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B cell non-Hodgkin's lymphoma xenograft response to bortezomib and vincristine

Abstract

Modern chemotherapy treatment has significantly increased the survival rate of pediatric B cell non-Hodgkin's lymphoma (B-NHL). Yet, researchers should continue seeking new treatments for pediatric B-NHL because there are still subgroups of pediatric patients of B-NHL with poor prognosis. Moreover, current drug therapy treatments remain toxic and dangerous. Current methods of studying B-NHL and drug therapy responses have involved xenografting human B-NHL into mouse models. This method, however, is problematic because the results are not directly translatable for use in clinical studies. Autografts may provide a more translatable model system, as transplanting a mouse tumor line into a mouse rather than a human cancer cell line will bring about a more comparable biological response.

The goal of this work was to develop xenograft models in which autografts models could be compared. To xenograft human tumors, Raji cells expressing luciferase were injected subcutaneously in both flanks of athymic nude mice. Tumors were treated with bortezomib, vincristine, or saline. Tumor progression and response to drug therapy was monitored through bioluminescence imaging (BLI) and caliper measurements. These techniques show bortezomib and vincristine appear to be successful in treating human Burkitt lymphoma (BL) xenografted into athymic nude mice. All tumors present in a mouse disappeared within 10 days after administration of bortezomib. Likewise, tumors shrank in a mouse receiving vincristine 4 days after administration. Upon continued vincristine administration (every 4-5 days), one tumor completely disappeared, while the other significantly shrank. These findings are important

because they show that both bortezomib and vincristine are successful in treating human BL and establishes a system in which the autografts can be compared.

Introduction

Unpublished data indicates that modern chemotherapy has greatly increased the prognosis for pediatric B cell non-Hodgkin's lymphoma (B-NHL) of all stages. Yet, much effort continues to be focused on improving treatment of pediatric B-NHL for several reasons. There remain subgroups of B-NHL pediatric patients that continually have a poor prognosis, particularly those involving the bone marrow and CNS at the time of diagnosis. In fact, the World Health Organization (WHO) says that more than 95 percent of pediatric B-NHL are classified as either intermediate or aggressive forms of cancer¹. Moreover, approximately 45 percent of pediatric B-NHL are classified as Burkitt lymphoma². Additionally, although successful chemotherapy drugs have been discovered to treat pediatric B-NHL, the drugs are highly toxic and thus affect the patient's quality of life while receiving treatment according to unpublished data. Moreover, many of the therapies also increase the risk of developing a second malignancy due to the chemotherapy.

Development and evaluation of new therapies for pediatric B-NHL is more difficult than for adult B-NHL because it occurs with decreased frequency in children. As such, the research community tends to focus more on adult therapy for the cancer. Additionally, pediatric B-NHLs respond better to chemotherapy. However, the need to pursue successful, less toxic treatments for pediatric B-NHL should continue to be explored.

Bioluminescence Imaging

To monitor tumor progression and drug responses in both the xenografts and autografts, we used bioluminescence imaging (BLI). The general chemical reaction occurs through a complex method, involving several key molecules³. There are four necessary molecules required for the reaction to occur: a luciferase enzyme, a luciferin substrate, oxygen, and ATP (Figure 1). In the first step, ATP binds to the carboxylic acid functional group on the luciferin substrate. This molecule forms a complex with the luciferase enzyme called luciferyl AMP, also known as luciferyl adenylate. The second step involves a proton, H^+ , being removed from the carbon adjacent to the nitrogen, but opposite the sulfur molecule in the pentane ring of the luciferyl AMP. When this step occurs, and the luciferyl AMP releases a pyrophosphate (PPi), forming an anhydride. Oxygen then oxidizes the remaining anhydride, yielding dioxetanone. Dioxetanone is very reactive due to angle strain caused by the 90 degree angles of the four-member ring present in the molecule. The ring is subsequently broken, yielding an anion. The anion stabilizes through the release of carbon dioxide and yields oxyluciferin in the keto form. However, when basic conditions are present, a proton is removed from the compound to neutralize the environment, yielding an enol isomer. The reaction emits light when oxyluciferin stabilizes. The type of oxyluciferin isomer present directly affects the wavelength of light emitted. For example, when the keto isomer is formed, the light emitted is in the 620nm range. Yet, when the enol isomer is formed, light in the 562nm range is emitted. However, not every reaction produces the same quality of light and is dependent on several factors including the amount of oxygen available, the degradation of the luciferase gene, and internal bleeding.

