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14. ABSTRACT

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 of a large series of breast cancer cells to the HDAC6i ricolinostat 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. Additionally, our mechanistic studies have recently discovered that HDAC6 inhibition compromised cell viability through down-regulation of c-MYC. Our discoveries represent an exciting framework to transition the use of HDAC6i to the clinic.

15. SUBJECT TERMS
Synergistic treatment, breast cancer, HDAC6, c-MYC, STAT3.

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Final Progress Report

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 the 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-Keywords

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.

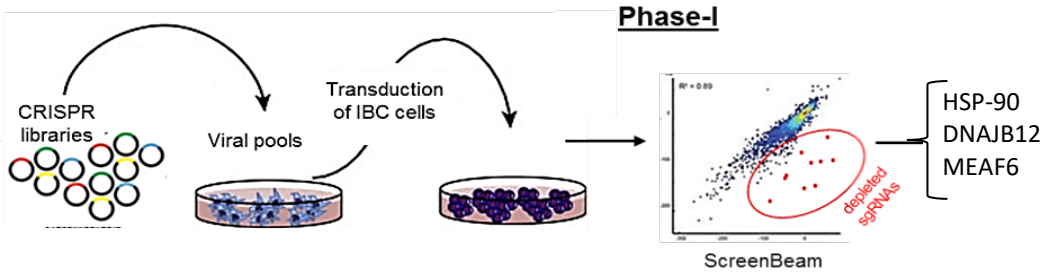


Fig 1. Representation of the completed phase-I of the genetic screen to Investigate HSP-90, DNAJB12 and MEAF6 as HDAC6 substrates that critically regulate the viability of IBC cells

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).

To complement our studies we performed proteomic profiling of IBC cells (SUM-149) treated with the HDAC6i ricolinoistat. This study confirmed the increase in the acetylation levels of HSP-90, DNAJB12, and MEAF6. Remarkably we also observed a strong signal in the acetylation of c-MYC at the residue K-148. This residue has been associated with the increase of the c-MYC turnover and degradation mediated by the proteasomal to be mediated the acetyltransferase EP300[5]. Interestingly, EP300 was also one of our top hits in the proteomics study. In this case, the acetylated residue was located in the autoinhibitory loop (AIL) which blocks the acetyltransferase activity of EP300 when hypoacetylated[6]. This generates a mechanistic model where HDAC6 inhibition acetylates multiple molecular substrates and this post-translational modification (PTM)

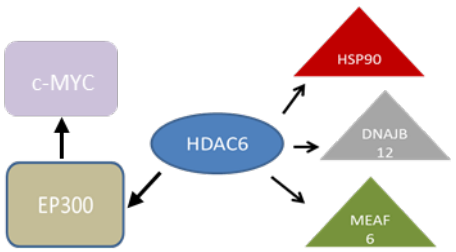


Fig 2. Models showing the downstream signaling of HDAC6.

impact the function of these substrates inducing loss of cell viability (Fig. 2). To confirm the involvement of these genes in the lethality induced by HDAC6 inhibition we performed rescue experiments. Here we overexpressed at high levels these genes in SUM-149 cells and compared the response of these lines to Ricolinostat. These studies showed that HSP-90, MEAF6, and C-MYC overexpression induce resistance to HDAC6 inhibitor (Fig. 3). Of all of them, overexpression of C-MYC presented the strongest effect.

Next, we evaluated the anticancer activity when the HDAC6 substrates HSP-90, MEAF6, and C-MYC are silenced utilizing RNAi (loss-of-function). Interestingly, RNAi-mediated inhibition of the individual genes compromised the fitness of the cells. As expected based on the overexpression data, knock-down of c-MYC induced the strongest reduction in cell growth (data not shown).

