THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF FOOD SCIENCE
and VETERINARY & BIOMEDICAL SCIENCES

PTEROSTILBENE INHIBITS THE GROWTH OF HUMAN NON-SMALL CELL LUNG CANCER CELLS BY INDUCING CELL CYCLE ARREST

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ABSTRACT

Pterostilbene is a dimethylated analog of resveratrol and is commonly found in blueberries. We tested the hypothesis that pterostilbene could inhibit the growth of non-small cell lung cancer cells *in vitro*. Pterostilbene dose-dependently reduced viability of H1299 human lung cancer cells (IC_{50} = 17.6 μM - 49.0 μM). Time-dependent studies with 25 μM and 50 μM pterostilbene showed that this loss of viability was due to growth inhibition rather than cytotoxicity. Cell cycle analysis showed that pterostilbene induced G_1 phase arrest. Induction of cell cycle arrest occurred in a p53-independent manner, through down-regulation of kRas. Further studies are needed to determine the underlying mechanism of these effects and determine if growth inhibition occurs *in vivo*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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1. INTRODUCTION

Lung Cancer

As the leading cause of cancer mortality both worldwide and in the United States, lung cancer is a major focus of cancer prevention efforts.[1] Lung cancer is closely associated with tobacco use, with approximately 80% of cases diagnosed in current or former smokers, and the remaining 20% in non-smokers.[1] Smoking increases the relative risk of lung cancer 10 to 30 times compared to nonsmokers.[2] Furthermore, when examining lung cancer deaths, smoking is implicated as the cause of 90% of male deaths and 75-80% of female deaths.[3]

The two major classifications of lung cancer, small cell and non-small cell lung cancer, are based on the cell-type from which the cancer arises. Non-small cell lung cancers can be further subdivided into squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma.[4] Small cell and non-small cell lung cancers are best differentiated through their contrasting molecular abnormalities. For example, point mutations in the small G-protein Ras are observed in less than 1% of small cell lung cancers, but are observed in 15-20% of non-small cell lung cancers.[5] Likewise, p53 inactivation occurs in approximately 90% of small cell lung cancers, but only 50% of non-small cell lung cancers.[5]

Common Oncogenic Abnormalities in Lung Cancer

Table 1.1. Frequently acquired molecular abnormalities in lung cancer.[5]

ABNORMALITIES	FREQUENCY Small Cell Lung Cancer	FREQUENCY Non-Small Cell Lung Cancer
Microsatellite instabilities	~35%	~22%
RAS point mutation	<1%	15%-22%
EGFR mutation	<1%	<10% (West), ~40% (Asia)
EML4-ALK	0%	3%-7%
MYC family overexpression	15%-30%	5%-10%
p53 inactivation	~90%	~50%
RB inactivation	~90%	15%-30%
$p16^{INK4A}$	0%-10%	30%-70%
LKB1 inactivation	~40%-60%	20%-40%
Telomerase activity	~100%	80%-85%
BCL2 expression	75%-95%	10%-35%

The frequency of common molecular abnormalities in lung cancer is shown in Table 1.1. Other molecular abnormalities include autocrine loops at the gastrin-releasing peptide (GRP)/GRP receptor and stem cell factor (SCF)/KIT in small cell lung cancer, and autocrine loops in transforming growth factor alpha (TGF-α)/epidermal growth factor receptor (EGFR), heregulin/ERBB2, and hepatocyte growth factor (HGF)/MET in non-small cell lung cancer. Frequently lost alleles in small cell lung cancer include: 3p, 4p, 4q, 5q, 8p, 10q, 13q, 17p, 22q. Frequently lost alleles in non-small cell lung cancer include: 3p, 6q, 8p, 9p, 13p, 17p, 19q.[5]

The most commonly mutated protooncogenes are EGFR and the rat sarcoma oncogene (RAS). EGFR is part of the ErbB protein family (specifically ErbB-1). A receptor tyrosine kinase, EGFR is activated upon binding with natural ligands such as epidermal growth factor (EGF), TGF-α, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epigen, and epiregulin.[6]

In non-small cell lung cancer, EGFR is overexpressed in 40 to 80% of cases.[7] Co-expression of EGFR and ligand TGF-α in lung cancer cells is indicative of a self-stimulated autocrine growth factor loop important for tumor formation.[8] Downstream signal cascades include MAPK, Akt, and JNK pathways that are responsible for DNA synthesis and consequent cell proliferation.[9] Mutations that constitutively activate EGFR tyrosine kinase cause the up-regulation of downstream signals that will activate cell survival and proliferative signals.[10] Thus, cancer may occur because EGFR activation can result in uncontrolled proliferation, invasion, and metastasis.

In lung cancer, Kirsten rat sarcoma viral oncogene (KRAS) is the most common RAS homolog that occurs from gene mutation.[11] The RAS gene is a downstream member of the EGFR signaling cascade. However, KRAS mutation is mutually exclusive from EGFR mutations.[12] In adenocarcinomas, KRAS mutations typically occur at codon 12, though also appear at codon 13 and 61.[13] In contrast, 90% of the EGFR mutations in lung cancer are usually amino acid deletions derived from codons 746 to 750 or due to a substitution at codon 858 (leucine-to-arginine).[14]

RAS is a GTPase activity, and is active when bound with GTP (versus inactive when bound with GDP). RAS is positively regulated by the recruitment of guanine-nucleotide exchange factors after growth factor receptor stimulation; this results in increased levels of active RAS-GTP. Negative regulation of RAS occurs through catalysis of RAS-GTP to RAS-GDP by RAS GTPase. RAS GTPase activity is enhanced by RAS GTPase activating proteins (RAS-GAPs). Oncogenic mutations that cause GAP-resistance in RAS result in the accumulation of active RAS-GTP and prolonged stimulation of signaling cascades that promote proliferation and cell survival.[12]

Lung Cancer Therapies

Common lung cancer therapies include cisplatin and etoposide for small cell lung cancer, and gemcitabine, paclitaxel, docetaxel, etoposide, and vinorelbine for non-small cell lung cancer.[15,16] Cisplatin disrupts cellular division and induces apoptosis by causing intrastrand cross-linking DNA, whereas etoposide forms a complex with topoisomerase II, preventing DNA from re-ligating and ultimately causing double-strand DNA strand breaks.[17,18] Gemcitabine is a diphosphate analog that inhibits ribonucleotide reductase, preventing deoxynucleotide formation required for DNA replication and repair.[19] Paclitaxel, docetaxel, and vinorelbine all disrupt tubulin turnover, and ultimately interfere with mitosis.[20,21] Currently, the five-year survival rate for non-small cell lung cancer, the most prevalent type, is 15%.[22] Cisplatin therapy, after five years, had an absolute survival benefit of 4.1%, with a 14% reduction in relative risk of death.[23] Adjuvant

chemotherapy had an absolute overall survival benefit of 8.6% after five years.[24] Such studies strongly support the use of chemotherapeutic treatments for lung cancer for increased long-term survival.