Bioluminescence imaging uses the same bioluminescent chemical reaction that occurs in animals to provide bioluminescence in cells of interest³. This bioluminescence is detectable and

quantifiable under an *in vivo* imaging system (IVIS) camera. Essentially, BLI is the measure of chemical luminescence of cells of interest in a living animal.

The use of bioluminescence imaging to monitor tumor and drug responses has several advantages to other methods used in biomedical research. One, bioluminescence imaging is non-invasive and does not require the animal to be euthanized in order to analyze a tumor. Keeping the animal alive in cancer research is critical, as the only way to watch tumor progression over a long period of time is to keep the animal living. Secondly, keeping the animal alive allows for the researcher to watch the drug response. Lastly, bioluminescence imaging is advantageous because it allows quantification of tumor size, allowing for a more precise tumor measurement. Data such as the total flux and total number of cells in the tumor can be obtained via bioluminescence imaging. Flux can be defined as the number of photons per second being admitted and is important because it quantifies the size of tumors transformed with the luciferase gene.

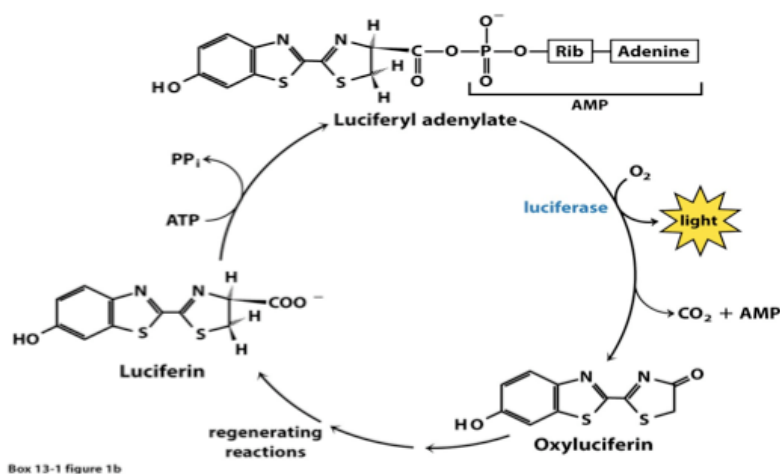


Figure 1: The firefly bioluminescence cycle. BLI involves the same mechanism with luciferin being injected *i.p.* The overall reaction emits light that is detected under the an *in vivo* imaging system (IVIS)⁹.

Cell Lines

The cell line used for our experiment was a human BL cell line called Raji, which were derived from a BL patient in Nigeria⁴. We transformed this cell line with the luciferase gene so we could elicit our desired bioluminescent reaction. After this transformation, we classified our Raji cells containing the luciferase gene as Raji-luc cells. Thus, the expression of luciferase would be used to simply monitor and quantify tumor growth.

Mouse Strains

We used athymic nude mice in this experiment. These mice lack a thymus gland, and because of this they also lack hair. Thus, when we xenografted Raji-luc cells into the mice, we did not have to be concerned about an immune response. Also, as we used BLI, we could not have any hair on the tumor site because the hair would absorb the emitted light rather than letting it be detected by the IVIS camera. Using naturally nude mice eliminated the need to shave the tumor area.

Method

Because Raji cells do not naturally express luciferase, our first step was to transform the cells with the gene. We did this transformation through use of recombinant DNA technology and retroviral vectors. The luciferase gene is cut from its original firefly host using restriction enzymes⁵. A section of the retroviral RNA is also cut using the same restriction enzymes. Thus, the area where the cuts were made in both organisms are analogous. The luciferase gene is then transformed into the retroviral RNA where DNA ligase covalently bonds it to adjacent nucleotides. The retrovirus then completes the process of reverse transcription to create cDNA out of the mRNA template. The process involves using the enzyme reverse transcriptase to help

create a cDNA strand. RNase then degrades the remaining mRNA while DNA polymerase lays down the nucleotides complementary to the single cDNA strand, allowing hydrogen bonding to occur between nucleotides. The luciferase gene, now present in the virus cDNA, is capable of entering the host cell where the DNA can replicate, further transcribing the luciferase gene.

