ANTICANCER MECHANISMS OF FLAXSEED AND ITS DERIVED MAMMALIAN LIGNAN ENTEROLACTONE IN LUNG

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program:
Cellular and Molecular Biology

April 2017

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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ABSTRACT

Whole flaxseed and its derived lignans have shown anti-cancer properties in a variety of malignancies. However, their potential remains uninvestigated in lung cancer, the leading cause of cancer-related deaths worldwide. We investigated the anti-tumor effects of flaxseed-derived mammalian lignan enterolactone (EL) in human lung cancer cell cultures and the chemopreventive potential of 10% whole flaxseed in a mouse model of lung carcinogenesis. We found that EL inhibits *in vitro* proliferation and motility of a panel of non-small cell lung cancer cell (NSCLC) lines. EL-mediated inhibition in lung cancer cell proliferation was due to a decrease in mRNA and protein expression levels of G_1-phase cell cycle promoters and a simultaneous increase in mRNA and protein expression levels of $p21^{WAF1/CIP1}$, a negative regulator of the G_1-phase. Similarly, EL decreased lung cancer cell motility by modulating cytoskeleton organization, inhibiting the activation of the FAK-Src-paxillin signaling cascade, and expression of down-stream motility regulators. Our *in vivo* investigation revealed that 10% whole flaxseed reduced the incidence, number, and size of lung tumor nodules in A/J mice exposed to the tobacco smoke carcinogen, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). RNA sequencing revealed altered expression of genes whose products modulate inflammation and oxidative stress, and are likely to be responsible for chemopreventive potential of whole flaxseed. The results from our *in vitro* studies highlight the anticancer potential of EL in lung cancer, while the results from our *in vivo* study show that whole flaxseed holds promise as a chemopreventive dietary agent in lung.

ACKNOWLEDGEMENTS

I started my PhD in Cellular and Molecular Biology program in the Biological Sciences Department at NDSU in Fall 2010 and have been fortunate to be surrounded by people who always had confidence in me. At the outset, I take this opportunity to thank Dr. Katie Reindl, my advisor, whose unwavering support and guidance helped me complete my research and made this thesis possible. The time and energy that she devoted to me, and the patience she has shown in helping me improve on my weaker areas have honed my research and scientific writing skills. Dr. Reindl, thank you so much for all your help and guidance over the past six years.

I am grateful to Drs. Jane Schuh, Yeong Rhee, and Steven Qian, members of my thesis committee, for their valuable suggestions and advice over the course of my research. I would also like to thank Dr. Pawel Borowicz for his insightful suggestions and guidance on various experiments and Jordon Flaten for assistance with the same. I would like to acknowledge Jeffery Kittilson for his support in trouble shooting with experiments. He is the go-to person for every graduate student in the department.

This section would be incomplete without fond appreciation of all graduate and undergraduate students in my lab who have been a part of my joyous graduate school ride. I would specifically like to mention Kaitlin Lindsey for being a dedicated undergraduate student and for assisting me in my experiments and my dear friend, Dr. Harsharan Dhillon, with whom I spent most of my time during my doctoral studies.

I have been fortunate to have a loving and supportive family, my father (Dr. Devendra Chikara), mother (Dr. Anita Chikara), and brother (Abhishek Chikara), who are my pillars of strength. My father's warm love and endless care for me is beyond description. This work would

not have seen the light of the day if he had not supported and encouraged me throughout my journey as a PhD student.

DEDICATION

This thesis is dedicated to my father, Dr. Devendra Chikara. His untiring support, and encouragement have made this dissertation possible. He has been the wind beneath my wings.

Thank you for everything!

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LIST OF ABBREVIATIONS

ABC……...ATP-binding cassette

ALA……α-linolenic acid

ATP……...Adenosine triphosphate

CDK……..Cyclin-dependent kinases

CDKI…….Cyclin dependent kinase inhibitors

cDNA……Complementary-deoxyribonucleic acid

CN…….Control normal

c-Src……..Steroid receptor coactivator c-Src

CT…….....Control tumor

CYP450…….Cytochrome P450

DAPI…….4',6-diamidino-2-phenylindole

DHM……..(+)-dihydromethysticin

DIM……...3,3- diindolylmethane

DMSO……...Dimethylsulfoxide

DNA……..Deoxyribonucleic acid

DN…….Dominant negative

ECM……..Extracellular matrix

ED…….Enterodiol

EGCG……...(-)-epigallocatechin gallate

EGFR…….Epidermal growth factor receptor

EL……..Enterolactone

ERK……...Extracellular signal-regulated kinase

FAK……...Focal adhesion kinase

F-actin……....Filamentous actin

FBS……Fetal bovine serum

FN……..Flaxseed normal

FPKM……Fragment per kilobase of exon per million fragments mapped

FT……..Flaxseed tumor

GOF……...Gain of function

HD…….Homozygous deletion

H&E……..Hematoxylin and eosin

HMOX-1……...Heme oxygenase-1

HPRT……Hypoxanthine guanine phosphoribosyl transferase

IARC…….International Agency for Research on Cancer

I3C……Indole-3-carbinol

IGTA2……..Integrin subunit alpha2

IGF-1……Insulin-like growth factor-1

i.p……..Intraperitoneal

KRAS……...Kirsten rat sarcoma viral oncogene homolog

LAR……..Laricinesol

MAPK……..Mitogen-activated protein kinase

MCM2……..Minichrosome maintenance gene 2

Met……...Hypermethylation

mM……...Millimolar

mg…….Milligram

mg/kg……....Milligram/kilogram

MMPs……...Matrix metalloproteinases

mRNA……..Messenger ribonucleic acid

MTT…….....3-(4, 5-dimethylthiazol-2-yl)-2, 5-dephenyltetrazolium bromide

Mut……...Mutation

N^7-mG……..N^7-methylguanine

nAChR…….Nicotinic acetylcholine receptors

ng…….Nanogram

NGS…….Normal goat serum

mAbs……...Monoclonal antibodies

NMU ……..N-nitrosomethyl-urea

NNAL ……4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

NNK……4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NSCLC……Non-small cell lung cancer

O^6-mG ……O^6-methylguanine

8-OHdG……...8-hydroxy-2' -deoxyguanosine

OP……Overexpressed

OS……Overall survival

PAGE……...Polyacrylamide gel electrophoresis

PARP……...Poly(ADP-ribose) polymerase

PBS……..Phosphate buffered saline

PDGF……...Platelet-derived growth factor

PEITC …….Phenethyl Isothiocyanate

PFS…….....Progression free survival

PI3K……...Phosphoinositide 3-kinase

PINO……..Pinoresinol

pRb……....Retinoblastoma protein

PP2A……..Protein phosphatase-2 alpha

PSA……Prostate specific antigen

RT-qPCR……...Reverse transcription-quantitative polymerase chain reaction

RNA……...Ribonucleic acid

RNA-Seq……....RNA sequencing

ROS……Reactive oxygen species

SCLC……..Small-cell lung cancer

SDG……Secoisolariciresinol diglucoside

SDS…….Sodium dodecyl sulfate

SECO……..Secoisolariciresinol

SOD-1…….Superoxide dismutase-1

SV……...Selected vegetables

TBS…….Tris-buffered saline

TK……...Tyrosine kinase

TKI……..Tyrosine kinase inhibitor

TP53……Tumor protein, p53

TRAMP……...Transgenic adenocarcinoma mouse prostate

rRNA……...Ribosomal ribonucleic acid

UGTs……...UDP-glucoronultransferases

µl……Microliter

µM…….Micromolar

µg/ml…….Microgram/milliliters

VEGF……Vascular endothelial growth factor

CHAPTER 1. GENERAL INTRODUCTION, OBJECTIVES AND SPECIFIC AIMS

Introduction

Lung cancer is a leading cause of cancer related morbidity and mortality worldwide and in the US [1]. Epidemiological studies have established that exposure to tobacco-specific carcinogens is a predominant risk factor for development of lung cancer [2]. Smoking cessation does lower the risk of lung cancer, which becomes evident only after 5 years post-abstinence, but the relative risk never returns back to that of a non-smoker [2]. This suggests that, in addition to cessation of tobacco smoking, understanding of the underlying molecular mechanisms by which tobacco consumption leads to increased risk of lung cancer is imperative to develop new treatment and chemoprevention modalities.