Lung cancer therapies are continuing to evolve, and have become much more specific. Targeted therapies have increased median survival time for patients who do not respond to second- or third-line treatment. For example, erlotinib had a statistical significant response rate of 8.9% compared to placebo, and a median survival time of 6.7 months versus 4.7 months.[25] Other target therapy drugs currently on the market, include bevacizumab, cetuximab, and gefitinib, specifically inhibit EGFR.[26] Other specific therapies that interfere with other oncogenic dysregulations in lung cancer, such as the Ras pathway, are also currently being developed and studied.[26]

Blueberries

A popular fruit native to North America, blueberries are a member of the genus *Vaccinium*.[27] The three major species of blueberries are high-bush (*Vaccinium corymbosum*), rabbiteye (*Vaccinium virgatum*), or low-bush (*Vaccinium angustifolium*); high-bush varieties account for 60% of blueberry production in North America and are grown commercially in Michigan, New Jersey, and North Carolina.[28] Between 1980 to 2007, production of high-bush blueberries has nearly doubled to over 200 million pounds, while production of low-bush blueberries has tripled to more than 150 million pounds.[29]

Blueberries may be prepared a variety of ways: fresh, frozen, juiced, pureed, concentrate, and dried. While all derived from the same general fruit, differences in preparation have been noted to lead to differences in nutritional properties. For example, while all blueberries are low in calories and high in fiber, canned and frozen blueberries have lower levels of vitamin A and C (Table 1.2).[28] Variation may also be explained by the variety of blueberry used in each process. Fresh blueberries sold are almost always of the high-bush variety, while low-bush blueberries are often frozen or processed.[30]

Table 1.2. Blueberry Nutrient Composition (per 100 g).[28]

Table 1. Nutrient Composition of Blueberries per 100 g Edible Portion (http://www.nal.usda.gov/fnic/cgi-bin/list_nut.p)

	Fresh (Raw) Blueberries	Frozen Blueberries (Unsweetened)	Canned Blueberries in Syrup
Nutrient			
Water, g	85	87	77
Energy, kcal	56	51	88
Protein, g	0.7	0.4	0.7
Fat, g	0.4	0.6	0.3
Carbohydrate, g	14	12	22
Dietary fiber, g	2.7	2.7	2.0
Minerals			
Calcium, mg	6	8	5
Potassium, mg	89	54	40
Sodium, mg	6	1	3
Iron, mg	0.2	0.2	0.3
Vitamins			
Vitamin C, mg	13	2.5	1.1
Niacin, mg	0.4	0.5	0.1
Folate, mcg	6	7	2
Vitamin A, IU	100	81	64

The antioxidant capacity of blueberries have been widely reported. Antioxidants may play a role in reducing cancer risk by attenuating cellular damage from oxygen free radicals.[26] Phenolic compounds, such as anthocyanins, proanthocyanidins, phenolic acids, and stilbenes, are the main contributor to the antioxidant capabilities of blueberries.

On average, low-bush blueberry varieties had higher ORAC antioxidant capacity and higher levels of anthocyanins and phenolic acids than highbush varieties.[30,31]

Stilbenes

Blueberries contain a number of stilbene compounds. Stilbenes has an ethylene double bond with phenyl groups on either end; stilbenoids, stilbene derivatives, have hydroxlated phenyl groups.[27]

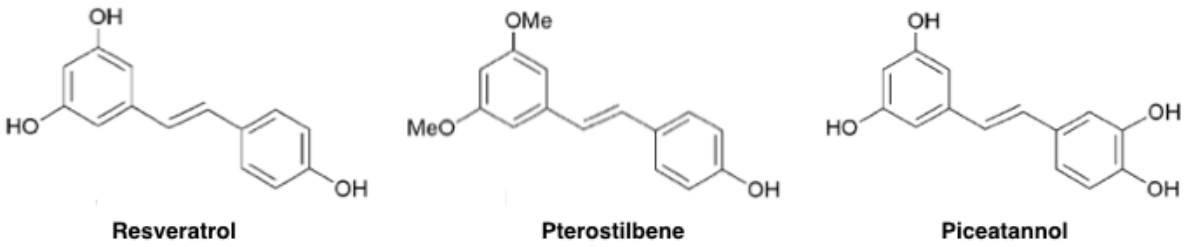

Figure 1.1. Chemical structures of various stilbenes.[27]

The major stilbenes in blueberries are resveratrol, pterostilbene, and piceatannol (Figure 1.2). *V. corymbosum*, *V. ashei*, and *V. anyustifolium* have been found to contain up to 853 ng of resveratrol per gram of dry fruit.[2] Pterostilbene and piceatannol, on the other hand, were found in the *V. ashei* and *V. corymbosum* species of blueberries, respectively, at concentrations of up to 151 ng per gram of dry fruit and 422 ng per gram of dry fruit.[32]

Resveratrol, the most well-studied stilbenoid, is a photoalexin and most commonly found in grapes and wine. Resveratrol has been shown to have anti-initiation, anti-promotion, and anti-progression activities in various animal models of carcinogenesis.[33] These effects are related in part to resveratrol-mediated induction of apoptosis. A possible molecular mechanism includes the kinase-mediated activation of p53 through extracellular-signal-regulated protein kinases (ERKs) and p38.[34] Phosphorylation of p53 at serine 15 by ERKs and p38 kinase stabilizes and upregulates the action of p53.[34,35] Resveratrol also activates c-Jun NH2-terminal kinases (JNKs) which phosphorylates p53 in vitro.[34] Together, ERKs, p38, and JNKs may mediate the activation of p53 and apoptosis.[35] Evidence also indicates that resveratrol's anti-cancer properties are related to cell-cycle G1 phase arrest.[36]

Pterostilbene, or 4-[(E)-2-(3,5-dimethoxyphenyl)ethenyl]phenol, is an analog of resveratrol.[37] Pterostilbene levels can be induced by ultraviolet light exposure or microbe infestation.[37,38] Like resveratrol, pterostilbene has putative antioxidant, anticancer, and anti-inflammatory properties.[39] However, pterostilbene has far greater bioavailability than resveratrol (95% compared to 20%, measured in plasma levels), so it may have more efficacious clinical applications in cancer prevention.[40,41]

