Northern Illinois University

The Effect of Agents on HIV1-LTR

A Thesis Submitted to the

University Honors Program

In Partial Fulfillment of the

Requirements of the Baccalaureate Degree

With University Honors

Department of Biological Sciences

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DeKalb, Illinois

December 2001

Abstract

The goal of these experiments is to determine the relative effects of various agents (Ptpt dinucleotide, lutein, and vitamin C) on the activity of long terminal repeat (LTR) in HIV 1 infected cells (HIV LTR) in both control and UV exposed cells. It is known that the LTR is important in transcription and therefore viral replication. A HeLa cell line transfected with a plasmid that has the HIV-LTR and an E. coli chloranphenicol acetyl transferase (CAT) reporter gene was used. The agents were applied and then the amount of CAT activity was measured doing a CAT assay. The more CAT activity means the more LTR activity.

The results showed that none of the agents used (ptpt dinucleotide, lutein, and vitamin C) inconclusively inhibited the LTR in HIV 1 infected cells. This doesn't mean that these agent don't have inhibitory elements. These experiments simply show that the agent doesn't inhibit the LTR directly or through NFkB. It may inhibit through another enzymatic signal cascade or through a method other than the LTR. Another explanation for the results are that the concentration was too low and/or the agent didn't have enough time to have an effect on the cells. Therefore, further experimentation is needed to discount these agents as a possible HIV treatment.

Acknowledgements

I would like to thank Dr. Griffiths for teaching me lab techniques and allowing me to work in his lab. I would also like to thank all others who work in the lab that create an enjoyable working environment. Especially Courtney Carter who helped me with many of my projects and ideas.

Approval Page

I, the undersigned, approve this project for its submittal to the honors and biology departments at Northern Illinois University for the purpose of fulfilling the requirements set forth to complete university and biological honors.

Dr. Griffiths

T Griffiths 12/5/01

signed date

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Introduction

Despite AIDS (acquired immunodeficiency syndrome) deaths going down in the mid 1990s due to combination treatments that help keep other infections at bay, there are about one million people in the United States infected with HIV (Boyles 2001). The Center for Disease Control and Prevention (CDC) reported as of June 30, 1997 AIDS is the second leading killer of people ages 25-44 in the United States with 612,078 cases of AIDS and 379,258 AIDS deaths. Worldwide the CDC reported an estimated 30.6 million people were living with HIV/AIDS as of December 1997. Through 1997 there have been 11.7 million (9 million adults and 2.7 million children) HIV/AIDS related deaths (National Institute of Allergy and Infectious Diseases 1998). AIDS is caused by HIV (human immunodeficiency virus). The virus attacks and kills CD4+T cells of the immune system, and the death of these immune cells leaves the host vulnerable to opportunistic infections. The virus has two single strands of RNA and at the ends of these strands is an RNA sequence called the long terminal repeat (LTR). This sequence controls the production of new viruses. The HIV-LTR is stimulated by the cellular trans-activator Nuclear Factor kB (NFkB) and the viral protein Tat (Biswas et. al 1993).

The production of new viruses can be triggered by many different stresses like proteins in the virus or host cell. UV light is also known to be a stress that induces LTR activity. In the following experiments UVC light (100-280 nm) was used. UVC is absorbed by nucleic acids and proteins (Tyrell, 1996) and causes DNA damage. This kind of light doesn't make to the earth's surface (it is blocked by the ozone layer) but it is used in all germicidal lamps found in labs, hospitals, etc. Valerie (1988) et al. report that UVC does indeed induce HIV type 1 LTR in HeLa cells.. It is not known how UV induces HIV-LTR but there are many theories.

UV light activates a variety of transcription factors and proteins (Devary et al., 1992; Sachsenmaier et al., 1994). One such transcription factor is NFkB (Devary et al., 1993). It moves from the cytoplasm to the nucleus and binds to the DNA inducing LTR

activity. UVB can also induce this transcription sequence but most of the following experiments will be using UVC. These HeLa cells were transfected with a plasmid that had the HIV-LTR and E. coli chloramphnicol acetyl tranferase (CAT) gene. Therefore when LTR is activated so is the CAT reporter gene. So the amount of LTR activity is measured indirectly by finding the amount of CAT activity. We obtained the HeLa cells engineered by Valerie from Gayle Woloschak of Argonne National Laboratory.

