AWARD NUMBER: W81XWH-16-1-0461
BC151500

TITLE: "Novel Targeted Therapies for Inflammatory Breast Cancer"

PRINCIPAL INVESTIGATOR: Jose Silva

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, NY 10029

REPORT DATE: October 2018

TYPE OF REPORT: Annual Progress Report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and
should not be construed as an official Department of the Army position, policy or decision
unless so designated by other documentation.
"Novel Targeted Therapies for Inflammatory Breast Cancer"

E-mail: jose.silva@mssm.edu

Mount Sinai School of Medicine, 1 Gustave L. Levy Pl, New York, NY 10029

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

Approved for Public Release; Distribution Unlimited

Inflammatory breast cancer (IBC, ~5% of all breast cancers) is the most lethal form of breast cancer, presenting a 5-year survival rate that is less than half of the non-IBC patients. Remarkably, we have found that survival of IBC cells depends on histone deacetylase 6 (HDAC6) function. Here, first, we used these state-of-the-art system biology approaches to evaluate the response to ACY-1215 of a large series of breast cancer cells (sensitive and resistance) to identify critical hubs associated with resistance to HDAC6 inhibition. Through our studies we have found that STAT3 signaling is strongly upregulated in resistant cell lines upon inhibition HDAC6 suggesting an adaptative survival mechanism of the treated cells. Importantly STAT3 inhibitors (such as Ruxolitinib) already exist and can be easily translated to the clinic. Thus, our studies identified STAT3 inhibition as the prime candidate to synergistically interact with Ricolinostat. Additionally to STAT3, other pathways such as P38, TGF-β, and AKT has also emerged as MRs.

Synergistic treatment, STAT3, IBC treatment, HDAC6.
Table of Contents:

- Table of contents 1
- 1.Introduction 2
- 2.Keywords 2
- 3.Accomplishments 2-5
-4.Impact 5
- 5.Changes/problems 5-8
- 6.Products 8
- 7.Participants and other collaborating organizations 8
- 8.Special reporting requirements 8
**Progress Report 1st year**

**1-Introduction**

Inflammatory breast cancer (IBC, ~5% of all breast cancers) is the most lethal form of breast cancer, presenting a 5-year survival rate that is less than half of the non-IBC patients. Despite these facts, IBC remains poorly understood and systemic disease management relies exclusively on chemotherapy. Remarkably, we have found that survival of IBC cells depends on histone deacetylase 6 (HDAC6) function, whereas HDAC6 is mainly dispensable in non-IBCs\(^1\). Importantly, we have demonstrated that the leading HDAC6 inhibitor (Rocilinostat, Acetylon Inc.), which is being tested in clinical trials for other tumor types, inhibits the growth of IBC cells *in vitro* and *in vivo*. Our findings represent an exciting opportunity to develop novel targeted therapies for IBC patients.

**2-Keyterms**
Inflammatory breast cancer, targeted therapy, HDAC6 inhibitor, Ricolinostat, Ruxolitinib, P38, STAT3.

**3-Accomplishments**

During the past period of support we have:

- **Task 1)** Investigate HSP-90, DNAJB12 and MEAF6 as HDAC6 substrates that critically regulate the viability of IBC cells: Despite its canonical roles in protein in proteostasis HDAC6\(^2\) could act through other unrelated substrates. Through our collaboration with Acetylon, we have identified several novel putative substrates of HDAC6. HSP-90, DNAJB12 and MEAF6 were identified as the top candidates. Thus, we are utilizing a genetic screening strategy to investigate the involvement of these genes in the lethal phenotype induced by HDAC6 inhibitors\(^3,4\). We have generated a CRISPR sgRNA library containing 10 guides for each of the selected genes and an additional set of 10negative controls. This library has been used to perform genetic screens in vitro using the SUM-149 cell line. This screen validated that the three candidate genes selected scored positive for synthetic lethality in IBC.cells (Fig.1).

In order to confirm the involvement of these genes in the lethality induced by HDAC6 inhibition we performed rescue experiments. Here we overexpressed at high levels this genes in SUM-149 cells and compared the response of these lines to Ricolinostat. These studies showed that HSP-90 and MEAF6 overexpression induce resistance to HDAC6 inhibitor. To further understand the lethality induced by inhibition of HDAC6 and its downstream targets we performed expression profiling followed by GSEA analysis of SUM cells where HDAC6 or the downstream substrates was silenced. Remarkably, these studies revealed that modulation of the chromatin remodelers CREBBP and EP300 were two critical hubs upstream to the HDAC6 signaling (Fig.2).
- **Task 2.1a and 2.1b** Design and evaluation of combination therapy with HDAC6 inhibition for IBC treatment.

We have pioneered the development of computational and experimental methods for identifying important hub/Master Regulators (MRs) of cancer cells. These MRs represent critical gene and pathways that modulate both cell viability and response to treatments. Thus, these methods allow us to rationally select tumor targets as novel anticaner treatment as well as new therapeutic combinations. Here, first, we used these state-of-the-art system biology approaches to evaluate the response to ACY-1215 of a large series of breast cancer cells (sensitive and resistance) to identify critical hubs associated with resistance to HDAC6 inhibition.