Continuous exposure to tobacco-specific carcinogens such as polycyclic aromatic hydrocarbons and *N*-nitrosamines damages DNA and leads to lung cancer [2, 3]. The imbalance between metabolic activation and detoxification of these carcinogens results in formation of DNA-adducts in the genome [2]. Persistent DNA adducts, due to reduced DNA repair capacity, in critical regions of oncogenes (such as *KRAS)* and/or tumor suppressor genes (such as *TP53)* may lead to mutations [2, 4-6]. Mutations in *KRAS* gene are found in 24-50% of human primary adenocarcinomas, and *TP53* is mutated in 50% of lung cancer cases [7-11]. The mutations in *KRAS* lead to constitutive expression of oncogenic K-ras protein which modulates signaling pathways that play a role in sustained lung cancer cell proliferation [12], while inactivation or expression of mutant p53 protein allows continued replication and proliferation of unrepaired DNA and further leads to accumulation of mutations [13]. This suggests that tobacco-specific carcinogens cause aberrant expression of critical genes which leads to lung cancer development.

Once diagnosed, the treatment for lung cancer requires a multi-disciplinary approach. Depending on the type and stage of the disease, in addition to the patient's performance status and preference, common treatment modalities are surgery, chemotherapy, radiotherapy or a combination of these [14, 15]. Traditional chemotherapeutic agents such as cisplatin [16, 17] and docetaxel [18] are cytotoxic to rapidly dividing healthy cells as well as tumor cells, and patients often develop resistance to treatment and relapse [19]. To circumvent these problems, targeted therapy was pursued. Targeted therapeutic agents aim to block signaling pathways that offer selective growth advantages to cancer cells due to driver mutations in specific genes while being non-toxic to normal cells [20]. Even though targeted therapies have shown modest success in patients harboring specific mutations, they often develop resistance after prolonged use [20-22]. Therefore, there is a need for newer strategies to supplement existing ones.

The use of whole foods and/or their individual bioactive components which possess anticancer properties is an attractive approach to combat lung cancer due to their selective effects on tumor cells. A number of plant-derived phytochemicals, such as lycopene (tomatoes), curcumin (turmeric), and piperlongumine (long peppers) readily available in nature, have demonstrated selective toxicity towards a variety of cancer types. In addition, a few of these plant-derived bioactive agents potentiate the antitumor activity of targeted chemotherapeutic agents in preclinical models of an array of cancers [23-26]. They exert their anticancer effects via their antioxidant properties, inhibition of cancer cell growth, induction of apoptosis, and inhibition of cell motility. Flaxseed, a whole grain rich in plant phytochemicals called lignans, has demonstrated significant anticancer effects in preclinical models of breast, colon, and prostate cancers. However, the anticancer potential for whole flaxseed and its derived mammalian lignan enterolactone remains uninvestigated in lung.

Lung cancer statistics

Globally, 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012. Lung cancer accounted for 13% (1.8 million) of new cancer cases and 19% (1.6 million) of cancer deaths, respectively [27]. This makes lung cancer the most common cause of cancer-related death in men and the second most common in women worldwide. Similarly, in the U.S., lung cancer is the leading cause of cancer-related deaths among men and women with an estimated 224,390 (18%) new cases diagnosed and 158,080 (37%) deaths in 2016 [27]. Annually, lung cancer accounts for more deaths than breast, colon, pancreas, and prostate cancers combined.

Lung cancer types

Lung cancer arises from epithelial cells lining the respiratory airway. It is divided into two broad categories on the basis of cell morphology, i.e., cell size and histological analysis of stained tumor sections [28]. Small cell lung cancer (SCLC) arising from neuroendocrine cells accounts for 15-20% of all cases and is highly malignant. On the other hand, non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancer cases and arises from respiratory epithelium [28]. NSCLC is further divided into 4 major pathological subtypes: adenocarcinoma (50%), squamous cell carcinoma (30%), large cell carcinoma (10%), and mixed cellularity NSCLC (10%) [28]. The different lung cancer subtypes harbor genetic alterations (mutations and epigenetics) in oncogenes and tumor suppressor genes responsible for deregulation of specific oncogenic pathways (Table 1) [28-32]. Therefore, SCLC and NSCLC have diverse responses to chemotherapy, demonstrating the importance of genetic characterization for their effective clinical management. A vast majority of SCLC (98%) and NSCLC (85%) cases are attributed to long-term tobacco consumption.

Table 1.Gene alterations in SCLC and NSCLC

Altered genes		Frequency	
		SCLC	**NSCLC**
Tumor suppressors	***TP53***	~ 90% (Mut)	~ 50% (Mut)
	pRb	~ 90% (Mut)	~ 15% (Mut)
	p16	~ rarely (Mut and/or Met)	~ 70% (Met and/or HD)
Oncogenes	***KRAS***	~ 16.2 (Mut)	~ 30% (Mut)
	EGFR	~ 4% (Mut)	~10-15 % (Mut), 40-80% (OP)

Mut=mutation, Met=Hypermethylation, HD=Homozygous deletion, OP=Overexpressed
pRb = Retinoblastoma protein, *KRAS* = Kirsten rat sarcoma viral oncogene homolog, *EGFR* = Epidermal growth factor receptor, and TP53=tumor protein, p53

History of tobacco smoking and risk of lung cancer

In 1912, lung cancer represented less than 0.5% of all cancer cases worldwide and primary malignant neoplasms of the lung were among the rarest form of the disease [33]. In 1920, lung cancer constituted only 1% of all malignancies in the US [1]. By 1950, several epidemiological studies established the role of tobacco in development of lung cancer and carcinogens such as polycyclic aromatic hydrocarbons (benzo[*a*]pyrene) and N-nitrosamines (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) etc. were discovered in cigarette smoke [34]. Thereafter, numerous studies evaluated the carcinogenicity of benzo[*a*]pyrene and NNK in laboratory animals. In 1953, it was observed that cigarette smoke tar rubbed on mouse skin led to lung tumor formation [35]. The Surgeon General's report in 1964 implicating tobacco smoking as the single most important cause of lung cancer led to a decline in per capita consumption of

tobacco in the U.S. [36]. Therefore, evidence from early epidemiological studies, animal experiments, and clinical observations has established a link between tobacco and lung cancer.

Tobacco-smoke constituents and their potential carcinogenicity in lung

Dependence on nicotine is the primary reason for continued tobacco consumption [1, 3]. One gram of tobacco contains 6-18 mg of nicotine. Nicotine levels in mainstream tobacco smoke entering lungs range from 0.5–1.63 mg/cigarette [37]. In addition to nicotine, the mainstream tobacco smoke consists of (i) vapor-phase components (95%) which consist of gases such as nitrogen, oxygen, and carbon monoxide, and (ii) particulate-phase components (5%) which consist of at least 3,000 compounds of which 70 are considered carcinogenic to laboratory animals and/or humans by the International Agency for Research on Cancer (IARC) [2]. The common tobacco-smoke carcinogens are listed in Table 2.

Table 2. List of carcinogens present in cigarette smoke

Categories of Carcinogens	**No. of compounds**
Polycyclic aromatic hydrocarbons	10
N-nitrosamines	7
Aza-arenes	3
Aromatic amines	3
Heterocyclic aromatic amines	8
Aldehydes	2
Miscellaneous organic compounds	15
Inorganic compounds	7
Total	55

Nicotine is not considered a carcinogen by IARC, and it does not cause lung cancer per se [38]. Rats treated for 2 years with daily inhalation of nicotine, at a concentration twice the plasma concentration found in heavy smokers, did not develop lung tumors [39]. Similarly, nicotine did not cause formation of lung tumors in A/J mice, while chronic tobacco smoke exposure did [40]. However, nicotine has been shown to bind and activate cell-surface receptors such as nicotinic acetylcholine receptors (nAChR). Activation of nAChR accelerates tumor progression by (i) inducing cell proliferation via protein kinase C [41], phosphoinositide 3-kinase (PI3K/Akt) [42], and RAF/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK)/myc [43] signaling pathways, (ii) inhibiting apoptosis by phosphorylating members of the Bcl-2 family of pro-apoptotic proteins [44], and (iii) inducing angiogenesis by regulating expression of pro-angiogenic vascular endothelial growth factor (VEGF) [45]. Therefore, nicotine is considered a co-carcinogen, facilitating the growth of already established tumors [46]. This suggests that tobacco smoke-induced lung cancer in humans and in rodent models may be due to the presence of multiple classes of carcinogens such as polycyclic aromatic hydrocarbons and tobacco-specific *N*-nitrosamines.