Materials and Methods

CAT assay: The media was poured off and the plates were rinsed once with cold PBS. Then 3 ml of trypsin was added to the plates and the cells were allowed to detach. 3 ml of PBS was then added and the suspension was put into 15 ml centrifuge tubes and put on ice. The plates were then washed with 1 ml PBS and added to the tubes. The tubes were centrifuged for 10 minutes at 1,000 rpm. The supernatant was discarded and 1 ml of cold PBS was added to the tubes. The supernatant was transferred to epindorf tubes. 0.1 ml of the supernatant was added to 10 ml of PBS to count the number of cells in a Coulter Counter. The remaining liquid in the epindorf tube was centrifuged for five minutes. The supernatant was removed and 150 ul of 0.25 M Tris-HCl (pH 8.0) was added. The pellet was resuspended and put through three freeze/thaw cycles using liquid nitrogen and a 37.0 degree Celsius water bath. The tubes were then heated in a 60 degree Celsius water bath for ten minutes to inactivate the endogenous acetylase. The tubes were spun again for four minutes. The supernatant was transferred to new tube and stored for 24 hours in a -80 degree Celsius freezer. During this 24 hour period the master mix was made. This contained 69.5 ul of 0.25 M Tris-HCl (pH 8.0), 5 ul n-butyryl Coenzyme A, and 0.5 ul 3H-chloramphenicol (0.025 mCi/ml). After 24 hours the supernatant was thawed and 50 ul is added to a new epindorf tube. 80 ul of the master mix was added under the radioactive hood. The tubes were then incubated at 37 degrees Celsius for 2.0 hours. Then 300 ul of xylene was added to terminate the labeling. The tubes were then vortexed

vigorously for 1-2 minutes and spun for 3 minutes. The aqueous layer was removed and 150 ul of 0.25 M Tris-HCl was added. The tubes were vortexed and spun as before. The aqueous layer (lower layer) was again removed and another 150 ul of Tris-HCl was added. The tubes were vortexed and spun again as before. Next 200 ul of the upper organic layer was removed and added to a scintillation vial with 10 ml of scintillation cocktail. A scintillation counter was used to determine cpm and the results were plotted in counts per 100,000 cells.

Incubator: The incubator used was a water-saturated incubator at 37.2 degrees Celsius and 2.5% CO2.

media: The media consisted of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, and antibiotics.

Chapter 1

The effect of ptpt dinucleotide on LTR in HIV 1 infected cells

Introduction: Ptpt thymidine dinucleotide has been shown to enhance DNA repair by activating p53 (Maeda, et. al. 1999). p53 is a tumor suppressor protein and transcription factor. Therefore it is hypothesized that since p53 inhibits transcription factor perhaps it also inhibits the long terminal repeat in HIV 1 infected cells.

Materials and Methods: The cells used were HeLa cells transfected with the HIV-LTR and CAT reporter gene on a plasmid. The cells were cultured in five plates at 300,000 cells per plate in 35 mm dishes. Each dish was given 5ml of media. The cells were placed in the incubator and allowed to attach. The plates were then exposed to various procedures as shown in Table 1.

Table 1: Plate treatment #1 of dinucleotide Ptpt

plate number	name	treatment
1	control	wash 2 times with 3 ml PBS and given 5 ml of media
2	UVC	washed 2 times with 3 ml PBS and exposed to 19 J/m2 of UVC light and given 5 ml of media
3	UVB	washed 2 times with 3 ml PBS and exposed to 200 J of UVB light and given 5 ml of media
4	ptpt	add 55.5 ul ptpt (oligodeoxythymidylate) and 5 ml of media
5	ptpt +pbs	washed 2 times with 3 ml PBS and add 55.5 ul ptpt and 5 ml of media

*the ptpt solution consisted of 0.5 ml of PBS and 50 units of ptpt dinucleotide (ptt oligodeoxythymidylate)

After this treatment the cells where again placed in the incubator for twenty-four hours. After twenty-four hours the cells were put through a CAT assay to determine the amount of CAT activity present.

Then to verify the results the experiment was performed again with the treatments shown in table 2 and then placed through a CAT assay. 400,000 cells were plated for each 35mm dish. They were allowed to attach then put through the treatments shown below.

Table 2: Treatment #2 using ptpt on HIV infected HeLa cells

plate number	Name	Treatment
1	control	wash twice with 3 ml PBS and add 5 ml media
2	control UV	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media
3	ptpt #1	wash twice with 3 ml PBS and add 5 ml media and 55.5 ul ptpt
4	ptpt #2	wash twice with 3 ml PBS and add 5 ml media and 55.5 ul ptpt

Results: The results of this experiment are shown in Tables 3 and 4 and Charts 1 and 2. These results seen to contradict each other. Treatment #1 showed no decrease (and actually a small increase) in LTR activity and Treatment #2 showed a decrease in LTR activity.