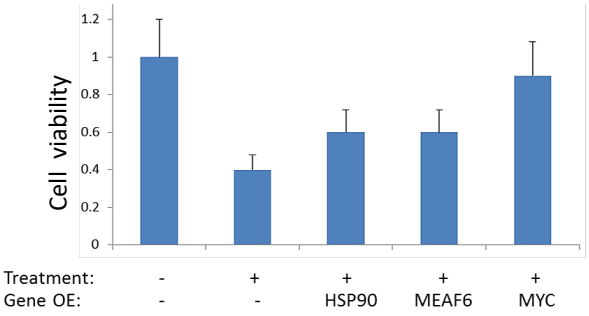


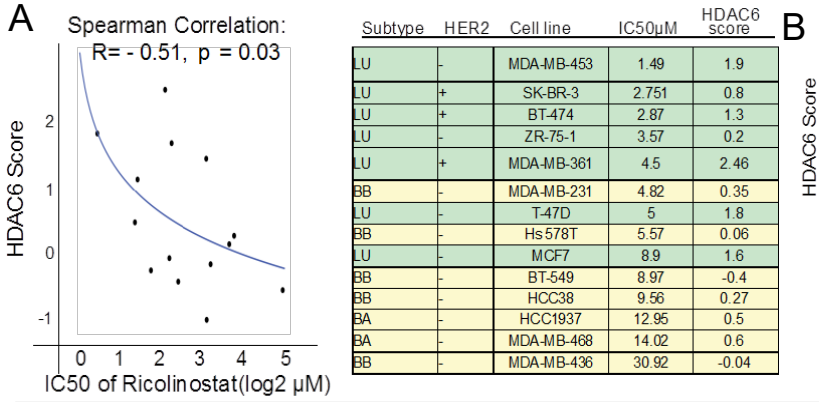
Fig 3. Overexpression of HDAC6 substrates induces resistance to HDAC6i. The bar graphic shows the effect in cell viability when SUM-149 cells that overexpress different HDAC6 substrates are treated with ricolinostat for 24 hours.

- *Task 2 Design and evaluation of combination therapy with HDAC6 inhibition for IBC treatment.*

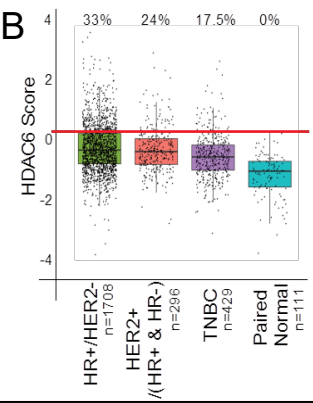
2.1-Candidate based therapy using chemotherapy plus HDAC6 inhibition.

We have pioneered the development of computational and experimental methods for identifying important hub/Master Regulators (MRs) of cancer cells. These MRs represent critical genes and pathways that modulate both cell viability and response to treatments[7,8]. Thus, these methods allow us to rationally select tumor targets as novel anticancer 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 resistant) 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. 4), whit 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. 4A). Importantly, similar results were found when primary breast cancer samples were evaluated METABRIC and TCGA data sets[9] (Fig 4B).



Subtype	HER2	Cell line	IC50µM	HDAC6 score
LU	-	MDA-MB-453	1.49	1.9
LU	+	SK-BR-3	2.751	0.8
LU	+	BT-474	2.87	1.3
LU	-	ZR-75-1	3.57	0.2
LU	+	MDA-MB-361	4.5	2.46
BB	-	MDA-MB-231	4.82	0.35
LU	-	T-47D	5	1.8
BB	-	Hs578T	5.57	0.06
LU	-	MCF7	8.9	1.6
BB	-	BT-549	8.97	-0.4
BB	-	HCC38	9.56	0.27
BA	-	HCC1937	12.95	0.5
BA	-	MDA-MB-468	14.02	0.6
BB	-	MDA-MB-436	30.92	-0.04



P-value	HR+/HER2-	HER2+ (HR+/-)	TNBC	Paired-Normal
HR+/HER2-	-			
HER2+ (HR+/-)	0.14	-		
TNBC	<2.2e-16	3.86e-05	-	
Paired-Normal	<2.22e-16	7.76e-12	1.63e-13	-

Fig. 4. MR analysis of HDAC6 response. Illustrative example of MR analysis (HDAC6 score) of cell lines and primary breast cancer samples. (A) The left panel shows the strong association between HDAC6-score and the response to the leading HDAC6 inhibitor Ricolinostat in cell lines. The right panel summarizes the result and the molecular subtype of the breast cancer lines analyzed. (B) The graphic show the HDAC6 score when the primary breast (METABRIC) samples are stratified based on molecular subtypes