In order to confirm that the luciferase gene had been successfully transfected into the Raji cell line and develop a per cell count that would eventually be used to calculate the total flux in photons per second through tumors, we ran a bioluminescence reaction *in vitro* with the Raji cells. The Raji cells were placed in a 96-well dish at different concentrations and a luciferin substrate was added in the presence of oxygen and ATP (Figure 2). If the luciferase gene is present and thus producing the luciferase enzyme, the luciferin substrate will interact with the luciferase in a chemical reaction that creates bioluminescence that is detectable under the Xenogen IVIS 200. Through this plate read we were able to confirm that our Raji cells had successfully acquired the luciferase gene, and had become Raji-luc cells. We were also able to quantify the average photon/second/cell count through the cells as 16.5.

A.

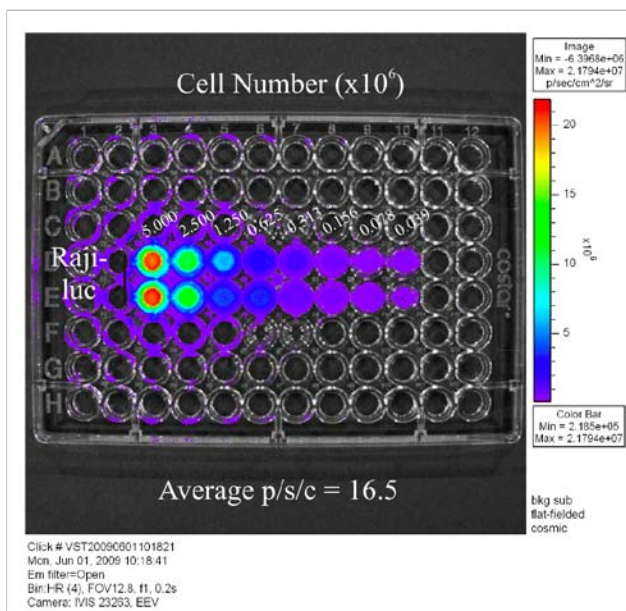


Figure 2: Assay to quantify the average photon/second/cell count emitted in the bioluminescence reaction of Raji-luc cells used in the experiment. Average p/s/c determined to be 16.5.

After running this experiment, we cultured our Raji-luc cells until we had enough viable cells to inject into 12 athymic nude mice. We determined cell counts and viability using Guava flow cytometry. This method essentially measures the number of nucleated cells in a given sample. We first removed a 10ul sample of our Raji-luc cells and placed them into an Eppendorf tube. We then added 50ul the Guava ViaCount solution, which allows the Guava machine to distinguish between nucleated living cells and non-nucleated dying or dead cells. An additional 40ul of isotonic PBS was added and the sample read in the Guava machine, yielding our number total cells, viable cells, and percentage of viable cells per sample. By multiplying the total volume of our cultured Raji-luc cells by the number of viable cells in the Raji-luc sample, we determined the total number of viable Raji-luc cells we had in culture.

The Raji-luc cells were collected by centrifuge at 14000x g for 5min. We aspirated off the media. The Raji-luc cells were washed with PBS and centrifuged for a second time. The PBS was aspirated and we resuspended the cells in PBS at a concentration of 5×10^7 cells/ml. We filtered this solution to assure that we had a homogenous suspension to ensure no large fragment of cells would stick that could prove to be fatal to the mouse upon injection.

We injected all 12 athymic nude mice subcutaneously. Each mouse received two injections, one in each flank, of 100ul each, essentially, 5×10^6 cells/injection. The mice were placed in three cages of four mice each. The mice in each cage were labeled by ear punch. Five days after, we imaged the mice using the Xenogen IVIS 200. Mice were first anesthetized using isoflurine and weighed on a scale. Luciferin (10mg/g) was then injected into the interperitium of each mouse based on the weight of the mouse. The mice were placed into the Xenogen IVIS 200 camera to detect presence of a bioluminescent reaction, and thus the presence of a tumor (Figure 3).

