Modeling the Effects of Vasoactive Intestinal Peptide on Pre-Osteoblast Differentiation

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Personal Statement

When I was a freshman, I learned that my grandfather had gotten diagnosed with Parkinson's Disease. This news came as a complete heartbreak and shock; I had heard of neurodegenerative diseases such as Alzheimer's and Parkinson's, but I never thought they would directly affect my family. I remember asking my grandfather about how he felt and whether he already noticed the effects of the condition. He responded that his handwriting was becoming smaller, his speech quieter, and his walking slower. My grandfather is an incredible person and a brilliant mathematician, and he has taught mathematics to hundreds of people throughout his life, including his five grandchildren. Parkinson's made it difficult for him to do what he loves, and that took the greatest toll of all.

Half a year later, while choosing a topic to research, I found myself poring over articles about Parkinson's Disease. After an upperclassman asked me about my specific interest in Parkinson's and I told him about my grandfather, he promptly advised me to study a similar disorder that was not as close to my heart. Thus, clinging to my fascination with neurodegenerative disorders, I settled on multiple sclerosis (MS). For the first two years of research, I investigated a cell type called microglia as a model system for MS. This project did not have the potential that I thought it would, so in the beginning of junior year, I decided to expand on the topic, specifically looking into the connections between MS and disorders in other organ systems. Once I came across a study describing a higher prevalence of osteoporosis in MS patients, I was intrigued and began researching the crosstalk between the skeletal and nervous systems. After spending time searching for similarities between the pathways of the two diseases, I came across the molecule vasoactive intestinal peptide (VIP), which turned out to be the perfect link between the conditions. VIP was beneficial in MS animal models and its

receptors were expressed by pre-osteoblasts. Thus, my project came to be the investigation of VIP as a common treatment of osteoporosis and MS.

I did all of my research in a biosafety level two lab at my high school. I am incredibly fortunate to have had such an advanced cellular biology research program at my disposal, and I utilized every possible aspect of the school's facilities. Toward the end of my junior year, after performing most of my data collection and analysis, I realized that the data I had would be well supplemented by a mathematical data model. Osteoblast differentiation is a time-sensitive dynamic process, so I knew that a model integrated over time would be an excellent way to analyze the data I already had as well as extrapolate potential results for the future. I had taken pre-calculus in sophomore year and calculus in junior year, so I used my newly acquired knowledge to build a system of three differential equations, each of which corresponded to a stage of differentiation. When I began considering a mathematical model, I did not know exactly which direction to take; thus, I created a simple model with few parameters. Ultimately, the model was built to determine the concentrations of cells in each stage of differentiation at a certain time.

The process of integrating mathematics into my cellular biology research project significantly strengthened the conclusions of my research and opened my eyes to the field of applied mathematics. It was daunting at first to venture into a new discipline. Mathematical modeling requires a completely different way of thinking from biology, and connecting the two fields was a challenge. While I was considering the connection between mathematics and biology, I had the idea to integrate the concentration of cells over time. As the model was meant to help me observe concentrations accumulated over time as opposed to instantaneous concentrations, it made sense to use integrated variables. This allowed me to model the dynamics

of the differentiation process and also fill the gaps that my data had previously left. The model provided my project with an entirely new dimension and brought a level of sophistication and creativity to my research, as I could now approach the same question from various angles. It was confusing and intimidating to try applying mathematics to my research at first, but it also completely transformed my research for the better.

One of the greatest aspects of working in a high school laboratory is that the students who spend several hours each day in the lab, as I do, become a tight-knit group of friends. Older students pass their wisdom onto students who are just beginning their research, and this cycle continues. As a graduating senior, the most valuable piece of advice that I can offer to a high school student is to not be afraid to venture outside of his or her "research comfort zone", or the field which the student is used to, and to keep an open mind in terms of research conclusions. Being inside a box with one correct outcome can prevent one from making discoveries and finding fascinating results; thus, it is important to be receptive to a number of outcomes. By leaving my comfort zone and accepting different results, my project became multi-dimensional and my conclusions became stronger.

Research

Multiple Sclerosis (MS) is a chronic, autoimmune disease of the central nervous system (CNS) that affects millions of people globally. It targets the neurons of the CNS and destroys their myelin sheaths, or the fatty layer of insulation around them. This can lead to loss of motor function, deteriorating vision, loss of balance, and muscle weakness. In addition, recent studies have shown

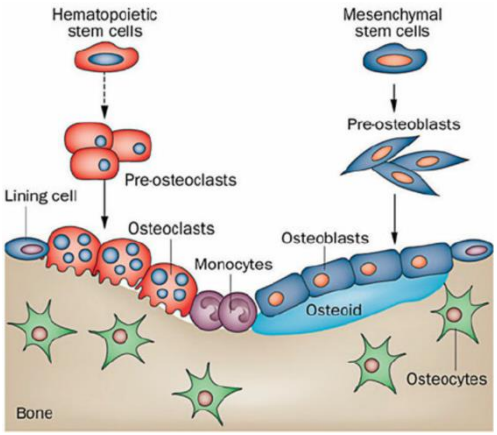


Figure 1. Bone formation and resorption (adapted from Nature Publishing Group (Lian et al., 2012). This diagram depicts the dynamic process of the bone life cycle.