Tobacco-specific *N*-nitrosamines, such as NNK, are formed by *N*-nitrosation of nicotine in tobacco during the post-harvesting processing (curing) [47]. A smoker of low-yield nicotine cigarettes (≤ 0.8 mg/cigarette) inhales to ~187 ng NNK/cigarette, in the mainstream smoke, while a smoker of medium-yield nicotine cigarettes (0.9 to 1.2 mg/cigarette) inhales ~251 ng NNK/cigarette [48]. Others have reported that mainstream tobacco smoke contains a wide range of NNK (80-770 ng/cigarette) [1, 2]. In addition to NNK already present in mainstream tobacco smoke, inhaled nicotine undergoes metabolic conversion via 2'-hydroxylation to 4-(methylamino)-1-(3-pyridyl)-1-butanone (aminoketone), a precursor of NNK by cytochrome

P450s (CYP450s) in humans [49-53]. Therefore, endogenous production of NNK may result in exposure to this carcinogen over and above the amounts already present in tobacco.

NNK is a potent carcinogen and has been shown to induce lung tumors in all the three (rats, mice, and hamsters) commonly used animal models of lung carcinogenesis since 1978. A/J treated with a single dose of NNK (2.5, 5 or 10 mumol of NNK in saline) developed lung tumor development in a dose-dependent manner [54]. After administration of NNK (100 mg/kg), the mice develop pulmonary hyperplasia in 6-14 weeks, which progresses to adenoma by 14-34 weeks, and adenocarcinoma after 34 weeks [55]. NNK has a remarkable affinity for the lung, causing pulmonary adenoma and adenocarcinoma independent of the route of administration [56].

Etiology of lung cancer

NNK is a pro-carcinogen that requires metabolic activation to exert its carcinogenic effects. Bioactivation of NNK by CYP 450 enzymes occurs via two pathways, (i) hydroxylation of NNK at the α-methylene position [57] and (ii) hydroxylation of NNK at the α-methyl position [58]. The methylene hydroxylation pathway generates pro-mutagenic O^6-methylguanine (O^6-mG) and N^7-methylguanine (N^7-mG) DNA-adducts which may result in base mispairing of guanine with thymidine, which is a critical determinant in the initiation of lung tumorigenesis [57]. On the other hand, the methyl hydroxylation pathway produces pyridyloxobutyl DNA adducts which also contribute to NNK-induced lung tumorigenesis by inhibiting O^6-alkylguanine-DNA-alkyltransferase, the enzyme that repairs O^6-mG adducts (Figure 1) [58]. At least eight human CYP enzymes (CYP 2A6, CYP2A13, CYP2B6, and CYP2E1) expressed in the respiratory tract can catalyze NNK metabolism [50-53, 59].

NNK can be metabolized through detoxification pathways that eliminate it from the body. Conversion of NNK into 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by carbonyl reduction, followed by glucuronidation of NNAL results in detoxification of NNK, which lowers the risk of developing mutagenic DNA adducts. The UDP- glucuronosyltransferase (UGTs) family of genes catalyzes glucoronidation, a major phase II metabolic reaction (Figure 1) [60].

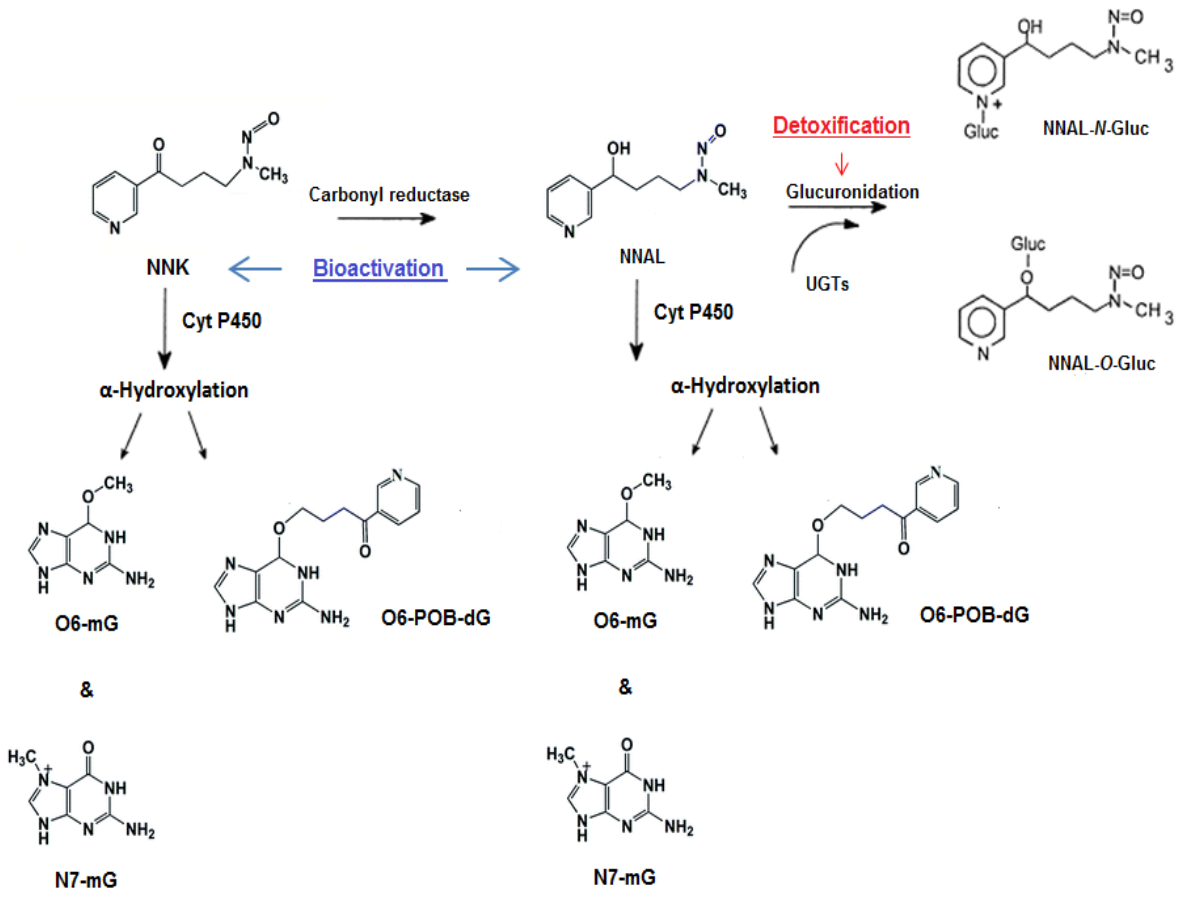

Figure 1. Pathway for bioactivation and detoxification of NNK. Two major routes of NNK metabolism include, carbonyl reduction and α-hydroxylation. Carbonyl reduction of NNK yields NNAL. Metabolic activation of NNK and NNAL to DNA adducts (O6-mG, N7-mG, and O6-POB-dG) proceeds via α-hydroxylation pathways catalyzed by CYP450s. Detoxification of NNAL occurs via UGT-catalyzed glucuronidation. Glucuronidation of NNAL at the pyridine nitrogen forms NNAL-*N*-Gluc, while that at the carbinol oxygen gives NNAL-*O*-Gluc. [56]

Only 15% of lifetime smokers develop lung cancer, hence, an individual's susceptibility may depend on a fine balance between metabolic activation and detoxification of NNK [1]. Pulmonary *CYP2A6* and *CYP2A13* genes mediate metabolic activation of NNK [61, 62]. However, there is no convincing evidence that *CYP2A6* gene deletion results in decreased risk of lung cancer [63, 64]. This indicates that other CYP genes such as *CYP2A13* may be pivotal in regulating metabolic activation of NNK. Furthermore, NNK alpha-hydroxylation by *CYP2A13* is higher than that by *CYP2A6* in human lung cells [65]. It has been shown that a functional polymorphism resulting in C-->T transition in the *CYP2A13* gene results in Arg257Cys amino acid substitution and significantly reduces its enzymatic activity toward NNK [61]. This indicates that an individual's susceptibility to lung cancer is attributed to the presence of genetic polymorphisms in these genes.