Table 3: CAT activity in HeLa Cells with Ptpt treatment #1

Treatment	CAT activity in counts/100,000 cells
Control	7,476.287
UVC	28,623.946
UVB	7,465.975
Ptpt dinucleotide	12,115.438
Ptpt and PBS wash	8,544.118

Chart #1: CAT Activity in HeLa Cells with Ptpt Treatment #1

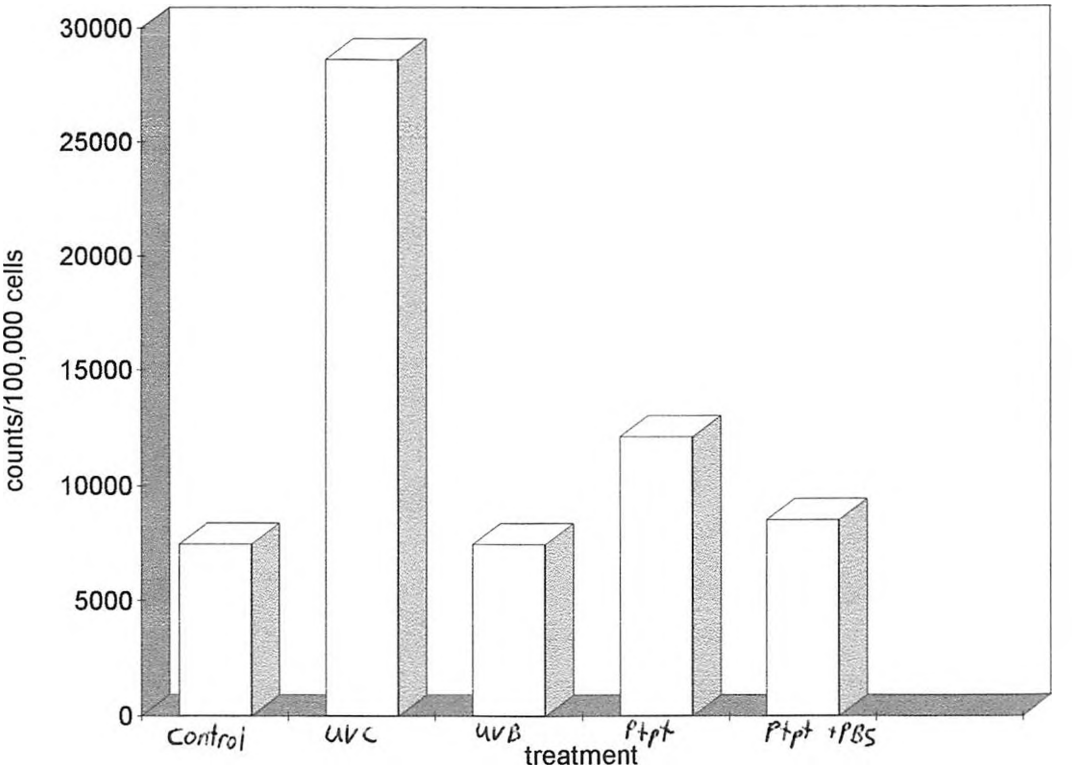
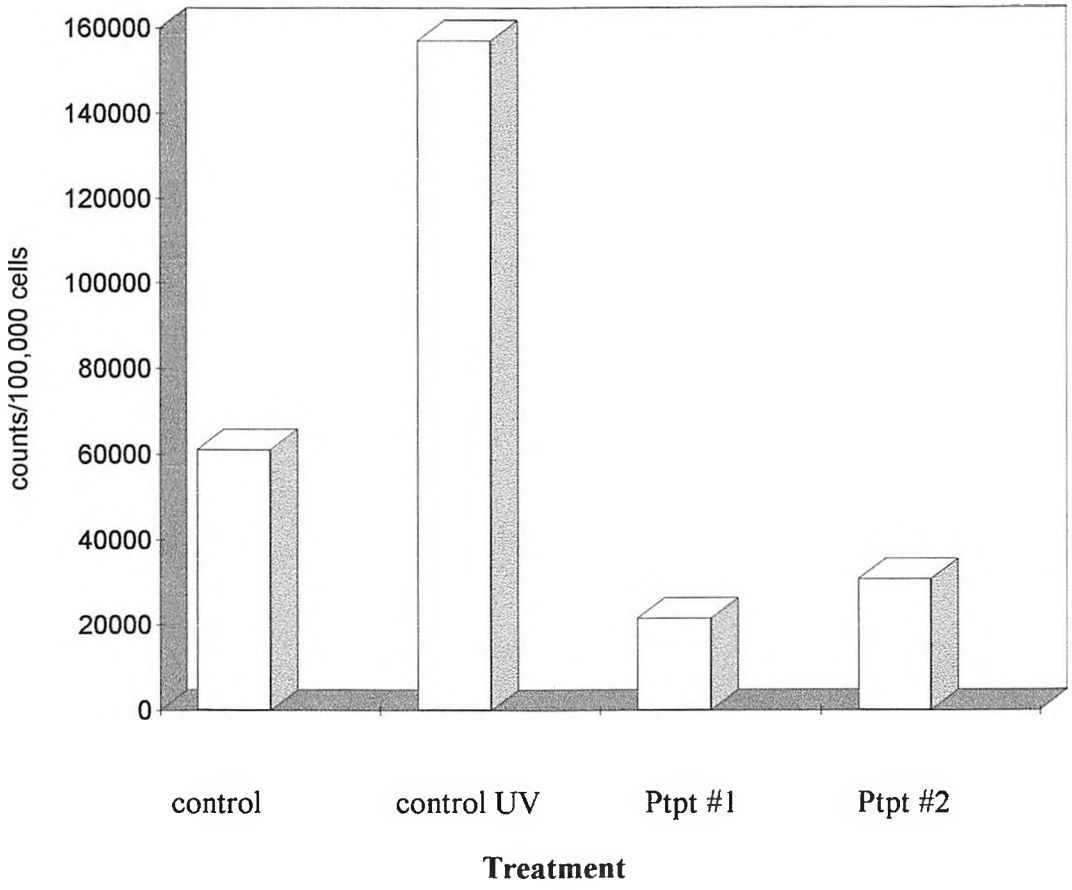