mounting evidence of crosstalk between the cellular pathways of MS and osteoporosis, a disease of the degeneration of bone and loss of bone density. These studies have demonstrated that the risk of osteoporosis in people with MS is far higher than that of the general population (Lian et al., 2012). Osteoporosis is a disease that originates from an imbalance in the bone remodeling cycle. Osteoblasts, which are the cells that form new bone, cannot maintain pace with osteoclasts, which break down bone, causing a loss in bone mass and increasing the chance of fractures. The elevated risk of osteoporosis in MS patients can be due to a number of factors. For example, due to the muscle degeneration seen in MS patients, the mechanical load that the bones receive is reduced, and physical inactivity can lead to decreased bone mass and density. With these observations, the increased incidence of osteoporosis in patients with MS warrants further research as well as a common therapy.

When investigating molecules of significance in these two diseases, one neuropeptide called vasoactive intestinal peptide (VIP) is distinctive. VIP is found in the nervous system as a transmitter of nervous signals, or a neuropeptide, and studies have demonstrated its beneficial effect in animal models of disease. In these animal models, VIP has shown the capacity to control immune cell function, which is significant in the brains of MS patients, as well as inhibit other immune responses (Tan and Waschek, 2011). Due to this, it is regarded as a molecule that prevents inflammation with the potential to be a therapeutic factor in MS. Interestingly, neuropeptides are not only responsible for their roles in the nervous system, but they have also been demonstrated to affect the skeletal system. VIP is one of the few neuropeptides which can be

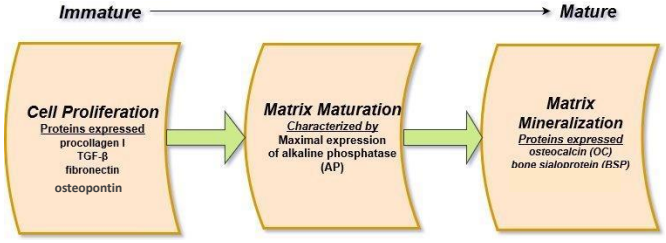


Figure 2. Diagram of the three stages of pre-osteoblast differentiation and the marker proteins in each stage.

interpreted by molecules on osteoblasts called VIP receptors. This indicates that certain neuropeptides, including VIP, may have significant effects on bone cell functions that have not been extensively studied (Lerner, 2000). Given VIP's positive effects in models of MS and the connection between MS and osteoporosis, VIP may have beneficial effects on bone formation and integrity and may represent a way to address the increased frequency of osteoporosis in MS patients.

Bone formation begins with mesenchymal stem cells, or MSCs, which have the potential to differentiate, or mature and become more specialized, into a number of different cell types, including osteoblasts. The MSCs differentiate into pre-osteoblasts, which then differentiate into osteoblasts, and lastly osteocytes, which are fully mature cells that form bone. The research reported here focuses specifically on the effects of VIP on the differentiation of pre-osteoblasts to osteoblasts, which occurs in three stages – cell proliferation, matrix maturation, and matrix mineralization. Each of these three stages is characterized by the expression of specific proteins: for example, in the first stage, fibronectin, TGF-β, osteopontin, and collagen 1 predominate; in the second stage, alkaline phosphatase; and in the third stage, osteocalcin. As the cell progresses through these stages to become a mature osteoblast, calcium and phosphate deposits can also be detected (Rutkovskiy et al., 2016).

In this study, the effect of VIP on pre-osteoblast differentiation is measured through the expression of these proteins, and untreated pre-osteoblasts are compared to pre-osteoblasts under varying treatments. Specifically, pre-osteoblasts are treated with exogenous VIP, ascorbic acid (Vitamin C), or the combination of VIP and ascorbic acid. Ascorbic acid has been shown in numerous

studies to have stimulating effects on osteoblast proliferation and differentiation, although the mechanism through which this occurs has not been fully established (Aghajanian et al., 2015). Additionally, a limitation of this study is that pre-osteoblasts spontaneously differentiate in culture; thus, the results obtained are evaluated in comparison to a control, in which pre-osteoblasts mature over time. Another limitation is that wet lab assays only demonstrate the cumulative effects of the treatments at a specific time point with no way to observe the dynamics of the differentiation process. Wet lab studies of this nature do not show the proportions of cells in the various stages of pre-osteoblast differentiation at the various time points. For this reason, a data model was constructed and tested in this study that could predict the proportion of cells in the varying stages of pre-osteoblast differentiation under the effects of the treatments for 96 hours and at 240 hours. Thus, this research investigates the role of VIP in promoting pre-osteoblast differentiation; it is hypothesized that *in vitro* VIP treatment of the pre-osteoblast cell line MC3T3-E1 Subclone 14 will result in protein expression indicative of pre-osteoblast differentiation, demonstrating its potential as a therapeutic option to be investigated in patients with osteoporosis as well as MS (based on previous research).