In addition to polymorphisms present in CYP genes, genetic polymorphisms in UGTs responsible for detoxification of NNAL have been shown to influence an individual's risk of lung cancer. Polymorphisms in *UGT1A6* increase its mRNA stability and glucuronidating activity of the protein resulting in increased detoxification of NNAL [66], on the other hand, polymorphisms in *UGT1A10* and *UGT2B17* decrease their activity and result in lowered glucuronidation, the major detoxification pathway for NNAL, which may then result in formation of NNAL-induced DNA adducts and initiate lung carcinogenesis [67, 68].

NNK-induced DNA adducts: mutations in critical genes

Compared to non-smokers, smokers exhibit significantly elevated levels of DNA-adducts in lungs [69]. DNA-adducts are a form of DNA damage, which may be mutagenic, and result from covalent attachment of a chemical moiety to DNA. DNA-adduct levels are 2.3-fold lower in former smokers than in current smokers, suggesting that levels of DNA adduct are indicative

of the extent of tobacco exposure and bioactivation of carcinogens present in it. Persistent DNA adducts lead to mispairing and may result in somatic mutations [2]. Studies have shown that smokers have a higher mutation frequency ranging from 8.1-24.3/Mb as compared to a mutation frequency of 0.6-1.7/Mb in non-smokers [70]. Studies have also shown that mutations occurring in oncogenes and tumor suppressor genes such as such *KRAS* and *TP53* known to play a role in lung carcinogenesis are more common in smoking-related lung tumors in humans [71-73].

TP53

Tumor suppressor gene *TP53* encodes the tumor suppresser protein p53 [72]. The most common mutations in *TP53* are G-->T transversions (34%), followed by G-->A transitions (21%), and A-->G transitions (19%) [72]. As in other cancers, a majority of these alterations are missense mutations, in which a single nucleotide is substituted by another. This results in accumulation of high levels of mutant p53 proteins with prolonged half-lives [74, 75]. The mutant p53 protein is unable to recognize wild-type p53 DNA binding sequences and results in repression of wild-type p53-mediated (i) mRNA expression of cell cycle inhibitor p21 which facilitates DNA repair in response to DNA damage [76] and (ii) apoptosis when DNA damage is irreparable [77]. Therefore, mutant p53 contributes to increased genomic instability and continued proliferation of cancer cells.

KRAS

Mutations in *KRAS*, which transduces signals from a cell surface receptor (EGFR) to intracellular proteins, are prominent early events observed in smoking-related lung carcinogenesis in human and mouse models of lung cancer [12, 78, 79]. *KRAS* mutations are predominantly found in smokers and are generally G-->T transversions, in contrast to the G-->A transitions found in non-smokers [80, 81]. Similarly, preclinical studies relying on A/J mice

sensitive to NNK have shown that a majority of tumors in these mice contain point mutations in *KRAS* [78, 82]. As a member of the ras gene family, *KRAS* encodes a ~21 kDa G-protein with intrinsic GTPase activity, which allows it to cycle between GTP-bound active and GDP-bound inactive states [83]. Oncogenic *KRAS* alleles, on the other hand, produce mutant K-ras proteins that lose their GTPase activity, rendering the proteins constitutively GTP-bound and activated. This leads to stimulus-independent, persistent activation of downstream effectors, such as the RAF/MEK/ERK signaling, which initiate transcription of genes that regulate cell survival, proliferation, and metastasis [81].

Cell cycle regulation

The mammalian cell cycle is composed of 5 phases. Each phase is controlled by cyclic expression of specific cyclins and cyclin-dependent kinases (CDKs) [84]. In the G_1-phase of the cell cycle, cyclin D1/CDK4 and cyclin D1/CDK6 complexes initiate phosphorylation (inactivation) of the retinoblastoma proteins (pRB), which leads to the release of E2F transcription factor, allowing expression of its target gene cyclin E [84, 85]. Cyclin E then forms a complex with CDK2 and leads to further phosphorylation and thereby, inactivation of pRb, resulting in its own expression through a positive feedback loop [84, 85]. Therefore, progressive phosphorylation of pRb by cyclin D-Cdk4/6 and cyclin E-Cdk2 releases E2F and promotes G_1-S phase transition [84, 85]. In the case of DNA damage, the transcription factor p53 is stabilized and activates transcription of cyclin dependent kinase inhibitors (CDKIs) belonging to Cip/Kip family (p21, p27, and p57) and the INK4 family (p16, p15, p18, and p19) [86]. These inhibitors bind CDKs and inhibit formation of cyclin D1/CDK4 and cyclin E/CDK2 complexes. This inhibits phosphorylation of pRb leading to G_1-phase cell cycle arrest,

which enables cells to repair DNA damage before its replication in the S-phase. Therefore, regulation of cell proliferation is one of the crucial mechanisms of action for *p53* [86].

Cell cycle deregulation in lung carcinogenesis

In addition to mutation in *TP53*, which impairs DNA damage G_1 checkpoints and facilitate DNA damage accumulation, cooperative deregulation of other genes is involved in progression of cells from G_1 to S phase, a frequent event that promotes cell survival and proliferation [87]. Deregulation in the p16/cyclin D1/CDK4-CDK6/pRb pathway is an early event in lung tumorigenesis. This occurs due to (i) loss of p16 protein as a consequence of gene deletion, promoter hypermethylation, and/or gene mutation [88, 89], (ii) amplification of the cyclin D1 locus and increased expression of cyclin D1 proteins [90, 91], and (iii) pRb inactivation (hyper-phosphorylation) [88]. NSCLC patients who have decreased expression of p16 and p21 have significantly shorter overall survival, indicating that these CDKI have different targets to inhibit lung tumorigenesis [92-94]. Similarly, decreased expression of p27 is seen in NSCLC patients, and correlates with reduced cancer cell differentiation [95, 96]. Increased expression of cyclin E and activation of cyclin E/CDK2 complexes in lung tumors, as a result of reduction in p27 levels, is also an important contributor to lung carcinogenesis [97]. In conclusion, many regulators of the G_1-phase are abnormally expressed in lung tumors. In NSCLC, the lack of CDKI such as p16, p21, and p27, and overexpression of cyclin D1 and cyclin E are the main mechanisms that trigger increased progression of cells through the G_1-phase of the cell cycle. These events play a critical role in initiating and promoting lung tumors.

NNK-induced lung carcinogenesis in rodents follows a similar deregulation of the cell cycle. Immuno-histochemical staining and western blot analyses have shown overexpression of cyclin D1 and CDK4 proteins in NNK-induced mouse lung adenocarcinomas [98]. NNK also

activates the Raf-1/MEK/ERK1/2 signaling pathway which has been implicated in regulation of the cell cycle by promoting phosphorylation and activation of Bcl-2, an anti-apoptotic protein, and the transcription factor c-myc [99]. Activated c-myc promotes cell proliferation by (i) increasing expression levels of cyclin D2, cyclin E1, CDK4, and E2F at mRNA and protein levels and (ii) repressing mRNA expression levels and catalytic activity of CDKI, p21. Further, NNK has been shown to stimulate proliferation of primary human airway epithelial cells by activating NFκB protein, which in turn up-regulates protein levels of cyclin D1 (within 3-6 h), followed by an increase in levels of p-pRb proteins (detected at 6-15 h) [100]. These findings indicate that aberrant expression of cyclins and other cyclin-related genes is a frequent occurrence in lung tumorigenesis in both rodents and humans.

