Histone deacetylase inhibitor therapy in graft-versus-host-disease

Melissa How

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Abstract

There is a high prevalence of blood cancer. Leukemia is an example of a common type of blood cancer. Treatment for leukemia is radiation and bone marrow transplants. However, there is a negative complication that may occur from receiving bone marrow from a mismatched donor. This complication is called graft-versus-host disease (GVHD). GVHD is a negative side effect of life-saving bone marrow transplant in leukemia patients. In GVHD, the cells from the donor bone marrow recognize the host's cells as foreign and attack the patient's skin, liver, and gastrointestinal tract. This results in the extensive cell damage seen in GVHD patients, which is caused by inflammation. However, there is a positive effect of GVHD seen in leukemia patients called graft-versus-tumor (GVT). In GVT, donor cells attack the remaining tumor cells that remain after chemotherapy. This results in a better prognosis for leukemia patients and a lower chance of a reoccurrence of cancer. Unfortunately, GVHD and GVT coincide—one cannot exist without the other. Finding a drug that limits the effect of GVHD, but preserves GVT is essential. High doses of this new type of drug called HDAC inhibitors (HDACi), is an effective anti-tumor agent. Moreover, further studies determined that at low doses the same drug causes a powerful anti-inflammatory effect. HDACi has the ability to significantly lessen the effect of GVHD while maintaining GVT. In order for these drugs to be more widely implemented as a better option for GVHD patients, further study must be done on the different classes of HDACi. Our objective is to better understand how HDACi works by performing a comparative study to specifically assess how HDACi inhibit GVHD while maintaining GVT. We will compare the different classes by measuring the differences in how they affect GVHD. Once we understand the processes, we can better determine which HDAC inhibitor is most effective against tumor cells and is best at hindering GVHD. Determining the best HDACi therapy will aid in translating this drug therapy into human models. We hope to find the best drug therapy with the least side effects that will be the most successful in patients with GVHD.

Background and Significance

Every four minutes, one person in America is diagnosed with a cancer of the blood. In the year 2013 alone, 48,610 new leukemia cases are expected and 23,720 of them are expected to be fatal as a result of the cancer.[10] Presently, treatment for leukemia consists of irradiation and bone marrow transplants. More than 5,000 Americans per year receive a bone marrow transplant from a mismatched donor, leading to a strong likelihood of the patient developing graft-versus-host disease (GVHD). Moreover, an estimated 500 to 1,000 Americans die each year[13] due to mismatched bone marrow donor complications like GVHD.