To transition our studies to an *in vivo* context MDA-MB-453 (sensitive) and MDA-MB-436 (resistance) cells were injected as mouse xenografts in the flanks of γ-SCID mice and treated several therapeutic regiments, including ricolinostat and paclitaxel as single agents and ricolinostat plus paclitaxel in combination (combo). Confirming *in vitro* results, ricolinostat demonstrated significant antitumor growth activity as a single agent in MDA-MB-453 but not in MDA-MB-436 xenografts. WT-blot measuring Ac-α-Tubulin and Ac-His-K27 in tumor extracts from treated animals confirmed that the effect was associated with specific HDAC6 inhibition, with minimal effect on other class-I HDACs. Interestingly, while a small tumor mass was still detectable in the sensitive cells at the end of the treatment period with ricolinostat (1 month), combination treatment with paclitaxel-induced complete response (Figure 4). Intratumoral evaluation of the treated animals showed that the ricolinostat response in MDA-MB-453 tumors was associated with higher apoptosis levels (activated Caspase-3) while no such effect was seen in ricolinostat resistant MDA-MB-436 cells (data not shown).

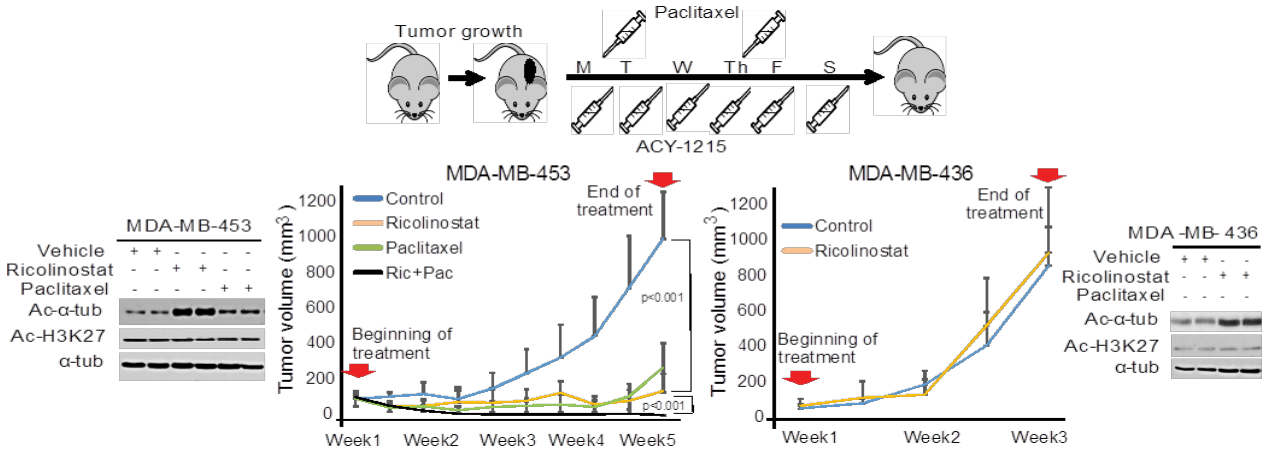
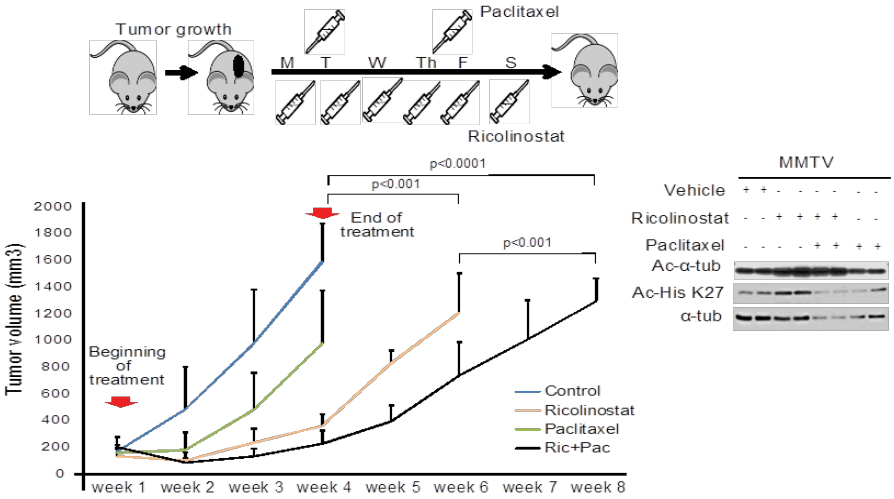


Fig. 4. Anticancer activity of ricolinostat in vivo. Treatment of ricolinostat sensitive (MDA-MB-453) and resistant (MDA-MB-436) growing as xenografts in SCID mice. The cartoon illustrates the treatment regimen. The combinatorial effect with paclitaxel was also investigated in sensitive cells. On resistant cells, only ricolinostat was used because no effect was observed with the combo in vitro. The western blots show the accumulation of acetylated tubulin in tumors treated with ricolinostat. Additionally, the absence of off-target effect in class-I HDACs is shown by the minimum changes seen on the levels of acetylated Histone-3-K27 (two independent tumor samples are shown).

