Children's Hospital of Philadelphia

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March 6, 2023

Ry Guy Foundation
398 Sherry Drive
Atlantic Beach, FL 32233

Dear Baker Family and Members of the Ry Guy Foundation:

Thank you so much for supporting our work with this generous grant from the Ry Guy foundation. Please find attached the final report for your foundation. I am asking that we get one last extension to complete the sequencing for this project.

Overall, this project has been a tremendous success. Years ago, it served as the inspiration to put down the ideas that I thought we needed to "punch out" Ewing sarcoma. There were substantial delays due to the move to CHOP and COVID-19 and the project evolved relative to what was originally proposed. However, I believe we have achieved the aims of the proposal. Further, these results are extremely timely and will drive the direction of our work. The most exciting thing we have seen in our work has been some exciting patient responses in our phase I trial called SARC037. However, we have not quite "punched out" the disease. While we have seen some impressive clinical responses, not every patient has responded. The most likely explanation for the differences in response is the heterogeneity in the target that **was the fundamental basis of this Ry Guy study**. Therefore, the completion of this project is extremely timely and will guide our approach moving forward. Further, the sequencing that we have completed in this study will allow us to better interpret sequencing of patient samples from our clinical study. Therefore, I am very grateful for your investment and believe we have returned meaningful results from that investment.

Please find enclosed a final report of the study objectives and remaining work. Additionally, I have included the original proposal. I am happy to schedule a call if you want to discuss the results and ask questions. The results are criticaly important for our lab.

Please don't hesitate to contact me with any questions.

Sincerely,

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Overview: Thank you very much for the support of the RyGuy foundation for this work. In this final report, I have included a summary of the work. The project evolved quite a bit based on ongoing laboratory observations. In addition, there were substantial delays due to the move to CHOP and the COVID-19 pandemic. Nevertheless, we have achieved the goals of the original proposal and are preparing the manuscript based on this support from the RyGuy foundation. In this summary, I will provide an explanation of the overall goals of the proposal and a description for how and why the aims evolved. I will cite notable data that led to this evolution in approach. We do need an extension to finish the proposed sequencing to complete the project. However, we will acknowledge the support of the RyGuy foundation and provide a copy of the manuscript when complete. Overall, while we did not complete exactly what we proposed, this support provided a catalyst for this paper. This paper will guide our direction of therapeutic development for years to come.

Background: Ewing sarcoma is absolutely defined by the EWS-FLI1 transcription factor. This is the only recurrent somatic mutation occurring in 20% or more of patients. In addition, it has been known for 25 Years that this tumor absolutely depends on EWS-FLI1 for cell survival. Unfortunately, although the tumor is known to be dependent on this oncogene, EWS-FLI1 is considered an "undruggable target". Nevertheless, there is a critical need for effective therapies for patients with Ewing sarcoma. Indeed, the objective response rate (the number of patients with tumors who shrunk on a phase II study) for the last 800+ patients treated on 62 clinical trials was 8.3%. This poor response rate is despite the fact that the tumor is driven by a well-defined and well-known driver mutation, EWS-FLI1. Therefore, our fundamental goal in the laboratory has been and continues to be to directly EWS-FLI1 to drive patient responses in the clinic. The goal of this proposed study was to generate data that would better inform the therapeutic targeting of EWS-FLI1

The goal of our work, and this proposal, is to develop an effective therapeutic approach focused on the defining molecular lesion of the tumor. In this proposal, we sought to better understand the therapeutic implications of transcriptional diversity we have seen in our preclinical data. We have found that even though EWS-FLI1 is the only recurrent mutation of the tumor, there is a substantial amount of heterogeneity in the transcriptional program of EWS-FLI1 from one cell line to another. Although it is the same fusion protein, it behaves differently in different cellular contexts. In this proposal, we seek to better understand the mechanism of this diversity and use a panel of small molecule inhibitors to understand the therapeutic implications of this transcriptional diversity.

Project goal: The goal of this study is to determine how the transcriptional heterogeneity of EWS-FLI1 impacts therapeutic development that is focused on this mutation.