Pterostilbene Biotransformation and Bioavailability

Pharmacokinetics

In rats, total serum clearance of pterostilbene was measured at 16.0 ± 0.4 mL/min/kg when given an intravenous dose of 20 mg/kg.[42] However, when given a dose of 5 mg/kg, clearance levels were measured at 37.0 ± 2.5 mL/min/kg.[43] The potentially difference may be due to saturation of elimination systems, lowering clearance and raising plasma exposure. Pterostilbene is mainly excreted by non-renal routes. Very little pterostilbene is excreted through urine (0.002 ± 0.001 L/h/kg or only 0.219 ± 0.088%). Therefore, it is assumed that pterostilbene is mainly cleared through hepatic clearance. Hepatic clearance was calculated ($CL_{hepatic} = CL_{total} - CL_{renal}$) at a value of 0.958 L/h/kg.[42]

While in the body, pterostilbene most likely predominantly resides in the central blood compartment. The volume of distribution for pterostilbene was measured at 2.41 ± 1.13 L/kg. Meanwhile, total drug exposure in the serum over time was measured by the mean area under the curve (AUC) at a concentration of 17.5 ± 6.6 μg.h/mL.[42] The half-life of pterostilbene has been consistently measured at approximately 1.5 hours (103.8 ± 46.8 min and 96.6 ± 23.7 min).[42,43] This half-life is seven times longer than the 14 minute half-life of resveratrol.[42]

Pterostilbene has a logP coefficient of 3.99, indicating that pterostilbene is lipophilic and enhancing potential cell membrane permeability.[44] The increased bioavailability of pterostilbene in comparison to resveratrol is due to the structural differences between both compounds. Resveratrol has three hydroxyl groups while pterostilbene has one hydroxyl group and two methoxy groups; the added lipophilicity due to the methoxy groups increases absorption and the potential for cellular uptake.[41]

However, this limited aqueous solubility may be a barrier to oral absorption. After oral administration of 5 gm/kg, AUC/dose, a measure of plasma exposure, was measured at 56.7 ± 21.4 g h/L.39 Regardless, pterostilbene has an oral bioavaliability seven-fold greater than that of resveratrol.[38]

Extensive first pass metabolism of pterostilbene resulted in a low maximum concentration (141 ± 71 ng/mL) measured within rats. Due to first pass metabolism, the absolute bioavailability of pterostilbene was measured at only 12.5 ± 4.7%.[43] However, other pterostilbene metabolites are present in plasma samples.

Phase II Metabolism

Pterostilbene is mainly metabolized by phase II enzymes, specifically undergoing conjugation with glucuronide and sulfate. Sulfate conjugates are more prevalent in rats, indicating that sulfate conjugation may be the preferred pathway.[38]

However, in humans, pterostilbene is a poorer substrate for human sulfotransferases (SULT). Sulfonation of the related stilbene, resveratrol, demonstrated a preference for sulfa conjugation of the 3 hydroxyl position, but pterostilbene is methylated at the 3 position and only has a hydroxyl group at position 4. In human isozymes, only SULT1E1 had specificity for the 4' hydroxyl group, and fittingly, sulfonation of the 4' hydroxyl group in resveratrol was less efficient.[45] While sulfonation may be more prevalent in rats, glucunoridation appears to be more important in humans.

UDP-glucuronosyltransferase (UGT) 1A1, UGT1A3, UGT1A8, UGT1A9, and UGT1A10 have all been implicated as the phase II glucuronidation enzymes responsible for metabolizing pterostilbene. UGT1A1 demonstrated the highest activity, yielding the primary glucuronide conjugation metabolite, pterostilbene-4'-O-glucuronide. UGT1A3 also had substantial activity, but UGT1A8, UGT1A9, and UGT1A10 showed discernible, but minimal activity. Slower UGT activity is observed in pterostilbene than resveratrol, backing up observations of pterostilbene's longer half-life. The longer half-life of pterostilbene is specifically attributed to the methylation of the 3 position, and thusly, cannot be glucuronidated on that position.[46]

Differences in pterostilbene glucuronidation between male and females were also assessed in human liver microsomes. While both sexes had equivalent amounts of UGT1A expression and similar K_M values, 180 ± 31 μM in males and 189 ± 31 μM in females, there were significant differences in V_{max} levels. Females had V_{max} levels measured at 2706 ± 100 $pmol \bullet min^{-1} \bullet mg^{-1}$ versus a V_{max} level of 1678 ± 123 $pmol \bullet min^{-1} \bullet mg^{-1}$. This significant difference translates into females having a 50% higher catalytic efficiency (V_{max}/K_M) than males when metabolizing pterostilbene.[46]

Phase II pterostilbene metabolites are found in higher concentrations in the body than the parent compound itself, perhaps serving as a storage pool for the parent compound.[38] Interestingly, glucuronidated-pterostilbene increases in concentration after 1-2 hours, also indicating that enterohepatic recycling occurs.[42]

Anticancer Mechanisms of Pterostilbene

Pterostilbene's anti-cancer effects derive from its ability to initiate apoptosis in numerous cancers, including breast cancer, colon cancer, leukemia, lung cancer, pancreatic cancer, prostate cancer, among others. Pterostilbene activates intrinsic (mitochondrial-derived) and extrinsic apoptotic pathways.[41] Intrinsic apoptotic pathways are regulated through mitochondrial proteins such as Bcl-2, caspase, Bax, etc., while the extrinsic apoptotic pathway works through the Fas-receptor pathway.[47]

However, elucidating the specific apoptotic pathways involved has been controversial. For instance, caspase-dependent apoptosis was observed in some cancer cell lines treated with pterostilbene, but in leukemia cells exposed to pterostilbene and a pancaspase inhibitor, inhibition of pterostilbene-mediated apoptosis was not demonstrated. Pterostilbene's mechanism of action may be caspase-dependent and/or independent depending on cell type.[32] For example, pterostilbene treatment demonstrated inhibition of Bcl-2 in metastatic cells, and subsequently led to sensitization of the cells to vascular endothelium-induced cytotoxicity.[48] Other studies have demonstrated that an alternative pterostilbene mechanism of tumor cell death may be a lysosomal cell death program that is dependent on HSP70 levels and is mediated through lysosomal membrane permeabilization.[48]

Pterostilbene treatment has also demonstrated an ability to inhibit cell growth by complete cell cycle phase arrest.[41] For example, in breast cancer cells, pterostilbene was found to be an effective cancer inhibitor by inducing both apoptosis and cell cycle arrest.