Our analysis of the HDAC6 score in primary breast cancer and cell line models has shown that HER2+ cells present high values suggesting 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 5). Remarkably, a significant positive response was observed.

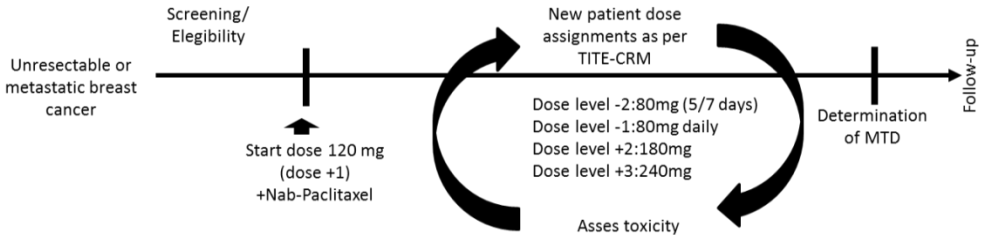


The data collected above revealed two

Figure 5. Anticancer activity of ricolinostat in HER2 transgenic animals. Growth of tumors emerging in the FVB/N-Tg(MMTVneu)202Mul/J model under difference treatment. ACY-1215 was administered five days per week as a single dose of 50mgr/kg. Paclitaxel was administered twice per week as a single dose of 10mgr/kg. The western blot illustrate the accumulation of Ac-tubulin in the tumors cells when the animals are dosed with Ricolinostat.

important findings. First, a group of breast cancers responds to anticancer regiments containing HDAC6 inhibitors. Second, we can identify these cancers by using our HDAC6 score as a predictive biomarker. Thus, in collaboration with Acetylon/Celgene (pharmaceutical company that manufactures Ricolinostat) and Dr. Kevin Kalinsky, we initiated an investigator-initiated phase-Ib clinical trial (NCT02632071).

The following data obtained from the clinical trial were collected using additional funds / resources that are not part of the DoD grant, but are included since they are complementary studies that help present the full scope of research.



- *Trial characteristics:*

1. Subjects have confirmed metastatic adenocarcinoma of the breast; all breast cancer subtypes are allowed.
2. Minimum number of prior treatments required given standard nab-paclitaxel dosing
   -If HER2 negative: none
   -If HER2 positive: two prior regimens containing HER2 targeted therapies in the inoperable locally advanced setting. No maximum number of prior treatments in the metastatic setting.
3. ECOG performance status of 0–1 and recovered from toxic effects of all prior therapy to grade 1 or less.
4. Women and men of all races and ethnic groups are eligible for this trial. Age >18 years.
5. Study design (adaptive phase-1b):
   -Ricolinostat/ACY1215 dosing 80mg, 120mg, 180mg, 240mg PO daily on days 1–21 in a 28-day cycle.
   -Nab-paclitaxel (Abraxane) at 100 mg/m2 30 minute IV infusion on days 1, 8, and 15 in a 28-day cycle.