Approach: The approach in this proposal is to carefully determine the differences in the EWS-FLI1 transcriptional program across 5-6 different models and determine how compounds identified as EWS-FLI1 inhibitors work in the different cellular contexts. We seek to understand if individual inhibitors alter the gene expression program in a manner that reflects the heterogeneity or if different compounds need to be paired to specific EWS-FLI1 transcriptional signatures.

Data generated throughout the course of the proposal that modified the goals:

The goal of this RyGuy foundation grant was to better understand exactly how EWS-FLI1 alters gene expression **from the standpoint of small molecules that target EWS-FLI1**. We proposed:

(1) A comprehensive analysis of the EWS-FLI1 transcriptome and molecular mechanisms responsible for the transcriptional diversity.
(2) Better understanding of how compounds perturb this transcriptome using a panel of small molecules.

Completed work:

(1) Confirmation of heterogeneity: In the first part of this project, we wanted to confirm the findings of transcriptional heterogeneity which served as the basis of the proposal. So, we carefully silenced EWS-FLI1 in 6 cell lines and indeed confirmed the heterogeneity. Network profiling revealed marked differences from cell line to cell line suggesting a therapeutic opportunity to optimize suppression for a given model. Next, the goal was to determine the molecular reasons for the transcriptional heterogeneity.

Data generated for this proposal: In our first update, we proposed using 3D-chromatin capture to determine if chromatin looping accounts for the heterogeneity. However, we determined that this would not yield usable information unless we knew exactly where EWS-FLI1 was bound in the genome. In order to do this and exclude artifact from CHIPseq, we developed the CUT and Tag assay for EWS-FLI1. This took about 1.5 years but has now been submitted for sequencing. Further, we reasoned that we needed to exclude transcriptional differences from cell to cell before interrogating looped chromatin. We needed to determine if there are cell population differences for a given cell line to account for differences in the transcriptional program. Since the RNA sequencing represents the average signal of a given gene across all of the cells in the entire population, it is possible that subsets of cells with a different transcriptional signal account for the marked heterogeneity. This meant we needed to develop single cell RNA sequencing which took considerable optimization but also has been submitted for sequencing in two different cell lines. These two projects would have exhausted the budget for the proposal, so we are supplementing both this sequencing and the sequencing below with internal funds to complete the proposal.

(2) To develop a panel of chemical probes of EWS-FLI1 inhibitors- In this sub-aim (aim 1 of the original proposal) we seek to see how different compounds perturb the transcriptional signature of EWS-FLI1 in different contexts. We therefore completed a PCR screen of all of our candidate compounds (see figure 4 below in the original proposal) at 1X and 2X the NR0B1-luciferase IC50. Unfortunately, there was marginal suppression. In the first update, we reasoned that the PCR most likely needs to be more like 5X to 10X the luciferase. So, we optimized the PCR for one of our established inhibitors, mithramycin. We treated all six cell lines with the optimized exposure of mithramycin and performed RNA sequencing. The analysis of this data is pending and will be included in the proposal. However, this made us recognize that probably the most important next question for this proposal is to determine how all of our EWS-FLI1 inhibitors **that are in the clinic (or soon to be translated to the clinic)** impact the EWS-FLI1 transcriptome across all six cell lines. Therefore, we optimized drug exposures, RNA collection, confirmed silencing at similar IC50 ratios by qPCR, and submitted for sequencing.

Remaining work: All of the drug treatments have been collected and submitted for sequencing. We do need a one last project extension to complete the sequencing. We are working through the analysis now, and will report the results at the AACR in two months and in a manuscript to be submitted by the summer. In addition, we have established some cellular phenotypes to make genotype-phenotype correlations which will be part of the study.

(3) Confirm the Specificity of Lead Compounds Using a Novel Next-Generation Sequencing Assay: We developed this novel assay and used it to sequence some of our compounds identified as EWS-FLI1 inhibitors. Unfortunately, the assay simply did not perform well, and was not worth the time or the cost to develop. Since the cost of RNA sequencing had come down substantially, we have determined that the most cost-effective way to complete the proposal is to simply perform bulk RNA sequencing. Therefore, all analyses will be based on bulk RNA sequencing which will provide additional information than just the impact on the EWS-FLI1 transcriptome.

