Selenotrisulfide Derivative of Alpha-Lipoic acid: Evaluation in a Cell Culture Model for Potential Use as a Topical Antioxidant

by

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B.S. University of Central Florida, 2002

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Molecular Biology and Microbiology
in the Burnett College of Biomedical Science
at the University of Central Florida
Orlando, Florida

Summer Term
2005

ABSTRACT

Selenium is a required micronutrient in mammalian cells. It is incorporated in the form of selenocysteine into selenoenzymes such as glutathione peroxidase and thioredoxin reductase, and is absolutely required for activity. Thioredoxin reductase is necessary for reduction of oxidized thioredoxin and therefore plays a major role in maintaining the redox status of the cell. Glutathione peroxidase is responsible for reducing peroxides into their corresponding alcohols and water. Together, these selenoenzymes constitute a significant part of the cell's arsenal to defend itself against oxidative stress. Exogenous sources of oxidative stress, such as UV radiation, are capable of generating reactive oxygen species (ROS). Elevated levels of ROS can lead to covalent modifications of lipids, nucleic acids, and proteins within a cell. This damage has been implicated in the development of cancer and degenerative diseases. As the skin is the first level of defense for UV radiation, skin cancer is an obvious concern.

Previous studies have demonstrated a protective effect against UV-induced cytotoxicity when selenium compounds were administered to skin cells in cell culture models. Topical selenium application to mice has also been shown to reduce UV damage to skin. Although a variety of chemical forms of selenium are available in nutritional supplements, the efficiency by which they are used for selenoprotein synthesis varies greatly. It is debated within the selenium research community which form is best for use as a supplement. In this study, we have focused on a selenotrisulfide derivative of alpha-lipoic acid (LASe). We have examined its utilization for selenoprotein synthesis through radiolabeling studies (^{75}Se) in a human keratinocyte cell line (HaCaT). We have determined that is incorporated into selenoproteins with nearly the same efficiency as selenite and L-selenocysteine. We have also determined that LASe is far more efficient as a supplement in cell culture than selenate or L-selenomethionine, two forms of selenium commonly used as supplements. LASe was also found to protect HaCaT keratinocytes from UV- induced cytotoxicity. Cells pretreated with LASe and exposed to 500J/m^2 and 750J/m^2 of broadband (UVA/UVB) UV radiation showed greater survival than untreated controls in a dose –dependent manner. Cells pre-treated either with lipoic acid or selenium in the form of selenite alone also observed protection. Nonetheless, these finding are significant given that LASe was previously shown to penetrate the skin better than other forms of selenium. These results indicate that LASe has the potential for use as a topical antioxidant upon further testing in animal studies.

I dedicate this work to my family, with special thanks to my father, for giving me the love, support, and encouragement necessary to complete this thesis.

ACKNOWLEDGMENTS

I am grateful to everyone who has helped me throughout this process. I would like to thank the following people for their assistance, without which, I would not have been able to complete this thesis:

Dr. William Self, for the opportunity to work on this thesis and for teaching me the skills necessary to do so. I am thankful of all his guidance, support, and time.

My committee members, Dr. Annette Khaled and Dr. Karl Chai, who have always been available to answer my questions and offer advice. I am very appreciative of their time.

Dr. Norberg Fusenig of the German Cancer Research Center, Heidelberg Germany for his gift of HaCaT ketatinocytes.

Dr. Kevin Belfield of the University of Central Florida- Department of Chemistry, for the loan of his UV reactor and UV radiometer.

Dr. Thressa Stadtman of the National Institutes of Health, Bethesda Maryland for the use of antibodies to thioredoxin reductase.

The members of the Self lab for their help, especially Fanta Konate.

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LIST OF ACRONYMS/ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl phosphate
DHLA	dihydrolipoic acid
DKM	defined keratinocyte medium
DMEM	Dulbecco's modification of Eagle's medium
EDTA	ethylene diamine tetra-acetate
FCS	fetal calf serum
Gpx	glutathione peroxidase
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
LA	lipoic acid
LASe	selenotrisulfide derivative of lipoic acid
L-SeCys	L-isomer of selenocysteine
L-SeMet	L-isomer of selenomethionine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenlytetrazolium bromide
NBT	nitro blue tetrazolium
PBS	phosphate buffered saline
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulfate polyacrylamide gel
Se	selenite
SECIS	selenocysteine incorporation sequence
TBS	tris buffered saline
TFA	trifluoroacetic acid
Trx	thioredoxin
TrxR	thioredoxin reductase
UV	ultraviolet

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Selenium is a trace mineral which has chemical properties similar to sulfur(1). Selenium has been shown to be required for optimal health in mammals. This has been known since Klaus Schwarz and Milton Scott discovered the harmful effects of selenium deficiency in farm animals in the 1950's, and how these effects could be ameliorated with selenium supplementation (2). It is now known that selenium is utilized in the diet for the synthesis of selenoenzymes. Each of these selenoenzymes contains selenocysteine at their active site, and is required for activity (3). The mechanism of the synthesis of bacterial selenoproteins is well understood, using *Escherichia coli* as a model (4). Synthesis of selenoproteins in eukaryotes, although not as well defined, is increasingly understood (5).

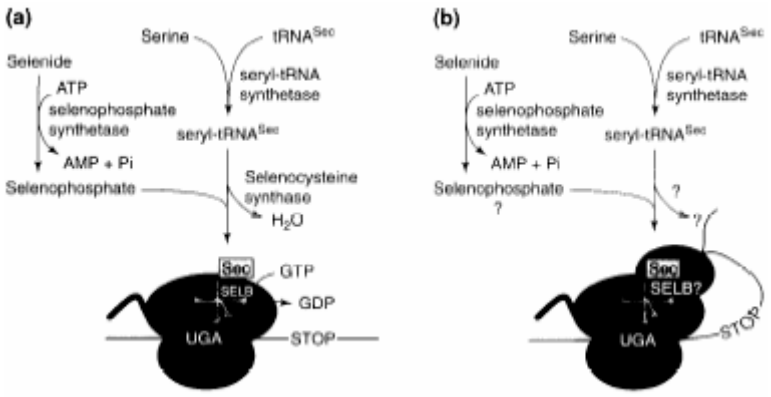

Figure 1
Selenocysteine insertion to selenoproteins. Picture (a) represents prokaryotic insertion and picture (b) represents eukaryotic insertion (5).

Selenocysteine is cotranslationally inserted at a UGA codon (usually a stop codon) by a selenocysteyl tRNA $^{[ser]\,sec}$ in the presence of specific elongation factors. Selenophosphate synthetase converts selenide to selenophosphate, which is the selenium donor responsible for converting a serine charged tRNA to selenocysteyl-tRNA. Stem-loop structures within the mRNA of selenoproteins, called selenocysteine insertion sequences (SECIS elements) are required for selenoprotein synthesis (5).

In the 1970's, selenium was shown to be a component of the bovine form of the antioxidant enzyme glutathione peroxidase, which is responsible for detoxifying peroxides (6). It was much later (1995) determined that selenium is a necessary component of the enzyme thioredoxin reductase, which is responsible for reducing thioredoxin (7) (8). Based on *in silico* analysis of the human genome, it is estimated that about 25 mammalian selenoproteins exist (9). A great deal of progress has been made in the selenium field regarding selenium deficiency and supplementation in humans. It is now known that severe deficiency of this mineral can lead to serious health problems, and that less severe deficiency also results in a number of adverse effects in humans (10). Consequently, the significance of research in this area is evident.

Lack of adequate selenium in the diet can lead to a number of adverse health conditions, and there are varying degrees to which a human's health may be compromised by inadequate selenium. One of the most severe conditions associated with selenium deficiency is Keshan disease. Keshan disease is a condition which is limited to individuals living in certain geographic areas of China in which the selenium levels in the soil are very low. The disease is classified as a cardiomyopathy, and is associated with infection by a cocksackie virus. It is known that selenium deficiency makes individuals

more susceptible to this virus, which is a causative agent of myocarditis (inflammatory heart disease). Furthermore, selenium deficiency in an infected individual results in a mutation to the virus itself, which has been shown to increase its virulence. Kashin-Beck disease is an arthritic condition associated with both selenium and iodine deficiency. The condition is characterized by enlarged joints and distorted growth of long bones. Kashin-Beck disease is also isolated to regions with low soil selenium (10).

Less severe selenium deficiency has also been linked to a number of health problems. Lower than adequate plasma selenium levels have been associated with increased susceptibility to viral infection, Alzheimer's disease, decreased viable sperm count in men, and increased incidence of death due to cardiovascular disease (11). It has been assumed that the primary effects of selenium deficiency result from a decrease in the antioxidant action of enzymes that contain selenium, such as glutathione peroxidase and thioredoxin reductase, but until all selenoproteins are identified and characterized this remains speculative.

There is a great deal of epidemiological data that suggests deficiency in selenium leads to increased risk of cancer. In the late 1960's it was determined that in the United States an inverse relationship exists between cancer deaths and the amount of selenium in crops within certain areas (12). A study published in 1971 reported that individuals living in areas with average or higher than average concentrations of selenium in the crops had a lower death rate from cancer of the gastrointestinal tract, lung and breast than those in low selenium areas (13). This inverse relationship was also found in a study examining the death rate due to colorectal cancer. Furthermore, it has been shown that the selenium status of individuals with cancer tends to be lower than that of healthy

controls (14). In a study involving the eventual development of non-melanoma skin cancer and the development of adenomatus polyps of the colon, the individuals with lower plasma selenium levels had a higher incidence of these cancers (15). Likewise, a Finnish study found that selenium in the serum was lower in individuals with cancer than in the healthy control individuals (16).

In addition to epidemiologic studies of deficiency in selenium status, significant effort in clinical research studies has focused on selenium supplementation. Due to the strong correlation between less than adequate selenium status and higher incidence of cancer, it seems logical that additional supplementation of selenium in the diet of individuals afflicted with such conditions would help to reduce the risk of cancer. It is from this line of reasoning that a number of studies involving the effect of selenium supplementation have arisen. In particular the Nutritional Prevention of Cancer (NPC) Trial, involving individuals with a history of basal or squamous cell carcinoma, demonstrated that selenium supplementation was efficient in reducing the incidence of lung, colon and prostate cancers by about 50% (17). Though the study was conducted in order to evaluate the use of selenium supplements in patients with a history of skin cancer, the results obtained did not show any effect of selenium supplementation on recurrence of skin cancer. The fact that there was a reduced incidence of other types of cancer in individuals who received selenium supplements (200 μg per day as selenized yeast) compared to those who received a placebo is strong evidence supporting the ability of selenium to reduce cancer risk. The individuals in this study who received the greatest benefit from selenium supplementation were those whose plasma selenium levels were the lowest. Individuals with the highest plasma selenium levels did not experience a

reduced incidence of cancer upon supplementation with selenium. Taken together, these results suggest that selenium supplementation is most effective in reducing cancer incidence in individuals with selenium deficiency.

Perhaps the largest clinical trial involving the supplementation of selenium and cancer is the Selenium and Vitamin E Cancer Prevention Trial (SELECT) (18). The SELECT trial is a subtrial begun from initial data elucidated in the NPC trial. SELECT is a clinical trial that examines the incidence of prostate cancer in individuals supplemented with both vitamin E and selenium. The effect of supplementation with selenium (in the form of selenomethionine) and Vitamin E alone as well as together will be examined against the effect of each supplement alone as well as compared to controls (19). Though it will not be complete until 2013, the evidence already available suggests that the two supplements are effective at preventing prostate cancer (18) .