Materials and Methods

Cell Culture

MC3T3-E1 Subclone 14 Mus musculus Pre-Osteoblast cells (ATCC #CRL-2594) were incubated at 37℃, at 5% CO_2 and 100% humidity and were fed with α-MEM (Gibco Life Technologies).

Trypsinization and Maintenance of Cell Cultures

When cells became 90% confluent, they were trypsinized with 0.25% trypsin (Gibco) until they became detached from the flask.

Indirect Antibody Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect ELISA was performed on lysates or supernatant in a 96-well plate according to the manufacturer (Abcam).

Alkaline Phosphatase Detection by Flow Cytometry

Confluent pre-osteoblasts were harvested with 0.25% wt/vol trypsin at 37˚C. The cell pellet was rinsed twice with 50 mmol/L Tris-HCl, 0.15 M NaCl pH 7.5 (TN) and 300,000 cells were incubated in 0.5 mL TN containing 6.25 µg fluorescein diphosphate (FDP, F 2999 Molecular Probes) for 10 min at 37˚C. The reaction was blocked by cooling in an ice bath. The cells were collected by centrifugation, washed twice with TN, and suspended in 0.3 mL TN for analysis. Single cell analysis was performed on a Facstar Plus cell sorter (Becton Dickinson) equipped with an argon laser emitting 250 mW excitation at 488 nm; 5000 cells were analyzed in each sample. Specific green fluorescence due to fluorescein emission was analyzed through a 530-540 nm band pass filter (protocol retrieved from Rattner et al., 1997).

Statistical Analysis

Data was analyzed using t-test for statistical significance ($\alpha = 0.05$). Data was analyzed with regard to cell number at the time of the assay. Most data is presented as percent of solvent control. Additionally, the significance of the combination treatment was compared to the ascorbic acid treatment to investigate the potential additive effects of VIP. Assays were performed 3 or more times with biological replicates of 4 and 5 (n = 4-5). All graphs and diagrams were made by author unless otherwise noted.

Results

Effects of Ascorbic Acid and VIP on Collagen I Expression (96 Hours)

While other first-stage proteins (osteopontin, TGF-β, fibronectin) were also measured, they had similar results to those of Collagen Type I; therefore, Collagen Type I is used a representative of all first-stage proteins in this paper. Collagen Type I is the most prevalent extracellular matrix protein in bone and it peaks in production in the proliferative stage. At the end of differentiation, the mature osteocyte lives in the environment of a mineralized extracellular matrix made of Collagen Type I mostly secreted early in the process of differentiation (Stein, 1993). The three treatments used in this study had a significant downregulation effect ($p < 0.001$***) on Collagen I expression, indicating, once again, that fewer cells are in the first stage of differentiation and more cells are in later stages under administration of the treatment as opposed to no treatment. No dramatic additive effect of VIP was observed.

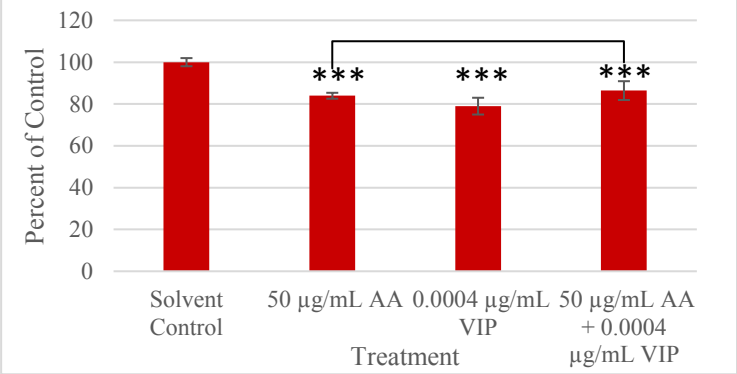


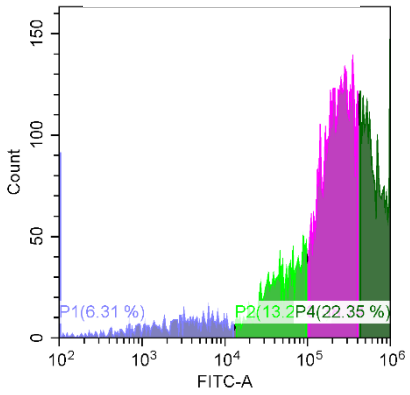
Figure 3. Collagen Type I Expression. The three treatments had similar effects on collagen expression after 96 hours, decreasing it significantly when compared to the control. However, VIP had no significant additive effect when the combination treatment is compared to the ascorbic acid treatment. Bars represent means ± SD (n = 4-5). Data with asterisk marks are significantly different from the values in the control group at $p<0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.05$◆; $p < 0.01$◆◆; and $p < 0.001$◆◆◆.