Treatment modalities for NSCLC

Surgery

Surgical resection is offered to early-stage (stages I and II) NSCLC patients. Most of these patients eventually experience recurrence, one third locally and two thirds systemically [101]. Hence, along with surgery, early-stage lung cancer patients are usually offered adjuvant chemotherapy. However, the majority of lung cancer patients (75-80%) are not diagnosed until the disease has advanced (stage III and IV) and has metastasized beyond the primary location. These patients, if untreated, have a dismal 1-year survival rate of 10 to 15% with a median survival rate of 4-6 months [102]. Therefore, chemotherapy is the preferred treatment option in advanced stage disease.

Traditional chemotherapy

Chemotherapy is a systemic treatment approach that targets actively proliferating cells [103]. The first chemotherapeutic agents used were alkylating agents such as cisplatin and its

analogue carboplatin [104]. In addition to exhibiting severe toxicity to healthy tissue, cisplatin and carboplatin-based chemotherapy led to only a modest 1.5-3 months prolongation of survival in patients with advanced disease [105]. Therefore, during the last decade, newer less toxic second line agents such as gemcitabine, vinorelbine, paclitaxel, and docetaxel have been studied, and these have demonstrated comparable anticancer efficacy to cisplatin-based therapy in NSCLC. A number of studies have shown that combinations of first and second line chemotherapeutic agents result in an improved progression free survival (PFS) ~ 4.6–6.9 months, overall survival (OS) ~8-10 months, and 1-year survival in 31-46% patients [106-109]. In addition, neo-adjuvant chemotherapy is recognized as an important treatment option for all stages of the disease, including patients with inoperable NSCLC to initially regress the growth and size of the tumor which may then become amenable to resection.

Targeted therapy

Advances in molecular biology have led to an improved understanding of cancer pathogenesis and have led to the advent of targeted therapies. The identification of driver mutations and molecular abnormalities in tumor tissue specimens and the administration of specific inhibitors to those targets are the basis of targeted therapies. In lung adenocarcinoma, along with mutations in *TP53* regardless of smoking status, at least two distinct major pathways contribute to its pathogenesis which include a nonsmoking-associated activation of EGFR signaling and a smoking-associated activation of KRAS signaling.

Anti-EGFR therapies. Never smokers with lung adenocarcinoma have the highest incidence of aberrant EGFR activation. In normal cells, activation of EGFR receptor via ligand binding leads to receptor dimerization, followed by phosphorylation of tyrosine residues within its intracellular COOH-terminal portion via the intracellular kinase domain. This provides

specific docking sites for proteins which mediate intracellular signaling to promote cell proliferation. Aberrant activation of the EGFR pathway in NSCLC has been observed, and is due to overexpression [110, 111] and amplification of the wild-type *EGFR* gene [112] and/or activating mutations (10-15%) [113]. In lung tissue samples, mutations in the tyrosine kinase (TK) domain of EGFR result in constitutive activation in the absence of ligand binding and mediate downstream growth signaling. EGFR inhibitors are categorized into two main classes: (i) small-molecule EGFR-TK inhibitors such as gefitinib and erlotinib (reversible inhibitors), and afatinib (irreversible) which compete with ATP for binding to the EGFR TK domain and inhibit its activity [114-116], and (ii) monoclonal antibodies (mAbs) such as cetuximab that target the extracellular portion of the EGFR and competitively inhibit ligand binding to the receptor [117].

Anti-KRAS therapies. K-ras protein is a downstream mediator of EGFR-induced cell signaling. Gene mutations in *KRAS* occur in 15%–30% of lung adenocarcinomas and are generally exclusive of mutations in *EGFR* [30]. Activating mutations in *KRAS* lead to constitutive activation of K-ras protein and persistent stimulation of downstream signaling pathways such as RAS/RAF/MEK/ERK which stimulate cell proliferation even in the presence of inactivated EGFR and/or EGFR tyrosine kinase inhibitors (TKIs) [118]. However, currently there is no targeted drug treatment for mutated K-ras proteins. Current treatments involve targeting proteins upstream and/or downstream of K-ras proteins.

Recent advances in chemotherapy and targeted therapy have undoubtedly improved the survival rate for lung cancer patients. Numerous studies have shown substantial advantages for TKIs in patients with mutant EGFR in terms of PFS and OS compared with chemotherapy alone. The median PFS has been reported to 9.2–13.1 versus 4.6–6.9 months, and OS ~21.6–34.8 versus ~8–10 months for TKIs and chemotherapy, respectively [119-123]. Similarly, in KRAS

mutant lung cancer patients who failed first line chemotherapy, MEK inhibition with selumetinib significantly increases the median PFS from 2.1 to 5.3 months and OS from 5.2 to 9.4 months [124]. However, targeted therapies have their own limitations.

Limitations of current NSCLC treatment modalities

There are several limitations of chemotherapies and targeted therapies. The common ones are as follows: First, drug resistance at multiple levels is commonly observed in cancer patients which may arise due to: (i) increased activity of membrane-bound transporter proteins such as P-glycoprotein, belonging to ATP-binding cassette (ABC) transporter superfamily, that act as efflux pumps to pump out intracellular chemotherapeutic drugs [125], (ii) decreased activity of solute carrier transporters that increases chemo-sensitivity by mediating the cellular uptake of chemotherapeutic drugs [125], (iii) tumor resistance, which may be intrinsic (that is, present before treatment), or acquired during treatment [126], (iv) heterogeneity of the tumors, where resistance may arise by positive selection of a drug-resistant tumor sub-population [127], and (v) parts of solid tumors may be poorly vascularized leaving tumor cells without drug exposure [128]. Second, drug toxicity which may be (i) general toxicity such as gastrointestinal disturbances, loss of hairs and myelosuppression, and susceptibility to infection etc.[129], (ii) specific toxicity which may be nephrotoxicity and cytotoxicity (platinum based therapy), pulmonary toxicity (cisplatin and cetuzimab), cardiotoxicity (imatinib and doxurubicin), and peripheral neuropathy (bortezomib) etc . Last, the cost of targeted therapy, often administered with chemotherapy, remains very high.

In view of the high incidence of lung cancer and high cost of treatment, together with modest outcome of current therapies, there is a need to look for alternative therapeutic strategies

for treatment and prevention of lung cancer. There is ample epidemiological evidence for a close association between cancer and diet which needs to be further explored.

Anticancer role of whole foods and their derived bioactive components in lung

Plant-based foods such as fruits and vegetables are rich in bioactive phytochemicals. In an exploratory study, NSCLC patients undergoing conventional therapies along with consumption of selected vegetable (SV) and freeze-dried commercial vegetable soup had a median survival time of 15-15.5 months as compared to median survival time of 4-4.8 months in patients who did not consume SV [130]. This suggests that components present in plant-based whole foods may positively influence lung carcinogenesis.

The anticancer potential of plant-based whole food is attributed to their rich phytochemical content. Bioactive phytochemicals such as resveratrol (grapes) [131, 132], epigallocatechin-3-gallate (EGCG; tea) [133], lycopene (tomato) [134], sulforaphane (cruciferous vegetables) [135, 136], curcumin (turmeric) [137] and myricetin (vegetables, fruits, nuts, and berries) [138] and others have been reported to modulate expression of genes and proteins that play a role in cancer cell proliferation and apoptosis. Table 3 lists *in vitro* anticancer properties of some of the bioactive components derived from plants in NSCLC and their mechanisms of action. In addition, a few of these plant-derived bioactive agents potentiate the antitumor activity of targeted chemotherapeutic agents in preclinical models for an array of cancer types [23, 25].

Polyphenols are ubiquitous in the plant kingdom and constitute one of the largest group of phytochemicals. These are secondary metabolites of plants and are believed to defend the plant against ultraviolet radiation and pathogens [139]. More than 8,000 polyphenolic

compounds have been identified in various plants, and lignans are an important subgroup of polyphenolics which exhibit multiple health-promoting properties [139].