Our studies have identified a series of breast cancer cell lines (~10%) that are sensitive (IC50>2.5uM) to HDAC6 inhibitors as well as a series (~50%) that are complete resistant (IC50>10 uM) to these treatments (Fig. 3), with the rest of the cell models somewhere in between. Interestingly, we found that HDAC6 function was a MR only for responsive cell lines and that these lines were enriched in hormone receptor positive and Her2 positive features (Fig. 3A). Importantly, similar results were found when primary breast cancer samples were evaluated (METABRIC data set).

![Fig. 3. MR analysis of HDAC6 response.](image)

Our analysis of the HDAC6 score in primary breast cancer and in cell line models have showed that HER2+ cells present high values suggesting an enhanced sensitivity to HDAC6 inhibitors. Thus, we also expanded our studies to a transgenic model where breast cancer is driven by oncogenic HER2 (FVB/N-Tg(MMTVneu)202Mul/J). We used this model to perform the same treatments described above (Fig 4).

Remarkably, a significant positive response was observed. Remarkably, these studies suggest that another breast cancer types, other than IBCs can benfice of HDAC6 inhibition therapy.

---

**Figure 4. Anticancer activity of ricolinostat in HER2 transgenic animals.**

Growth of tumors emerging in the FVB/N-Tg(MMTVneu)202Mul/J model under different treatment. ACY-1215 was administered five days per week as a single dose of 50mg/kg. Paclitaxel was administered twice per week as a single dose of 10mg/kg. The western blot illustrate the accumulation of Ac-tubulin in the tumors cells when the animals are dosed with Ricolinostat.
To investigate the mechanism of anticancer activity we performed a comparison of MR between the resistant and resistant cell lines we have found that STAT3 signaling is strongly upregulated in resistant cell lines upon inhibition HDAC6 suggesting an adaptative survival mechanism of the treated cells. Importantly stat3 inhibitors (such as ruxolitinib) already exist and can be easily translated to the clinic. Thus, our studies identified STAT3 inhibition as the prime candidate to synergistically interact with Ricolinostat. Additionally to STAT3, other pathways such as P38, TGF-β, and AKT has also emerged as MRs.

Our additional studies regarding MRs of IBC cells have also identified additional targets that enhance the activity of HDAC6 only in the presence of chemotherapy. In those studies, not covered by this grant, we have used a different computational approach to evaluate the response of IBC cells through time after exposure to Ricolinostat and chemotherapy. Interestingly those studies have suggested that proteasome inhibitors (Bortezomib), as well as mTOR inhibitors (Rapamycin), may have also synergistic anticancer activity when combined with HDAC6 inhibitors. These targets are not overlapping with the ones described above and may also expand our repertoire of putative novel targets. However, those will not be investigated under this grant funding which will be focused to the four inhibitors described above.

In order to evaluate the synergistic activity of some of the identified hubs with HDAC6 inhibitors we have evaluated combinatorial therapies using specific inhibitor and Ricolinostat (Fig.5). Multiple inhibitors for the identified pathways are available. Based on their reported selectivity, safety profile and anticancer activity we have selected:

- Ruxolitinib for STAT3 modulation
- LY2228820 for P38 modulation
- LY2109761 for TGF-Beta modulation
- AT7867 for AKT-modulation

Remarkably our studies have already validated the synergistic anticancer activity of some of the identified MRs (STAT3 and P38).

During the next period of support we will:

a) **Task1**: Evaluate the synergism anticancer activity when the HDAC6 substrates HSP-90, DNAJB12 and MEAF6 are silenced utilizing RNAi (loss-of-function). Additionally, we will also perform rescue experiments overexpressing c-DNAs to investigate whether this experimental modulation of the substrates reverses the growth inhibition mediated by Ricolinostat. These studies will be performed in vitro and in vivo.

b) **Task2**: Investigate the top synergistic candidates (STAT3 and P38) for in vivo validation. Here we will evaluate the growth inhibitory response of orthotopic xenograft mouse model of SUM-149 (when treated with small molecule inhibitors for the selected candidates plus Ricolinostat. Additionally, we will complete
our studies by comparing these data with the growth inhibitory response of orthotopic xenograft mouse model of SUM-149 when combinatorial therapeutic regiments containing chemotherapy plus the small molecule inhibitor for the selected candidate.

References

4-Impact
The data that we have generated during the last years have generated two main clinically relevant findings. First we have found that HDAC6 is a master Regulator of Hormone Receptor and HER2 positive breast cancer cells and seconds that resistance to the anticancer activity of HDAC6 inhibitors is associated with activation of the STAT3 and P38 pathway. This opens the exciting opportunity of combining STAT3 inhibitors with HDAC6 inhibitors.