It is clear that a great deal of evidence exists to support the idea that selenium is effective at reducing certain types of cancer. It is generally agreed upon that selenium's effect on cancer is due to its antioxidant properties (i.e. increasing the levels of thioredoxin reductase and glutathione peroxidase). Oxidative damage results in modification of cellular proteins, lipids, and DNA, and it is thought that excess modification of this kind leads to many of the diseases associated with aging, including cancer (20). There are a number of sources of reactive oxygen species such as superoxides, hydrogen peroxides and hydroxyl radicals. Oxidative stress may come from an endogenous source, such as the normal metabolic processes within a cell, but exogenous sources of oxidative stress also exist. One exogenous source of oxidative stress is the sun, specifically, the ultraviolet radiation it emits. Concern of skin cancer

risk is growing as the ozone layer is increasingly depleted (21). The ozone absorbs most UVC rays (200-280nm), but we are left susceptible to UVA and UVB rays. Most of the UV rays responsible for damage to skin are in the UVA (320-400nm) and UVB (290-320nm) range. It is known that UVB radiation causes direct damage to DNA, while UVA radiation generates reactive oxygen species (22).

Though the NPC trial showed that oral selenium supplementation did not significantly reduce the risk of recurrent skin cancer, there is evidence that topical selenium supplementation can help to protect from skin cancer. Several studies involving topical application of selenium to animals as well as the addition of selenium compounds to skin cells in culture have shown selenium supplementation to be protective. A study by Burke (23) has shown that topical selenomethionine, combined with vitamin E, reduced the incidence of skin damage due to blistering and inflammation in a mouse model (hairless mice) treated with UV. In addition, a number of cell culture studies have demonstrated that selenium is protective from UV-induced cell death. Selenite and selenomethionine pretreatment of human melanocytes and keratinocytes reduced UVB induced death (24) (25). Furthermore, selenite and selenomethionine have shown protection from UV induced apoptosis of human primary keratinocytes (26).

Even though there are certainly advantages to selenium supplementation, certain forms of selenium can also prove to be toxic. Selenium toxicity, or selenosis, has been observed in animals as well as humans, but was first identified in farm animals (horses and cattle in the 1930's in South Dakota). These animals had conditions called "blind staggers" and "alkali disease" (27). Alkali disease is a less chronic poisoning than

blind staggers, but both are the result of animals consuming plants which are known to accumulate high levels of selenium.

Toxicity in humans is rare, but can occur. Because there are several chemical forms of selenium, and each is metabolized differently, it is necessary to determine the potential toxicity of a selenium compound used as a supplement. The most toxic form of selenium in selenite. Selenite is reduced to selenide and then incorporated into selenoenzymes (28) (3) (5). Selenocysteine is an efficient nutritional source of selenium, but is not used in supplements due to the high cost of chemical synthesis. Less toxic forms of selenium, such as selenomethionine and selenate exist, though they are less efficient at selenoenzyme synthesis. Additionally, selenomethionine has the ability to be incorporated non-specifically into methionine residues of proteins (29). Such non-specific incorporation could cause changes to a protein's structure, and consequently, its function. Long-term supplementation in cell culture models or animal studies with L-selenomethionine has yet to be carried out.

Selenocysteine protein synthesis requires that selenite is reduced to selenide, selenide being the selenium donor for selenophosphate synthetase (5). It is believed that glutathione, a small cysteine containing peptide, is involved in this reduction process in vivo. Glutathione has been shown to react with selenite to form a selenotrisulfide in which selenium is substituted for sulfur (30). This spontaneous chemical reaction was initially described by Painter, which requires a sulfur to selenium ratio of 4:1 (28) . The formation of selenotrisulfides was also examined by Ganther. Glutathione-based selenotrisulfides are not stable at physiological pH (30) . Selenotrisulfides are intermediates which are formed in the reduction of selenite to selenide. Consequently,

selenotrisulfides are further in the metabolic pathway to selenoenzyme synthesis than other forms of selenium.

Due to the protection that selenium provides against UV induced oxidative damage, as well as the close relationship between selenium's toxicity and its utilization in selenoenzyme synthesis, it is clear that a need exists for an efficiently utilized, non-toxic selenium compound. In addition to the ability of such a compound to be utilized for selenoprotein synthesis, an ideal compound would be absorbed into the skin, as an oral supplement is not the most effective. In 2000, a unique selenotrisulfide derivative of alpha-lipoic acid was initially characterized and is shown in figure 2 (31). This compound (LASe) was formed by the same spontaneous reaction previously described by Painter and Ganther (28) (32). Unlike other known selenotrisulfides, this lipoic acid derivative of a selenotrisulfide has been shown to be stable at physiological pH (31). Furthermore, due to its hydrophobic lipoic acid component, LASe has the potential to penetrate hydrophobic barriers, such as human skin. Furthermore, the established antioxidant properties of lipoic acid make this compound an excellent *potential* topical antioxidant (33) (34). In this study, we have examined the capacity of the selenium derived from LASe to be utilized by cells in culture for selenoenzyme synthesis and determined whether this compound has the ability to protect cells from UV induced cytotoxicity.

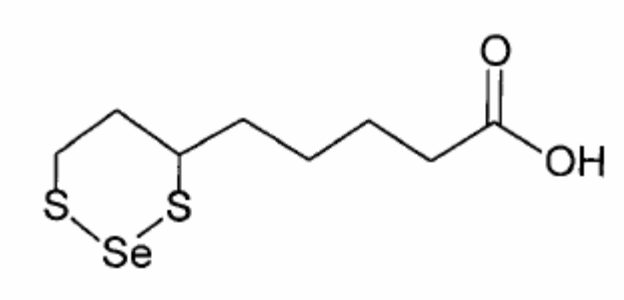

Figure 2
The chemical structure of LASe (31)

CHAPTER 2: MATERIALS AND METHODS

Synthesis and Purification of LASe

The Painter reaction for synthesis of the LASe was carried out as previously described (31), with modifications. The reaction was carried out by combining two solutions: a 50 mL solution of 4 mM dihydrolipoic acid (DHLA) made in 100% ethanol and a 50mL solution of 2 mM sodium selenite (Na_2SeO_3) in water. Upon mixing the solution thus contains a 2:1 ratio of DHLA: Na_2SeO_3 in 50% ethanol. This 2:1 DHLA: Na_2SeO_3 results in a 4:1 ratio of sulfur to selenium. The reaction mixture was kept at room temperature for 30 minutes to allow the reaction to go to completion.

The entire reaction mixture was loaded in 5 mL increments using a 5 mL loading loop (Rheodyne) onto a preparative C_{18} high pressure liquid chromatography (HPLC) column (Vydac) to which it bound. The column was washed for 25 minutes at a flow rate of 2 mL/minute with dH_2O-TFA solvent to allow the compound (LASe) to adsorb to the column. The LASe was then eluted using a linear gradient of methanol -TFA (0.1%) for 30 minutes at a flow rate of 2 mL/minute. The UV-visible spectrum of the fractions (analyzed using a Hewlett-Packard 8453 spectrophotometer) eluted in the range where LASe was expected to elute from the column, based on previous studies(31) . Fractions were pooled and the column and lines were washed with dH_2O, pH 2.0. The pooled fractions were again loaded onto the preparative C_{18} column. The column was washed with dH_2O (pH 2.0) to ensure binding of LASe. An ethanol gradient was used (0%-100%) to elute the compound. Fractions containing LASe were pooled, based on their

absorbance in the correct region (288 nm). The sample was then stored at -20°C until use.

Cell Culture Methods

Hela S3 cultivation. HeLa S3 cells, obtained from the laboratory of T.C. Stadtman (NHLBI, NIH) were cultured in sterile, gamma irradiated, tissue culture treated 12- well plates (growth surface 3.66 cm^2 per well) or screw cap flasks with venting position (growth surface 25 cm^2). Tissue culture plates and flasks were purchased from Techno Plastic Products (TPP). Cells were cultivated in Dulbecco's Modification of Eagle's Medium (DMEM) containing 4.5 g/L- glucose, L-glutamine (Cellgro) with 10% fetal calf serum (FCS) (ICN Biomedicals), 100 units/mL penicillin and 100 µg/mL streptomycin (Cellgro) at 37 ºC in a humidified 5% CO_2 atmosphere (Revco Elite II incubator). Five milliliters (mL) of cell culture medium (DMEM) was used in 25 cm^2 flasks and 2 mL was used in 12-well plates. To maintain cells for continuous culture, cells were harvested and re-plated when they reached confluence. Culture medium was exchanged every 2-3 days.

To harvest HeLa cells, culture medium was removed and replaced with enough Trypsin-EDTA (Cellgro) to cover the cells (2 mL in the 25 cm^2 and 0.5 mL in 12-well dishes). Cells were incubated at 37 degrees for 5-10 minutes, or until cells were visibly detached from growth surface when viewed under an inverted microscope. Cells in trypsin were transferred to a sterile centrifuge tube, and DMEM was added to inhibit further action by trypsin. Cells were pelleted by centrifugation for 2 minutes at 1, 000 rpm (Hermle Labnet Z383K). Medium was then decanted and the cell pellet was

resuspended in DMEM. The cells were again harvested by centrifugation for 2 minutes at 1, 000 rpm. DMEM was decanted, and cells were resuspended again in culture medium if they were being plated, or in phosphate buffered saline (PBS) with calcium and magnesium (Cellgro) if they were to be used for further analyses such as SDS-PAGE, immunoblot, or radioisotope analysis.

HaCaT cultivation. Spontaneously transformed HaCaT keratinocytes (gift of Dr. Norbert Fusenig) were cultured in 25cm² flasks in 5 mL of DMEM containing 4.5 g/L glucose, L-Glutamine, 10% FCS, 100 units/mL penicillin and 100 µg/mL streptomycin at 37 ºC in a humidified 5% CO_2 atmosphere (Revco Elite II incubator). To maintain cells for continuous culture, cells were harvested and re-plated 5 days after reaching confluence, at no higher than 1 to 5 ratio. Culture medium was exchanged at least every 2-3 days.

To harvest HaCaT cells, culture medium was removed and cells were covered with a 0.05% ethylenediaminetetraacetic acid (EDTA) solution made in PBS without calcium and magnesium. Cells were incubated at 37°C for 15-20 minutes to allow the EDTA solution to disintegrate the desmosomes present in the culture monolayer. The EDTA solution was then removed and the cells were incubated for 5-10 minutes at 37°C with Trypsin-versene to allow detachment of cells from the monolayer. DMEM was added, and detached cells were harvested by centrifugation for 2 minutes at 1,000 rpm. Cells were resuspended in DMEM and again harvested by centrifugation for 2 minutes at 1,000 rpm. The culture medium was removed, and cells were resuspended in fresh culture medium if being plated, or in PBS if being used for analysis of radiolabeled selenium incorporation.