Effects of Ascorbic Acid and VIP on Alkaline Phosphatase Expression Flow Cytometry (240 Hours)

Following the procedure specified in materials and methods, the results obtained from the Facstar Plus Cell Sorter (Becton Dickinson) were gated according to the visible peaks in the control, and these gates were applied to the rest of the samples. The fluorescence that is presented on a

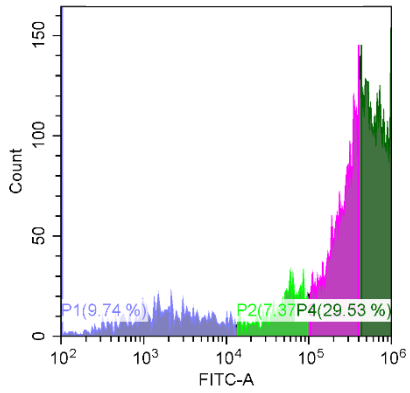
logarithmic scale on the x-axis is proportional to the intensity of alkaline phosphatase expression, which is maximized in the second stage of differentiation (Lian and Stein, 1993). In reference to the control data, the peaks demonstrate spontaneous differentiation, meaning that after ten days the osteoblasts naturally produced a certain amount of alkaline phosphatase.

Control



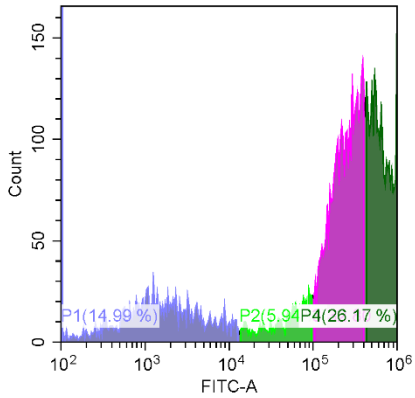
Population	Events	% Total	% Parent
All Events	10000	100.00 %	100.00 %
P1	631	6.31 %	6.31 %
P2	1328	13.28 %	13.28 %
P3	3874	38.74 %	38.74 %
P4	2235	22.35 %	22.35 %

AA



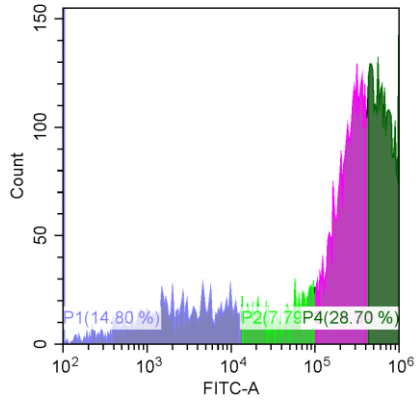
Population	Events	% Total	% Parent
All Events	10000	100.00 %	100.00 %
P1	974	9.74 %	9.74 %
P2	737	7.37 %	7.37 %
P3	2230	22.30 %	22.30 %
P4	2953	29.53 %	29.53 %

VIP



Population	Events	% Total	% Parent
All Events	10000	100.00 %	100.00 %
P1	1499	14.99 %	14.99 %
P2	594	5.94 %	5.94 %
P3	3280	32.80 %	32.80 %
P4	2617	26.17 %	26.17 %

AA+VIP

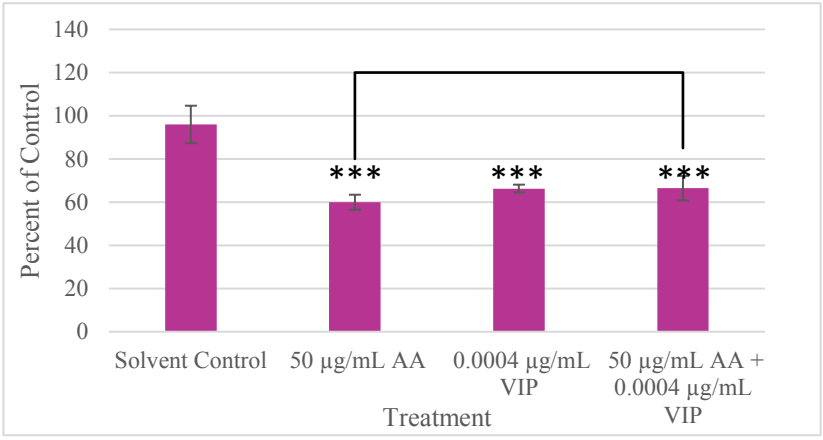


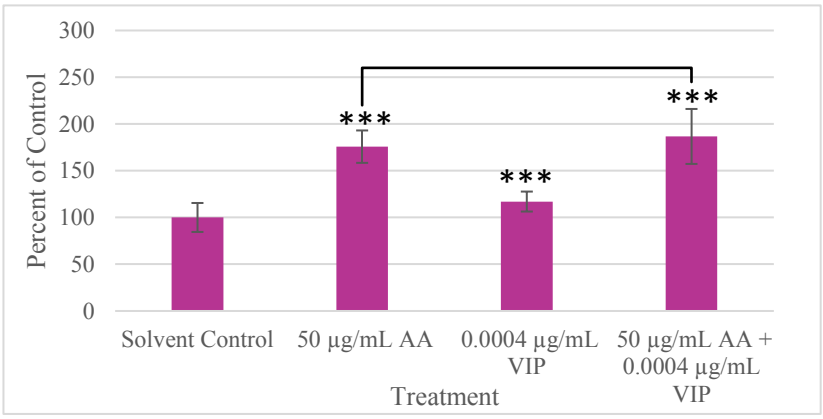
Population	Events	% Total	% Parent
All Events	10000	100.00 %	100.00 %
P1	1480	14.80 %	14.80 %
P2	779	7.79 %	7.79 %
P3	2934	29.34 %	29.34 %
P4	2870	28.70 %	28.70 %