Summary: In summary, this is an exciting project that seeks to understand and directly target the transcriptional heterogeneity of EWS-FLI1. Although the proposal ended up not being exactly what we proposed because the gene signature assay was not necessary and the panel of compounds did not achieve the suppression needed for sequencing, we have maximized the impact of the funds. We will determine (and report) how different compounds that we are moving to the clinic impact this heterogeneity across all six cell lines. These results will provide the basis for our therapeutic approach moving forward. Critically, our clinical trial has demonstrated striking tumor regressions in some (but not all) patients. The likely explanation for the disparity in response is the transcriptional heterogeneity that we have observed. Therefore, the results of this study will provide the basis of our approach moving forward. We will seek to broaden the impact and hopefully

increase the percentage of patients responding to our drug by establishing more effective combination therapies. We have coupled these findings with support from another foundation to try to identify these combination therapies. Nevertheless, these findings will provide the fundamental basis for our approach and therefore will be highly impactful. Thank you for supporting this very important study.

Please see below for the original proposal.

Lay Summary:

The goal of this proposal is to take the next step towards developing an **effective molecularly targeted** therapy for Ewing sarcoma. Many tumors have gene(s) that they absolutely depend on to sustain cell growth. The principle of molecularly targeted is to target the exact genes the tumor depends on for survival with small molecules to use the strength of the cancer as its weakness. The number of tumors that respond to therapies of this type is growing and the guiding principle that has emerged is that these therapies only work if they target the right gene in the right tumor, also known as the dominant oncogene.

It has been known for more than 25 years that Ewing sarcoma cells are absolutely dependent on the continued activity of the EWS-FLI1 transcription factor for cell survival.[1] EWS-FLI1 is the only recurrent mutation and is responsible for the continued growth of the tumor.[2-5] It has been shown in multiple independent studies that turning off EWS-FLI1 using laboratory techniques leads to the inability of the cells to continue to grow. Unfortunately, EWS-FLI1 is a type of gene called a transcription factor that is a challenging drug target and considered by many to be "undruggable". Nevertheless, EWS-FLI1 is the dominant oncogene of this tumor and therefore it is likely that a small molecule that is able to block EWS-FLI1 will be effective in the clinic.

The goal of our laboratory is to identify and clinically translate small molecules that target the EWS-FLI1 transcription factor to impact patients with Ewing sarcoma. We have a number of compounds in various stages of clinical translation (see Fig. 1). The goal of this proposal is to identify a series of compounds that will serve as the next generation of clinical lead compounds (discovery in Fig 1). These compounds will be prioritized for clinical development. In addition, we will use these compounds as "chemical probes" to determine what is the most effective way to target EWS-FLI1. In order to accomplish these goals, we need a series of compounds that are highly specific inhibitors of EWS-FLI1. To identify this panel of compounds, we will use data generated through a series of high-throughput screens to identify lead compounds.[6,7] We will confirm the specificity of these compounds using a novel next-generation sequencing assay that will be funded by this mechanism. Subsequent work will focus on understanding exactly how these drugs target EWS-FLI1; efforts that will last for several years. Nevertheless, by including a library of FDA approved compounds in the pipeline, we will have a number of novel compounds and hits that could be repurposed and translated alone or in combination to patients.

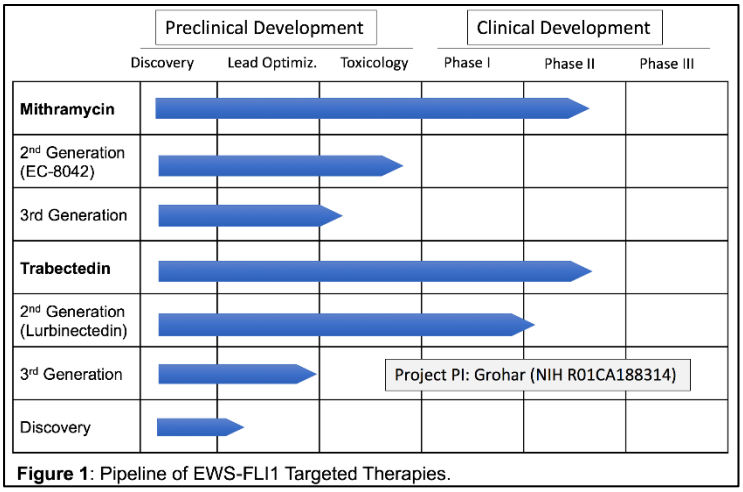

Figure 1: Pipeline of EWS-FLI1 Targeted Therapies.