Table 4: CAT activity in treatment #2 HeLa Cells

Treatment	Amount of CAT activity (counts/100,000 cells)
control	61,043.35
control UV	156,976.70
ptpt #1	21,554.40
ptpt #2	30,622.16

Chart #2: CAT activity in Treatment #2 HeLa Cells



Discussion: The results for the ptpt dinucleotide were mixed. Treatment #1 showed that ptpt didn't inhibit HIV-LTR (in fact it showed a slight increase). Then treatment #2 showed that there was a slight inhibition of HIV-LTR. However, there was an anomaly in the second treatment. The cell count for the dishes treated with the ptpt was about double that of the control. This brings up the possibility of systematic error where perhaps the Coulter Counter was miscalculating. If this is the case and there are actually fewer cells on the plates treated with the ptpt then the numbers for the CAT activity is less than it really is. Another interesting source of error is that UVB in treatment #1 did not increase the LTR activity. This could be due to the treatment being too low. However, this doesn't affect the results obtained.

If the results from treatment #1 is the case, this lack of decrease (and slight increase) in LTR activity could be due to many various reasons. One is that it indeed doesn't inhibit LTR. The other reason is that the ptpt inhibits LTR but indirectly through a method other than NFkB. NFkB is a transcription factor (Devary et al., 1993) found in the HeLa cells that when it is released by inhibitory kB (IkB) travels from the cytoplasm to the nucleus where it binds to the DNA to induce transcription and therefore cell proliferation. Perhaps it stimulates an enzyme that inhibits LTR not transfected into the HeLa cell. The slight increase in CAT activity may be due to the possibility that ptpt dinucleotide causes stress on the cell. And as mentioned before stresses on the cell cause activation of LTR. But regardless of the effect of ptpt, more experimentation is needed to reach a true conclusion on its abilities.

Chapter 2

The effect of lutein on LTR in HIV 1 infected cells

Introduction: It is known that oxidative stresses may be important for the progression of the HIV disease because it activates NFkB which is needed for HIV replication (Treitinger et al. 2000). Therefore it is reasonable to think that antioxidants like lutein would slow down HIV replication. In this experiment the effect of lutein on HIV-LTR activity was examined. It has been found that there are significant depletions of carotenoids (like lutein) in those infected with HIV (Lacey et al. 1996). So there is a metabolic change that occurs in those affected with HIV. The body could be using carotenoids to try and fight the disease. Another finding that supports this hypothesis is that low concentrations of plasma carotenoids were associated with increased risk of death in infants with HIV (Melikian et al. 2001). Chauvin (1999) showed that vitamin E, another antioxidant, was shown to inhibit UV-induced CAT activity. It was hypothesized that this was due to its antioxidant properties. Therefore it would be logical to assume lutein, a stronger antioxidant, would have a greater inhibitory effect.

Materials and Methods: Six 35mm plates were plated with 503,040 cells per plate. The cells were kept in an incubator and allowed to attach. Then the cells were exposed to the treatments according to table 3.