[38] Cell cycle arrest was also observed in pancreatic cancer cell lines MIA PaCa and PANC-1. However, the phase of arrest differed between cell lines: in PANC cells, S phase cell cycle arrest was exhibited at all concentrations, at 24 hours, while in MIA cells, cell cycle arrest appeared in S phase at lower concentrations (25 and 50 μM) and G_0/G_1 phase at higher concentrations.[49] Thus, pterostilbene's pathway of mechanism appears to be cell line-dependent.

Studies on the related polyphenol, resveratrol, have indicated G_1/S and G_2/M cell cycle arrest effects in prostate cancer cell lines.[50] The transition that is favored was cell line dependent. Resveratrol can interact with both androgen and estrogen receptors; androgen-responsive cell lines, like LNCaP cells were more sensitive to apoptosis and favored G_1/S cell cycle inhibition.[50] Furthermore, in LNCaP cells, resveratrol activated NFkB and increased expression of p53, a key cell cycle regulator that may induce apoptosis, and p21 and p27, cyclin-dependent kinase inhibitors.[50] Pterostilbene also up-regulated p21 expression and p53 expression in LNCaP cells, blocking G_1/S cell cycle progression.[51] However, the balance between cell cycle arrest and apoptosis appeared to be determined by p53 expression. p53 negative prostate cancer cells, such as PC3, underwent apoptosis after pterostilbene treatment, but p53 positive prostate cancer cells, such as LNCaP, favored cell cycle arrest.[51]

There is the possibility that these effects are cell line-dependent or tissue dependent, but even so, are worth exploring. In lung cancer cells, there have been relatively few studies on the effects of pterostilbene. It has been suggested that

pterostilbene was an anti-proliferative agent due to apoptosis induction in lung cancer cell lines NCI-H460 and SK-MES-1.[40] However, H1299 (non-small cell lung cancer) cells do not express the tumor suppressor p53, and do not exhibit apoptotic signals when treated with pterostilbene. p53 is an important regulator of cells because it can induce autophagy, perhaps saving cells from apoptosis.

Purpose

The anticancer effects of pterostilbene remain understudied and further experiments in more cancer cell types are required. The purpose of the present research was to test the hypothesis that pterostilbene can inhibit the growth of and induce cell cycle arrest in human non-small-cell lung cancer cells, *in vitro*.

2.

MATERIALS AND METHODS

Chemicals

Pterostilbene was purchased from Sigma-Aldrich (St. Louis, MO). All other reagents used were of the highest quality, commercially available.

Cell Culture

H1299 (American Type Culture Collection, Manassas, VA) non-small-cell lung cancer cells were maintained in RPMI 1640 media (Cellgro, Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 1% Penicillin Streptomycin (Sigma, St. Louis, MO). Flasks were incubated at 37^{o} under a 5% CO_2 atmosphere. Cells were passed at 75-80% confluence using 0.25% trypsin-EDTA.

Cell Viability Assay

Cells were seeded at a concentration of 50,000 cells/mL in 96-well plates and allowed to attach overnight. Cells were then treated with pterostilbene (final concentration 0 – 100 μM) for 24 -72 h. Cell viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay.

Western Blot Analysis

Cells were seeded at a concentration of 170,000 cells/mL in 50 mm cell culture dishes and attached overnight. Cells were treated with pterostilbene (0 - 50 μM) for 6 hours and 24 hours. The cells were then harvested using a cell scraper. Samples were lysed using Meng lysis buffer with 1/100 phosphatase inhibitor 2 and 3 and 1/100 protease inhibitor. The protein was quantified by the Bradford assay. Protein samples were combined with Laemelli buffer and separated by polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with primary antibodies (Cell Signaling Technology, Danvers, MA) for caspase 3 (1:1000 dilution), cleaved caspase 3 (1:1000 dilution), cleaved PARP (1:1000 dilution), Cyclin E2 (1:1000 dilution), cyclin D1 (1:1000 dilution), p-15 (1:1000 dilution), β-tubulin (1:1000 dilution), kRas (1:500 dilution), and β-actin (1:1000) overnight at 4^{o}C. After incubation with a fluorescently labeled secondary antibody (LI-COR Biosciences, Lincoln, NE), the proteins were imaged using an Odyssey imaging system (LI-COR Biosciences).

Cell Cycle Analysis

Cells were seeded at a concentration of 50,000 cells/mL in 50 mm cell culture dishes and allowed to attach overnight. They were then treated with pterostilbene in serum-complete or serum-free medium for 6 – 72 hours. Cells were harvested by trypsinization, washed with PBS, and fixed in 70% methanol. Cells were then stained

with in PBS containing 40 μg/mL propidium iodine and 0.5 mg/mL RNAase for30 min at 37°C . Cell populations were determined using a Coulter XL-MCL flow cytometer and then analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

3.

RESULTS

Cell growth viability activity of pterostilbene

A significant concentration and time-dependent decrease in the number of viable H1299 cells was induced by pterostilbene treatment (Figure 3.1). The IC_{50}s were 49.0, 25.6, and 17.6 μM at 24, 48, and 72 hours, respectively. Inclusion of catalase and SOD with pterostilbene treatment had no effect, suggesting that oxidative stress does not significantly inhibit growth (data not shown).