Aim 1: To Establish a Pipeline of EWS-FLI1 Inhibitors and Chemical Probes

Aim 2: Confirm the Specificity of Lead Compounds Using a Novel Next-Generation Sequencing Assay

Background:

The goal of this proposal is to establish a pipeline of EWS-FLI1 inhibitors and at the same time identify a series of compounds to be used as chemical probes to discover the therapeutic vulnerabilities of the EWS-FLI1 transcription factor. The **unifying hypothesis** is that the major challenge to developing effective small molecule inhibitors of EWS-FLI1 is the network of protein interactions EWS-FLI1 uses to direct the transcriptional program.[8] Since this network of interactors is cell context specific, this leads to considerable transcriptional heterogeneity across different tumors. Indeed, recent preliminary data confirms widely variable changes in gene expression that occur across cell lines when EWS-FLI1 is silenced. While very specific targets of EWS-FLI1 exist that are well-established and common across several cell lines, such as NR0B1 (see below), a subset of genes show large magnitude expression changes that are quite unique to individual cell lines but specific for EWS-FLI1 (Fig. 2). In this proposal, we will exploit these unique gene expression changes as an assay to identify compounds that are highly specific for EWS-FLI1. We will establish a 500-gene next generation sequencing assay of EWS-FLI1 target genes that includes targets that are common across all cell lines and ones that are unique to individual cell lines. We will confirm the specificity of the assay for EWS-FLI1 using siRNA silencing and identify compounds that exactly mirror the gene expression changes induced by siRNA silencing of EWS-FLI1. By including targets that are cell-line specific, we will identify compounds that

reverse both the common targets and the unique targets **ONLY** in the cell line in which they are known to change.

The transcriptional heterogeneity of EWS-FLI1: Despite the known dependence of Ewing sarcoma on EWS-FLI1, the exact identity of all of the down-stream targets of EWS-FLI1 is not known. There are a number of downstream targets that have been previously identified. A small number have been thoroughly characterized by traditional methods including, knock-down rescue approaches, CHIP and mutational studies. These targets are well-established and include genes like NR0B1, FOXO1, LOX, NKX2.2, EZH2, PHLDA1, EGR2.[9-14] We will refer to these as the "well-established" targets.

Many more targets have been identified by innovative studies that utilize shRNA silencing of EWS-FLI1 and genome-wide technologies to characterize common sets of gene expression changes across cell lines and tumor samples.[15-21] These studies arrive at a gene signature of EWS-FLI1 where the confidence in the targets stems from the identification of the same target in different cell lines. However, by definition, this means that these studies do not characterize gene expression changes unique to individual cell lines. These cell-line specific targets are in fact more numerous than the common targets and likely to be equally important to the biology of that tumor. Indeed, perhaps the best EWS-FLI1 gene signature characterized to date was identified by Steve Lessnick.[16] In this study, he used shRNA knockdown of EWS-FLI1 with "rescue" with cDNA, coupled to microarray and RNAseq analysis that included other investigators data sets of both cell lines in culture, Ewing sarcoma primary tumors and normal tissues. Dr. Lessnick noted surprisingly few similarities across these data sets (*Lessnick, SL, personal communication)*. Therefore, in order to derive a "gold standard" gene set, he needed to employ an innovative bio-informatics approach and a meta-analysis.[16] Therefore, while this approach and others identified gene signatures that have offered much insight into the biology of the tumor and have driven many important discoveries, there is clearly a need to understand the contribution of the unique gene expression changes to the biology of the tumor; particularly when developing EWS-FLI1 directed therapeutics.