5-Changes/Problems
All specific tasks are moving forward according to the original experimental plan and no changes are proposed for the next period of support (see tables below).
<table>
<thead>
<tr>
<th>Specific Aim 1 (specified in proposal)</th>
<th>Timeline</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specific Aim 1 tasks</strong></td>
<td>Months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 – Investigate Novel Putative Targets (HSP-90, DNAJB12 and MEAF6).</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c) Generation and validation of shRNA and c-DNA library targeting the three selected genes</td>
<td>0-6</td>
<td>Erin Nekritz and Dr. Silva (MSSM)</td>
<td></td>
<td>Completed</td>
</tr>
<tr>
<td>d) Screens in vitro using the shRNA and c-DNA libraries in the SUM-149 cell line.</td>
<td>6-12</td>
<td>Erin Nekritz and Dr. Silva (MSSM)</td>
<td></td>
<td>Completed</td>
</tr>
<tr>
<td>e) Validation of shRNA/cDNA screens hits by lethality rescue experiments in SUM-149, Sum-190 and IBC3 cell lines</td>
<td>12-30</td>
<td>Erin Nekritz and Dr. Silva (MSSM)</td>
<td></td>
<td>COMPLETED</td>
</tr>
<tr>
<td>f) Synergism studies for HSP-90, DNAJB12 and MEAF6 loss/gain-of-function studies combining two genes at a time (rescue experiments combination of two at a time)</td>
<td>24-36</td>
<td>Erin Nekritz and Dr. Silva (MSSM)</td>
<td></td>
<td>IN PROGRESS</td>
</tr>
<tr>
<td>g) In vitro, genome-wide level studies evaluating the consequence of inhibiting HSP-90, DNAJB12 and MEAF6 in IBC cells. These studies will consist of expression profiling followed by GSEA of SUM-149, Sum-190 and IBC3 cell lines after the three candidate genes have been knock-down by RNAi.</td>
<td>12-24</td>
<td>Erin Nekritz and Dr. Silva (MSSM)</td>
<td></td>
<td>COMPLETED</td>
</tr>
<tr>
<td>h) The studies from e) will be complemented by in vivo studies in the cell line SUM-149 (25 SCID mice will be used).</td>
<td>24-36</td>
<td>Erin Nekritz and Dr. Silva (MSSM)</td>
<td>Dr. Mundi and Dr. Califano (Columbia Un.)</td>
<td>IN PROGRESS</td>
</tr>
<tr>
<td><strong>Specific Aim 2 tasks</strong></td>
<td>Months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candidate based therapy using chemotherapy plus HDAC6 inhibition.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Dose-response studies with ACY-1215 in 45 breast cancer cell lines to identify sensitive vs resistant breast cancer cells.</td>
<td>0-3</td>
<td>Egin Nekritz and Dr. Silva (MSSM)</td>
<td></td>
<td>Completed</td>
</tr>
<tr>
<td>b) Generate expression profiles in the selected resistant and sensitive cell lines in dose-response experiment with ACY-1215.</td>
<td>3-9</td>
<td>and Dr. Silva (MSSM)</td>
<td>Completed</td>
<td></td>
</tr>
<tr>
<td>c) Identify Master Regulators (MRs) that define responsive vs resistant cell lines to ACY-1215 (candidate driven studies). (Phase-I)</td>
<td>9-15</td>
<td>Erin Nekritz and Dr. Silva (MSSM)</td>
<td>Completed</td>
<td></td>
</tr>
<tr>
<td>Evaluate combinatorial regimens HDAC6 and MRs inhibitors in preclinical in vitro. (Phase-II).</td>
<td>12-24</td>
<td>Erin Nekritz, Dr. Silva (MSSM)</td>
<td>COMPLETED</td>
<td></td>
</tr>
<tr>
<td>a) MR analysis normally yields a few dozen putative candidates. Here we will utilize compound inhibitors for five of the top-ranked candidates will be evaluated by dose-response experiment in vitro in SUM-149, SUM-190 and IBC-3 cell lines as well as the resistant cell lines previously identified.</td>
<td>20-36</td>
<td>Erin Nekritz, Dr. Silva (MSSM)</td>
<td>IN PROGRESS</td>
<td></td>
</tr>
<tr>
<td>Selected candidates are:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>- Ruxolitinib for STAT3 modulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>- LY2228820 for P38 modulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>- LY2109761 for TGF -Beta modulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>- AT7867 for AKT-modulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Validation of the top candidate from a) with an additional independent inhibitor in vitro in SUM-149, SUM-190 and IBC-3 cell lines.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evaluate combinatorial regimens HDAC6 and MRs inhibitors in preclinical in vivo. (Phase-III).</td>
<td>0-6</td>
<td>Erin Nekritz, Dr. Silva (MSSM)</td>
<td>Completed</td>
<td></td>
</tr>
<tr>
<td>a) Obtain ACURO approval for animal work</td>
<td>24-30</td>
<td>Erin Nekritz, Dr. Silva (MSSM)</td>
<td>IN</td>
<td></td>
</tr>
<tr>
<td>6- Products</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-Special Reporting Requirements</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3-30</th>
<th>Dr. Silva (MSSM)</th>
<th>Dr. Mundi and Dr. Califano (Columbia Un.)</th>
<th>PROGRESS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erin Nekritz, Dr. Silva (MSSM)</td>
<td>Dr. Mundi and Dr. Califano (Columbia Un.)</td>
<td>Not started yet</td>
<td></td>
</tr>
</tbody>
</table>