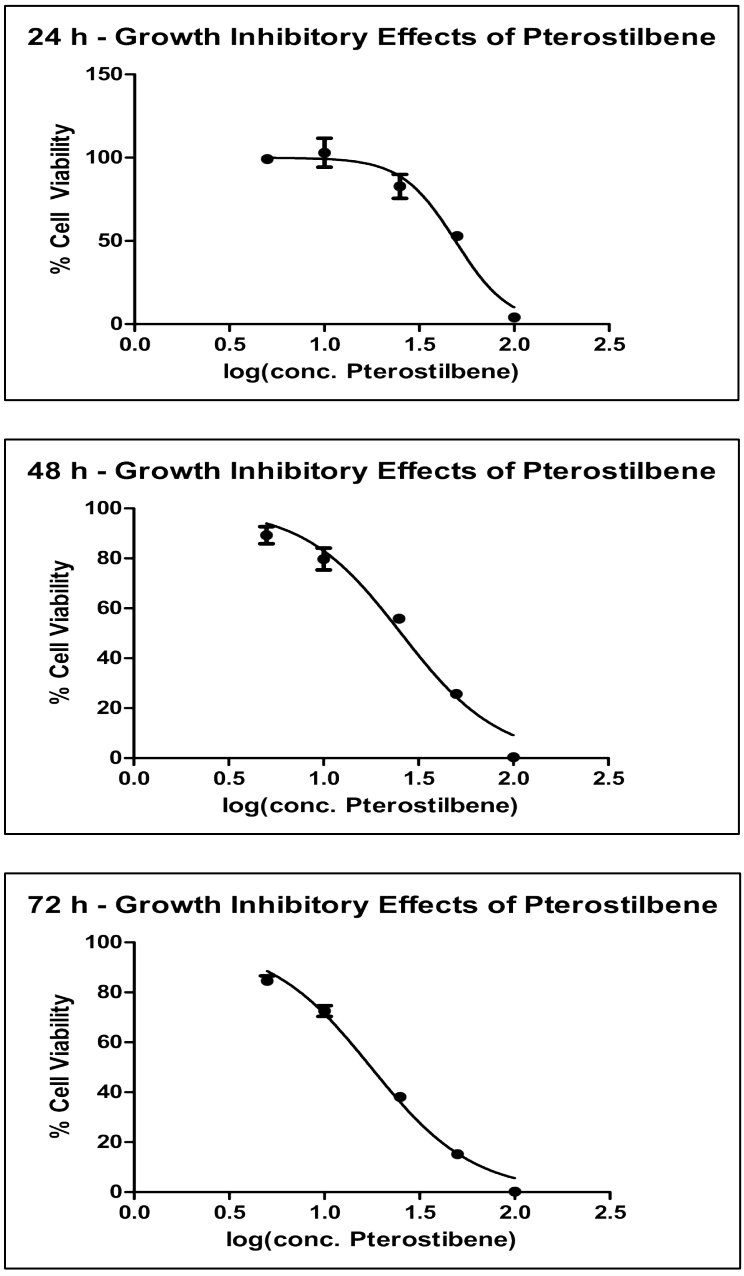

Figure 3.1

Effect of pterostilbene on the number of viable H1299 human lung cancer cells. Cell viability assay. Pterostilbene treatment (5 μM - 100 μM) resulted in significant concentration-dependent decrease in the number of viable H1299 cells. (Experimental, representative data run in triplicate.)

Cell Cycle

Cell cycle analysis of synchronized H1299 cells treated with pterostilbene showed dose-dependent cell cycle arrest in G_0/G_1 phase that was maintained for 24 h at 25 μM and 72 h at 50 μM (Figure 3.2).

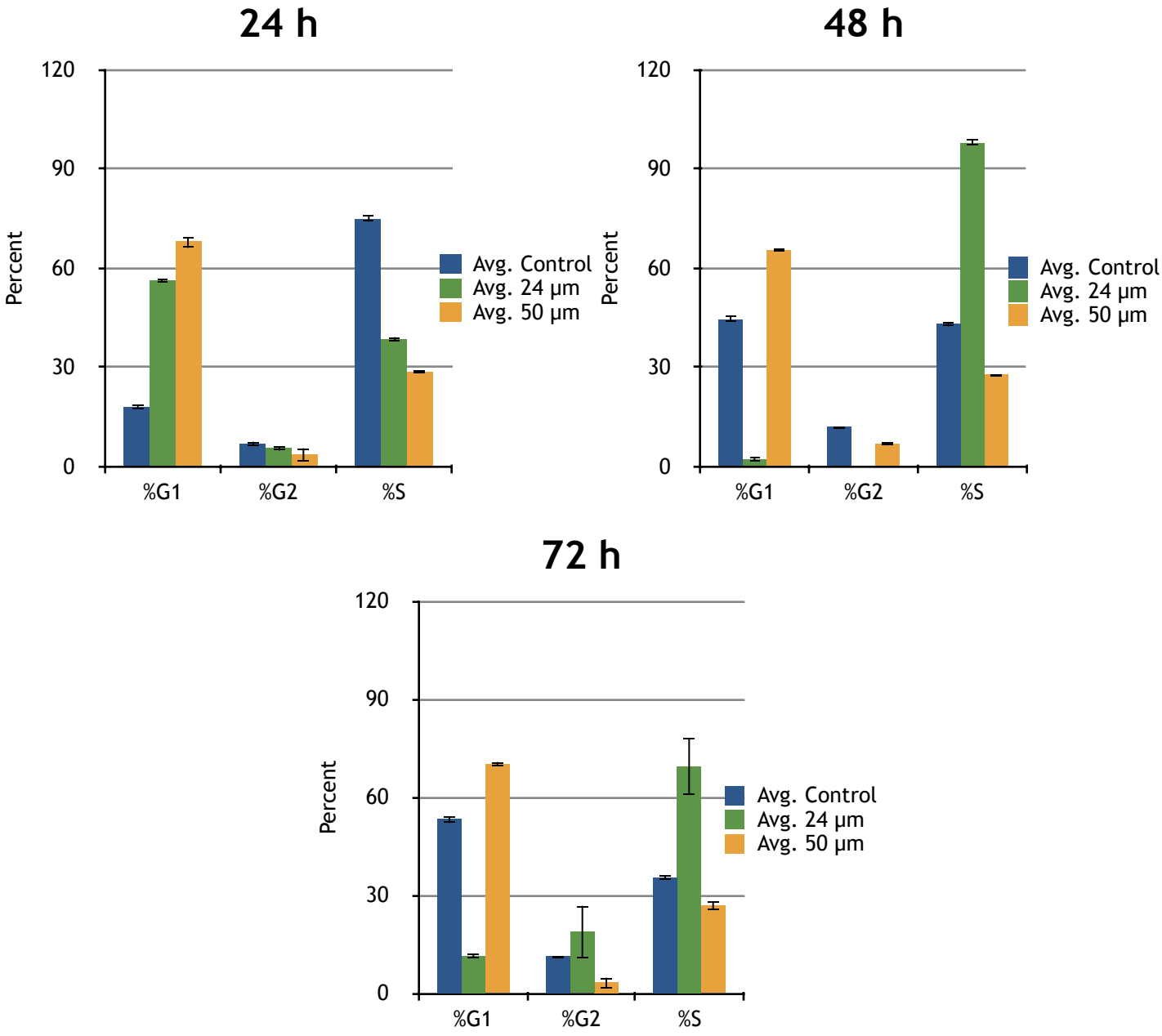

Figure 3.2

Effect of pterostilbene on the cell cycle progression of serum-starved (synchronized) H1299 human lung cancer cells. Pterostilbene treatment resulted in significant dose-dependent G_0/G_1 cell cycle arrest.

Cell cycle analysis of H1299 cells treated with pterostilbene showed cell cycle arrest in G_0/G_1 phase after treatment with 25 μM of pterostilbene for 6 h and 12 h in cells that were not serum-starved. S phase arrest was appreciable at 50 μM (Figure 3.3).