Figures 4 (Top left, Control), 5 (Top right, AA), 6 (Bottom left, VIP), 7 (Bottom right, AA and VIP). Flow cytometry analysis of the expression of alkaline phosphatase after 240 hours of treatment. Treated osteoblasts were incubated in fluorescein diphosphate for 10 minutes at 37˚C. Fluorescence is displayed on a logarithmic scale. Corresponding tables represent the percentages in each of the colored gates.

Taking note of the dark green area on each graph as the most intense alkaline phosphatase, all three treatments induced the production of alkaline phosphatase after ten days, as the percentage of cells with highly intense alkaline phosphatase expression increased (29.53% for ascorbic acid, 26.17% for VIP, and 28.70% for the combination treatment, as opposed to 22.53% for the control). Ascorbic acid had the most significant effect in upregulating production of alkaline phosphatase and the VIP and combination treatments followed closely, demonstrating that VIP follows a similar trend as ascorbic acid.

Effects of Ascorbic Acid and VIP on Osteocalcin Expression (96 Hours and 240 Hours)





Figures 8 and 9. *Figure 8:* Osteocalcin Expression 96 Hours of Treatment. There was a significant decrease in the expression of osteocalcin under all three treatments, and the effect was approximately equivalent. *Figure 9:* Osteocalcin Expression 240 Hours of Treatment. All three treatments significantly induced the expression of osteocalcin. Bars represent means ± SD (n = 4-5). Data with asterisk marks are significantly different from the values in the control group at $p<0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.05$◆; $p < 0.01$◆◆; and $p < 0.001$◆◆◆.

Osteocalcin, a non-collagenous protein that regulates mineral deposition in bone and inhibits "mineral nucleation" *in vitro* was evaluated in the presence of the three treatments after 96 hours and 240 hours (Lian and Stein, 1993). This protein increases in concentration when the density of bone increases, indicating that it is present in late stages of differentiation. Specifically, its expression peaks in the mature bone cell, or in the third stage of differentiation. After the first

four days of differentiation, osteocalcin expression was significantly lower in the treatment groups than in the control group, indicating the lower proportion of cells in the third stage. However, after ten days, there was a dramatic rise in osteocalcin expression in all three of the treatments, demonstrating VIP's benefit as a differentiating factor and its potential as a treatment for osteoporosis.

Modeling the Results

Description

It is evident that delineating the stages of pre-osteoblast differentiation is difficult and creates issues when attempting to develop therapies for MS and osteoporosis. While the research reported here demonstrates that VIP may be a significant therapeutic agent for osteoporosis in MS patients, *in vitro* research such as this may be verified and furthered by creating a data model. Therefore, a data model was built to represent the dynamics of pre-osteoblast differentiation in an effort to understand the differentiation process under treatment more fully. This model presents fold change of cell numbers in the three stages of differentiation – cell proliferation, matrix maturation, and matrix mineralization – as independent variables. The dependent variable is denoted as fold change because the final number of cells (at the time of measurement) is normalized by the initial number of cells. $X(t)$ denotes the number of pre-osteoblasts at time t, normalized by this number at time 0. $Y(t)$ and $Z(t)$ represent the fold change of partially differentiated osteoblasts and fully mature osteoblasts, respectively. Additionally, the only cells that can proliferate in this model are pre-osteoblasts.

The proportional term aX represents the rate of proliferation. The transitions between the stages are modeled in a simple way, with pre-osteoblasts only able to progress to the second stage,

and partially differentiated cells only able to enter the third stage, or the fully mature stage. The linear terms αY and βZ denote the rates of the two transitions. Additionally, due to the 96-hour treatment period, mortality is assumed to play a role, and it is proportional to the square of the total number of cells, so that the higher the number of cells, the greater the rate of mortality. Between the three stages, the mortality is also proportional to the number of cells. All experiments were begun with a set number of pre-osteoblasts and no differentiated cells, so the model can be summarized by the following equations with the initial conditions: X(*0*) = 1, Y(*0*) = 0, Z(*0*) = 0.