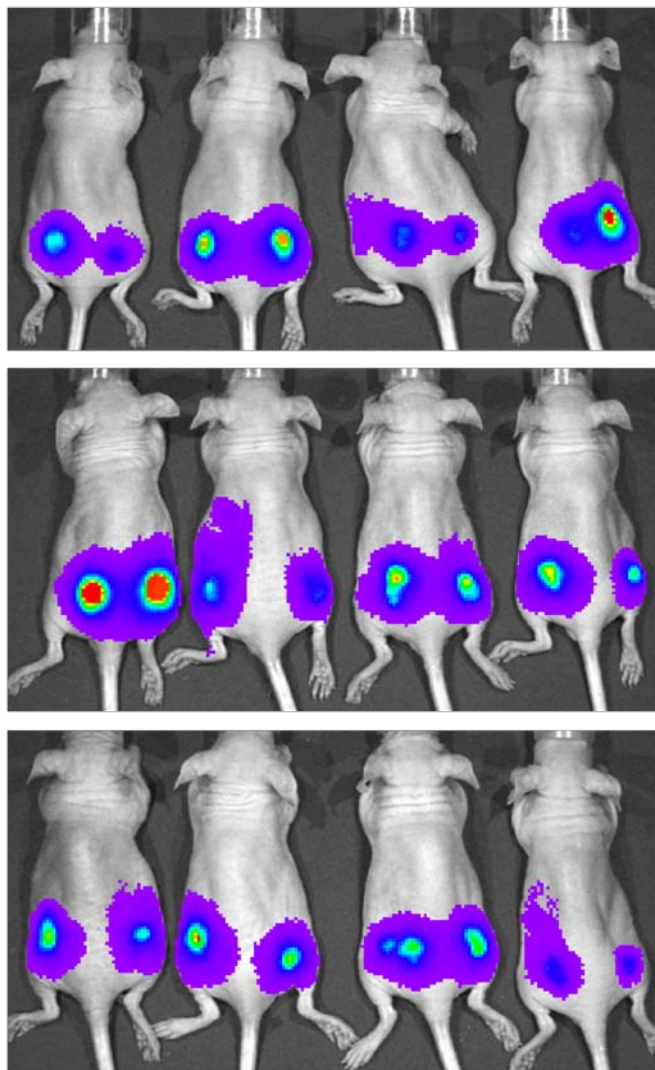


Figure 3: Initial BLI screening done on Day 5. Tumor cells present in both flanks of nearly all 12 mice.

Because the mice were anesthetized when we injected with luciferin, their hearts and circulatory systems operated at a slower pace under the drug, requiring more time to develop high-quality saturated images under the Xenogen IVIS 200. On average, we obtained optimal images at 25min post-injection time. We did follow-up imaging sessions to monitor tumor progression on Days 14, 17, 21, 33, and 36 following the same protocol. In addition to using bioluminescence to quantify our tumors, we used caliper measurements as a secondary method to measure and

quantify tumors. This was done by using calipers to measure the length and width of the tumors on both flanks. Caliper measurements were done on our mice on Days 17, 21, 26, 28, and 37. Once the tumors in the mice reached a certain size according to the Xenogen IVIS 200, we then divided the mice into treatment groups receiving vincristine, bortezomib, or saline. Bortezomib (1mg/ml) and vincristine (1mg/ml) were administered in 1ul amounts every 4-5 days.

Vincristine is a chemotherapeutic drug that acts by inhibiting mitosis, thus hindering malignant cells from dividing. The drug binds tubulin, a protein responsible for the formation microtubules⁶. Microtubules, in turn, are responsible for creating the spindle fibers necessary for moving chromosomes during mitosis. Thus, by preventing the spindle fiber formation, vincristine is able to arrest the cell cycle before anaphase, telophase, and cytokinesis can occur.