Table 5: Lutein treatments #1

Plate number	Name	Treatment
1	control	wash 2 times with 3 ml PBS and add 5 ml media
2	control UV	wash 2 times with 3 ml PBS, expose to 19 J/m2 of UVC, and add 5 ml media
3	ethanol	wash 2 times with 3 ml PBS and add 5 ml media and 30ul ethanol

4	ethanol UV	wash 2 times with 3 ml PBS, expose to 19 J/m2 of UVC, and add 5 ml media and 30ul ethanol
5	lutein	wash 2 times with 3 ml PBS and add 5 ml media and 30 ul of lutein/ethanol mixture*
6	lutein UV	wash 2 times with 3 ml PBS, expose to 30 seconds of UVC, and add 5 ml media and 30 ul of lutein/ethanol mixture*

*The lutein/ethanol mixture composed of 1 mg xanthophyll (lutein) and 0.439 ml of ethanol giving a concentration of 2.27 mg/ml.

After twenty-four hours the cells were put through a CAT assay to determine the amount of CAT activity present.

Next, to test the effect of a change in concentration and time to react, eight 35mm plates were plated with 600,000 cells. These cells were placed in the incubator and allowed to attach. They were then subjected to the treatments in table 6.

Table 6: Lutein treatments #2

Plate number	name	treatment
1	control	wash twice with 3 ml PBS and add 5 ml media
2	control UV	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media
3	ethanol	wash twice with 3 ml PBS and add 5 ml media and 40 ul of ethanol
4	ethanol UV	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media and 40 ul ethanol
5	24 hour lutein	wash twice with 3 ml PBS and add 5 ml media and 40 ul lutein solution
6	24 hour lutein UV	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media and 40 ul lutein solution

7	48 hour lutein	wash twice with 3 ml PBS and add 5 ml media and 40 ul lutein solution
8	48 hour lutein UV	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media and 40 ul lutein solution

*The lutein/ethanol mixture composed of 1 mg xanthophyll and 0.439 ml of ethanol giving a concentration of 2.27 mg/ml.

**All cells were plated on the same day however plates 1-4 and 7-8 were treated 48 hours before the CAT assay and plates 5 and 6 were treated 24 hours before the CAT assay

Results: Both treatments didn't show a decrease in LTR activity in HIV 1 infected cells. The results of the first treatment are shown in table seven and chart three; and the second treatment in table eight and chart four. Ethanol alone (plates 3 and 4) was used to test the effect of the solvent used for lutein on the results. As can be seen in the following charts, the ethanol had no effect on the amount of CAT activity.

Table 7: CAT activity in HeLa cells with Treatment #1 of lutein

Treatment	CAT activity (counts/100,000 cells)
Control	1,538.5
ethanol UV	7,557.272
lutein	18,728.395
lutein UV	38,653.846