Assessment of Cell Viability for UV Irradiation Studies

For UV irradiation studies HaCaT cells were cultivated in 12-well dishes. Cell viability was assayed using the trypan blue exclusion method (as explained below). The number of viable cells was determined by counting of cells using an Improved Neubauer hemocytometer (Hausser Scientific). Cells for UV irradiation were plated to achieve approximately 80% confluence. Cells were initially plated in 1 mL of DMEM, and 24 hours after plating, medium was exchanged for 2 mL of fresh DMEM. HaCaT cells were also grown in 96-well dishes for UV studies when cell proliferation was assayed for by MTT (see below). Cells were plated at 5,000 cells per well with 100 µL of DMEM. Media was exchanged 24 hours after plating of cells.

Assessment of Cell Viability Using Trypan Blue. Trypan blue dye in 0.4% phosphate buffered saline solution was purchased from MP Biomedicals. The dye was used to stain cells for visualization under an inverted microscope (Nikon) using the 20X objective lens. The cells were viewed on an Improved Neubauer hemocytometer (Hausser Scientific). Cells were counted using a cell counter (Fisher). To prepare cells for counting, cells were harvested as previously described, and resuspended in PBS with calcium and magnesium. This cell suspension was mixed with 100% trypan blue dye at a 1:1 ratio. The number of live (not absorbing dye) and dead cells were counted on each side of the hemocytometer was recorded. Both sides of the hemocytometer were counted, and these two numbers were averaged. The number of cells per mL of culture

were determined using the manufacturer's instructions for the hemocytometer (Hausser Scientific).

Assessment of Cell Viability Using MTT Assay. MTT reagent was purchased from Midwest Scientific. A working solution of 5mg/mL was made in PBS and filter sterilized. Solutions were stored at 4ºC in the dark, and were kept for no more than 2 weeks. Cells were cultivated in 96 well plates at a density of 5,000 cells/well in 100µL of medium which consisted of 90% DMEM with serum and 10% defined keratinocyte medium (DKM). The protocol used for the assay was adapted from the Molecular Probes Vybrant® MTT Cell Proliferation Assay Kit. At the appropriate time point for assessing cell viability for each experiment (either 24 or 48 hours), 10µL of the 5mg/mL MTT was added to each well. The plates were then returned to the incubator so that the dye could form a precipitate in the presence of metabolically active cells. The formation of a purple formazan precipitate occurred within 2- 4 hours. After four hours incubation, 100 µL of a detergent solution (10% SDS, 10nM HCl) was added to solubilize the precipitated crystals. Following overnight incubation the absorbance at 570nm was determined using aa Dynex Technologies Opsys MR microplate reader.

Figure 3 shows the average absorbance of MTT plotted versus the number of cells plated per well, in order to determine the optimum concentration of cells for experiments using MTT as a marker. Determination of optimal cell counts for use in the 96-well plates was performed as described in the ATCC® MTT cell proliferation assay instructions. First, HaCaT cells from a confluent 25cm^2 flask were harvested and resuspended in 1mL of DMEM. The average number of HaCaT cells in one confluent flask was counted by trypan blue dye method (as previously described), and was found to

be 5.5 X 10^6 cells. Consequently, when resuspended in 1 mL of DMEM, the cells are at a density of 5.5 X 10^6 cells/mL. From this suspension, dilutions were made in DMEM. First, a dilution to 1 X 10^6 cells/mL was made in DMEM. From this dilution, subsequent dilutions were made in DMEM of 500,000cells/mL, 100,000cells/mL, 50,000cells/mL, 25,000 cells/mL and 10,000 cells/mL. Next 100µL of each suspension was plated in triplicate in a 96-well microplate, so that there was triplicate plating of cells in the following amounts; 50,000cells/well, 10,000cells/well, 5,000cells/well, 2,500cells/well, and 1,000cells/well.

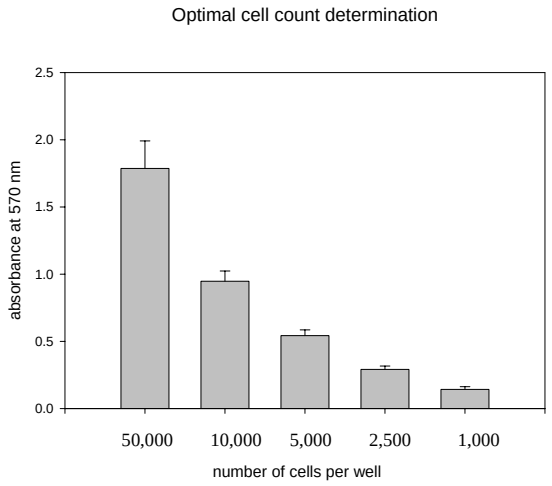

Figure 3
Calibration of MTT Assay. Each bar represents at least 3 independent cultures, with the standard deviation plotted as error.

From these results, it was concluded that plating 5,000 cells per well would yield an absorbance reading which is accurate for control (untreated) cells. When cells were

cultured in 24-well plates, a proportionally larger number of cells (48,000 cells/well) was used. When cells were cultivated in 24-well plates, volumes of culture medium, MTT, and detergent were increased so that they were proportional to those used in the 96-well plate experiments. Cells were cultured in 1mL of culture media, and 100 µL of MTT was added to each well at the time which cell viability was to be assessed. After overnight incubation with SDS-HCl solution, the contents of each well were resuspended and 200 µL from each well containing solubilized MTT precipitate was transferred to the well of a 96-well microplate. The absorbance was then read at 570nm on the Dynex microplate reader.

Nutritional Utilization Studies

Treatment with Selenium Compounds and Radiolabeling. For radiolabeling studies, cells were cultivated as a monolayer to approximately 60% confluence. Culture medium was removed and replaced with fresh media containing a basal concentration (10 nM) of unlabeled sodium selenite. Cells were radiolabeled using equal amounts of ^{75}Se (University of Missouri), usually 10µCi. Selenium compounds were added to cells in concentrations ranging from 25nM-1µM. Sodium selenite, L-selenocysteine , and LASe (31) were added to cells in concentrations ranging from 25 nM-150nM in HeLa cells and 25nM-200nM in HaCat cells. L-selenomethionine, selenate, and LASe were added to cells in concentrations ranging from 250nM- 1µM in HeLa and 200nM-1µM in HaCaT. Cells were incubated for 48 hours following these additions and each experiment was performed in triplicate for statistical analysis of the data.

Protein Concentration Determination. Protein concentration was determined as previously described (35). Concentrated Bradford reagent was purchased from BioRad. One mL of the Bradford reagent was added to a 1.5 mL microcentrifuge tube. Either the cell extract itself or a dilution of the cell extract (made in water) was added to the 1mL of Bradford reagent. Tubes were vortexed to mix the contents and then allowed to sit at room temperature for 10 minutes to allow the coomassie dye to bind to the cellular proteins. After 10 minutes, the contents of each tube were transferred to disposable visible cuvettes (Fisherbrand), and the absorbance of each sample was read at 595nm using a model 8453 Hewlett-Packard spectrophotometer. A standard curve was generated using bovine serum albumin (Pierce) for each experiment.

Measurement of ^{75}Se Present in Cellular Protein Following Radiolabeling. Following radiolabeling and treatment with selenium compounds, the cell culture medium was removed and cells were harvested, as described above. Cells were resuspended in 100 µl of buffer (50 mM tricine buffer containing 0.1 mM benzamadine, 0.5 mM EDTA and 1mM dithiothreitol) and lysed by sonication for 5-10 seconds (Fisher Scientific model 100). Protein concentration was determined using the Bradford method. The amount of labeled selenoprotein in the cell extract was determined by assaying for ^{75}Se (Perkin Elmer model 1470) and reported with respect to total protein (cpm/µg).

Equal amounts of protein (indicated in figure legends) from crude cell extracts were applied to 12% polyacrylamide gels, which were made using Bio-Rad casting stands and 0.75mm glass plates. Gels were electrophoresed at 150 volts for 55 minutes. Gels were stained with coomassie dye solution (50% methanol, 10% acetic acid, 1gm/L coomassie blue G250) and de-stained with a methanol/acetic acid solution (50%

methanol, 10% acetic acid). Gels were subsequently dried using a gel drying system (DryEase Minigel Drying System, Invitrogen). The stained, dried gels were then exposed for 48 hours on phosphor screen (Molecular Dynamics) to identify the radiolabeled selenoprotein bands. Image analysis was performed using Image Quant software (Molecular Dynamics).

Western Blot Analysis of Protein Levels

Equal protein amounts were separated with 12% SDS PAGE as described in the previous section. Following electrophoresis, the gels were removed from the glass plates, soaked in transfer buffer (25 mM Tris, 80 mM glycine, 20% methanol) and placed in a transfer cassette with PVDF membrane (Bio-Rad). The PVDF membrane was pre-wet with ethanol, and then soaked for at least five minutes in the transfer buffer, and then assembled with the gel as described by the manufacturer (Bio-Rad). Proteins were transferred to PVDF at a constant voltage (100 volts). Following the transfer, the membrane was removed from the transfer cassette and incubated with blocking solution (TBS with .01% Tween 20 containing 2% BSA) for 1 hour at room temperature. Once blocked, the membrane was incubated with the primary antibody (rabbit anti-human thioredoxin reductase- gift of T.C. Stadtman) at a 1:500 dilution made in blocking solution overnight at 4°C. Following incubation with the primary antibody, the membrane was washed 3 times for 5 minutes each wash with TBS-Tween. The secondary antibody (goat anti-rabbit conjugated with alkaline phosphatase) was diluted 1:2,500 in blocking solution and was incubated with the membrane for one hour at room temperature. After one hour, the secondary antibody was removed by three subsequent

washes with TBS-Tween, and then rinsed briefly in alkaline phosphatase buffer (0.1M Tris, 100mM NaCl, 5mM MgCl, pH 9.5). The alkaline phosphatase substrate was dissolved in 10 mL of alkaline phosphate buffer and contained 0.34 mL 5-bromo, 4-chloro, 3-indolyl phosphate (BCIP) and 0.67 mL of nitroblue tetrazolium (NBT). The membrane was incubated with substrate until the product was visibly detectable. The reaction was then stopped by washing with dH_20 and allowed to dry.

UV Irradiation Studies

HaCaT cells were cultured in 12- well dishes (TPP) and 96 well dishes (TPP) for UV studies. A Rayonet Photochemical Reactor was used with broadband UVA-UVB bulbs with a peak emission at 305 nm. The UV reactor was the gift of Dr. Belfield (University of Central Florida- Department of Chemistry). The reactor was turned on at least 20 minutes before treating cells to allow bulbs to warm up. An internal fan was also used inside the UV reactor to ensure the proper temperature was maintained (35 degrees) and this was verified using a thermometer. A radiometer/photometer (International Light) was used to detect the UV irradiance from the lamps before treatment of the cells. Before treatment with UV, cell medium was removed and set aside in sterile tubes. Cells were covered with PBS containing calcium and magnesium (Cellgro). One mL of PBS was used for cells in 12-well dishes, and 50µL of PBS was used for cells in 96-well dishes. Cells which were not being irradiated were protected from UV light by covering wells with several layers of aluminum foil (Fisher Scientific). Following UV irradiation of cells, original culture media was replaced, and cells were returned to incubator.