$$\frac{dX}{dt} = (a - \alpha)X(t) - bX(t)\big(X(t) + Y(t) + Z(t)\big),$$

$$\frac{dY}{dt} = \alpha X(t) - \beta Y(t) - bY(t)\big(X(t) + Y(t) + Z(t)\big),$$

$$\frac{dZ}{dt} = \beta Y(t) - bZ(t)\big(X(t) + Y(t) + Z(t)\big).$$

Representation of Experimental Data

The proteins that were measured through ELISA are either intracellular or extracellular. Intracellular protein concentrations at a time *t* are reflective of the cell numbers, and are represented as being proportional to cell numbers at the time of measurement, but extracellular measurements are the results of 96 hours of cellular activity, and are therefore represented by a time-integrated version of cell counts. Most of the experimental data is in percent of control, and the data model follows the same pattern. It creates a control set of parameters, and then estimates the actual parameter sets for the different treatments so that the observed percent of control ratios are fitted. The rate of mortality is assumed to be independent of the treatments and remains the same throughout the numerical procedure. For the estimation done by this model, alkaline phosphatase staining data and ELISA data for osteocalcin, fibronectin, and TGF-β are used to represent the stages of the differentiation process. The control set of parameters is chosen to fit the

data obtained for total cell count as well as the expected time for cells to begin to mature, reported by scientific literature to happen after about ten days (Lian and Stein, 1993). More precise description of the control set would be possible if more data was available, and it is a target of future stages of research.

Model Estimation Results (96 Hours)

The model results for the differentiation process under the three treatments after 96 hours as compared to the control are presented below. It is important to note both the estimated parameters and the time-dynamic graphs. The estimated parameters for the treatments displayed in the table below indicate that the rate of proliferation in pre-osteoblasts after being treated for 96 hours was positively affected by both ascorbic acid and VIP, with ascorbic acid having a stronger effect on proliferation than VIP alone, and having the strongest effect while combined. The graph for the fold change of cell numbers in the first stage demonstrates that VIP had the lowest fold change, indicating its insignificant effect on proliferation. Similarly, the rate of pre-osteoblast transition to the second stage was positively affected by all three treatments, VIP alone being more effective than ascorbic acid alone and most effective in combination. The combination treatment caused the greatest fold change in the second stage as well, demonstrating its potential to stimulate differentiation. Finally, the rate of transition to the fully differentiated stage was significantly affected by all three treatments, as was the fold change in the third stage. The combination treatment group had the highest fold change of cell numbers in the latest stage.

	Proliferation Rate	Rate of Transition to Stage 2	Rate of Transition to Stage 3
Control, Untreated	0.042000	0.019100	0.025200
Ascorbic Acid 50 µg/ml	0.051030	0.022309	0.031186
VIP 0.0004 µg/ml	0.046565	0.023491	0.031289
Ascorbic Acid 50 µg/ml, mix VIP 0.0004µg/ml	0.052396	0.024075	0.030345

Table 1. Table of Parameters of the Stages of Differentiation After 96 Hours of Treatment. This is a table of the rates of transitions between stages of the three treatments as compared to the control.

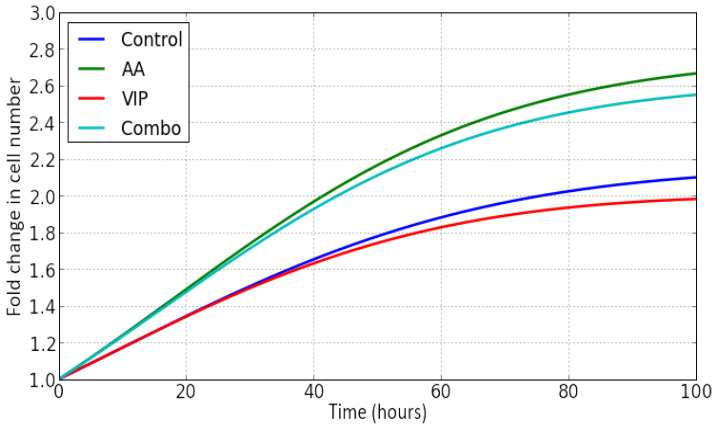
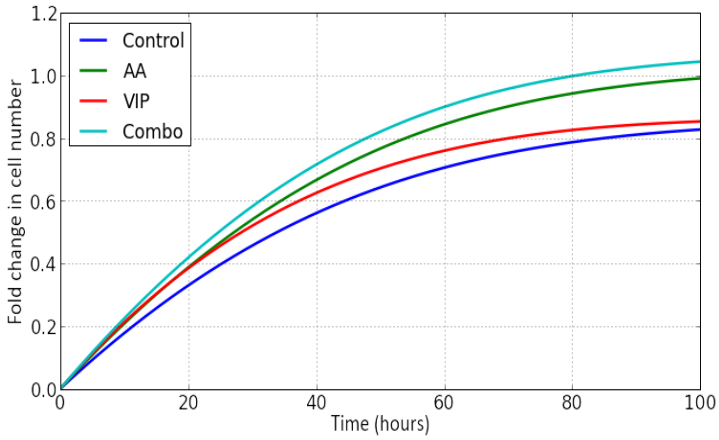


Figure 10. Graph Depicting Fold Change of Cell Numbers in the First Stage After 96 Hours of Treatment. The four lines represent the treatments and the control, and the fold change is relative to the initial number of pre-osteoblasts. It can be seen that ascorbic acid and the combination treatments had the greatest impact on fold change in the first stage, while the VIP-treated group had a lower fold change in the first stage than the control. As ascorbic acid is a proliferative agent, these results are expected.