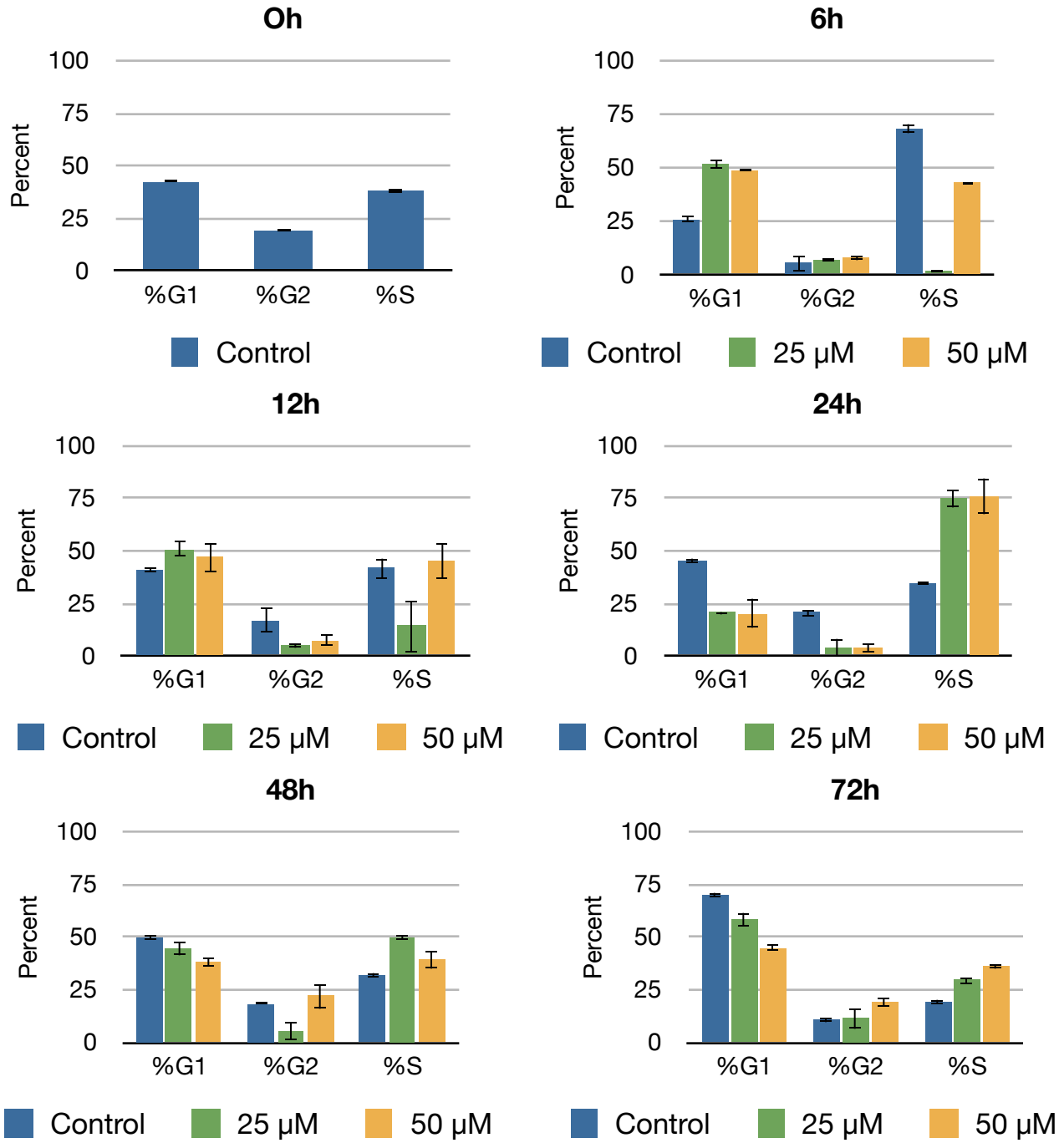

Figure 3.3

Effect of pterostilbene on the cell cycle progression of non-serum-starved H1299 human lung cancer cells. 25 μM and 50 μM treatment resulted in S phase arrest. At 6 h and 12 h, 25 μM treatment resulted in G_1 phase arrest.

Expression of Proteins Involved in Apoptosis and Cell Cycle Progression

Markers involved in apoptotic pathways were not significantly induced by pterostilbene treatment (Figure 3.4). Cleaved caspase-3 was not increased by pterostilbene treatment, nor was cleaved PARP. Pterostilbene did not appear to reduce the expression of Cyclin E2.

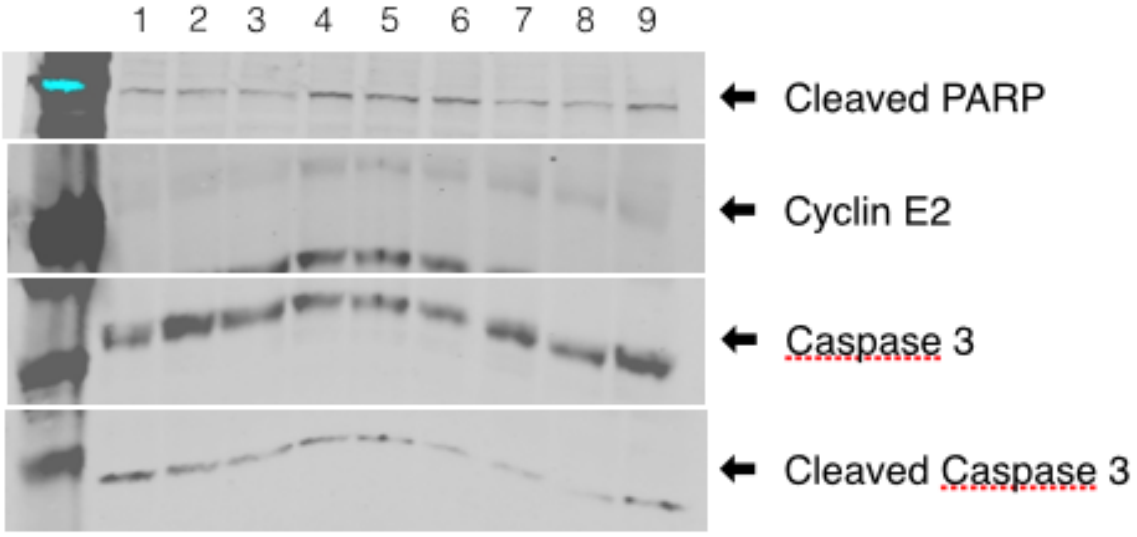

Figure 3.4
Effect of pterostilbene on the expression of proteins related to apoptosis and cell cycle arrest. Western blot analysis showed that 25 µM pterostilbene (wells 4-6) and 50 µM pterostilbene (wells 7-9) had no significant effect on markers for apoptosis (cleaved caspase-3 and cleaved PARP) or the expression of cyclin E2 compared to control treatment (wells 1-3).