Table 3. List of phytochemicals and their *in vitro* anticancer effects in lung cancer cell lines

Phytochemical	**Source**	**Lung cancer cell lines**	**Mechanism of action**	**Reference**
Resveratrol	Grapes	A549, H460	↑ Double strand DNA breaks, ↑ROS levels, ↑p53 and p21	[131]
		A549	↓ Cell proliferation, ↑ Apoptosis (↑ cleaved caspase-3 protein expression, ↓ Bcl-2 protein expression, and ↓ Bcl-2/Bax ratio)	[132]
Curcumin	Turmeric	PC-14, H1299	↓ Cell proliferation, ↑ Apoptosis, ↓ p-ERK ½ expression, ↓ EGFR expression	[140]
		H460	↑ Apoptosis, ↑ Reactive oxygen species (ROS)	[137]
Quercetin	Fruits, vegetables, leaves and grains	H209	↓ Cell proliferation, ↑ Cell cycle arrest (G2/M phase), ↑ Apoptosis (release of cytochrome c, ↑ cleaved caspase-3 protein expression, ↑ cleavage of PARP)	[141]
Sulforaphane	Cruciferous vegetables	A549	↓ Cell proliferation, ↑ Cell cycle arrest (G2/M phase),	[135]
Osthole	*Cnidium monnieri* (L.) Cusson	A549	↓ Cell proliferation, ↑ Apoptosis (↓ Bcl-2 and ↑ Bax proteins levels), ↑ Cell cycle arrest (G2/M phase; ↓ Cyclin B1 and p-Cdc25A proteins expression), ↓ PI3K/Akt signaling pathway	[142]

Table 3. List of phytochemicals and their *in vitro* anticancer effects in lung cancer cell lines (continued)

Phytochemical	Source	Lung cancer cell lines	Mechanism of action	Reference
Silibinin	*Silybum marianum* (L.) Gaertn	H1299, H460, H322	↓ Cell proliferation, ↑ Cell cycle arrest (G0/G1 phase; ↓ CDK 2,4,6, cyclin D1, D3, and E proteins levels, ↑protein levels of CDKIs, reduced pRb phosphorylation)	[143]
Diallyl trisulfide	Garlic-derived organosulfur	H358, H460	↓ Cell proliferation, ↑ Cell cycle arrest (G2/M phase; ↓ CDK1 and p-Cdc25C protein levels), ↑ Apoptosis (↓ Bcl-2 and ↑ Bax, Bak, and BID protein expression)	[144]
Salidroside	phenylpropanoid glycoside from *Rhodiola rosea* (L.)	A549	↓ Cell proliferation, ↑ Cell cycle arrest (G0/G1-phase), ↑ Apoptosis, ↓ ROS, ↓ TGF-β cell invasion (↓ protein levels of Snail)	[145]

Flaxseed and its phytochemicals

Flaxseed is a nutritional whole grain that has demonstrated health benefits in the pathophysiology of several diseases including cancer [146]. The health benefits of flaxseed are attributed to its phytonutrient content. Flaxseed is the richest source of the plant lignan secoisolariciresinol diglucoside (SDG) a polyphenolic compound, with levels reported to be 100–800 times higher than that of other oilseeds, cereals, legumes, and fruit and vegetables [147]. Additionally, flaxseed is a rich source of n-3 polyunsaturated fatty acid α-linolenic acid (ALA), proteins, and fibers [146, 148]. Flaxseed is available for human consumption in various forms with different nutritional compositions such as whole flaxseed (rich in both ALA and SDG), flaxseed oil (rich in ALA), or defatted flaxseed (rich in SDG) [146]. The isolation of

bioactive components from flaxseed has allowed researchers to distinguish the health-promoting effects of flaxseed-derived lignans from its ALA component [146].

Upon flaxseed ingestion, plant lignan SDG undergoes deglucosylation by the action of intestinal bacteria to form secoisolariciresinol (SECO), which undergoes further demethylation and dehydroxylation to give rise to enterodiol (ED), a mammalian lignan [149]. Finally, ED is dehydrogenated and gets converted into enterolactone (EL), another mammalian lignan [149]. In addition, minor amounts of pinoresinol (PINO) and laricinesol (LAR) present in flaxseed also get converted into SECO and then into ED and EL (Figure 2) [149]. Many studies have concluded that the SDG-derived mammalian lignan EL is effective at inhibiting cancer cell growth [150-

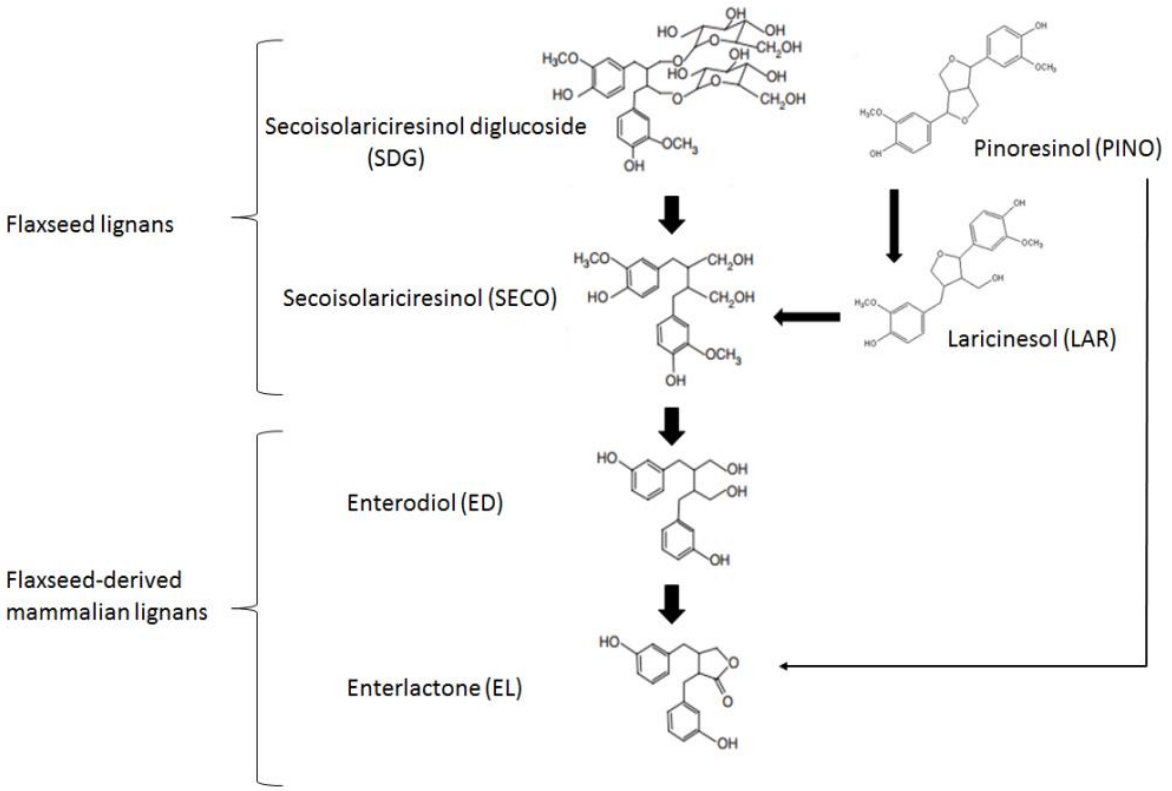

Figure 2. Metabolism of flaxseed lignans (SDG, SECO, PINO, LAR) into mammalian lignans (ED and EL).

152].

Flaxseed and its derived lignans in treatment of cancer

The anticancer potential of whole flaxseed, its lignan SDG, and SDG-derived mammalian lignans ED and EL have been documented in various xenograft mouse models of human breast carcinoma (MDA-MB-435 and MCF-7) [153-156], colon carcinoma (colo201) [151], and prostate carcinoma (LNCaP, in transgenic mice model of prostate cancer) [157]. In addition, *in vitro* studies have shown that EL inhibits growth of breast [158, 159], colon [151, 160], and prostate [150, 161, 162] cancer cell lines. Some of the reported anticancer mechanisms of SDG and EL include induction of cell cycle arrest in the G_0/G_1 and S-phases; suppression of adhesion, migration and invasion; and activation of apoptosis.

Breast cancer

Flaxseed exerts anti-proliferative effects in various mouse models of established breast cancer. Dietary supplementation of 10% flaxseed reduces the growth of established estrogen receptor-negative human breast tumors (MDA-MB-435) by significantly lowering levels of VEGF as compared to the control mice [163]. In addition, flaxseed has been reported to enhance the tumor inhibitory effects of tamoxifen, an established chemotherapy for breast cancer, in an estrogen-dependent xenograft (MCF-7) mouse model of breast cancer [154]. Furthermore, the components of flaxseed, SDG and oil have been shown to reduce the growth and metastasis of cancer cells in a xenograft mouse model of breast cancer [164]. These findings correlate with previous observations which show that the anti-tumorigenic effects of flaxseed are largely dependent on its SDG component [165, 166].