Unfortunately, the only sure way to prevent GVHD is to receive bone marrow from the patient's twin. Since not everyone has a twin an important question arises: How can we limit the negative effects of GVHD? Graft-versus-host disease (GVHD) is a deadly complication from life-saving bone marrow transplants. Unless the patient receives donor bone marrow from their twin, most patients receive donor bone marrow with slightly mismatched major histocompatibility complexes (MHC). MHC is what determines the compatibility of donors for transplants of any kind. The mismatch in MHC causes immune rejection and the development of GVHD. In leukemia patients, MHC plays an important role in the success of bone marrow transplants. Leukemia is a blood and bone marrow cancer treated with radiation and a donor bone marrow transplant. Donor bone marrow repopulates the areas where the old marrow cells have been killed by radiation. As a side effect, however; GVHD causes inflammation and the donor bone marrow attack host cells. In most types of transplants, GVHD is an unwanted and dreaded possibility. Fortunately, for some leukemia patients, GVHD can also be a welcome result of bone marrow transplantation. Studies have shown that mild cases of GVHD in leukemia patients lessen the possibility for a recurrence of cancer—a phenomenon known as graft-versus-tumor (GVT) effect. [5] In GVT, the effector cells target and attack the remaining cancerous cells that were not eradicated by chemotherapy. More recent studies have even concluded the possibility of limiting GVHD activity while preserving and even enhancing the effect of GVT. [3,12] Fortunately, research into a new type of drug aids in fighting GVHD. Histone deacetylase inhibitors (HDACi) are a new class of anti-cancer drug for the treatment of both solid and hematological malignancies. They inhibit the buildup of tumor cells in culture and in mouse models by inducing the disruption of the cell cycle, differentiation, and/or forcing the cell to die. There are five classes of HDAC inhibitors: hydroxamic acids (e.g., trichostatin A (TsA), suberoylianilide hydroxamic acid (SAHA)), benzamides (e.g., MS275), electrophilic ketones (e.g., trapoxin), cyclic tetrapeptides (e.g., depsipeptide (FK228), apicidin), and short chain fatty acids (e.g., butyrate, valproic acid). [4] Studies show that inflammatory cytokines are the major cause of GVHD-induced cell damage. [6] High doses of HDAC inhibitors are anti-proliferative against tumor cells. Low doses of the same drug have a powerful anti-inflammatory effect and limit the effect of GVHD while maintaining GVT. Earlier studies determined that hydroxamate-based HDACi compounds such as TsA and SAHA have inhibitory effects on production of tumor necrosis factor (TNF) α, interleukin (IL)-1α and IL-1β. [6,15] HDACi provide an answer to the problem of GVHD. They have the ability to limit the effects of GVHD while maintaining GVT. Research on HDAC inhibitor therapy on mouse models prove that HDAC inhibitors are an effective therapy in the reduction of the severity of graft-versus-host disease while maintaining GVT. However, much remains unknown about the mechanisms by which HDACi affect GVHD. We know HDACi effects about 2% of the genes in regulatory T cells and about 1-2% of the genes in non-T cells, [4] but we do not know exactly how they affect them. Understanding the mechanism and determining any possible toxicity is important in the utility of HDACi as a widely implemented therapy in human patients. With more research into the mechanisms of HDACi we can determine the most effective HDACi therapy with the least side effects. For leukemia patients this means there will be no fear of bone marrow transplantation because GVHD will no longer pose a serious threat.

Specific Aims

We will assess the effect of the different classes of HDAC inhibitors on GVHD and GVT. Then we will assess how gene expression changes in regulatory T cells due to HDACi therapy.In this project, we hypothesize that since HDACi have different targets or mechanisms of action, different HDACi will have variable success at preventing GVHD while maintaining GVT and that their effects on regulatory T cells will be an important factor in their success.

Aim 1: Assess graft-versus-host disease (GVHD) and graft-versus-tumor (GVT) using diverse HDAC inhibitors. By comparing the effect of the different classes of HDAC inhibitors in GVHD, we can better understand the mechanisms by which HDACi affect mouse models of GVHD. In order to assess the effect of HDACi on GVHD and GVT, we will measure tumor size to determine the effectiveness of the HDACi therapy. Mouse models are important to this study because mice are a model organism for humans.

Aim 2: Assess how gene expression changes in regulatory T cells due to HDACi therapy. By analyzing the spleen and lymph nodes we can assess the gene expression in regulatory T cells. We will use flow cytometry, a machine used to count cells, to evaluate the percentage of donor regulatory T cells in the treatment mouse. A cell sorter will then sort out the regulatory T cells and extract their RNA. By microarray, which maps proteins, we will determine gene expression changes in the regulatory T cells due to our HDACi treatment.