Bortezomib works as a proteasome inhibitor in the ubiquitin-proteasome system (UPS). The proteasome is a protein complex responsible for the breakdown of mutated proteins⁷. Proteasome inhibitors (bortezomib) are successfully block the 20S proteasome. Current research suggests that inhibiting the proteasome using bortezomib impacts NF- κ b, a group of nuclear factors responsible for regulating expression and growth of cancer cells⁸. The proteasome is important for the translocation process of NF- κ b during translation; thus, blocking the proteasome inhibits expression of NF- κ b. The cell is then signaled to undergo apoptosis. Treatments of all three groups began on Day 26 for the bortezomib mouse and Day 17 for the vincristine mouse because these are the days the mice tumors reached a significant enough size to treat (Table 1-2). Mice were euthanized if a tumor reached 2cm in diameter or the mouse seemed to be suffering as a result of the tumor.

Table 1

Bortezomib Administration	
<u>Day</u>	<u>Amount (ul of 1 mg/kg)</u>
26	240
33	240
37	240

Table 2

Vincristine Administration	
<u>Day</u>	<u>Amount (ul of 1mg/kg)</u>
17	120
22	80
26	270
33	270
37	260

Results

The administration of 1ul of bortezomib at 1mg/kg every 4-5 days was a successful treatment for B-NHL xenografted in athymic nude mice. This is confirmed by the tumor measurement data collected both through BLI and caliper measurements. Prior to initiating bortezomib treatment on Day 26, the bortezomib treated mouse had two sizable tumors (Figure 4). Through BLI, we determined that the total flux through the left flank tumor was 1.02×10^8 p/s and 1.72×10^8 p/s through the right. Additionally, through caliper measurements, we determined that the left flank tumor had an area of 0.35cm^2 , while the right flank tumor was too deep to measure. Post-bortezomib administration, both the total flux and tumor area decreased significantly. By Day 33 of the experiments, the total flux through the mouse's left flank tumor was 4.75×10^5 p/s and 6.59×10^6 through the right tumor. By days 36 and 37, no flux or measureable tumors were seen.

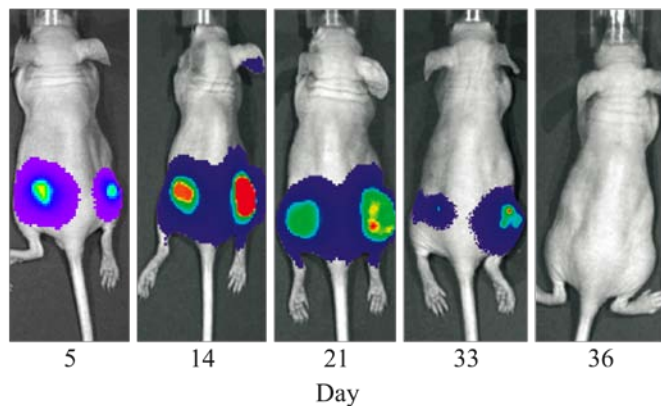


Figure 4: Bortezomib treated mouse. Bortezomib treatment began on Day 26. Total flux reached its peak at Day 21 (left tumor 1.02×10^8 p/s and right tumor 1.72×10^8 p/s). The mouse previously had gotten saline. A steady decline was seen after bortezomib took full effect. Tumors are absent by Day 36.

To demonstrate that 1ul of bortezomib at 1mg/kg every 4-5 days to successfully treat the tumors, a negative control was used for comparison. In a separate mouse, we lessened the amount of bortezomib the mouse received, giving the mouse only one-third of the required amount of bortezomib. Prior to drug treatment (Day 14 after Raji-luc injection), the mouse had a total flux of 1.12×10^6 through the left tumor and 1.39×10^6 through the right. Bortezomib treatment began on Day 17 at one-third the needed amount. By day 21 the total flux had climbed to 2.80×10^7 through the left tumor and 9.14×10^7 through the right and the mouse was ultimately euthanized.

Vincristine, the positive control, was also successful in reducing tumor size in our experiment, although to a lesser extent than bortezomib (Figure 5). We administered 1ul of vincristine at 1mg/kg into the peritoneum every 4-5 days. On Day 14, a mouse had a total flux of 2.28×10^7 through the left tumor and 5.65×10^7 through the right tumor. Additionally, caliper measurements showed the left tumor had an area of 0.81cm^2 and the right with an area of 0.72cm^2 . Vincristine treatment began on Day 17. By Day 28, improvement was already being seen through caliper measurements. By Day 33, there was no flux through the left tumor and a

reduced flux of 1.30×10^4 through the right. These fluxes remained constant in both tumors when images were taken again on Day 36.