Figure 11. Graph Depicting Fold Change in Cell Numbers in the Second Stage After 96 Hours of Treatment. The four lines represent the treatments and the control, and the fold change is relative to the initial number of pre-osteoblasts. The data of all three treatments suggest that there was a higher fold change in the second stage as compared to the control, and the combination treatment had the greatest fold change in the second stage, indicating induced differentiation.



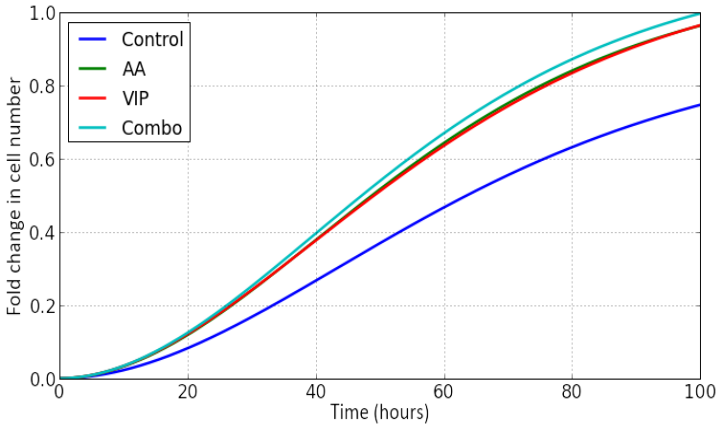


Figure 12. Graph Depicting Fold Change in Cell Numbers in the Third Stage After 96 Hours of Treatment. The four lines represent the treatments and the control, and the fold change is relative to the initial number of pre-osteoblasts. The three treatments caused a higher number of cells to progress to the third stage than the control. The combination treatment was more effective than the other two treatments and the control.

Model Estimation Results (240 Hours)

According to the table of parameters below, treating the cells with all three agents for ten days increased the rate of proliferation; however, the VIP-treated cells did not have a significant effect on proliferation. Interestingly, the VIP-treated group had a lower number of cells in the first stage than the control, which is in agreement with previous results obtained in this study about VIP's lack of efficacy in early differentiation stages. In the second stage, all three treatments caused the cells to transition to the second stage faster than the control, demonstrating an increased rate of differentiation, and there was a higher number of cells in the second stage the combination and ascorbic acid treated cells, while the VIP treatment first had a higher number than the control, then a lower number after the 100-hour mark. The results of the third-stage rates were notable, as only VIP increased the rate of transition to the third stage and the cell count in the third stage. This demonstrates VIP's significant effects in later stages of differentiation.

	Proliferation Rate	Rate of Transition to Stage 2	Rate of Transition to Stage 3
Control, Untreated	0.028800	0.015640	0.010080
Ascorbic Acid 50 µg/ml	0.030918	0.016421	0.009052
VIP 0.0004 µg/ml	0.028897	0.017827	0.010383
Ascorbic Acid 50 µg/ml, mix VIP 0.0004µg/ml	0.031606	0.017527	0.008550

Table 2. Table of Parameters of the Stages of Differentiation After 240 Hours of Treatment. This is a table of the rates of transitions between stages of the three treatments as compared to the control.

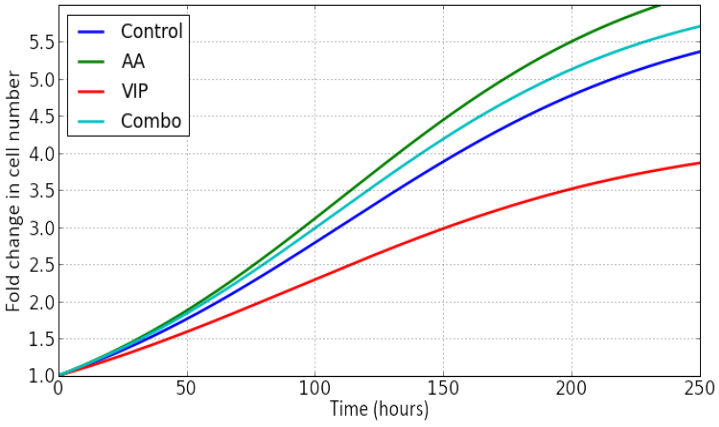


Figure 13. Graph Depicting Fold Change of Cell Numbers in the First Stage After 240 Hours of Treatment. The four lines represent the treatments and the control, and the fold change is relative to the initial number of pre-osteoblasts. This graph shows that after ten days in treatment, ascorbic acid treatment caused a greater number of cells to be in the first stage, followed closely by the combination treatment, and then control and VIP with the lowest rates. This is expected, due to ascorbic acid being a proliferative factor and VIP lacking an effect in the early stages in other results of this study.