Research Design and Methods

Although there is much research on HDAC inhibitors and their effect on GVHD and GVT, the mechanisms are not well understood. In our hypothesis we stated that different HDACi therapies will have different rates of success at preventing GVHD while maintaining GVT since they each work differently. The following methods will allow us to perform a comparative study to specifically assess HDAC inhibition of GVHD while preserving GVT. To compare the effect of each class of HDACi on GVHD, we will perform three trials on mouse models over a period of 1 year. From the five classes of HDAC inhibitors, we have chosen eight different HDAC inhibitors we want to compare: trichostatin A (TsA), suberoylanilid hydroxamic acid (SAHA), MS275, trapoxin, depsipeptide (FK228), apicidin, butyrate, and valproic acid. In order to prepare the mice for different HDACi therapies, GVHD will be induced in them by the following methods. We will use the B16 melanoma transplantable tumor cell line. This tumor will be transplanted into our recipient C57BL/6 mouse line to induce cancer in our mouse model. To induce GVHD, we will use FOXP3^{GFP} mice on the BALB/c background as the donor mouse at a 1donor mouse to one recipient mouse (1:1) ratio. Because of the mismatched MHCs in the two mouse lines, immune rejection and the development of GVHD occurs in the recipient (C57BL/6) mice. Throughout the experiment, maintenance and treatment of lab mice will follow the ethics and regulations established by the University of California, Merced. We applied for and received IACUC approval for use of mouse models and will follow any and all guidelines for the humane use of mice. All laboratory personnel have been trained on the safe and correct handling of mice.

Experimental Design

As stated above, we will perform three trials. Three C57BL/6 mice will be dedicated to each of the eight classes of HDACi (for a total of 24 mice per trial) to compare

	Trial 1 HDACi Therapy	Trial 2 # of Mice	Trial 3 HDACi Therapy	# of Mice	HDACi Therapy	# of Mice
C57BL/6 (Recipient Mice)	TsA	3	TsA	3	TsA	3
SAHA	3	SAHA	3	SAHA	3	
MS275	3	MS275	3	MS275	3	
Trapoxin	3	Trapoxin	3	Trapoxin	3	
FK228	3	FK228	3	FK228	3	
Apicidi	3	Apicidi	3	Apicidi	3	
Butyrate	3	Butyrate	3	Butyrate	3	
Valproic Acid	3	Valproic Acid	3	Valproic Acid	3	
No Treatment	3	No Treatment	3	No Treatment	3	
Total: 27 mice	Total: 27 mice	Total: 27 mice				
		# of mice		# of mice		# of mice
FOXP3GFP (Bone Marrow Donor Mice)		27		27		27

Figure 2: Table 1 This is a table representation of the number of mice that will be necessary for each type of drug therapy in each of the three trials.

the results of each HDACi therapy on its ability to prevent GVHD while maintaining GVT. An equal number of FOXP3^{GFP} mice will be used as a mismatched bone marrow donor to induce GVHD/GVT in each of the C57BL/6 mice.

Each trial requires 27 C57BL/6 mice and 27 FOXP3^{GFP} mice for a total of 51 mice per trial. In all, this experiment will require 156 mice.

Animals

Male and female FOXP3^{GFP} mice on a BALB/c background, bred (from a breeding pair provided by Hoyer Lab) and maintained in our animal facilities at UC Merced, were used at the age of 6 weeks as the donor mice. Male and female C57BL/6 (The Jackson Laboratory; Bar Harbor, Maine), bred and maintained in our animal facilities at the University of California Merced, were used at the age of 6 weeks as our GVHD/GVT model.

B16 Melanoma Transplantation

To prepare the B16 cells (Sigma-Aldrich; Germany), we will ensure that they are in the logarithmic growth phase when harvesting for inject (the flasks should be $\leq$ 50% confluent). We will draw out a portion of the medium, rinse the flask briefly with 3mL trypsin/EDTA, and draw it out again. 5mL of trypsin/EDTA will be added and the flask tilted to ensure all the cells are covered. We will add 5mL of the cold complete medium (CM) (See Appendix A) and pipet vigorously to obtain a single-cell suspension. 50mL will be transferred to a centrifuge tube and 40mL of cold CM will be added to neutralize trypsin. Cells will be spun to form a pellet for 10 minutes at 663 x g at 4 °C in a Beckman Coulter Allegra X-14R Centrifuge. The supernatant will be poured slowly into another container and