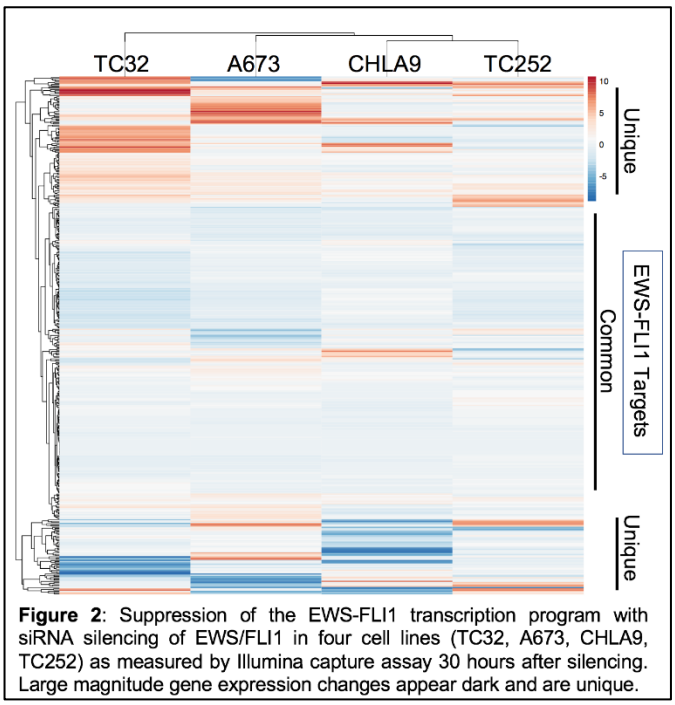

Figure 2: Suppression of the EWS-FLI1 transcription program with siRNA silencing of EWS/FLI1 in four cell lines (TC32, A673, CHLA9, TC252) as measured by Illumina capture assay 30 hours after silencing. Large magnitude gene expression changes appear dark and are unique.

We have recently utilized next generation RNA sequencing with deep coverage to characterize the transcriptome of EWS-FLI1 in 5 cell lines. We used siRNA instead of shRNA in order to better control kinetics and selected the earliest time point that showed a reduction in EWS-FLI1 protein in order to limit the contribution of indirect targets. We found tremendous heterogeneity among the 5 cell lines. Although, we identified previously described common targets of EWS-FLI1, many of these exhibited small magnitude gene expression changes with log fold changes (LogFC) of <2. In contrast, many more genes that exhibited Log FC >2 were unique to individual cell lines and therefore only 13 EWS-FLI1 common repressed genes and 4 EWS-FLI1 induced genes changed in expression (LogFC >2) across all 5 cell lines (Fig. 3). Furthermore, even when we restricted the analysis to the three P53 wild-type cell lines, we only found 54 common induced targets and 118 common repressed targets that exhibited a LogFC >2. In contrast, silencing of EWS-FLI1 in the TC32 cell line alone led to the induction of 732 EWS-FLI1 repressed targets and repression of 165 induced targets with a LogFC >2 or more.

The biology of transcription drives the heterogeneity: Transcriptional heterogeneity reflects the biology of eukaryotic transcription and is the major challenge to developing a transcription factor directed therapy.[22-25] Like most transcription factors, EWS-FLI1 interacts with a number of proteins to activate RNAPII, remodel chromatin, block differentiation and establish the oncogenic program.[8,26-28] These networks of interactions are highly context-dependent and subject to widely variable differences in chromatin structure and cofactor expression patterns. Therefore, from a drug development standpoint, it is unclear where to dock a small molecule to reverse activity or even if there is a strong enough protein-protein interaction such as between

BAX and BCL2 suitable for a small molecule inhibitor.[29] Indeed, it is not clear if a **single** small molecule is sufficient to completely inhibit EWS-FLI1 to drive a specific therapeutic endpoint.

In this proposal, we contend that all of these challenges are evident in the transcriptional heterogeneity of EWS-FLI1 that we have observed. We believe that by focusing on this transcriptional heterogeneity we can perhaps identify vulnerabilities that are common across all cell types and yet yield different expression changes in unique cellular contexts. Since these vulnerabilities are unknown, we will employ an unbiased screening approach. We have previously screened more than 50,000 compounds to identify a hit list of more than 50 compounds. In this proposal, we will complete additional screens to increase the number of compounds that reverse EWS-FLI1 activity. We will confirm the specificity of the compounds for EWS-FLI1 using a novel assay that interrogates the blockade using the transcriptional heterogeneity we have observed. Finally, we will use the compounds both as clinical lead candidates and as tools to understand the most effective way to target this fusion protein.