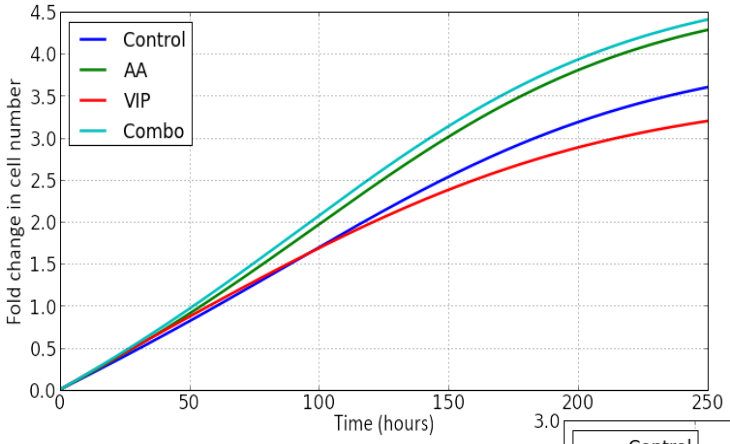
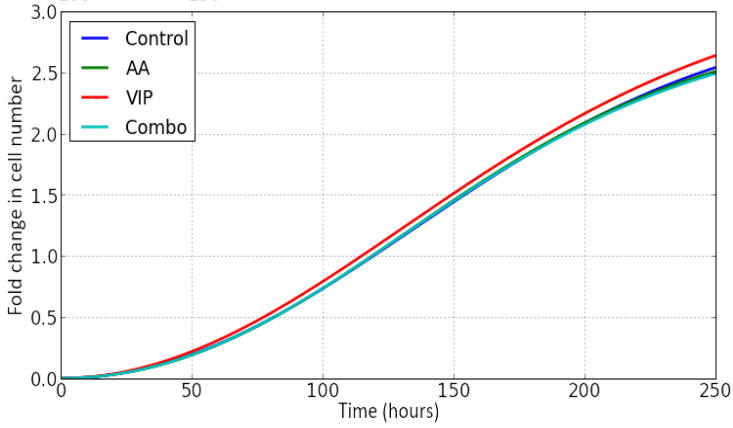


Figure 14. Graph Depicting Fold Change in Cell Numbers the Second Stage After 240 Hours of Treatment. The four lines represent the treatments and the control, and the fold change is relative to the initial number of pre-osteoblasts. The results here are interesting to note, as the combination-treated group had the greatest number of cells in the second stage, while the VIP-treated group had a higher cell count than the control group in the first 100 hours, and a lower cell count later on, indicating its increasing efficacy as the stages occur.

Figure 15. Graph Depicting Fold Change in Cell Numbers in the Third Stage After 240 Hours of Treatment. The four lines represent the treatments and the control, and the fold change is relative to the initial number of pre-osteoblasts. Interestingly, VIP had the greatest number of cells in the third stage throughout the majority of the ten-day period. This further supports the proposal that VIP is effective in later stages of differentiation.



Conclusion and Future Research

After analysis of both the experimental data and the data model, it can be concluded that VIP is a potential treatment of MS as well as an alternative for ascorbic acid as a treatment for osteoporosis. Throughout the results from the study, it can be seen that VIP has similar effects – on the expression of the characteristic proteins – to those of the ascorbic acid treatment. The first stage proteins have consistent results showing that all of the treatments progress the pre-osteoblasts to further stages of maturation. The results shown through different stage protein expression and the data models after 96 hours and 240 hours indicate that VIP does not play a significant role in early stages of differentiation; however, it becomes extremely effective in later stages, as shown by osteocalcin expression, alkaline phosphatase results, and the third-stage graph of the 240-hour data model. Ascorbic acid is known to be a proliferative and differentiating factor, and the data

from this study support this. Therefore, a combination treatment of ascorbic acid and VIP is the most advantageous treatment, as ascorbic acid induces proliferation and early stages of differentiation and VIP stimulates later stages and maturation.

Many of the results indicate that the combination treatment of ascorbic acid and VIP is more effective than either of the treatments in isolation, and future research will investigate the synergistic effects of the combination treatment. Given that the cells were treated for a maximum of ten days and pre-osteoblast differentiation, in its entirety, takes from 16 to 20 days, it is also valuable to consider the potential long-term effects of the combination treatment (Lian and Stein, 1993).

As mentioned before, the data model was built on the premise that although the experimental results offered the final concentration of each protein after 96 hours and 240 hours, they did not indicate the proportions of the cells in different stages. This limitation exists due to the fact that pre-osteoblast differentiation is a dynamic process that does not always occur one stage at a time, and it is addressed by the time-integrated approach of the protein expression data. As a note, the parameters in which the data model resulted do not indicate that the majority of cells under the treatments are in later stages of differentiation; they represent that a greater percentage of pre-osteoblasts under the treatments have progressed to the later stages, suggesting that the treatments induced differentiation.

Ultimately, using the model, it is possible to predict what will occur in the late stages of differentiation – in two or three weeks – and the next step would be to experimentally test these predictions. Another molecule of interest is cyclic adenosine monophosphate (cAMP), a secondary messenger that regulates osteoblastic function and gap junction assembly. It would be beneficial to know the effects of the treatments on cAMP expression as gap junctions are crucial structural

components of bone. Finally, a valuable direction of future research would be to test these treatments *in vivo*; however, it is crucial to note that a limitation exists due to the short half-life ascorbic acid possesses in an organism. Thus, a strategy of these treatments would be to administer them more frequently.

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