-*Trial results:* While this is a non-randomized phase Ib trial with the main goal of determining the maximum tolerated dose (MTD) and evaluating the safety and tolerability of Ricolinostat/ACY1215 with nab-paclitaxel, it also has the secondary goal of investigating the correlation of the HDAC6 score with patient (pt) response. Between 3/2016 and 2/2018, 17 patients were accrued; 16 were evaluable. Of evaluable pts, the median age was 57.5 (range: 41-78), 3 were TNBCs, and 13 (HR)+/HER2-. The mean number of prior lines was 4 (range: 0-9). The first patient started at 120 mg, the second at 180 mg, and the rest at 240 mg. No dose-limiting toxicities were seen, and MTD was not reached. Grade III events related to nab-paclitaxel included neutropenia (n=1), peripheral neuropathy (n=1), and 1 grade IV neutropenia. Grade III syncope related to ACY-1215 was observed in 2 pts. In the 16 evaluable patients, the following were best responses: 2 partial response (PR), 10 stable diseases (SD), and 4 progressive diseases (PD: 2 TNBC, 2 HR+/HER2-). We were able to obtain tumor specimens in the form of paraffin sections (FFPE) with >50% in tumor content for 10 of the 16 evaluable patients (3 achieving PD and 7 showing SD or PR). RNA was obtained from these samples, subject to genome-wide RNA-seq and the expression profiles obtained were used to calculate the HDAC6 scores. Interestingly, when we compared the HDCA6-scores between patients showing PD (non-responders) and those with either SD or PR (responders), a statistically significant higher HDAC6 score was seen in responder patients (Fig. 6A). Importantly, patients with high HDAC6 score had a significantly improved progression-free survival (PFS) compared to low HDAC6 score. Patients with high HDAC6 score had a median PFS of 6.51 months (95% CI: 5.19-NA), which was significantly better (p = 8.0E-4, Figure 3E) than patients with low HDAC6 score who had a median PFS of 1.84 months (95% CI: 1.08-NA).

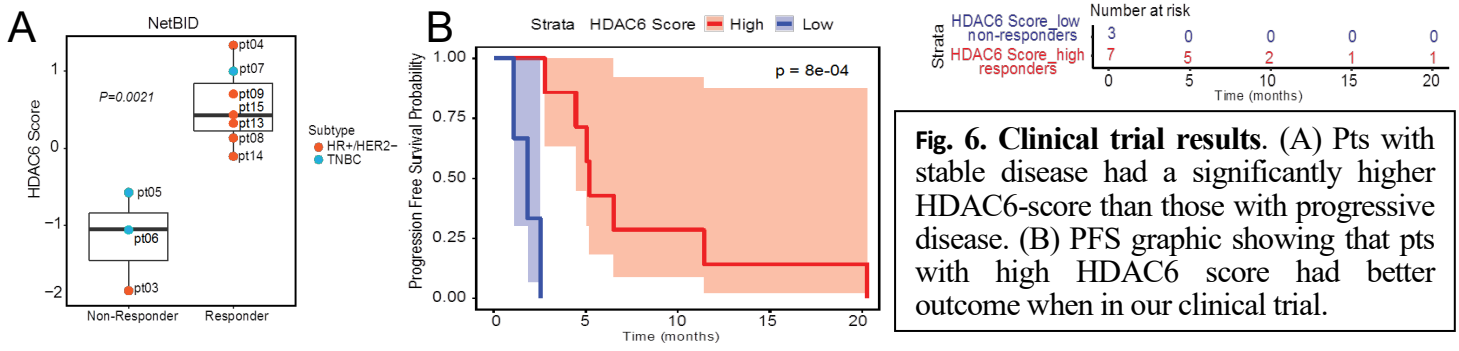


Fig. 6. Clinical trial results. (A) Pts with stable disease had a significantly higher HDAC6-score than those with progressive disease. (B) PFS graphic showing that pts with high HDAC6 score had better outcome when in our clinical trial.

-*Trial conclusions*: Ricolinostat 240mg daily is safe and tolerable with weekly nab-paclitaxel. Clinical activity has been observed, with the majority of pts demonstrating SD and PR. High HDAC6 score associates with longer PFS. HDAC6 score should be evaluated in larger trials as a predictor of response to HDAC6 inhibition.

To investigate the molecular mechanism involved in the response to ricolinostat we compared the transcriptional profiling of three ricolinostat sensitive cell lines as well as MMTV-Neu tumors treated with ricolinostat for 12 hours with control counterparts. Interestingly, gene set enrichment analysis (GSEA) revealed that hallmark signatures associated with c-MYC activity were the topmost downregulated signatures in cells treated with ricolinostat (Fig. 7A). Prompted by these results we compared the MYC expression at a protein level between a series of ricolinostat resistant and sensitive cell lines (Fig. 7B). This study revealed a strong reduction in MYC protein in ricolinostat sensitive cell lines while these were unchanged in resistant cells (Fig. 7B). Loss of Myc activity and protein expression was associated with a mild reduction in c-MYC mRNA in some cell lines but not in others (Figure S7D). As expected, the same specific loss of protein was observed when the cells were treated with other HDAC6 inhibitors or when HDAC6 was silenced by RNAi (Fig. 7C).
Genome-wide CRISPR screens have indicated that a large majority of breast cancer cells depend on MYC expression [10] and we validated this dependency on ricolinostat sensitive cells using RNAi (Figure 7D). Based on these data we conclude that the loss of MYC expression induced by ricolinostat treatment is involved in the loss of viability seen in ricolinostat sensitive cells.