UV Induced Cytotoxicity Studies. HaCaT cells were cultured in 24 well dishes in media that consisted of 90% defined keratinocyte media (DKM) and 10% DMEM. The HaCaT cells underwent a gradual transition to this media by increasing the percentage of DKM and decreasing the percentage of DMEM (with 10% serum) with each passage of the cells. The first passage of cells was done performed in media that consisted of 25% DKM and 75% DMEM. The next passage of the cells was performed in a 50% mixture of each type of medium, and so on. An attempt was made at transitioning cells to 100% DKM, however these cells did not recover well from this transition and were very slow growing. It was therefore determined that the optimal ratio of defined media with DMEM was 90% DKM and 10% DMEM with serum. Thus the final concentration of FCS was 1%.

Cells gradually transitioned to 90% DKM and 10% DMEM with serum (hereafter termed 90:10) were plated in 24-well plates at a density of 48,400 cells per well. Twenty four hours following the initial plating, the media was exchanged for fresh 90:10 media. Fresh dilutions of the LASe were prepared in 95% ethanol. The LASe was added to the cells in the following concentrations: 10 nM, 25 nM, 50 nM, 100 nM, 500 nM. Twenty four hours after the LASe was added, the media was pooled for each concentration and set aside. Each well was covered with 500 µL of PBS containing calcium and magnesium. The cells were then UV irradiated with a dose of UVA/UVB of 500 J/m^2, 750 J/m^2, 1000J/ m^2. The pooled media was then replaced and the cells were returned to the incubator. A control was also performed in which the media was pooled and set aside and the cells were covered with 500 µL of PBS containing calcium and magnesium and set in the dark for one minute. Media was then replaced in the control cells and they were

returned to the incubator. Forty eight hours after UV irradiation, cell viability was assessed using the MTT assay.

Toxicity of LASe Compared to Other Selenium Compounds. Cells were cultured in 96 well dishes in 90:10 and approximately 2,100 cells per well were initially plated. Following a 24 hour incubation, to allow cells to adhere as a monolayer, approximately 5,000 cells per well are expected based on cell viability assessed using Trypan Blue (see previous section). After 24 hours of incubation, medium was removed and replaced with fresh medium. At this time, LASe, L- selenocysteine, and selenite were added to the cells in triplicate in the following concentrations 1 µM, 5 µM, 10 µM, 15 µM, 25 µM, with the appropriate control (0 µM selenium compound). After incubation for twenty four hours, cell viability was assessed using MTT assay.

Ethanol Toxicity. HaCaT cells were plated in 24-well plates (TPP) at 48,400 cells per well. This number was chosen because this is proportionate to the amount of cells plated in a 96-well plate in other analysis. The ratio of the increased amount of media added to the 12-well plates compared to the 96-well plates would be the ratio to use in determining the amount of cells to plate. Cells were initially plated in 1mL of DMEM, media was exchanged 24 hours after plating, and 95% ethanol was added in the following percentages: 0.25%, 0.35%, 0.5%, 0.75%, 1.0%, and 2.0%. Cell viability was assessed by MTT assay as described above, with adaptations made in the volumes of reagents used so that the amounts would be proportional to the amounts used in 96 well plates. Instead of 10µL of MTT solution, 100µL was used. Similarly, 1mL of the SDS-HCl solution was used instead of 100µL. Aliquots (200uL) were then transferred to 96-

well plates and the absorbance at 570nm was determined using the microplate reader, as previously described.

CHAPTER 3: RESULTS

Nutritional Utilization in HeLa Cells

To determine whether or not the selenium derived from LASe would be utilized efficiently for selenoprotein synthesis in mammalian cells, we first used a cell line that is well established, HeLa S3. To allow for cell proliferation, and, in turn, significant protein synthesis, cells were cultivated to 60% confluence and then labeled with ^{75}Se. At the same time unlabeled selenium compounds were added to the cells in increasing concentrations. Unlabeled LASe, L-selenocysteine, and selenite were added in concentrations from 25nM to 150nM. These experiments were carried out in order to compare the nutritional utilization of the selenium in LASe to the utilization of two other well-studied and efficient forms of selenium. In addition to the low concentrations (25nM-150nM), LASe was added to labeled cells in higher concentrations. The range for this study was from 250nM-1μM. Unlabeled L-selenomethionine and selenate were also added to labeled cells in the same concentrations. This experiment was carried out to assess the utilization of the compound when supplied to cells in a higher dose, as well as to compare its utilization to the 2 other well-studied selenium compounds. A much higher dose was required for such a comparison, as L-selenomethionine and selenate are not utilized with as high efficiency as L-selenocysteine and selenite for specific selenoprotein biosynthesis. Cells were cultured for 48 hours after the compounds were added to allow sufficient time for new selenoprotein synthesis and labeling.

As shown in Figure 4, radiolabeling experiments in HeLa reveal that unlabeled selenium in the form of LASe, L-selenocysteine , and selenite are efficiently utilized for selenoprotein synthesis. This experiment assumes that the ^{75}Se comes from radiolabeled selenium which was incorporated during selenoprotein synthesis, as culture medium was removed before harvesting cells, and cells were lysed prior to analysis of protein and ^{75}Se content.

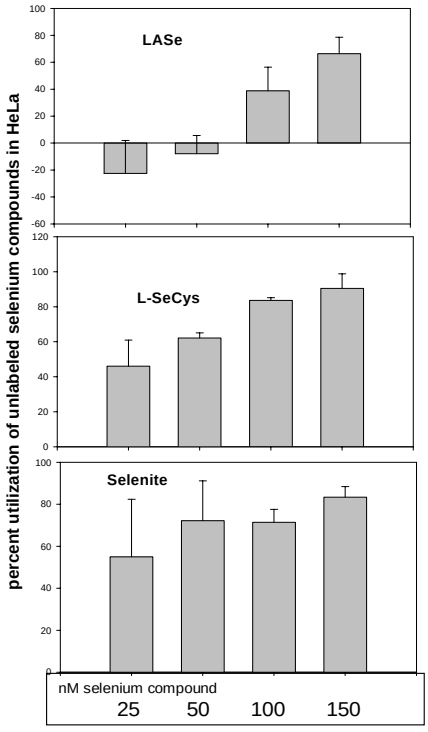

Figure 4
Efficiency of Utilization of LASe, L-SeCys and Selenite in HeLa Cells. Radiolabeled selenoprotein levels were determined in extract of HeLa cells treated with concentrations of selenium compounds from 25nM-150nM and labeled with with ^{75}Se. Data represent the average of at least three separate cultures with standard deviation shown as the error. The cells were incubated with compounds for 48 hours.

Because L-selenocysteine and selenite are both very efficient as nutritional sources of selenium in mammalian cells, these unlabeled selenium compounds readily compete with ^{75}Se in the form of selenite in the cells. LASe is able to compete with the ^{75}Se in the cells, as an upward trend is also seen in the ^{75}Se efficieny of utilization with increasing concentrations of the compound, though the effect is not as dramatic as that seen with addition of L-selenocysteine and selenite. In Figure 5, the decreased radiolabeling effect is shown for a specific selenoprotein, thioredoxin reductase (indicated by arrow).

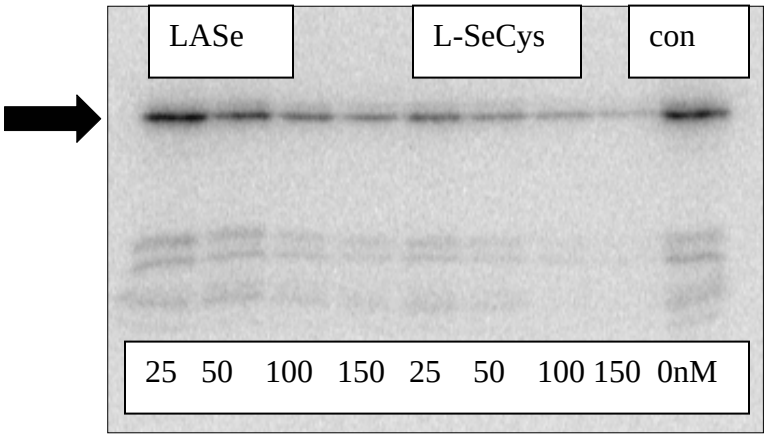

Figure 5
Autoradiography of a Representative SDS-PAGE of Cell Extracts from HeLa Cells Treated with LASe and L-SeCys. 15 µg of protein was loaded in each lane. Arrow shows the band representing thioredoxin reductase, the most abundant selenoenzyme in keratinocytes.

Utilization of LASe and L-selenocysteine are compared side- by -side in this representative figure. Triplicate experiments with cells treated with all three compounds in the four concentrations (25nM-150nM) were performed, with results similar to those

shown in the representative gel shown above. Decreased selenoprotein radiolabeleing with increasing concentrations of unlabeled LASe and L-selenocysteine is seen following SDS-PAGE analysis of cellular proteins from treated HeLa cell extracts and autoradiography of the gel.

Quantification of the labeled thioredoxin reductase band from the gels was performed using Image Quant analysis and correlated directly with the analysis of total isotope incorporation (data not shown). The amount of ^{75}Se present in the thioredoxin reductase from the cell extract decreased with increasing concentrations of all three selenium compounds. LASe was shown to compare well with the other two forms of selenium in terms of its ability to be incorporated into thioredoxin reductase in this analysis. Since the data for the ^{75}Se counts per μg of protein and the quantification of radiolabeled thioredoxin reductase are similar, one may assume that the ^{75}Se counts per μg of protein corresponds to the specific ^{75}Se radiolabeling of selenoenzymes.

Two forms of selenium which are present in most nutritional supplements of selenium due to their low toxicity are selenomethionine and selenate. Comparing the nutritional availability of the selenium in LASe to these two selenium compounds that are commonly used as supplements, LASe was shown to be a more efficient source of selenium for selenoprotein synthesis. As seen in figure 6, when HeLa cells were treated with the same concentrations of LASe, selenomethionine, and selenate, selenium from LASe was most effective as a nutritional source of selenium.

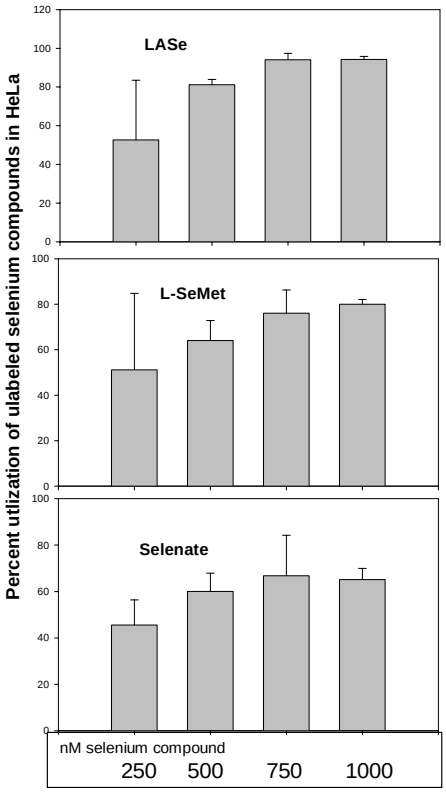

Figure 6
Incorporation of Selenium from LASe, L-SeMet and Selenate into Selenoproteins. HeLa cells were cultured in the presence of selenium compounds and were labeled with ^{75} Se (selenite, 10 nM) as in figure 4.