Upon consumption, SDG gets metabolized into mammalian lignans, ED and EL. ED and EL have been shown to inhibit adhesion, migration, and invasion of breast cancer cell lines

(MDA-MB-435 and MDA-MB-231) in a dose-dependent manner [167]. EL-mediated decrease in cell motility is attributed to its ability to remodel actin cytoskeleton, downregulate gene expression of matrix metalloproteinases (MMP-2, -9, and -14), and inhibit FAK signaling [158, 159]. This *in vitro* data is in agreement with previous *in vivo* studies, which suggest that consumption of flaxseed or its purified lignan inhibit breast tumor progression and metastasis.

Colon cancer

A number of studies have shown EL exhibit growth-inhibitory effects in colon cancer. EL suppresses the growth of colo201 human colon cancer cells *in vitro* and *in vivo* by downregulating expression levels of proliferation-related protein (PCNA) and the anti-apoptotic protein (BCL-2), and upregulating expression of cleaved caspase-3, an apoptosis enhancing protein [151]. In addition, subcutaneous administration of EL (10 mg/kg body weight), three times a week, in the same study observed a significant reduction in the growth of colo201 xenografts in athymic mice. In another study, it was shown that both EL (IC_{50}=50 μM) and ED (IC_{50}=150 μM) significantly inhibit the growth of the human colon cancer cell line Caco-2 by inducing apoptosis, with EL being a more potent apoptosis inducer as compared to ED [160]. Therefore, it can be concluded that EL has chemotherapeutic potential in colon cancer

Prostate cancer

The anticancer effects of ED and EL have also been studied in the context of prostate cancer. ED and EL suppress *in vitro* proliferation of prostate cancer cell lines (PC-3, DU-145, and LNCaP) [168]. EL-mediated inhibition of prostate cancer cell (LNCaP) growth has been attributed to its ability to (i) initiate apoptosis characterized by dose-dependent loss of mitochondrial membrane potential, release of cytochrome c, and cleavage of procaspase-3 and PARP, (ii) upregulate expression of survival genes such as survivin, and (iii) downregulate cell

cycle related genes such as minichrosome maintenance gene (MCM 2), CDK2, and E2F [150, 161]. An *in vitro* model of prostate cancer has demonstrated that EL (20 μM) significantly reduces the proliferation of mid and late stage models of prostate cancer cell lines by (i) inducing changes in the expression of the DNA licensing genes (GMNN, CDT1, MCM 2 and 7), (ii) reducing expression of the miR-106b cluster, and (iii) increasing expression of the PTEN tumor suppressor gene [169]. Evidence from literature, therefore, suggests that EL exerts anti-tumor effects against prostate cancer pre-clinical models.

Flaxseed and its chemopreventive potential

Breast cancer

The effects of whole flaxseed and its lignan on different stages of breast carcinogenesis (pre-initiation, initiation, promotion, and progression) have been investigated in several rodent models. These studies have demonstrated that consumption of flaxseed or purified SDG has potential to protect against carcinogen-induced tumor incidence, size, and volume at various stages of carcinogenesis.

Early life exposure to 5-10% flaxseed diet and its equivalent amount of SDG reduces the number of targets for malignant transformation, terminal end buds (TEBs) and promotes their differentiation to lobulo-alveolar structures that are resistant to malignant breast tumor formation [170]. Intake of 5 and 10% flaxseed by rats for 4 weeks (pre-initiation stage) before DMBA administration has been shown to significantly reduce epithelial cell proliferation (38.8-55.4%), and nuclear aberrations in TEB, which are highly proliferative and are susceptible to carcinogen inflicted DNA damage [171]. Further, consumption of 5% flaxseed during initiation stage reduces tumor burden, while flaxseed supplementation during the promotion stage (flaxseed given after DMBA administration) does not affect tumor burden but reduces tumor size [172]. In

addition, intake of 2.5% and 5% flaxseed-supplemented diet in the promotion stage of breast tumorigenesis, starting 13 weeks after DMBA administration, significantly regresses growth of already established breast tumors in mice [166]. 2.5% and 5% flaxseed-supplemented diet also reduces mammary carcinogen N-nitrosomethyl-urea (NMU)-induced breast tumor development and tumor invasiveness in Sprague–Dawley rats, as measured by the number of micro-invasions and invasion to neighboring tissues, [173].

The association between flaxseed intake and breast cancer risk has been evaluated in the Ontario Women's Diet and Health Study, a case-control study. It was concluded that monthly and daily/weekly consumption of flaxseed (1/4 cup serving, approximately 32.5 g) and flax bread (1 slice, approximately 2.5–5.0 g flaxseed) consumption is associated with a significant reduction (18%–24%) in breast cancer risk in all women [174]. In a randomized control trial in human breast cancer cases, 25 g/d flaxseed consumption was found to result in a decrease in tumor cell proliferation and an increase in tumor cell apoptosis control trial [175].

Colon cancer

Flaxseed and defatted flaxseed (lignan-rich) have shown protective effects in rodent models of carcinogen-induced colon cancer. The chemopreventive potential of flaxseed is evident in its ability to (i) reduce epithelial cell proliferation, (ii) decrease the number of aberrant crypts and aberrant crypt foci, and (iii) decrease colon tumor incidence, multiplicity, and size in rodent models of carcinogen-induced colon cancer [176-179]. The observed chemopreventive potential of flaxseed against colon cancer is attributed to its ability to induce cell cycle arrest by upregulating mRNA expression of p53 and p21, and decrease inflammation by lowering protein expression levels of cyclooxygenase (COX)-1 and COX-2 [179, 180].

Prostate cancer

Several epidemiologic studies have revealed a relatively consistent association between higher intake of phytoestrogens and reduced risk of prostate cancer in humans. Low-fat flaxseed-supplemented (30 g/day) diet, for 21-77 days (average 34 days) significantly increased apoptosis and decreased prostate specific antigen (PSA) in a pilot study of 25 cases of prostate cancer awaiting surgery compared to matched historical controls [181]. Further, 5% flaxseed inhibits the growth and development of prostate cancer in the transgenic adenocarcinoma mouse prostate (TRAMP) model [157]. In addition, there is evidence that EL can accumulate to high levels (>600 ng/ml) in prostate tissue and fluid, suggesting a biological function for EL derived from flaxseed consumption in its observed chemopreventive role in the prostate [182].

Lung cancer chemoprevention

Approximately 90% of lung cancer cases are attributed to tobacco smoking. There are approximately 1.3 billion smokers in the world [2], and although each year 70% smokers attempt to quit smoking, less than 5% succeed [183]. Even though smoking cessation is the ideal intervention to reduce the risk of lung cancer, former smokers still carry a significant risk. Therefore, lung cancer chemoprevention is an ideal strategy to combat the disease as high-risk population (current and former smokers) is easily identifiable.

Chemoprevention is defined as the use of plant-derived dietary agent or pharmaceutical interventions to inhibit, slow, or reverse the process of carcinogenesis. Chemopreventive agents are classified chemopreventive agents into two broad categories: (i) blocking agents, which prevent the interaction of carcinogens with DNA, and (ii) suppressing agents, which prevent post-carcinogen treatment downstream effects [184].

Several epidemiological studies have documented the cancer-preventive effects of cruciferous vegetables of brassica family in lung, colorectal, stomach, breast, prostate, and other cancers [185-187]. Cruciferous vegetables contain glucosinolates which, when consumed raw and to some extent cooked, yield isothiocyanates and indole-3-carbinol (I3C) among other products [185]. A significant inverse association has been found between lung cancer risk and consumption of cruciferous vegetables in smokers and ex-smokers, respectively [188, 189].