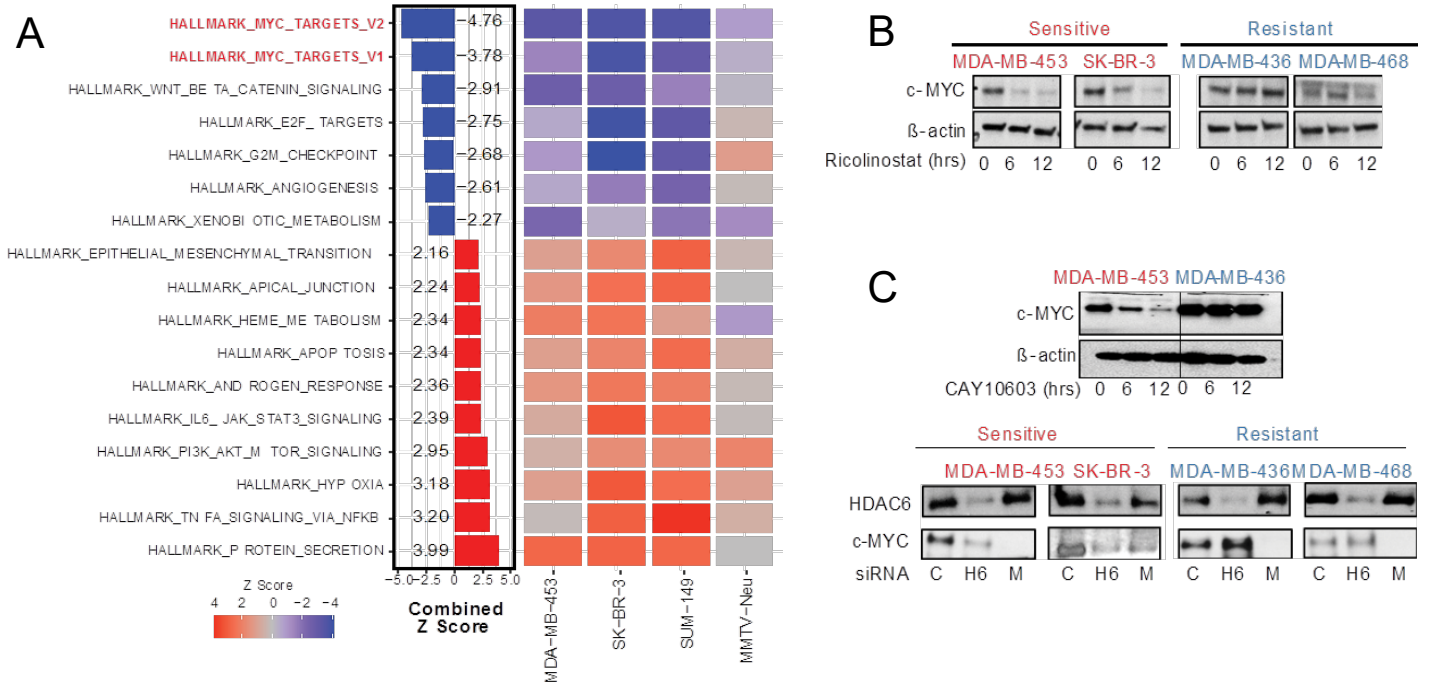


Fig. 7. Treatment with ricolinostat induces a robust reduction of Myc expression and activity. (A) Plot representing GSEA analysis of hallmark signatures during ricolinostat exposure in sensitive breast cancer cells. (B) The WT-blots show the reduction of c-MYC protein expression after ricolinostat treatment in sensitive but not resistant cancer cells. (C) WT-blots showing the reduction of c-MYC in sensitive cells lines when HDAC6 was inhibited by the small molecule inhibitor CAY10603 (upper panel) or by RNAi (lower panel) (c=non-targeting siRNA control, H6=siRNA targeting HDAC6, M= siRNA targeting c-MYC).