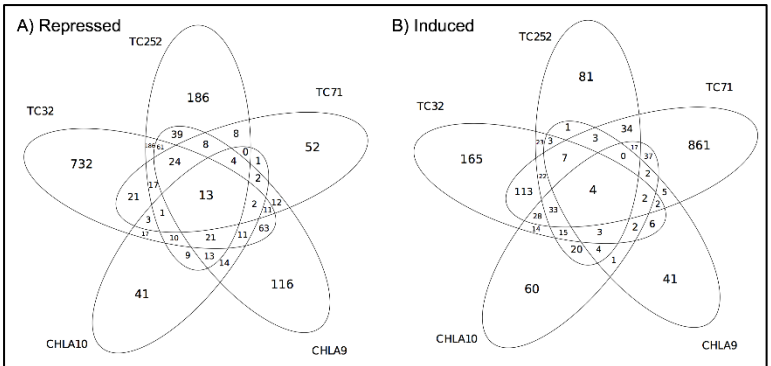

Figure 3: Venn diagram showing genes that are (A) repressed or (B) induced targets of EWS-FLI1 and show a log FC of >2 in the identified cell line 30 hours following siRNA silencing of EWS-FLI.

Defining an EWS-FLI1 inhibitor: The challenge of characterizing compounds as EWS-FLI1 inhibitors is that transcription factors lack a singular assay (such as a kinase phosphorylation assay) to define them as inhibitors. This necessitates a reliance on surrogate assays such as luciferase assays or gene set enrichment analysis to define inhibitors.[30,31] In addition, there is no gold standard criteria to define a compound as an EWS-FLI1 inhibitor.

In this proposal, we establish rigorous criteria for chemical probes and clinical candidates based on:
(1) Inhibition of "well-established targets" identified in multiple independent studies (see above)
(2) Reversal of the "gene signature" of EWS-FLI1
(3) Characterization of mechanism of EWS-FLI1 suppression
A chemical probe fulfills (1) and (2) and a clinical candidate fulfills criteria (1), (2) and (3).

Therefore, in order to enter the pipeline for clinical development, all three criteria will need to be met**. It is notable that we have already established a number of clinical candidates including, trabectedin, lurbinectedin and mithramycin, all of whom have second and third generation inhibitors. These prior discoveries are the foundation of this proposal (Fig. 1**).[6,32-38]

Approach:

Aim 1: To Establish a Pipeline of EWS-FLI1 Inhibitors and Chemical Probes

Rationale: In order to identify compounds as highly specific EWS-FLI1 inhibitors, we have previously performed unbiased functional drug screening of 50,000 compounds.[6] We screened a library of compounds using a NR0B1 luciferase reporter construct and a gene-signature secondary screen, identified mithramycin and translated the compound to the clinic. [6,35,39] This screen was successful because expression of NR0B1 and therefore the activity of this reporter is highly specific for EWS-FLI1.[14] Although both FLI1 and EWS-FLI1 can bind to the GGAA-microsatellite-containing promoter, only EWS-FLI1 can drive expression.[40,41] Indeed, the expression of NR0B1 has been definitively linked to EWS-FLI1 binding to the GGAA-microsatellite as disruption of the locus by CRISPR/Cas9 leads to a loss of the ability of EWS-FLI1 to transactivate.[42]

Experimental Design and Preliminary Results:

In this aim, we returned to the hit list from the above screen, selected the top 140 compounds and rescreened them in parallel with a constitutively active luciferase control and a viability assay to exclude general non-specific causes of luciferase suppression. As shown in Fig. 4, we found 62 compounds that suppressed NR0B1 driven luciferase by 25% or more *(P = 0.0005).* In addition, 55 compounds showed selectivity over the CMV driven control (Data is shown as percentage of CMV to visualize viability and constitutively active luciferase control).