Figure 7 shows that the selenium from the unlabeled selenium compounds is being efficiently utilized by the cells, as it is incorporated into selenoproteins. In particular, the autoradiography demonstrates that the cells can rapidly utilize the selenium from the unlabeled selenium compounds for synthesis of the 60 kDa selenoprotein thioredoxin reductase (indicated with arrow) at different efficiencies. Selenium supplied from LASe was a better nutritional source than selenium supplied from either L-selenomethionine or selenate. Consequently, selenium from LASe is more efficient as a nutritional source of the micronutrient than these forms in a HeLa cell culture model.

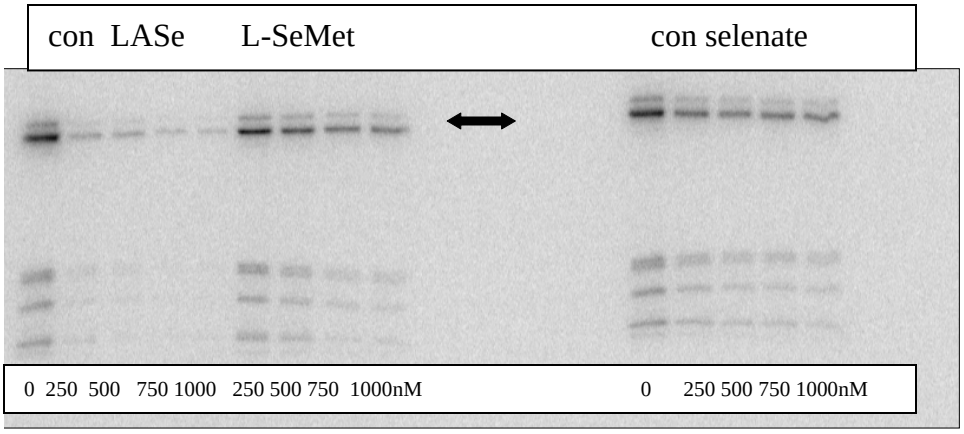

Figure 7
Representative SDS-PAGE to Compare Efficiency of Selenium Incorporation into HeLa Cell Extracts Treated with LASe, L-SeMet and Selenate. TrxR (arrow) decreases as increasing concentrations of unlabeled selenium compounds are incorporated into selenoproteins. Cells were cultured in the presence of Se compounds (250nM-1µM) for 48 hours. 20 µg of protein was loaded in each lane.

Overall Protein Levels in HeLa Cells do not Decrease

To confirm that the changes seen in the levels of radiolabeled selenoproteins were not due to an overall decrease in selenoprotein synthesis, the protein levels of the cytosolic TrxR were determined by a Western blot. Thioredoxin reductase was chosen as the specific selenoprotein to probe for, as it is the most abundant selenoprotein in HeLa cells. As shown in figure 8, the bands corresponding to thioredoxin reductase in cell extracts treated with all concentrations of selenate are of similar intensity. This

demonstrates that thioredoxin reductase was continiuously synthesized at a similar level in the cells treated with selenate in increasing concentrations. The cells were utilizing the unlabeled selenate as a source of selenium for synthesis of new thioredoxin reductase (figure 8).

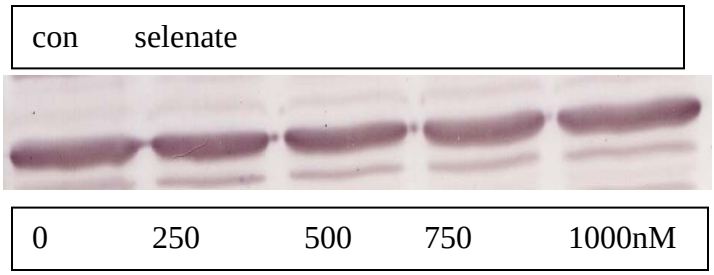

Figure 8
Western Blot Detecting Thioredoxin Reductase in HeLa Cells Treated with Increasing Concentrations of Selenate.

Similar results were obtained in cells treated with selenate is seen in those treated with LASe and L-selenomethionine. This is summarized in figure 9, as the level of thioredoxin reductase detected by immunoblot in cells treated with LASe and L-selenomethionine remains constant.

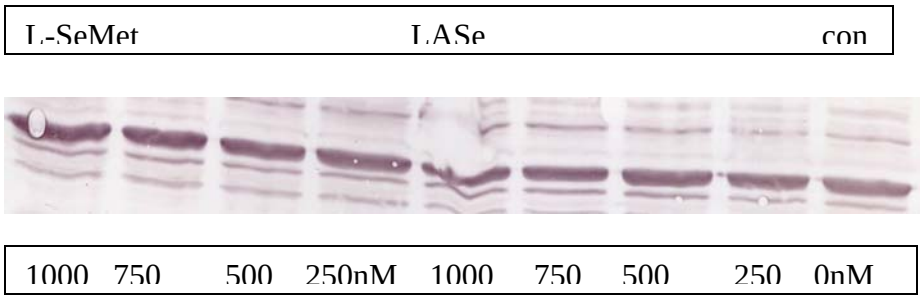

Figure 9
Western Blot to Detect Thioredoxin Reductase in HeLa Cells Treated with LASe and L-SeMet.

These results confirm the assumption that changes in the radiolabeling of selenoproteins were reflecting only the incorporation of unlabeled selenium from the compound of interest in the experiment.

Nutritional Utilization in HaCaT Keratinocytes

Following upon the results from the HeLa cell model, we decided to test the LASe compound in a second mammalian cell line to determine if similar results would be obtained and to test a cell type that is relevant for topical skin use of LASe. Due to the ease with which this compound crosses hydrophobic barriers, such as skin (unpublished data) we chose the skin cell line HaCaT. This is a spontaneously transformed keratinocyte cell line which is widely used in studies requiring a skin cell model. The

method for determining utilization of the compound for selenoprotein synthesis in HaCaT cells was the same as that used with HeLa S3 cells. Cells at approximately 60% confluence were treated with ^{75}Se, and at the same time increasing concentrations of unlabeled selenium compounds were added. For the comparison of more efficiently utilized selenium forms, LASe, L-selenocysteine, and selenite were added to the cells in concentrations from 25nM to 200nM. To compare LASe utilization in HaCaT to the less efficiently utilized selenium compounds L-selenomethionine and selenate, LASe, L-selenomethionine, and selenate were added to the cells in concentrations from 200nM to 1μM.

The nutritional utilization of the compounds in HaCaT cells is shown in figures 10 and 11. Similar to results from experiments in HeLa cells there was good utilization of selenium from LASe for selenoprotein synthesis.

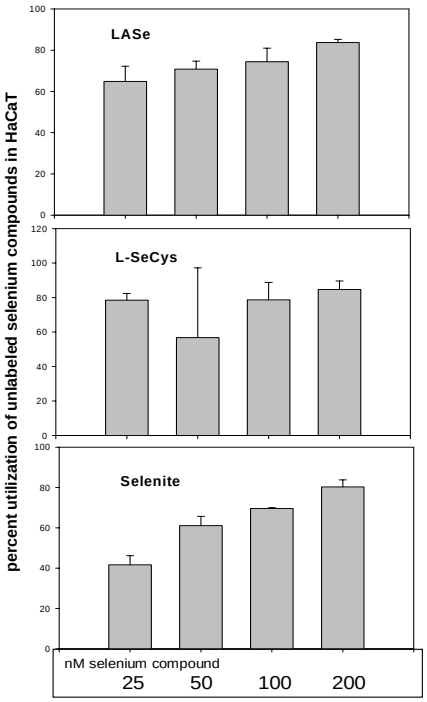

Figure 10
Incorporation of Selenium from LASe, L-SeCys, and Selenite in HaCaT. HaCaT cells were cultured in the presence of selenium compounds and were labeled with ^{75}Se (selenite 10nM).

In fact, it appears that the selenium in LASe is used more efficiently in HaCaT cells when supplied in low dosages, than in HeLa cells when it was supplied in similar dosages (figure 4). It appears that in HaCaT the selenium from LASe is utilized with nearly the same efficiency as selenocysteine, and with higher efficiency than selenite. As shown in figure 10, the data from the radiolabeling of cell protein in treated HaCaT (figure 11) corresponds to the radiolabeling of thioredoxin reductase in this representative autoradiography of a gel run with treated HaCaT cell extracts.

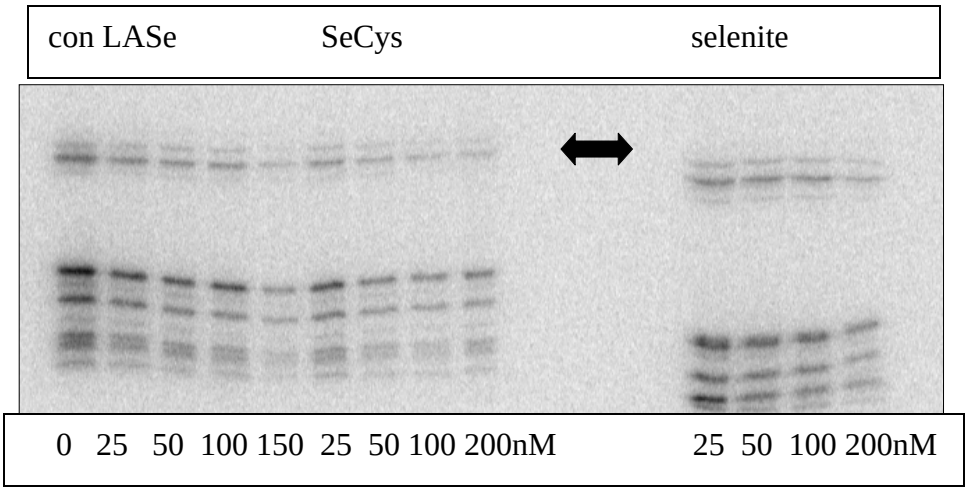

Figure 11
Autoradiography of SDS-PAGE Separating Extracts of HaCaT Cells Treated with 25nM-200nM Unlabeled Se Compounds and Labeled with 75 Se. 40µg of cell protein was loaded in each well. Arrow indicates TrxR.

As with the HeLa cells, the ability of HaCaT cells to utilize the selenium from LASe when supplied in higher dosages was compared to the less efficiently utilized forms of selenium, selenomethionine and selenate. Figure 11 shows the efficient incorporation of LASe into selenoproteins.

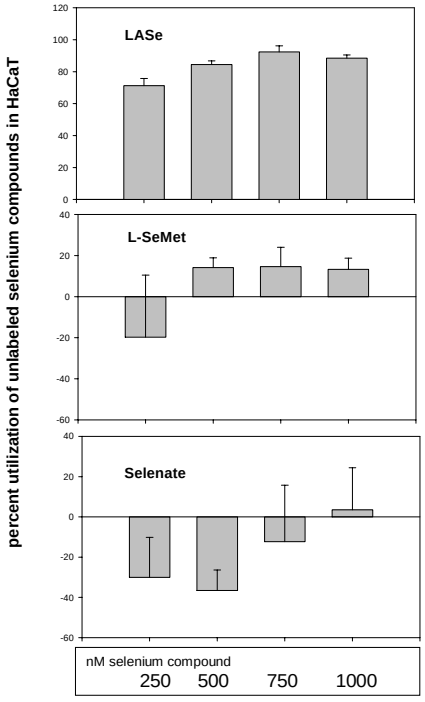

Figure 12

Incorporation of Selenium from LASe, L-SeMet, and Selenate in HaCaT. HaCaT cells were cultured in the presence of selenium compounds and were labeled with ^{75}Se (selenite 10nM).