2.2-Evaluate combinatorial regimens HDAC6 and MRs inhibitors

To investigate the mechanism of anticancer activity we performed a comparison of MR between the resistant and resistant cell lines and found that STAT3 signaling is strongly upregulated in resistant cell lines upon

inhibition HDAC6 suggesting an adaptive 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.

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), may have also synergistic anticancer activity when combined with HDAC6 inhibitors.

To evaluate the synergistic activity of STAT3 inhibitors with HDAC6 inhibitors we have evaluated combinatorial therapies using the specific inhibitors Ruxolutinib and Ricolinostat (Fig.8). Importantly, a similar combinatorial effect was seen in vivo when these studies were repeated using xenografts in immunocompromised mice (data not shown).

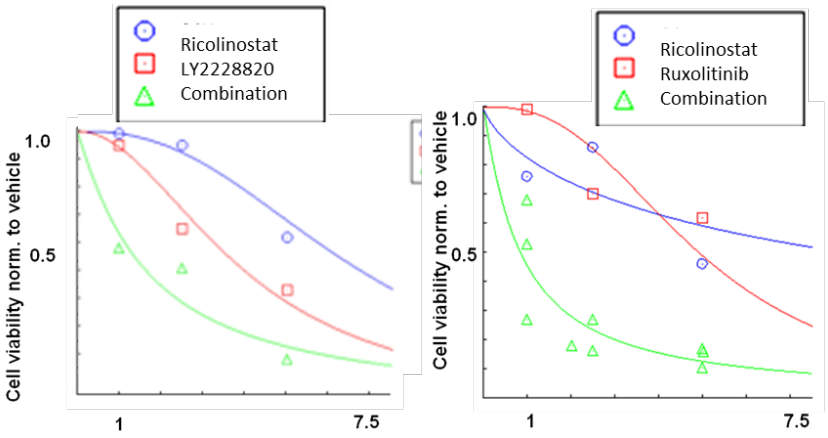


Figure 8. Synergistic activity of Ricolinostat and small molecule inhibitors targeting MR hubs. . The figure shows two examples of synergistic activity when Ricolinostat was combined with inhibitors targeting the identified MR in SUM-149 cells. .

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4-Impact

The data that we have generated during the period of support covered by this grant have generated several clinically relevant findings:

1- Approximately 30% of all BCs (enriched in HR+ and HER2+) present high HDAC6 activity

2- The HDAC6 score shows predictive biomarker potential in pre-clinical models of BC, including cell lines, xenografts, and transgenic mice, and it can identify BCs that respond to the leading HDAC6 inhibitor ricolinostat.

3- We also showed that ricolinostat has synergistic activity in BC cells when combined with standard chemotherapy.

4- In an open-label, single-arm phase Ib trial using ricolinostat in combination with nab-paclitaxel for patients with metastatic breast cancer, we show that the two agents can be safely combined, that clinical activity is identified specifically in patients with HR+/HER2- disease, and that the HDAC6 score was predictive of response.

5- The anticancer activity of ricolinostat was associated with its ability to reduce c-MYC protein expression and activity in sensitive cancer cells.

6-We have found that resistance to the anticancer activity of HDAC6 inhibitors is associated with activation of the STAT3 pathway. This opens the exciting opportunity of combining STAT3 inhibitors with HDAC6 inhibitors.

5-Changes/Problems

During the past period of support, we experienced significant issues related to the spread of the cov-19 pandemic (the laboratory was closed for several months. However, we were able to manage to finalize the proposed research.

6- Products

Some of our findings have been presented in the following international scientific meetings:

- **ASCO-2020**: Title "Phase IB trial of ACY-1215 (Ricolinostat) combined with nab-paclitaxel in metastatic breast cancer (MBC).
- **San Antonio Breast Cancer Symposium 2020**: Title "Phase IB trial of ACY-1215 (Ricolinostat) combined with nab-paclitaxel in metastatic breast cancer (MBC)."