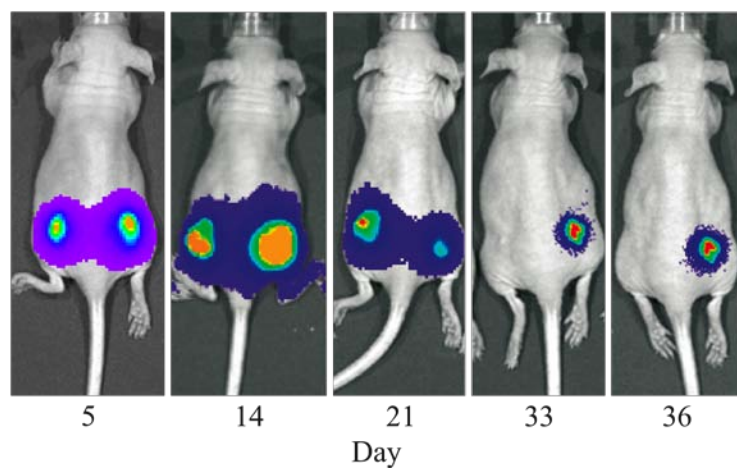


Figure 5: Vincristine treated mouse. Vincristine treatment began on Day 17. Maximum tumor flux was seen on Day 14 (left tumor 2.28×10^7 p/s and right tumor 565×10^7 p/s). A steady decrease in flux was seen after vincristine administration with the left tumor completely disappearing and the right decreasing to 3.33×10^5 p/s).

Left Flank Tumor Flux

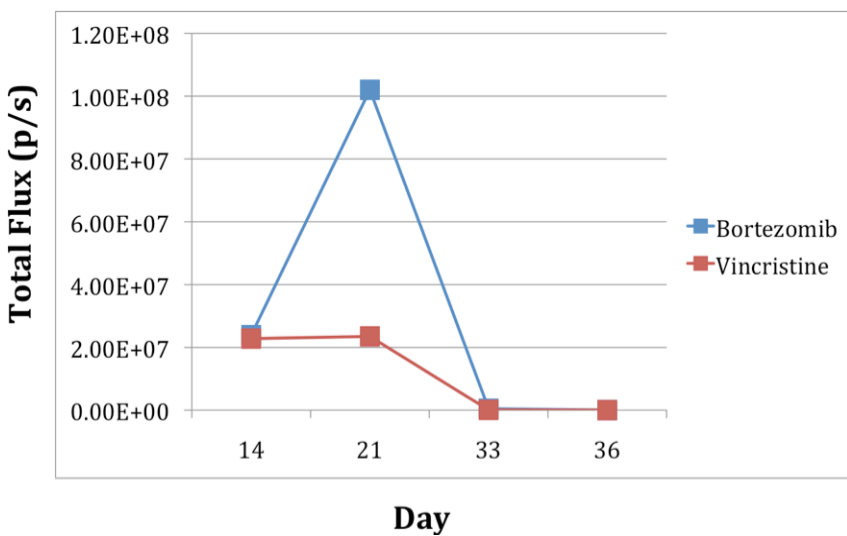


Figure 6: Total flux through the left tumor of both the bortezomib and vincristine treated mice throughout the experiment. Total flux determined through BLI.