the B16 cells will be resuspended in ice-cold PBS. The suspension will then be passed through a disposable strainer to remove any clumps. We should have a cell concentration of 1 x 10^6 cells/mL in ice-cold PBS [7]. At 6 weeks old C57BL/6 mice will be injected with 100 μl of B16 the melanoma cell suspension with a 27 ^{frac12} - gauge needle to induce cancer. B16 melanoma cells will be injected subcutaneously into each of the mice and the appearance of a bubble will form just under the skin. If no bubble forms, the mouse will be sacrificed and a new one will be used. Mice will be observed for tumor growth and the first set of tumor measurements will be taken after 5 days with the use of calipers. Measurements of the tumors will be taken daily to ensure tumor growth and a successful transplant [7]. At 6 weeks old C57BL/6 mice will be injected with 100 μl of B16 the melanoma cell suspension with a 27 ^{frac12} - gauge needle to induce cancer. B16 melanoma cells will be injected subcutaneously into each of the mice and the appearance of a bubble will form just under the skin. If no bubble forms, the mouse will be sacrificed and a new one will be used. Mice will be observed for tumor growth and the first set of tumor measurements will be taken after 5 days with the use of calipers. Measurements of the tumors will be taken daily to ensure tumor growth and a successful transplant 7.

Irradiation

Irradiation will occur 12 days after B16 injection. Recipient (C57BL/6) mice will be placed in an animal restrainer and into a J.L. Shepherd Self-contained Category I Cesium-137, Mark I series irradiator. Mice will be given a one-time does of 450 rads of radiation. Mice will then be placed back in their cages with an antibiotic tablet.

Bone Marrow Transplantation

At 6 weeks old donor mice (FOXP3^{GFP}) will be injected with 5-fluorouracil using a 27 gauge needle to enrich bone marrow stem cells. Each mouse will receive 0.150 mg for every 1 gram that the mouse weighs. Four days after injection the mouse will be sacrificed and we will remove the tibias and femurs. A 25 gauge needle will be inserted into the bones and the bone marrow cells (BMCs) will be flushed out with PBS. BMCs will then be suspended in PBS containing 2% heat-inactivated fetal bovine serum (FBS).

One day after irradiation, recipient mice (C57BL/6) will be injected with 3 x 10^6 BMCs from a donor via tail vein using a 27 gauge needle. Female donor mice BMCs will be injected into female recipient mice and male donor mice BMCs will be injected into male recipient mice.

The following experiments will allow us to accomplish our first aim, which is to measure the effect of our HDAC inhibitors on GVHD and GVT.

HDACi Therapies

Three mice will be used for each of the eight types of HDAC inhibitor treatment therapies. HDAC inhibitors come as a crystalline solid so it must be put into a solution of a stock concentration of 0.01 mg/mL (See Appendix B) in order to inject the drug into mice. Mice must be weighed before injection of the therapy because 0.1 mg of the drug is given for every 1 kg the mouse weighs. The average mouse weighs about 20 g so on average the mice will be injected with about 2 micrograms of the drug each time. In each trial another group of three mice will serve as a control group and receive injections of PBS. Injections of PBS will not affect the mice at all. Mice will receive daily injections over the span of two weeks.

Measuring GVHD

From day one of treatment, mice will be assessed for the effect of each respective HDACi therapy. We will measure the mice in each therapy type for tumor size and

weight. Tumor size will be measured daily by the use of calipers to record the effect of the HDACi therapy on tumor size. If the HDACi therapy is effective, we will notice the tumor decreases in size. Mice will be weighed every day for the entirety of the experiment, as weight loss is indicative of GVHD; weight gain will show us that the HDACi therapy has an effect on GVHD.

The following experiments will allow us to accomplish our second aim, which is to identify any changes in gene expression as a result of our HDACi therapies.