Publications:

Network-based assessment of HDAC6 activity is highly predictive of pre-clinical and clinical responses to the HDAC6 inhibitor ricolinostat (currently under review in Cancer Cell and posted inmedRxiv 2020.04.23.20066928; doi: https://doi.org/10.1101/2020.04.23.20066928)

Tizita Z. Zeleke1,§, Qingfei Pan2,§, Cody Chiuzan3, Maika Onishi6, Mariano J. Alvarez4,8, Erin Honan3, Min Yang5, Pei Ling Chia1, Partha Mukhopadhyay1, Sean Kelly3, Ruby Wu3, Kathleen Fenn3, Meghna S. Trivedi3, Melissa Accordino3, Katherine D Crew3, Dawn L Hershman3, Matthew Maurer7, Simon Jones10, Andrea Califano4, Kevin Kalinsky3*§, Jiyang Yu2*§, Jose Silva9*§&.

7- Participants & other Collaborating Organizations

-Jose Silva: No Change.

-Prabhjot S. Mundi: No Change.

Specific Aim 1(specified in proposal)	Timeline	Site 1	Site 2	Status
Specific Aim 1 tasks	Months			
1 – Investigate Novel Putative Targets (HSP-90, DNAJB12 and MEAF6).				
a) Generation and validation of shRNA and c-DNA library targeting the three selected genes	0-6	Erin Nekritz and Dr. Silva (MSSM)		Completed
b) Screens in vitro using the shRNA and c-DNA libraries in the SUM-149 cell line.	6-12	Erin Nekritz and Dr. Silva (MSSM)		Completed
c) Validation of shRNA/cDNA screens hits by lethality rescue experiments in SUM-149, Sum-190 and IBC3 cell lines	12-30	Erin Nekritz and Dr. Silva (MSSM)		Completed
d) 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)	24-36	Erin Nekritz and Dr. Silva (MSSM)		Completed
e) 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.	12-24	Erin Nekritz and Dr. Silva (MSSM		Completed
f) The studies from e) will be complemented by in vivo studies in the cell line SUM-149 (25 SCID mice will be used).	24-36	Dr. Mukhopadhya y and Dr. Silva (MSSM)		Completed
Specific Aim 2 tasks	Months			
Candidate based therapy using chemotherapy plus HDAC6				

inhibition. a) Dose-response studies with ACY-1215 in 45 breast cancer cell lines to identify sensitive vs resistant breast cancer cells. b) Generate expression profiles in the selected resistant and sensitive cell lines in dose-response experiment with ACY-1215. c) Identify Master Regulators (MRs) that define responsive vs resistant cell lines to ACY-1215 (candidate driven studies). (Phase-I)	0-3 3-9 9-15	Erin Nekritz and Dr. Silva (MSSM Erin Nekritz and Dr. Silva (MSSM) Erin Nekritz and Dr. Silva (MSSM)	Dr. Mundi and Dr. Califano (Columbia Un.)	Completed Completed Completed
Evaluate combinatorial regimens HDAC6 and MRs inhibitors in preclinical in vitro. (Phase-II). 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. Selected candidates are: <u>-Ruxolitinib for STAT3 modulation</u> <u>- LY2228820 for P38 modulation</u> <u>-LY2109761 for TGF-Beta modulation</u> <u>- AT7867 for AKT-modulation</u> Validation of the top candidate from a) with an additional independent inhibitor in vitro in SUM-149, SUM-190 and IBC-3 cell lines.	12-24 20-36	Erin Nekritz , Dr. Silva (MSSM) Erin Nekritz and Dr. Silva (MSSM)	Dr. Mundi and Dr. Califano (Columbia Un.)	Completed Completed
Evaluate combinatorial regimens HDAC6 and MRs inhibitors in				

preclinical in vivo. (Phase-III). a) Obtain ACURO approval for animal work b) Select the top candidate for in vivo validation. Evaluation of growth inhibitory response of orthotopic xenograft mouse model of SUM-149 (we will use 10 SCID mice) when treated with small molecule inhibitor for the selected candidate. c) Evaluation of growth inhibitory response of orthotopic xenograft mouse model of SUM-149 (we will use 15 SCID mice) when combinatorial therapeutic regiments containing chemotherapy plus the small molecule inhibitor for the selected candidate.	0-6 24-30 30-36	 Erin Nekritz , Dr. Silva (MSSM) Dr. Mukhopadhyay and Dr. Silva (MSSM) Dr. Mukhopadhyay and Dr. Silva (MSSM)	 Dr. Mundi and Dr. Califano (Columbia	 Completed Completed Completed

8-Spetial Reporting Requirements

N/A