Right Flank Tumor Flux

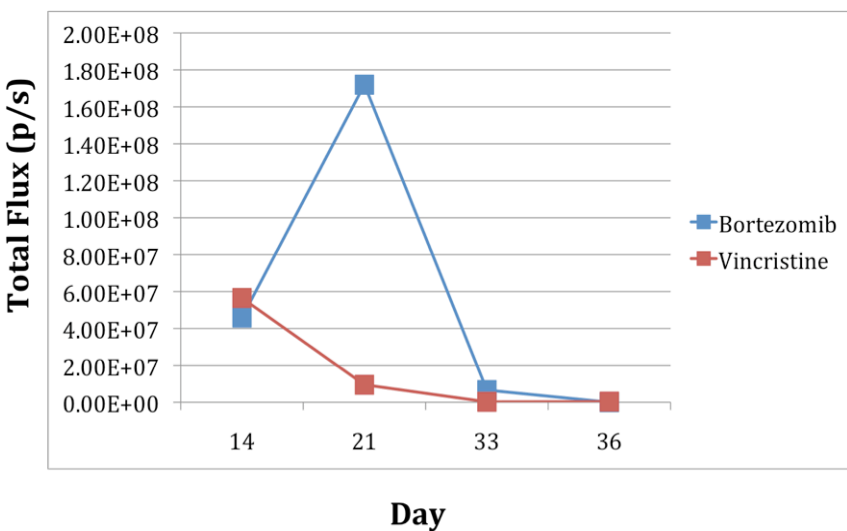


Figure 7: Total flux through the left tumor of both the bortezomib and vincristine treated mice throughout the experiment. Total flux determined through BLI.

Left Flank Caliper Measurements

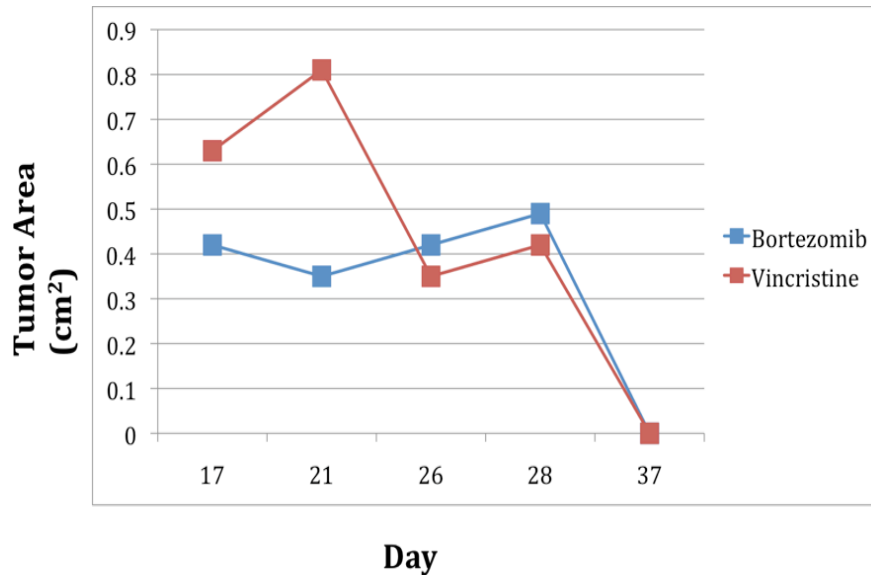


Figure 8: Left flank caliper measurements of both the bortezomib and vincristine treated mice throughout the experiment

Right Flank Caliper Measurements

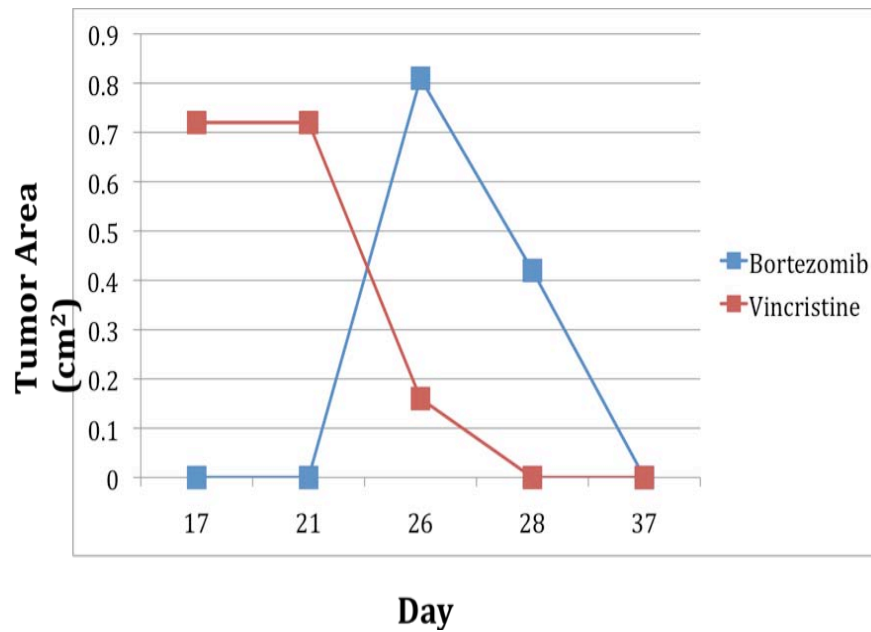


Figure 9: Right flank caliper measurements of both the bortezomib and vincristine treated mice throughout the experiment