In this aim, we will add to this collection of lead compound hits by screening two additional libraries. We will first screen a library of FDA approved compounds. These compounds may lack specificity but because of their approved status, would provide the fastest route to the clinic. In addition, we will screen a

library of natural products. Natural products are compounds identified as the biosynthetic product in a variety of organisms. These compounds are structurally rich and will provide a large number of unique chemical scaffolds to interrogate a wide range of molecular interactions. These unique compounds will increase the likelihood a given compound could work by a unique mechanism of action.

Results/Alternatives/Impact: With the completion of this aim, we hope to have at least 100 compounds in hand to be evaluated in the secondary screen of EWS-FLI1 inhibitors. All of these compounds will have in common that they reverse the activity of the NR0B1 promoter. As stated above, the expression of NR0B1 and therefore the activity of the NR0B1 promoter is highly specific for EWS-FLI1 in Ewing sarcoma cells. Nevertheless, we expect that a large percentage of these compounds will be false positives. In order to eliminate these false positives, we will employ the novel sequencing assay in aim 2 for the top 50 compounds. The ultimate confirmation of a compound as an EWS-FLI1 inhibitor, will only come with the identification of a mechanism of action which will be the subject of ongoing investigations. Nevertheless, the ultimate goal of this aim is to provide a list of clinical candidates to be validated for clinical translation AND a set of chemical probes to be used for future studies.

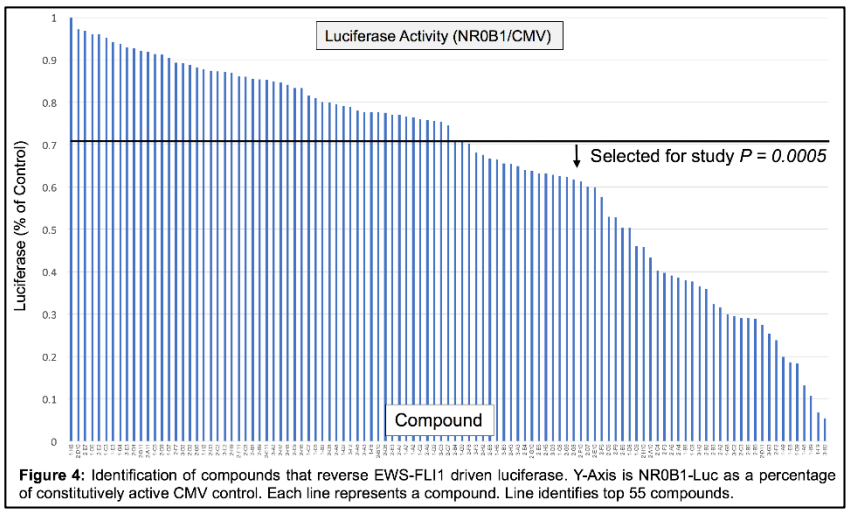

Figure 4: Identification of compounds that reverse EWS-FLI1 driven luciferase. Y-Axis is NR0B1-Luc as a percentage of constitutively active CMV control. Each line represents a compound. Line identifies top 55 compounds.

An alternative possibility is that we do not arrive at 50 compounds that meet the criteria for evaluation by gene signature. If this is the case, we will evaluate genomic targets. We have completed a genome wide siRNA screen using the same NR0B1 luciferase reporter as for the small molecule screen. We have sorted the top hits from this screen and have hundreds of target genes that block NR0B1 luciferase activity. If we do not have enough compounds to evaluate in the gene signature, we will prioritize these hits based on druggability, silence the target in multiple cell lines and perform gene signature screening with these hits.

Aim 2: Confirm the Specificity of Lead Compounds Using a Novel Next-Generation Sequencing Assay

Rationale: As stated above, a major challenge of transcription factor drug development is the transcriptional heterogeneity that exists across cell lines. In this aim, we will exploit this heterogeneity as an assay to identify highly specific inhibitors of EWS-FLI1. As shown in the heat map in figure 2, it is quite clear that silencing of EWS-FLI1 in multiple cell lines generates both cell line specific and common gene expression changes across several cell lines. In this aim, we will generate, a 500-gene assay that captures both the unique and common changes. This will increase confidence in the hits because gene A will change in expression with drug treatment regardless of cell line, while, for example, gene Y will only change in A673s, gene z only in CHLA9s and gene Z only in TC32s while NR0B1 will change in all cell lines with the same compound treatment.