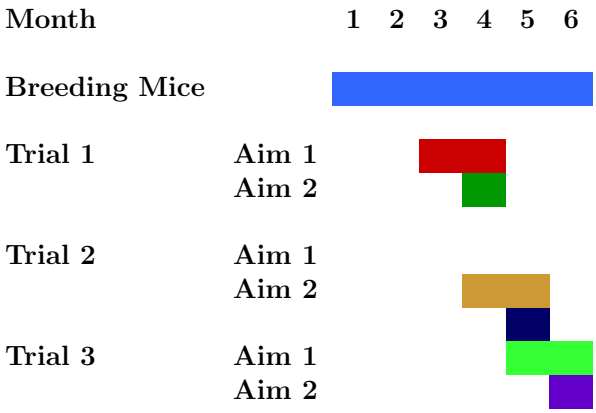
Flow Cytometry and Cell Sorting

After HDAC inhibitor treatment, all mice will be sacrificed and we will remove their lymph nodes and spleens. Lymph nodes and spleens will be mashed on a wire mesh in PBS with 2% FBS to disrupt the tissue. Samples will be filtered through a 70 μm strainer to remove any large clumps. In a 15 mL conical tube, we will centrifuge the samples at 400 x g for 10 minutes at 4 °C in a Beckman Coulter Allegra X-14R Centrifuge to pellet the cells. We will then draw up a portion of the supernatant and resuspend the cells in a buffer that will cause the red blood cells to burst. The cells will be suspended in this buffer for 1 minute. Then, dilute the red blood cell lysis buffer in 10 volumes of PBS. Spin the tubes at 400 x g for 10 minutes at 4 °C in a Beckman Coulter Allegra X-14R Centrifuge to form a pellet consisting of the cell membranes. Draw up the supernatant and resuspend the cells in PBS containing anti-CD4 antibody at a ratio of 1 μL of antibody per 500 μL of PBS. We will then transfer the cells to a 5 mL polystyrene round bottom tube. Flow cytometry will tell us the percentage of donor regulatory T cells within each mouse and show whether or not they are activated. We will use the ARIA II cell sorter to sort out the donor fluorescent regulatory T cells into a tube. The cells will be pelleted using a centrifuge and frozen over dried ice.

RNA Sequencing

After sacrificing the mice in Trial 3, we will send all 27 samples including the 3 "no treatment" samples. The regulatory T cells from these nine mice in addition to the three "no treatment" mice will be sent to Illumina for RNA sequencing. Freeze dried pellet will be sent to Illumina for RNA sequencing. From this data we can determine the effect of different HDACi on the genes of regulatory T cells and the most effective one to use against GVHD.

Table 2: Time line of experiments



Data Management

All data will be stored on Microsoft Excel into a shared drive on a password protected desktop computer, which all lab personnel will have access to. In addition, each lab personnel will maintain their own lab notebook for data and procedures they have accumulated throughout the experiment.

Rationale

The FOXP3^{GFP} mice on the BALB/c background were chosen because of the fluorescent properties of their regulatory T cells. Donor regulatory T cells in the recipient mice will be easily distinguishable through flow cytometry and a cell sort by

Mice and Animal Maintenance	**Supplier**	**Amount**	**Price Per Unit**	**Total Cost**
C57BL/6 strain	The Jackson Laboratory	5 Male 5 Female	Male:$18.15 Female:$19.85	$114.00
FOX3GFP	Hoyer Lab	5 Breeding Pairs	$0	$0
Maintenance (Includes personnel and all supplies needed for care)	UC Merced	30 cages	$365/cage	$10,950.00
	Subtotal:	**$11,064.00**		
Reagents/Cells	**Supplier**	**Amount**	Price Per Unit	**Total Cost**
B16 Melanoma	Sigma-Aldrich	1 vial	$425/vial	$425.00
TsA	Sigma-Aldrich	1 5mg vial	$138.00	$138.00
SAHA	Sigma-Aldrich	1 1mg vial	$62.90	$62.90
MS275	Sigma-Aldrich	1 1mg vial	$285.00	$285.00
Trapoxin	Sigma-Aldrich	1 1mg vial	$150.00	$150.00
FK228	Adooq	1 1mg vial	$450.00	$450.00
Apicidin	Sigma-Aldrich	1 1mg vial	$94.80	$94.80
Butyrate	Sigma-Aldrich	250 mg vial	$26.30	$26.30
Valproic Acid	Sigma-Aldrich	1 1g vial	$50.00	$50.00
	Subtotal:	**$1,667.00**		
Labratory Experiment	**Supplier**	**Amount**	**Price Per Unit**	**Total Cost**
Irradiator	J.L. Shepherd (Self-contained Category I CS-137)	Supplied by UC Merced	$0	$0
Flow Cytometer	BD (LSR II)	6 Hours	$35/hour	$210.00
Cell sorter	BD (Aria II)	36 Hours	$50/hour	$1,800.00
Centrifuge	Beckman Coulter (Allegra X-14R Centrifuge)	Supplied by Hoyer Lab	$0	$0
Supplied by Hoyer Lab	Illumina	27 samples	900/*sample*+530 Fee	$24,830.00
Miscellaneous lab supplies		Supplied by Hoyer Lab	$0	$0
	Subtotal:	**$26,840.00**		
Lab Personnel	**Time Spent on Project**	**Amount**	**Salary (Full time/1 year)**	**Total Cost (Full time/6 mo.)**
Principal Investigator	6 months	1	$51940.00	$25,470.00
Postdoctoral Fellow	6 months	1	$49,329.00	$24,664.50
Lab Technician		1	$38,000.00	$19,000.00
Undergraduate Lab Assistants		2	$0	$0
	Subtotal:	**$69,134.00**		
Miscellaneous	**Supplier**	**Amount**	**Rent**	**Total Cot**
Lab space	UC Merced	Supplied by Hoyer Lab	$0/year	$0
			Subtotal:	**$0**