Conclusion

Bortezomib is highly effective in treating B-NHL xenografted in athymic nude mice when administered approximately every 4-5 days. This is significant as bortezomib is an emerging drug for treating B-NHL. Knowing this will help in studies using autograft mouse models that would elicit a biological response similar to humans. This, in turn, could lead to a chemotherapy drug able to treat a wide variety of B cell cancers.

Vincristine also was effective in treating B-NHL in athymic nude mice when administered approximately every 4-5 days. It was necessary to show this in this experiment, as it gave us a positive control to compare bortezomib treated mice with. Our results obtained from vincristine seem to further support our findings of bortezomib's efficacy. This is because when compared to vincristine, bortezomib seems to act more rapidly in reducing tumor size. However, with both treatment groups only having a sample size of one due to mechanical errors, it is necessary to repeat such work to fully demonstrate that both drugs are effective in treating B-NHL.

Furthermore, this experiment shows the necessity of injecting appropriate amounts of luciferin when using bioluminescence imaging as a means of monitoring tumorigenesis. Our initial imaging session after injecting the Raji-luc cells showed luminescence in all 12 mice. In theory, this meant that the cells had taken in all 12 mice. As our imaging progressed, we noticed that several mice with very large tumors in previous imaging sessions no longer had visible tumors under the IVIS 200 in further sessions. Yet, the mice that exhibited this phenomenon were untreated. We hypothesized that this could be due to a scale weighing error. Our mice were being injected with 10ul/g of the luciferin substrate because this is what has been used in the literature. We noticed that our scale seemed to be registering a weight much lighter than

what our mice appeared to the naked eye. Therefore, we began using a different scale and noticed that our mice were in fact 3 times the weight as our other scale was indicating. More importantly, this meant that we were injecting only one-third of the luciferin needed to obtain a maximum luminescence reaction. When we began injecting the proper amount of luciferin we began to notice that tumors that we thought had disappeared had now reappeared.

Similarly, proper amounts of bortezomib and vincristine must be administered to see tumor shrinkage. The amount of both chemotherapy drugs being administered was based on mouse weight. Therefore, when we discovered that our scale was reading an improper weight for our mice, we realized that we were too little of the drugs needed to be effective. This was shown very early on with a mouse being treated with bortezomib. As the tumors of this mouse began to develop, we began administering bortezomib to the mouse. Yet, because the mouse was receiving only one-third of the amount it should have, the drug was ineffective in treating the tumor, and the tumor continued to grow. If the proper amount of bortezomib was administered, the mouse may have lived.

Future Directions

There are several different directions this project can now proceed. One involves the xenograft experiment, which must be repeated to insure that the tumors develop and remain in the mice, instead of spontaneously dying. Although many of the tumors we saw in the xenograft experiment did not actually die (rather, they were simply undetectable because of insufficient luciferin injections) we believe many of the tumor cells did spontaneously perish because they were only detectable for one or two imaging sessions. This action can be explained through two different hypothesis.

One, because the cells were injected into athymic mice, this simply meant the mice were only missing T cells that would reject the tumor take. These mice, however, were not completely immunocompromised because they still had NK cells and exhibit an inflammation response. Repeating this experiment using a more immunocompromised mouse, such as a SCID mouse, would eliminate the possibility of an immune response fighting off the tumor cells.

Secondly, Raji-luc cells can be injected using a Matra gel to improve tumor take. The dense gel is injected with the tumor cells, helping improve the binding affinity of the cells. Increased cell binding would create a greater number of cancer cells through mitosis, thus allowing for a greater signal. Moreover, the gel would help prevent the cancer cells from detaching themselves during the experiment.

References

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