Autoradiography of cell extracts separated by SDS-PAGE and treated with LASe, L-SeMet and selenate demonstrates that the selenium from the compounds was utilized for synthesis of selenoproteins (figure 13).

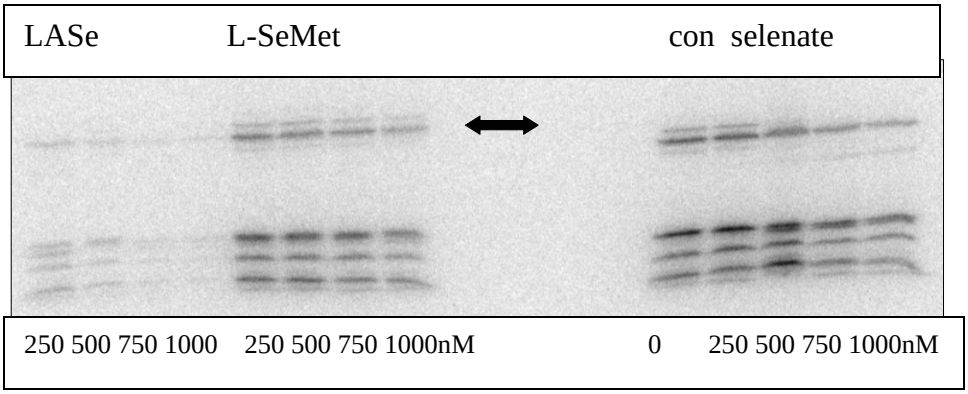

Figure 13
Autoradiography of Cell Extracts Separated by SDS-PAGE of HaCaT Cells Treated with 250 nM- 1μM Unlabeled Se Compounds and Radiolabeled with 75 Se. 100μg of cell protein was loaded in each well. Arrow indicates TrxR.

Toxicity of LASe in HaCaT /Comparison to other selenium compounds

The first experiment performed to compare the toxicity of LASe in HaCaT cells was performed in 96-well microplate. The results for both selenite and L-SeCys are similar in the effect that 1μM of each had on the cells. It appears as if this addition of selenium has a growth enhancing effect on the cells, as the amount of viable cells (by MTT assay) at 24 hours after treatment with both compounds in 1μM concentrations is about 20-30% higher than the untreated (control) cells. As the concentration of selenium, in the form of selenite and L-selenocysteine increases, there is gradual decrease in cell viability at 24 hours after treatment.

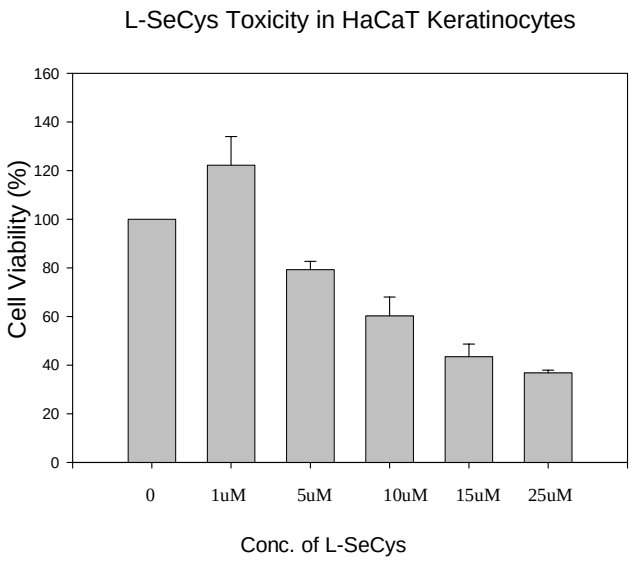

Figure 14
Toxicity of L-SeCys in HaCaT.
Graph represents cell viability (compared to control) assessed by MTT assay 48 hours after pretreatment with selenocysteine in various concentrations. Each bar is the result of at least 3 independent cultures, with standard deviation plotted as the error.

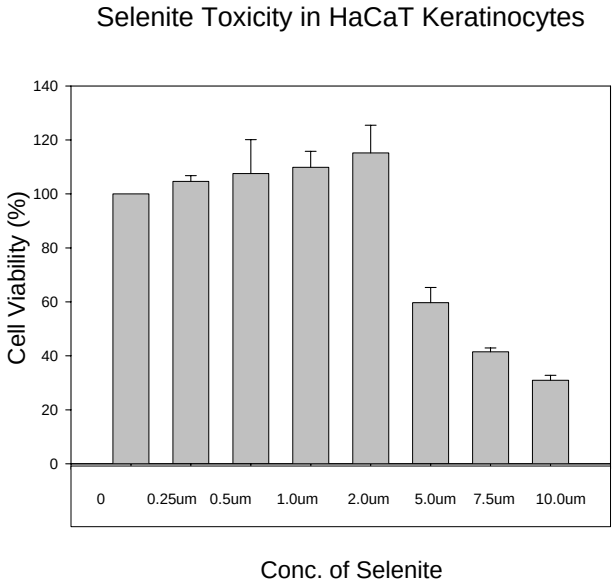

Figure 15.
Toxicity of Selenite in HaCaT
Graph represents cell viability 48 hours after treatment with increasing concentrations of selenite, assayed for by MTT reduction. Each bar is the result of at least 3 independent cultures, with standard deviation plotted as error.

After adding LASe to the cells in the same way in which selenite and L-SeCys were added, a different set of results was obtained. The initial addition of LASe in concentrations above 1µM had a negative effect on the viability of the cells, as determined by the MTT assay. Moreover, the negative effect that the LASe compound had was much more dramatic than the effect of the other two compounds**.** Because the nutritional studies with this compound have shown that it is utilized with nearly the same efficiency as selenite and selenocysteine, we did not expect that LASe itself would be more toxic than the other selenium forms being tested. To further investigate this effect,

we decided to test the toxicity of ethanol in HaCaT cell culture since this was the solvent added to the cells with LASe. The LASe is eluted in ethanol from the HPLC, and dilutions from the stock solution of LASe are made in 95% ethanol as well. In 96 well plates, cells are grown in 100µL of medium. The addition of just one microliter of the diluted LASe compound adds ethanol in a concentration that is equal to 1% of the total volume of the cell's medium. To determine if the ethanol, not the LASe was having a negative effect on cell viability, we added ethanol by itself in concentrations ranging from 0.25%-2.0%, and assessed the viability of the cells by measuring the reduction of MTT 24 hours after treatment. Based on the results of the MTT assay, we can assume that the ethanol was toxic to the cells and that only up to 0.5% ethanol in the total culture medium is safe to add to the cells. The addition of ethanol in concentrations higher than 0.5% of the total culture medium appears to have a negative effect on cell viability, as assayed by MTT reduction.

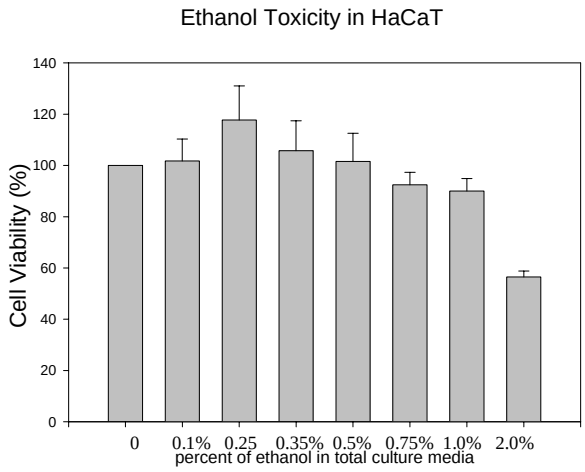

Figure 16
Ethanol Toxicity in HaCaT
Graph represents the cell viability 48 hours after pretreatment with ethanol in concentrations from 0.1%-2.0%. Each bar is the result of 3 independent cultures, with standard deviation plotted as the error.

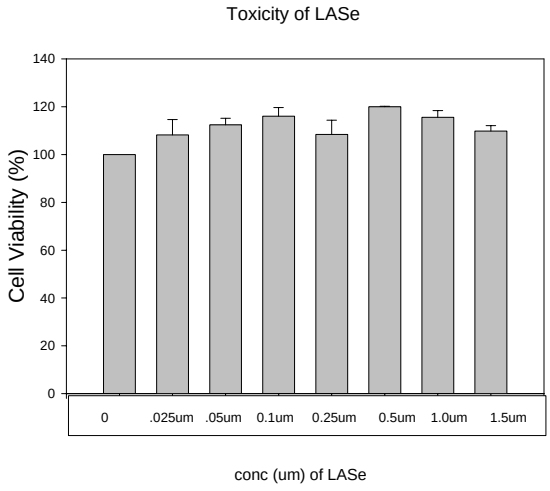

Figure 17
Toxicity of LASe (diluted into 5% ethanol) in HaCaT
Graph shows cell viability following pretreatnment with various concentrations of LASe . The final dilutions of LASe were made into 5% ethanol. Each bar is the result of three independent cultures, with standard deviation plotted as the error.

Once LASe was added to cells after being diluted in 5% ethanol, it was evident that LASe itself was not toxic to the cells. In fact, because these cells were deficient in selenium, due to their continuous culture in 90% defined media and 10% DMEM supplemented with serum, the addition of the selenium had a slight proliferative effect, indicated by an increase in MTT reduction. To determine if perhaps the lipoic acid component of the LASe were toxic to the cells, LA alone was diluted in 5% ethanol and added to the cells. The results are shown in figure 18.

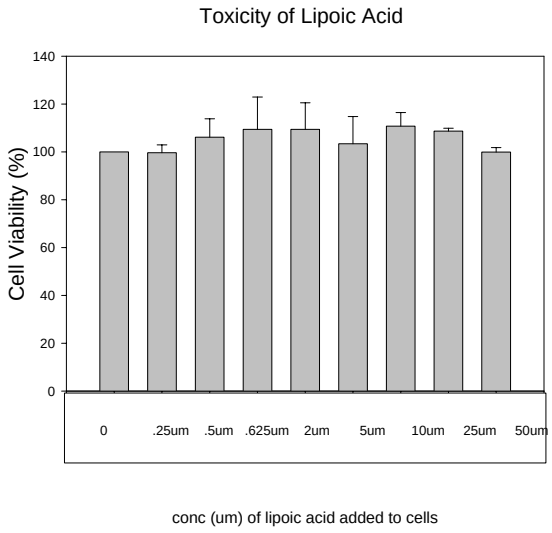

Figure 18
Toxicity of LA in HaCaT
The graph shows the % of cells alive after being pre-treated with lipoic acid at various concentrations. Each bar is the result of three independent cultures, with standard deviation plotted as the error.

It is clear LA, like LASe is not toxic to HaCaT cells. Furthermore, the same proliferative effect seen in the cells pretreated with LASe was seen in cells treated with LA. According to our studies, selenite is not toxic in concentrations up to 2 μM (figure 15). Taken together, this indicates that LASe and its individual components are not toxic to HaCaT cell in sub-micromolar concentrations. Thus, we began testing LASe in sub-micromolar concentrations to determine whether it could protect cells from UV induced cytotoxicity.