*There was contamination in the control UV and ethanol treatments

Chart #3: CAT activity in HeLa cells with treatment #1 of lutein

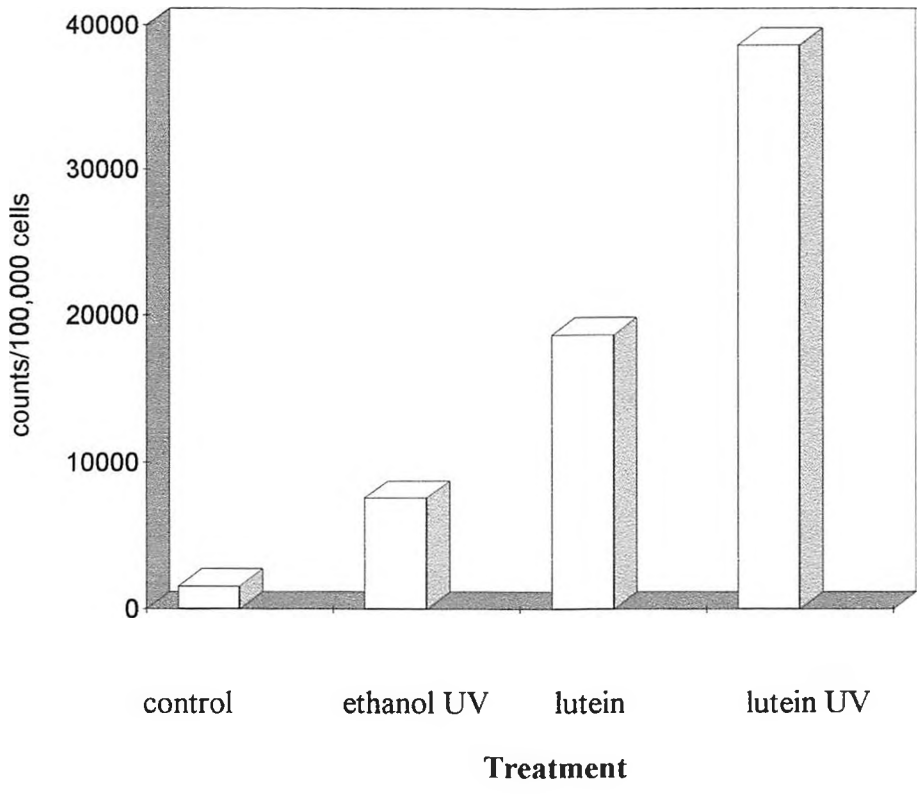
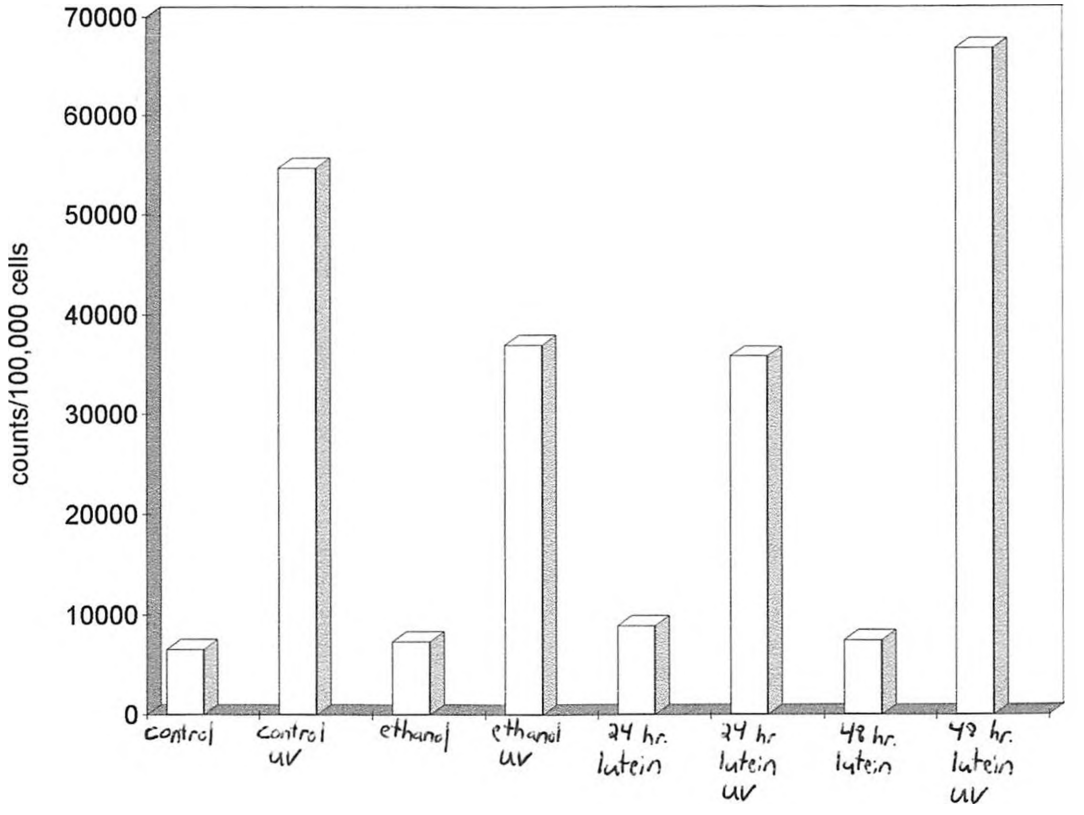


Table 8: CAT activity in HeLa Cells with treatment #2 of lutein

Treatment	CAT activity (counts/100,000 cells)
Control	6,557.32
Control UV	54,688.74
ethanol	7,312.02
ethanol UV	37,001.76
24 hour lutein	8,934.46
24 hour lutein UV	35,768.45
48 hour lutein	7,44.88
48 hour lutein	66,806.14

Chart #5: CAT Activity in HeLa Cells with treatment #2 of lutein



Treatment

**due to the light sensitivity of lutein, the plates treated with lutein were covered in a paper towel to prevent light exposure.

Conclusion: It has been shown that antioxidant supplementation helped in oxidation defense for those with HIV but there was no benefit in using high doses of it (Batterham et al. 2001). Therefore this can't be a cure for HIV but it can slow the progression of the disease. The results of treatment #1 did not decrease the amount of LTR activity in HIV 1 infected HeLa cells (as seen in table five and chart two). This effect may have been due to too little of a concentration or not enough time for the lutein to affect the cellular output. So in the second treatment I increased the lutein concentration and time of incubation. The results of this were similar to treatment one (as seen in table seven and chart number

four). There was once again little effect on the LTR activity. It is doubtful that the lack of effect was due to a low concentration since an observation was the cells turned an orangish color (the color of lutein). This suggests that the cells were saturated with lutein.