Pterostilbene treatment did appear to reduce the expression of cyclin D1 after 6 and 24 h compared to vehicle-treated control cells (Figure 3.5).

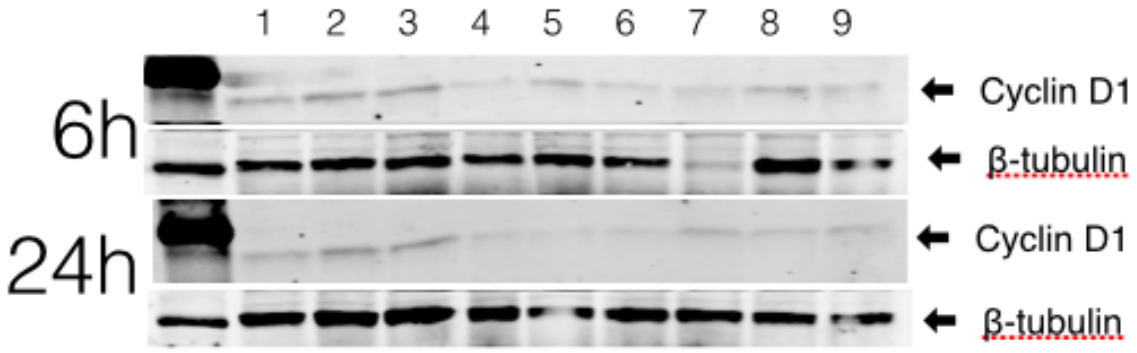

Figure 3.5
Effect of pterostilbene on the expression of proteins related to cell cycle arrest. Control treatment (wells 1-3), 25 μM pterostilbene (wells 4-6) and 50 μM pterostilbene (wells 7-9) showed significant markers for G_1 phase cell cycle arrest (inhibition of cyclin D1). β-tubulin (used as a housekeeping protein) showed consistent protein expression.

Pterostilbene treatment did appear to reduce the expression of kRas after 24 h compared to vehicle-treated control cells (Figure 3.6).

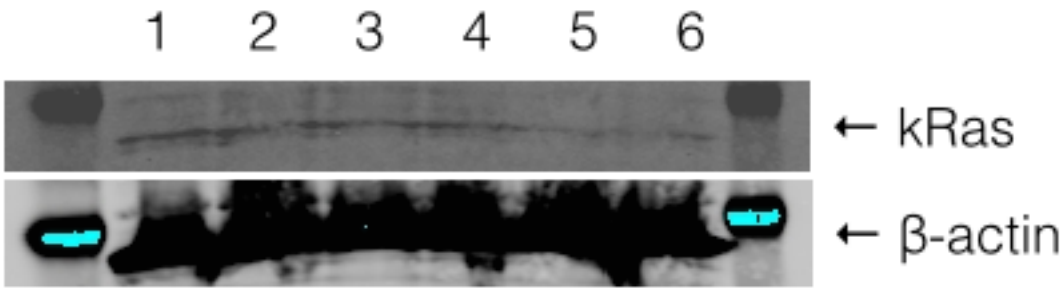

Figure 3.6
Effect of pterostilbene on the expression of proteins related to cell cycle arrest. Control treatment (wells 1-2), 25 μM pterostilbene (wells 3-4) and 50 μM pterostilbene (wells 5-6) showed significant markers for promotion of autophagy (down-regulation of kRas). β-actin (used as a housekeeping protein) showed consistent protein expression.

4.

DISCUSSION

In the present study, I found that pterostilbene dose-dependently reduced the number of viable H1299 human non-small cell lung cancer cells *in vitro*. This effect appeared to be related to growth inhibition and cell cycle arrest rather than induction of apoptosis.

After initial cell viability studies, we hypothesized that pterostilbene worked through apoptosis induction. However, the Western Blot analysis of proteins from cells treated with pterostilbene indicated that while complete caspase 3 was present, there was no indication of caspase-3 or PARP cleavage. This is consistent with the cell viability studies which demonstrated decreased cell viability from 24 to 72 hours. This is a potential indication that no cellular growth or death occurred. While this is contradictory to the findings in other lung cancer studies, it is probable that the mechanism of pterostilbene differs between cell lines.[40]

When examining the H1299 cell cycle after pterostilbene treatment in serum-starved cells, G_0/G_1 and S phase arrest was found. G_1 phase arrest was observed at concentrations of 50 μM pterostilbene, while S phase arrest was observed at concentrations of 25 μM pterostilbene. The percentage of cells in G_1 phase was consistently higher in cells treated with 50 μM pterostilbene compared to controls from 24 to 72 hours. Concentrations at 25 μM resulted in higher percentages of cells in S

phase from 48-72 hours, compared to controls. This may be due to serum-starvation. Serum-starved cells, after 24 hours are only just resuming cell cycle progression and have yet to progress far enough for most cells to reach S phase. Furthermore, a greater percent of G_2 phase was seen at 72 hours with treatment with 25 μM of pterostilbene than at 48 hours. This may indicate that lower concentrations of pterostilbene slow the cell cycle progression but do not completely arrest cell growth.

In non-serum starved H1299 cells, cell cycle arrest occurred mostly in the S phase, but also occurred in G_1 phase. At concentrations of both 25 μM and 50 μM, S phase arrest was observed. The percentage of cells in S phase was higher in 50 μM pterostilbene treated cells at 72 h, was equal to the percentage of 25 μM pterostilbene cells in S phase at 24 h, and was slightly less than the percentage of 25 μM pterostilbene cells in S phase at 48 h. Appreciable G_1 phase cell cycle arrest was noticeable at 6 h and 12 h timepoints at 25 μM pterostilbene concentrations. Cells were not serum-starved, which may contribute to a distribution of cells in various cell cycle states at the time of treatment. Especially at early timepoints, pterostilbene treatment may not have enough time to allow completion of the cell cycle back to G_1 phase.