UV Irradiation of HaCaT Cells

Before moving to studies involving the ability of selenium compounds to protect HaCaT cells from UV induced cell death, it was first necessary to determine the correct range of UV light to expose the cell to in order to induce cell death as a measurable endpoint. To determine the amount of UV necessary to cause cytotoxicity, cells were cultured in 12-well dishes until approximately 80% confluent. Cells were irradiated as described, and then incubated for 24 hours before assessing cytotoxicity by counting cells using trypan blue dye exclusion with a hemocytometer. Approximately 70% of the cells were killed with 750J/m^2 of broadband UV irradiation, 50% with 500J/ m^2, 18% with 250J/m^2. Figure 19 summarizes the changes in of cell viability 24 hours after UV irradiation, compared to untreated cells, 24 hours after UV irradiation.

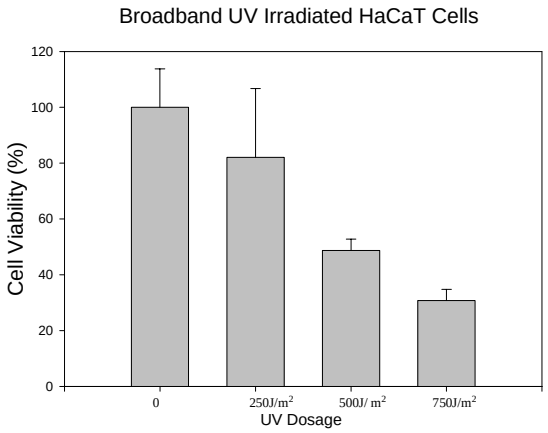

Figure 19
Cell Viability Following UV Irradiation, Determined by Trypan Blue Exclusion
Graph represents the percentage of cells, compared to untreated cultures viable 24 hours after UV irradiation. Each bar represents the average of three independent cultures, with the standard deviation plotted as the error.

In addition to cultivating cells in the 12 well dishes and determining the effective UV dosage to kill the cells using trypan blue staining and cell counting using a hemocytometer, cells were also cultured in 96-well plates and cell death was assessed by MTT assay. This was carried out to establish the UV dosage required to kill approximately 80% of the cells in these plates. This UV dosage, once established would be used for future protection studies. Cells were exposed to 100J/m^2, 250J/m^2, 500J/m^2, 750J/m^2 and 1000J/m^2 of broadband UVA-UVB light. As summarized in figure 20, similar results were obtained in the 96 well plates when the viability of cells was assayed for using MTT. This validates the use of MTT to assess cell viability upon UV exposure to keratinocytes.

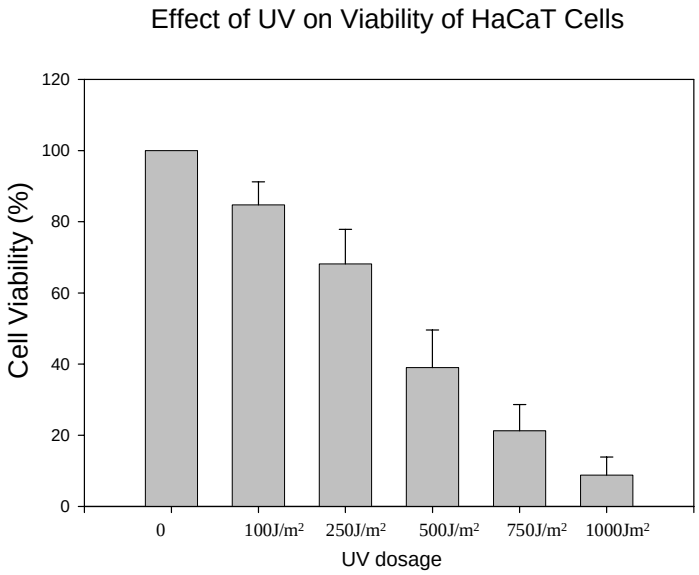

Figure 20
Cell Viability Following UV Irradiation, Determined by MTT Assay
Graph represents cell viability 48 hours after UV irradiation with dosages ranging from 100J/m2-10000J/m2. Each bar represents at least 3 independent cultures, with the standard deviation plotted as the error.

Determination of lethal UV dose for HaCaT cells cultured in 90% defined media and 10% DMEM

Since serum contains selenoprotein P and undefined forms of selenium, we chose to carry out experiments defined keratinocyte media (36). This modified DKM contains 1% serum (as defined in the methods section 90:10), which reduces the presence of other forms of selenium which are present in serum. Cells grown in 90:10 media, then, have only one predominant form and amount of selenium available to them, 7nM selenite. The comparison studies performed in this media are much more representative of the effects of compounds which are added to the cells, than if they were added to cells cultured in

DMEM with 10% serum. To confirm that the UV dosage effective to result in cytotoxicity of HaCaT cells cultured in 90% DKM and 10% DMEM, cells were treated with varying doses of UV in the same manner as in figure 20.

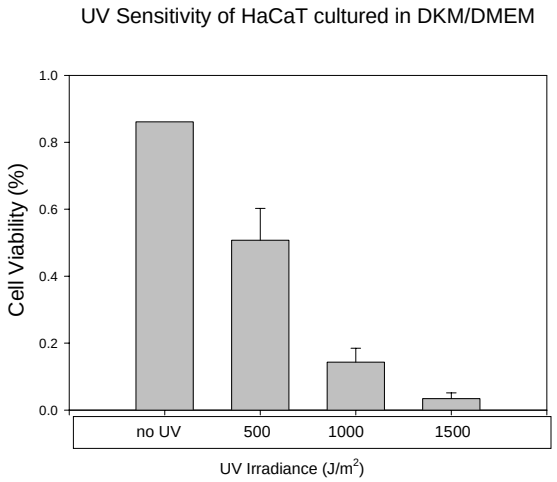

Figure 21
Cell Viability of HaCaT in 90%DKM and 10% DMEM following UV Irradiation
The graph represents cell viability 48 hours after UV irradiation with increasing doses of UV. Cells were cultured in 90% DKM and 10% DMEM (final conc. of serum 1%). Each bar represents at least 3 independent cultures, with the standard deviation plotted as the error.

Cells cultured in 90:10 displayed similar susceptibility to UV irradiation. Further UV studies were carried out using cells that had acclimated to the 90:10 media for several more passages. These cells exhibited nearly the same response to UV as those cultured in DMEM with 10% FCS. A UV dose of (figure 20) 750J/m^2 resulted in a decrease in cell viability by approximately 80%, and a dose of 500J/m^2 resulted in nearly 60% cell reduction in cell viability.

Protection of HaCaT Cells From UV Induced Cell Death

HaCaT cells pretreated for 24 hours with LASe as well as lipoic acid and selenite for comparison, were protected from UVA/UVB induced cell death. The 24 hour incubation period was selected in order to give the cells adequate time to metabolize the selenium and incorporate it into selenoproteins. The protective effect is represented in the figures below. Though the cells were treated with a range of UV ($250J/m^2$-$1000J/m^2$), the protective effect of the compounds was more dramatic at higher UV dosages ($500J/m^2$ and $750\ J/m^2$). Furthermore, this effect was concentration dependent, as there was a gradual increase in protection observed with increasing concentrations of the compounds. This increase in protection eventually reached a maximum value and protection was not as great for cells to which higher concentrations of compounds were supplied.

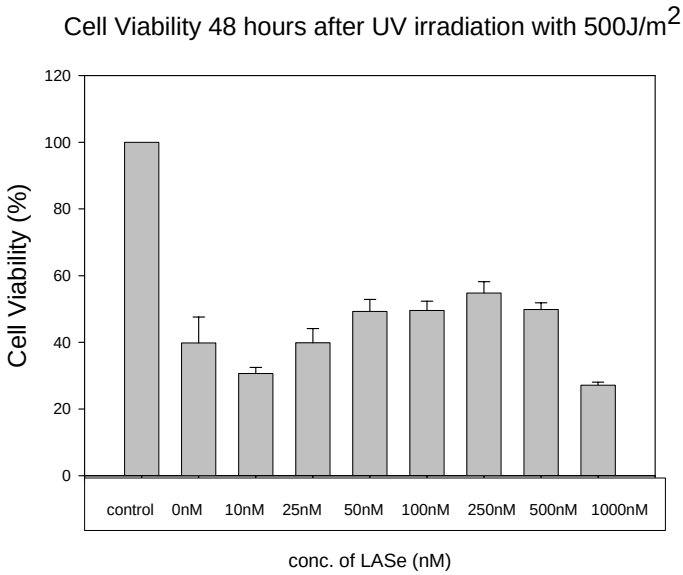

Figure 22
Cell Viability 48 Hours After UV Irradiation with 500J/m^2 Following Pretreatment with LASe
Graph represents the percentage of HaCaT cells alive 48 hours after being irradiated with 500J/m^2 of UVA-UVB. Cells were pretreated with increasing concentrations of LASe. Each bar represents at least 3 independent cultures, with the standard deviation plotted as error.

As represented in figure 22, LASe treatment before UV irradiation is protective. HaCaT show an increase in cell viability when exposed to 500J/m^2 when LASe has been supplied in concentrations from 25nM-500nM. The greatest amount of protection was achieved when LASe was supplied at a concentration of 250nM. The protection observed following pretreatment with this dose was an approximately 15% increase in cell viability.

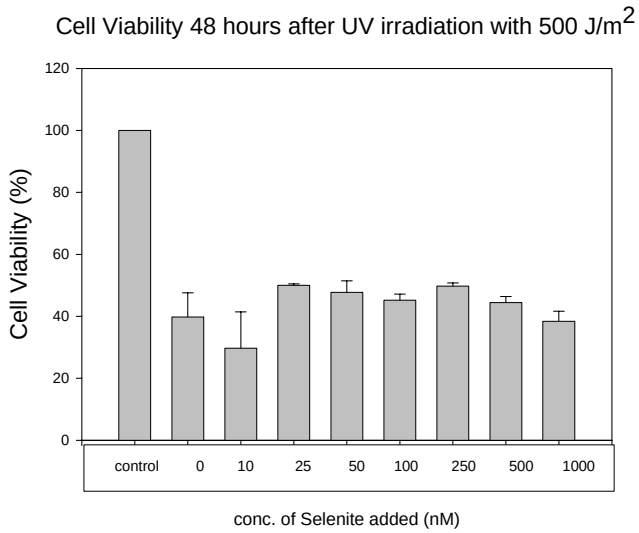

Figure 23

Cell Viability 48 Hours After UV Irradiation with 500J/m^2 Following Pretreatment with Selenite

Graph represents the percentage of HaCaT cells alive 48 hours after being irradiated with 500J/m^2 of UVA-UVB. Cells were pretreated with increasing concentrations of selenite. Each bar represents at least 3 independent cultures, with the standard deviation plotted as error.

Figure 23 represents the protection achieved when HaCaT were pretreated with selenite. In this instance, the concentration of selenite which offered the greatest amount of protection from UV irradiation with 500J/m^2 was 25nM. Pretreatment with 25nM selenite resulted in an approximate 10% increase in cell viability.