These results are still of interest because vitamin E has been shown to inhibit LTR using the same methods (Chauvin 1999). It was thought that the effect of vitamin E was due to its antioxidant effects. However lutein is a stronger antioxidant than vitamin E. This suggests that there is something else in vitamin E that prevents LTR activity. So this experiment didn't show that lutein inhibited LTR but more experimentation needs to be done before it is discounted as useful therapy to those who are HIV infected. Just because there is no effect of lutein on HIV-LTR doesn't mean that it isn't an effective inhibitor of HIV progression.

Chapter 3

The effect of Vitamin C on the LTR of HIV 1 infected cells

Introduction: It is possible that the oxidative stress that is associated with HIV infection might be important in the progression of the disease because the reactive oxygen species activates the nuclear transcription factor (NFkB) (Treitinger, et al 2000). Another reason to believe this is that lower concentrations of ascorbate (vitamin C) were found in HIV infected patients (Treitinger, et al 2000; Lacey et al 1996). This could be perhaps the body's way to fight the disease and a deficiency of vitamin C would speed up HIV progression. It has been shown that EPC-K1 (a compound of vitamin C and vitamin E) decreases NF-kappaB activity more than vitamin C or vitamin E alone (Hirano, et al 1998). Therefore it is our hypothesis that vitamin C will inhibit HIV1-LTR activity.

Materials and Methods: The cells used were HeLa cells transfected with the HIV-LTR and CAT reporter gene on a plasmid. The cells were cultured in four 35 mm plates at 600,000 cells per plate. Each dish was given 5 ml media. The cells were placed in the incubator and allowed to attach. The plates were then exposed to the procedures shown in table nine.

Table 9: Plate treatment for Vitamin C

Plate number	name	Treatment
1	control	wash twice with 3 ml PBS and add 5 ml media
2	control UV	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media
3	Vit. C	wash twice with 3 ml PBS and add 5 ml media and 50ul ascorbic acid solution*
4	Vit. C UV	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media and 50 ul ascorbic acid solution*

*Ascorbic acid (vitamin C) solution is composed of vitamin C dissolved in water with a concentration of 5 mg/ml.

After twenty-four hours the cells were put through a CAT assay to determine the amount of CAT activity present.

Then to test the variation of concentration and effectiveness over time, ten 35 mm plates were plated with 300,000 cells and placed in the incubator to attach. The cells were then submitted to the treatments shown in table ten.

Table 10: Plate treatment #2 for Vitamin C

Plate number	name	Treatment
1	control	wash twice with 3 ml PBS
2	control UV 96 hours	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media
3	Vit. C 96 hours	wash twice with 3 ml PBS and add 5 ml media and 55 ul of Vitamin C solution.
4	Vit. C 96 hours	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media and 55 ul of vitamin C solution
5	control UV 72 hours	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml of media
6	Vit. C 72 hours	wash twice with 3 ml PBS and add 5 ml media and 55 ul of vitamin C solution
7	Vit. C UV 72 hours	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media and 55 ul ascorbic acid solution
8	control UV 48 hours	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media
9	Vit. C 48 hours	wash twice with 3 ml PBS and add 5 ml media and 55 ul ascorbic acid solution

10	Vit. C UV 48 hours	wash twice with 3 ml PBS, expose to 19 J/m2 UVC, and add 5 ml media and 55 ul ascorbic acid solution

Results: The results of this experiment were not as expected. The vitamin C did not effectively inhibit the LTR in HIV 1 infected cells.

Table 11: CAT activity from vit. C treatment in HeLa cells

Treatment	CAT activity (counts/100,000 cells)
control	6,557.32
control UV	54,688.74
vitamin C	7,754.11
vitamin C UV	61,154.11

