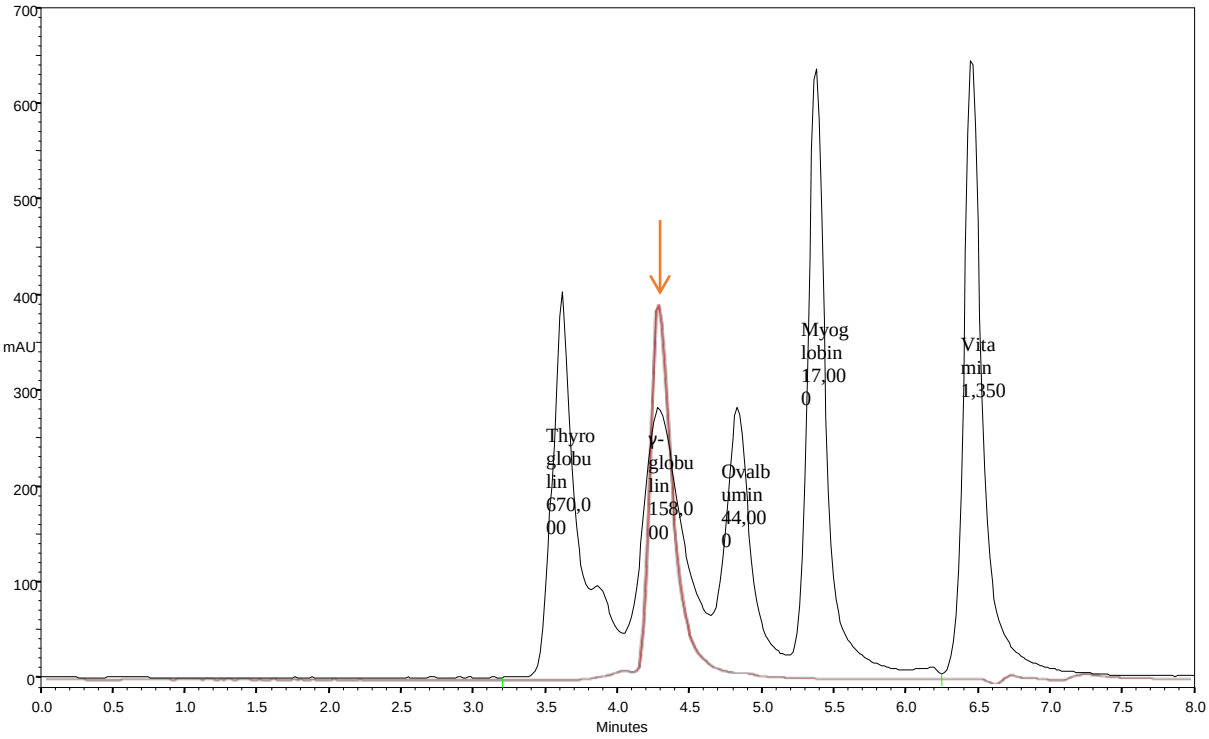






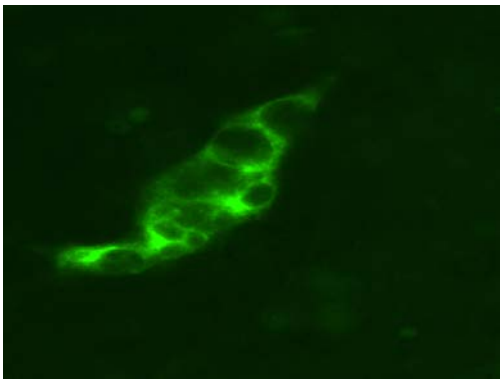
Subtask 2: To conjugate hMAb173 with a visible range fluorophore for the invitro microscopy studies and conjugate hMAb173 with 64Cu for in vivo imaging.
Conjugation of fluorophore has been conjugated in place of cytotoxic pay load. Protocol for the conduct of in vivo studies have been approved by the animal care (IACUC) committee. The approval letter is attached. We have not been able to conduct the in vivo studies due to restriction from the covid-19 pandemic. We are prepared to conduct the studies as soon as the in vivo studies are permitted.