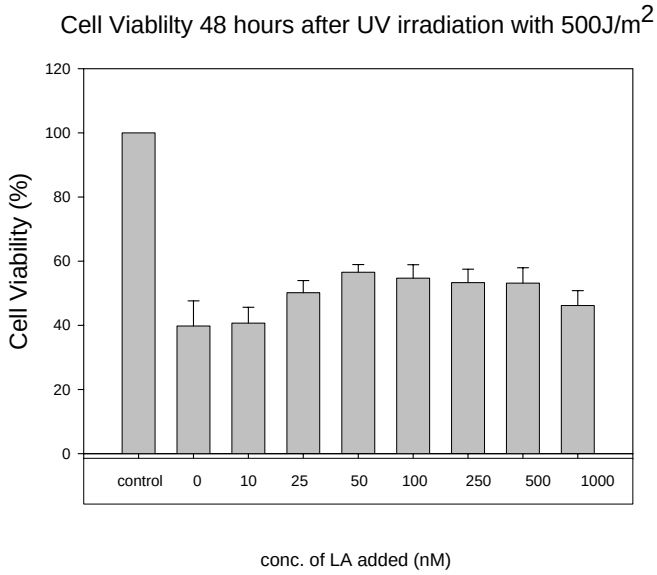

Figure 24
Cell Viability 48 Hours After UV Irradiation with 500J/m^2 Following Pretreatment with LA
Graph represents the percentage of HaCaT cells alive 48 hours after being irradiated with 750J/m2 of UVA-UVB. Cells were pretreated with increasing concentrations of LA. Each bar represents at least 3 independent cultures, with the standard deviation plotted as error.

Figures 24 demonstrates that pretreatment with lipoic acid also offers protection from UV induced cytotoxicity. Pretreatement with 50nM lipoic acid offered the greatest protection. The increase in cell viability in HaCaT treated with lipoic acid was approximately 15% above control values.

Cells were also UV irradiated with 750J/m^2. As could be expected, cell death of UV treated cells was higher compared to the cells not treated with UV. As in the cells treated with 500J/ m^2 of UV, protection from cell death by selenium compounds as well as LA was observed. In the cells treated with higher UV dosage, the greatest protective effect observed was from pretreatment with 25nM of all of the compounds.

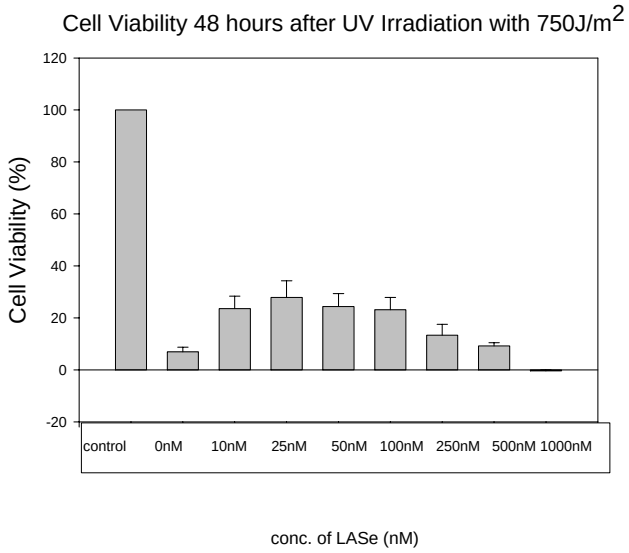

Figure 25

Cell Viability 48 Hours After UV Irradiation with 750J/m^2 Following Pretreatment with LASe

Graph represents the percentage of HaCaT cells alive 48 hours after being irradiated with 750J/m^2 of UVA-UVB. Cells were pretreated with increasing concentrations of LASe. Each bar represents at least 3 independent cultures, with the standard deviation plotted as error.

Figure 25 summarizes the protection achieved when HaCaT were pretreated with LASe and irradiated with a high dose of UV (750J/m^2). There was a dose dependent response to the level of protection offered by LASe, with the greatest protection achieved when

LASe was supplied at 25nM. At this dose of LASe, there was an approximate 21% increase in cell viability as compared to the control values.

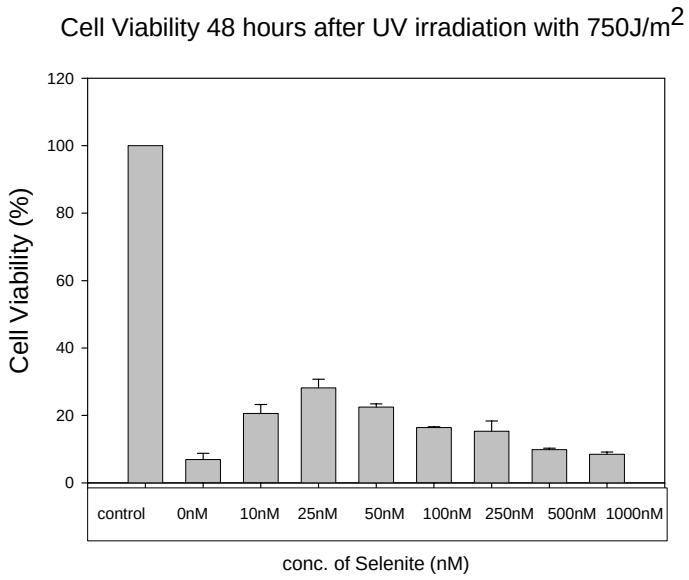

Figure 26

Cell Viability 48 Hours After UV Irradiation with 750J/m^2 Following Pretreatment with Selenite

Graph represents the percentage of HaCaT cells alive 48 hours after being irradiated with 750J/m^2 of UVA-UVB. Cells were pretreated with increasing concentrations of Selenite. Each bar represents at least 3 independent cultures, with the standard deviation plotted as error.

As presented in figure 26, 25nM selenite was the dose which offered the highest protection for cells irradiated at 750J/m^2. This is the same dose of LASe which had provided the highest level of protection from the same UV irradiance. Likewise, the level of protection was nearly the same (21% increase in cell viability).

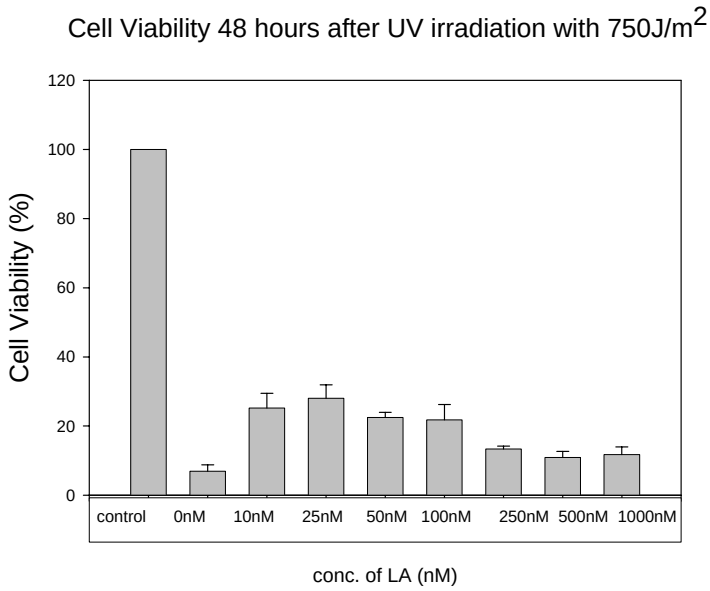

Figure 27
Cell Viability 48 Hours After UV Irradiation with 750J/m^2 Following Pretreatment with LA
Graph represents the percentage of HaCaT cells alive 48 hours after being irradiated with 750J/m^2 of UVA-UVB. Cells were pretreated with increasing concentrations of LA. Each bar represents at least 3 independent cultures, with the standard deviation plotted as error.

Similar results were observed regarding the level of protection offered by lipoic acid as in the studies examining LASe and selenite at 750J/m^2. Lipoic acid supplied at 25nM offered the greatest protection from UV, and the protection observed was also a 21% increase in cell viability upon pretreatment.

CHAPTER 4: DISCUSSION

This study has demonstrated the potential of a novel selenium compound (LASe) to provide selenium as a nutritional source and to protect skin cells in culture from UV induced cell death. Since the selenium from LASe was efficiently utilized for selenoprotein synthesis it has potential as a nutritional supplement The protective effect of the compound in the UV protection experiments, then, is likely due to its ability to increase the level of antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase. The radiolabeling competition studies demonstrated that LASe was more efficient than selenomethionine and selenate, especially in the target cell line, HaCaT. This finding is significant, as selenomethionine is a commonly used form of selenium as a supplement. Because selenomethionine has the potential to be non-specifically incorporated into methionine residues of proteins, its use as a supplement may not be ideal. In addition to demonstrating far greater efficiency than selenomethionine and selenate, LASe compared well with the two most efficient forms of selenium, selenite and selenocysteine. This nutritional evidence indicates that LASe has the potential for use as a supplement.

To more accurately study the effects of LASe in cell culture, a defined medium was used for protection studies. This medium eliminated most of the serum present in standard DMEM culture medium, which was necessary for these studies, as serum contains a number of different selenium sources, including selenoprotein P and one form of Gpx, as well as a small molecule form of selenium that has yet to be identified.

Consequently, the effects of the selenium compounds in this study could be studied individually, without the presence of unknown forms. Protection studies performed by other groups examining protection by selenium compounds other than LASe in cell culture were done in standard culture medium (DMEM).

The protective effect of LASe was comparable to the effect shown by its individual components, lipoic acid and selenite. This was not expected, since selenite is a good nutritional form of selenium and lipoic acid has been shown to be a potent antioxidant. The expected result would have been protection by LASe which was more than that achieved by selenite or lipoic acid treatment alone, however, this synergistic effect was not observed. The graphs representing the protection of HaCaT from 750J/m^2 most clearly show the protection from these compounds, and indicate that the protective effect is dose dependent. It appears that when HaCaT are pretreated with lipoic acid, selenite and LASe, the common dosage offering the greatest amount of protection is 25nM. The amount of protection offered by the three compounds was very similar and resulted in approximately a 21% increase in cell survival following UV irradiation. The graphs of the protection observed at 500J/m^2 resemble the graphs in the toxicity study. Consequently, the proliferative effect observed following the addition of these antioxidant compounds to slightly stressed cells in culture (due to elimination of serum from medium) is evident in cells treated with lower dosages of UV. There is, however, a protective effect observed at the lower dosage (500J/m^2) of UV as well as higher (750J/ m^2) dosage.

The fact that LASe is not toxic to cells in culture at concentrations up to one micromolar is significant. Furthermore, it has been demonstrated that LASe offers

protection to skin cells in culture from UV induced cell death. Combined with the evidence that this compound is absorbed into skin, it presents itself as a possible topical supplement which can offer protection from UV generated oxidative stress. To further investigate the mechanism by which LASe is protective of UV induced cytotoxixity, we would like to determine if supplementation results in increased activity of TrxR and Gpx. Furthermore, we would like to determine if any increase in the levels of these antioxidant enzymes correlates with increased UV protection. In addition, we would like examine any protection that this compound may offer from specific forms of oxidative stress, such as protein carbonylation and production of peroxides. Finally, our goal is to move our studies involving LASe's UV protective ability from cell culture models to an animal model.

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