Chart 5: CAT activity in Vitamin C treated HeLa cells

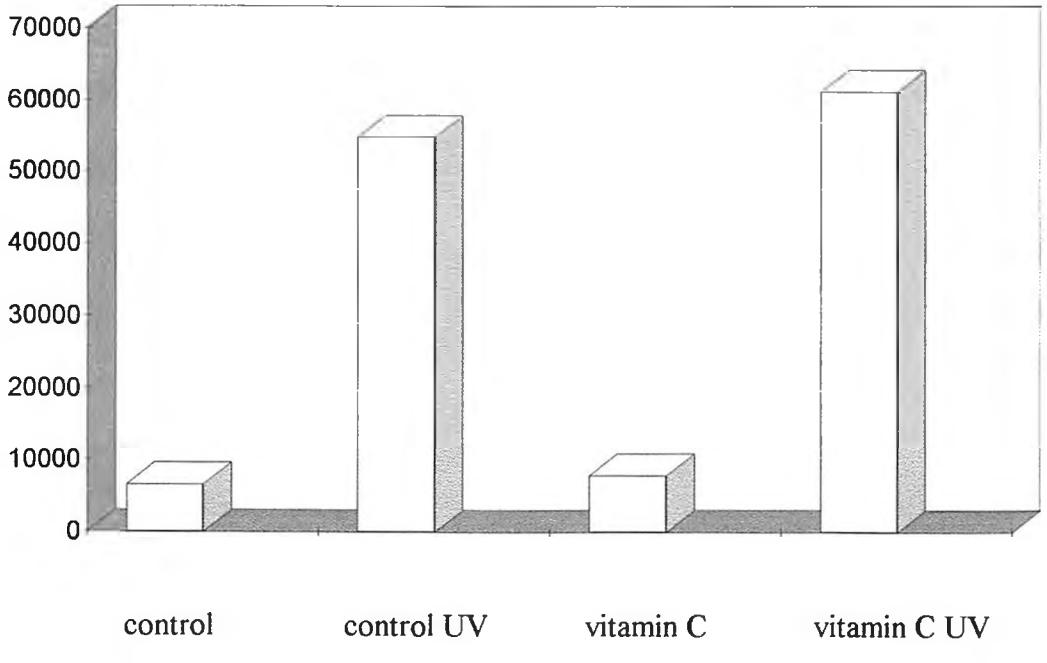
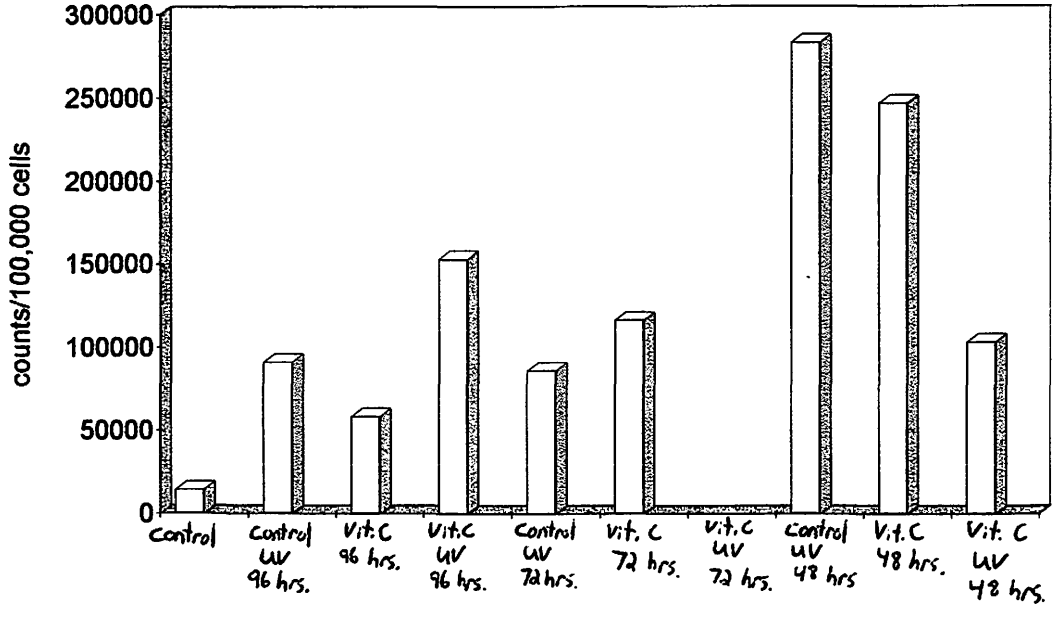


Table 12: CAT activity in HeLa cells treatment #2 with Vitamin C

Treatment	CAT activity (counts/100,000 cells)
control	14,299.17
control UV; 96 hours	90,976.89
Vit. C; 96 hours	58,195.83
Vit. C UV; 96 hours	153,198.53
control UV; 72 hours	85,823.17
Vit. C; 72 hours	116,492.28
Vit. C UV; 72 hours	*
control UV; 48 hours	283,144.07
Vit. C; 48 hours	247,111.98
Vit. C UV; 48 hours	103,131.19

*These results had to be thrown out due a large amount of cell death (so not enough cells to count).

Chart #6: CAT activity in Vitamin C Treatment #2 in HeLa Cells



Treatment

Discussion: These results were contrary to the hypothesis that vitamin C would inhibit the HIV-LTR activity. As can be seen in tables 11 and 12 and charts five and six vitamin C didn't inhibit HIV-LTR activity. A possible reason for this is that the effects of vitamin C are long term. The previous tests were performed for only the effects of four days. The results also show in treatment #2 that the treatment actually increased the amount of CAT activity. This may be due to the increased concentration of vitamin C caused a greater effect of its acidic properties (ascorbic acid). This acidic property may cause stress on the cell activating the LTR. Therefore further experimentation is needed to determine the effects of ascorbic acid as a possible treatment for those with HIV.

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