Experimental Design and Preliminary Results: The top compounds from the above screen will be sorted based on the magnitude of NR0B1 luciferase suppression relative to the constitutively active CMV control. We will subsequently optimize the concentration and duration of exposure needed to maximally suppress NR0B1 luciferase activity. Once this has been established for the top compounds, we will treat cells with compound and collect RNA in triplicate in three cell lines for the top 50 compounds. We will prepare the RNA and use next generation sequencing and a novel 500-gene assay that we will build based using funds from this proposal. We will interrogate each of the top compounds to identify a list of compounds that reverse both the common and unique gene expression changes in our assay. These compounds will exactly mirror the transcriptional heterogeneity EWS-FLI1 silencing induces in multiple cell lines. Any compound that reverses NR0B1 activity without changing the constitutively active CMV control and reverses the gene signature of EWS-FLI1 will be a chemical probe of EWS-FLI1 activity.

Tertiary screens of compounds**:** The compounds will be sorted based on the magnitude of suppression of the EWS-FLI1 gene signature. All of the compounds that completely reverse the gene signature of EWS-FLI1 including both unique and common targets will be selected for further evaluation. At minimum, the top 30 compounds will be confirmed using standard quantitative PCR. Each compound will be evaluated for its ability

to suppress EWS-FLI1, NR0B1 and induce the EWS-FLI1 repressed target, PHLDA1. In order to confirm the activity of the compound, new compound will be purchased from a commercial vendor. It is common that compounds in screening plates are not always the same as what is annotated in the library. Therefore, re-purchasing the lead compound is standard practice.

Once, suppression of NR0B1 is confirmed, these compounds will be also evaluated by standard assays. The effect of each compound on viability will be confirmed in multiple Ewing sarcoma cell lines including TC32, CHLA9, TC252, A673 cells. As a control the compounds will also be evaluated in translocation positive (RH30) and translocation negative (U20S) sarcoma cell lines. The effect on viability will be evaluated using our incucyte zoom microscope and confirmed by additional assays such as MTS assays.

Finally, the hits from the FDA library will be prioritized for further development as these have the fastest route to the clinic. Since almost all of these compounds have defined mechanisms of action, we will confirm the effect of drug treatment on the EWS-FLI1 using genetic approaches. For example, if a lead compound is known to poison a Ca^{2+} transport channel, we will use CRISPR/Cas9 or siRNA to silence the transport channel and see what the impact is on the EWS-FLI1 transcriptional program. Next, we will confirm the RNA effects at the protein level and see how the compound impacts the Ewing sarcoma cells relative to control including evaluating the effects on apoptosis and senescence to determine if the compound phenocopies EWS-FLI1 suppression. Finally, we will evaluate the ability of the compound to combine with other EWS-FLI1 directed small molecules that we have already described such as mithramycin, trabectedin, EC8042 and lurbinectedin. We will determine if the compound is able to amplify or sustain suppression of EWS-FLI1 by the other compound.

Results/Alternatives/Impact With the completion of this aim, we will identify the most comprehensive list of EWS-FLI1 chemical probes characterized to date. If we are able to successfully identify an FDA approved compound that works by a defined mechanism and link that mechanism to EWS-FLI1, we will also identify a bona fide clinical lead compound that we will work to translate to patients. In the absence of this result, we will identify a series of chemical probes that we can use as lead compounds to better understand the most effective way to suppress the EWS-FLI1 transcription factor. It is likely that these compounds will require additional chemistry to improve activity. However, it is possible that we identify a strong candidate compound that works by a defined mechanism to suppress EWS-FLI1. If this is the case, we will move to the in vivo setting and evaluate the compound in our models. The completion of these studies is likely beyond the scope of this particular grant. Nevertheless, since EWS-FLI1 is the dominant oncogene of the tumor, it follows that this will be important step towards the development of an effective molecularly targeted therapy for Ewing sarcoma.

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