its fluorescent properties. There is a foreseeable amount of human error in the injection of the B16 melanoma cells. If the cells are not correctly subcutaneously injected, the mouse must be sacrificed and a new mouse will be used. Therefore, we must keep a supply of extra mice on hand. For this reason, we made the decision to purchase three breeding pairs of each of the two mouse strains in order to keep a mouse colony.

Budget

These experiments will be conducted at the facilities at the University of California, Merced for the use of the established research lab of Dr. Katrina Hoyer and the animal facilities which contain all necessary equipment for the experiment. The budget report includes experimental equipment purchased, salaries of lab personal, and all equipment we already have access to. The budget is calculated for 1 year based on having an established lab already with most of the equipment needed for the experiment. Any material unused or still in working condition will be donated to the University of California, Merced. The estimated cost for this entire experiment is $108,705 for a time frame of 6 months.

Public Health Impact Statement

In leukemia patients, the ability to manage the effects of graft-versus-host disease (GVHD) while maintaining graft-versus-tumor is an important step towards complete remission. Further understanding of this new therapy will help us determine the most effective HDAC inhibitor drug therapy with the least side effects. side effects.

B. Reconstitution of HDACi

1. Dissolve the solid HDACi to get a concentration of 0.5 mg of the drug per 1 mL in DMSO. 2. Dilute the solution to 0.1 mg/mL using PBS. 0.1 mg of HDACi per 1 mL is the desired stock concentration for each HDACi drug therapy.

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Appendix

A. Complete Medium 10% (v/v) fetal bovine serum (FBS) 1 mM sodium pyruvate (final concentration) 2 mM L-glutamine 100 μ M nonessential amino acids. 100 U/ml penicillin 100 μ /ml streptomycin 50 μ /ml gentamicin 0.05 mM 2-mercaptoethanol Bring up to 500 ml with RPMI 1640 (Life Technologies)
Complete medium can be stored up to 2 weeks in the dark at 4 °C.

Melissa How

Melissa How is a junior majoring in Biology with an emphasis in human biology at the University of California, Merced. She is set to graduate in 2016 and hopes to also complete a minor in Psychology. She is a member of Dr. Katrina Hoyer's immunology research lab where she aids in various experiments. She is also an active member of UC Merced's chapter of Kappa Kappa Gamma, a nationally recognized women's fraternity.When she isn't doing school work or working in the lab, she participates in social and philanthropic events hosted by Kappa Kappa Gamma and other Greek organizations at UC Merced. After college she hopes to attend medical school and specialize in reconstructive surgery. She plans to dedicate some time to the nonprofit organization Operation Smile after receiving her medical degree.