Inhibition of cell cycle may be an important target for chemotherapy drugs. Cyclins and cyclin-dependent kinases regulate cell cycle.[52] Cyclin E2, the gatekeeper of G_1 cell cycle transition, promotes expression of cyclin A. Cyclin A allows for progression into S phase.[52] Cyclin D1 also promotes G_1-S phase cell cycle progression, and its dysregulation is critical in the development of human tumorigenesis.[53] Western

Blot analysis showed that pterostilbene reduced the expression of cyclin D1, but had no effect on the expression of cyclin E2. The prevalence of S phase cell cycle arrest coupled with cyclin D1 inhibition may indicate that pterostilbene slows cell cycle progression but does not completely arrest cell growth. Reports of arrest in other cell cycle arrest have been identified in other studies, indicating that cell cycle arrest may be cell line dependent and may act through different pathways.[34,38]

H1299 cells have mutated p53 and pterostilbene does not appear to induce apoptosis in these cells. Pterostilbene treatment of H1299 at 24 h down-regulated kRas expression, demonstrating the promotion of autophagy. Further studies that may be conducted on the potential role of p53-independent autophagy included JNK, AKT, and ERK1/2. The inhibition of JNK and AKT-mediated activation of ERK1/2 by pterostilbene in an in A549 human lung cancer cell line promoted autophagy.[54] It is important to note that A549 does express wild-type p53, so the question of what gears cancer cells toward cell cycle arrest, p53 activation or the aforementioned combination of the ERK1/2, AKT, and JNK pathways, must still be answered.

The efficacy of pterostilbene's lung cancer-preventative effect has also yet to be evaluated in vivo in animal and human models. Differences in metabolism between rat models and human models could be indicative of differing effectiveness depending on rate of the metabolism and the activity of the metabolite.

5.

CONCLUSION

Treatment of H1299 human non-small cell lung cancer cells with pterostilbene *in vitro*, resulted in growth inhibition and G_1 cell cycle arrest but not apoptosis. The exact mechanism through which pterostilbene induces cell cycle arrest has not yet to be elucidated, but a pterostilbene-mediated reduction of cyclin D1 signals was observed. This indicates that pterostilbene interacts with protein(s) upstream of cyclin D1 to inhibit its expression, and thus, the progression of cell cycle. Further experiments need to be done to clarify the mechanism of pterostilbene in non-small cell lung cancer.

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ACADEMIC VITA

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Education:

Pennsylvania State University, *Schreyer Honors College*, Class of 2014
University Park, PA, USA

- Major: *Immunology & Infectious Diseases*, *Toxicology* (B.S.)
- Minor: *Global Health*

Study Abroad: University of Limpopo, *Mankweng Hospital*, Summer 2013
Polokwane, Limpopo, South Africa

- Observed medical practices and clinical diagnoses in an HIV/AIDS clinic, community outreach programs, outpatient medicine, and pediatric medicine
- Participated in lectures and meetings at various public health facilities to learn about the South Africa health system and how it impacts and is impacted by the greater community

Relevant Coursework:

Bacterial Pathogenesis	Introductory Microbiology
Chemical Principles I/II	Immunotoxicology
Current Topics in Immunology	Immune System & Disease
Environmental Toxicology	Principles of Toxicology
Evolution of Infectious Diseases	Mammalian Physiology
General Biochemistry I/II	Mechanisms of Disease
Honors Biology: Concepts & Biodiversity	Molecular Toxicology
Honors Developmental Biology	Molecular Pharmacology
Honors Freshman Composition	Organic Chemistry I/II
Honors Introductory Global Health	Technical Physics I/II
Honors Molecular & Cellular Biology	Viral Pathogenesis
Honors Principles of Immunology	

Undergraduate Research Experience:

Dr. Joshua D. Lambert: Food Science, Toxicology; Pennsylvania State University; Fall 2011-Present

- Conducted biochemical and immunological analytic procedures to understand the effects of pterostilbene on lung cancer cells.

Work Experience:

Merck, Intern - Vaccine Immunology; Summer 2012

- Performed immunoassays to understand and analyze the effects of various viruses on the immune system and immune system response
- Used cell culture techniques to maintain a variety of cell lines

Dairy Queen, Server; Summer 2011

- Provided friendly service to customers

Golden City, Waitress; Summer 2011

- Waited and cleared tables while utilizing multicultural skills

Other Career Related Experiences

Lewistown Hospital, Shadowed Dr. Duilio Valdivia; January 2013-April 2013

- Observed medical practices and clinical diagnoses in the fields of internal medicine, oncology, and hematology

Elmcroft Assisted Living, Volunteer; January 2013-April 2013

- Provided assistance to nurses, conducted recreational activities for residents, conducted exercise activities for residents in the general unit and the Alzheimer's unit

Hospital of the University of Pennsylvania, Volunteer; Summer 2010-2011

- General Medicine Department, Cancer Center, and Pharmacy: provided care for patients, assistance to nurses, restocked critical hospital supplies, delivered medicine to appropriate locations

Leadership and Activities:

Association of Residence Hall Students, Pennsylvania State University
Activities and Social Chair, Fall 2012-Present

- Managed and planned several campus-wide activities that drew hundreds of students together and benefit the Penn State community
- Collaborated with the *Student Programming Association*, *LateNight Penn State*, and *University Park Undergraduate Association* to organize and execute programs in the Residence Halls and across campus
- Coordinated activities and social outings to encourage camaraderie among the association membership

Diversity Chair and South Halls Representative, Fall 2011-Spring 2012

- Managed and planned several activities to encourage diversity and acceptance across the Penn State community
- Attended regular *ARHS* meetings as a liaison to the *South Halls Residence Association.*

East Halls Residence Association, Pennsylvania State University
Programming and Activities Chair, Fall 2010-Spring 2011

- Managed and planned activities for 14 Co-ed Residence Halls with over 7000 East Hall student residents

Biomedical Club, Pennsylvania State University
Member, 2010 - Present

Awards and Achievements:

- Bunton-Waller Fellowship, Pennsylvania State University, Fall 2010-Present
- Dean's List, Pennsylvania State University, Fall 2010-Present

- *Academic Excellence Scholarship*, Pennsylvania State University, Fall 2010-Present
- *Oswald Scholarship*, College of Agriculture, Pennsylvania State University, Fall 2013-Spring 2014
- *Young SCHP Agricultural Scholarship*, Pennsylvania State University, Fall 2012-Spring 2013
- Honor Roll (Top 1%), College of Agriculture, Pennsylvania State University, Fall 2010, Spring 2011, Spring 2012
- *John N. Adam Jr. Scholarship for Excellence in Agriculture*, College of Agriculture, Pennsylvania State University, Fall 2011-Spring 2012
- *STARs Nominee*, Pennsylvania State University, April 2011
- *President's Freshman Award*, Pennsylvania State University, March 2011
- *The Raymond F. Russell Scholarship, College of Agriculture,* Pennsylvania State University, Fall 2010-Spring 2011