Subtask 3: To characterize and optimize the ADC for solubility, stability, specificity, affinity, and potency by several chemical linkers and ratio of Ab to toxin ratio.
We have optimized and characterized the ADC. Ligand-payload binds to six sites in the antibody. The purified antibody-drug conjugate is homogenous as shown below (arrow indicates the antibody drug conjugate). There is no evidence of aggregation. Antibody-drug conjugate is stable in plasma for 15 days. Antibody drug conjugate retains binding to the target as shown in figure 1. Potency of the antibody is shown below.

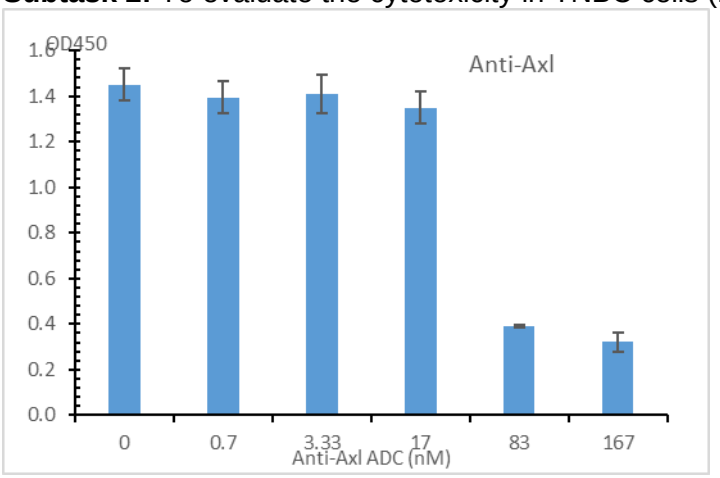


Subtask 5: To generate a batch of best performing ADC in the range of 500-1000 mg for the in vitro and in vivo testing in TNBC models.
Antibody drug conjugate has been generated for in vitro studies. Larger batch in the amount of 500-1000 mg will be produced once the in vivo studies are permitted by the university/IACUC.

Major Task2: To test ADCs in vitro in a panel of breast cancer cell lines and select lead compound for efficacy in AXL-expressing TNBC cells
Subtask 1: To test the cell membrane binding and internalization kinetics of the anti-AXL-ADC using confocal microscopy and FACS:
Antibody drug conjugate localizes to the tumor cell membrane detected by fluorescent labeled secondary antibody as seen below. No localization is observed in Axl negative cell lines, not shown.



Subtask 2: To evaluate the cytotoxicity in TNBC cells (MDA MB 268) treated by anti-AXL-ADC



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.

Subtask 3: To modify, if necessary linkage chemistry and modification ratios: We currently do not fee the need to modify the chemistry.
Specific Aim 2: to test the efficacy of the AXL-targeted ADC in preclinical animals
This work will be performed once we are able to conduct in vivo studies following COVID 19 restrictions are lifted.
Subtask 3: To set up TNBC patient-derived xenografts (PDX) in immune- deficient mouse models for the study of evaluating therapeutic efficacy of ADCs: PDX from triple negative breast cancer have been generated at Mayo Clinic. In vivo studies for efficacy will be performed when the access to the vivarium to conduct these studies are permitted. This work has been delated due to COVID-19.

◦**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?**
Next year, we will generate larger batch of the AXL ADC. We will conduct efficacy studies in organoids and in vivo studies for safety and efficacy.
We will optimize in vivo imaging of the tumor using fluorescent- antibody conjugate
We will study the downstream alterations including apoptosis, especially immune cell death of the AXL high TNBC.

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
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.

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

7. Participants & Other Collaborating Organizations

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, oversee and perform experiment
Funding Support:	None

Name:	Peter Conti
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.24
Contribution to Project:	Dr. Conti oversees the development of imaging reagents for in vivo studies
Funding Support:	None
Name:	Shuanglong Liu
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-0342-5325
Nearest person month worked:	1.2
Contribution to Project:	Dr. Shuanglong develops imaging reagents in vitro and in vivo studies.
Funding Support:	None
Name:	Binyun Ma
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	0000-0002-7745-1614
Nearest person month worked:	6.0 months
Contribution to Project:	Dr. Ma produces the antibody and in vitro studies
Funding Support:	None

8. **Special Reporting Requirements:**
Nothing to report

9. Appendices
Nothing to report