Isothiocyanates such as phenethyl isothiocyanate (PEITC) and sulforaphane have been widely studied as chemopreventive dietary agents in lung [190]. They inhibit the activity of several CYP450s which are phase I enzymes involved in carcinogen activation. By inhibiting the activity of CYP450s, isothiocyanates prevent DNA adduct formation and the subsequent mutations leading, thereby preventing carcinogenesis [191]. Isothiocyanates also induce certain phase II enzymes like GSTs and UGTs through activation of the Keap-1/Nrf2 pathway [191, 192]. The induction of phase II enzymes helps remove activated carcinogens by transforming them into a water-soluble compound that is excreted in the urine. PEITC prevents NNK oxidative bioactivation in A/J mice by inhibiting CYP2A1 and 2B1 enzymes by 13-27% and 30-50%, respectively [193]. Similarly, phenylbutyl isoselenocyanate (ISC-4), designed by modifying naturally occurring PEITC by replacing sulfur with selenium, significantly decreases O6-mG DNA adducts in NNK-treated A/J mice [191]. This decrease in DNA adduct formation is accompanied by increased expression and activity of several phase II NNK-detoxifying enzymes (GST1, GSTM3, GSTP1, UGT1A6A, and UGT1A9) [191]. Further, consumption of watercress, rich in PEITC, by smokers inhibits oxidative metabolism of NNK by inhibiting CYP1A2 and inducing glucuronidation of NNAL, resulting in increased excretion of NNK [194]. These studies suggest that isothiocyanates may act as potential lung chemopreventive agents.

I3C, also present in cruciferous vegetables, has been shown to inhibit NNK-induced lung tumors in A/J mice [195]. Administration of I3C during lung tumor-initiation phase, i.e. at the 50% in the carcinogen treatment and immediately post-carcinogen treatment, reduces NNK plus BaP-induced lung tumorigenesis in A/J mice [196]. I3C-mediated its lung tumor inhibitory effects by (i) reducing the number of Ki-67-positive cells, (ii) lowering levels of phosphorylated Akt and BAD, and (iii) increasing cleavage of poly(ADP-ribose) polymerase (PARP). I3C has also been shown to inhibit the progression of NNK-induced adenomas to adenocarcinomas, the most critical stage in lung cancer development [195]. I3C undergoes condensation reactions in the acidic milieu of the stomach and forms 3,3- diindolylmethane (DIM) [197]. DIM is considered to be responsible for the chemopreventive efficacy of I3C. DIM, along with I3C protect against NNK-induced lung tumorigenesis by inducing expression of Nrf2-mediated phase II drug metabolizing (*GSTm2*, *UGT1A1*, and *NQO1*) and antioxidant (heme oxygenase-1 (*HMOX-1*) and superoxide dismutase-1 (*SOD*1-)) genes [198].

Green tea and its major components (-)-epigallocatechin gallate (EGCG) have shown chemopreventive potential during the initiation and promotion stages of NNK-induced lung tumorigenesis in A/J mice [199]. Decaffeinated green tea (4 cups/day) significantly reduces levels of urinary 8-hydroxy-2' -deoxyguanosine (8-OHdG), a product of oxidative DNA damage and biomarker for cellular oxidative stress among smokers [200]. In NNK-induced mice model of lung tumorigenesis, consumption of green tea and EGCG alone has been shown to significantly reduce the number of lung tumors by decreasing the levels of 8-OHdG DNA lesions [201]. It was concluded that the antitumor effects of green tea and EGCG was due to their antioxidant potential. Further, polyphenon E (a green tea polyphenol) has been shown to

significantly decrease progression of lung adenomas to adenocarcinomas in NNK-treated A/J mice [202].

Kava, an aqueous root extract of *piper methysticum*, consumed as a beverage by South Pacific Islanders, has also demonstrated chemopreventive potential in NNK-induced lung carcinogenesis in A/J mice [203]. Kava given at a daily dose of 5 mg/g of diet, before NNK treatment, has been found effective in reducing adenoma incidence by 67% and adenoma multiplicity by ~99% [204]. The anti-tumor initiation efficacy of kava is attributed to the preferential reduction in NNK-induced O6-mG DNA adducts. The anti-tumor efficacy of kava is attributed to the presence of an active compound called fraction B [204]. Kavalactones, especially (+)-dihydromethysticin (DHM), is considered to be a potential chemopreventive agent which in a dose dependent (0.01-1mg/g) manner reduces O6-mG, 7-mG, 7-phbG, O6-phbdG and O2-phbdT DNA adducts in the lung tissues leading to reduced adenoma formation, and, thus, inhibits lung carcinogenesis [205].

These are some of the naturally occurring chemopreventive agents which have shown promising results in experimental animals; however, so far, none of these have proved useful in humans.

Rationale for the use of enterolactone in lung cancer treatment and flaxseed in chemoprevention of lung cancer

The rationale for investigating the anticancer efficacy of flaxseed-derived mammalian lignan EL in treatment of lung cancer stems from its convincing *in vitro* and *in vivo* anti-tumor effects in breast, colon, and prostate cancers. These studies have delineated the molecular mechanisms by which EL regulates apoptosis, cell proliferation, and motility *in vitro*, and *in*

vivo. However, the anticancer effects and the associated molecular mechanisms of EL remain unexplored in lung.

The chemopreventive potential of flaxseed in lung has been attributed to the potent anti-oxidant and anti-inflammatory properties of its lignan component (SDG) [206, 207]. Persistent oxidative stress and inflammation may be responsible for development of lung cancer in current and former smokers [208]. The lung protective anti-oxidant and anti-inflammatory effects of flaxseed have been documented in murine models of ischemia-reperfusion injury [207, 209]. In addition, the flaxseed lignan component (SDG) reduces murine lung inflammation and oxidative damage inflicted by radiation, a standard treatment for metastatic lung cancer [210,211]. It is important to note that flaxseed protects healthy murine lung tissue against adverse effects of radiation and, at the same time, does not decrease the effect of radiation on cancer cells. Lastly, flaxseed has been shown to alter the expression of several genes, the deregulation of which is commonly seen in lung cancer [206].

A plethora of research suggests the anticancer potential of flaxseed and EL in various cancers, and their protective effects for lung tissue against inflammation and oxidative stress. However, their anti-tumor effects and the underlying molecular mechanisms have not been investigated in lung cancer. Based on published literature, we predicted that EL would inhibit lung cancer cell growth *in vitro* and flaxseed would inhibit lung tumor development in a chemically-induced animal model of lung cancer.

Objectives and specific aims

The objectives of this study were to investigate the *in vitro* anticancer properties of EL in lung cancer cells along with the associated molecular mechanism, and to investigate the chemopreventive potential of flaxseed, a whole food, in a mouse model of lung carcinogenesis.

We hypothesized that: (i) EL decreases *in vitro* proliferation of lung cancer cells by initiating cell cycle arrest and inducing apoptosis, (ii) EL inhibits *in vitro* lung cancer cell motility by altering expression levels of cytoskeletal proteins and decreasing FAK-Src signaling, (iii) flaxseed, reduces lung tumor incidence and multiplicity by suppressing NNK-induced inflammation and oxidative stress in A/J mouse model of NNK-induced lung carcinogenesis. Therefore, the following specific aims were:

Specific aim 1: To determine the *in vitro* anti-proliferative ability of EL in NSCLC cells and the associated mechanisms. This aim has been addressed in Chapter 2, and the paper has been published in *Nutrition and Cancer: An International Journal*.

Specific aim 2: To identify the potential of EL to inhibit *in vitro* NSCLC cell motility and the associated mechanisms. This aim has been addressed in Chapter 3, and the paper has been published in *BMC Complementary and Alternative Medicine.*

Specific aim 3: To establish the potential of flaxseed as a lung chemopreventive dietary food in A/J mouse of NNK-induced lung carcinogenesis. This aim has been addressed in Chapter 4, and we plan to submit the paper to *Cancer Prevention Research*.

The specific aims proposed are likely to help us identify the role of EL in the treatment and the potential of flaxseed in chemoprevention of lung cancer.

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CHAPTER 2. ENTEROLACTONE INDUCES G1-PHASE CELL CYCLE ARREST IN NON-SMALL CELL LUNG CANCER CELLS BY DOWN-REGULATING CYCLINS AND CYCLIN-DEPENDENT KINASES

Abstract

Flaxseed is a rich source of the plant lignan secoisolariciresinol diglucoside (SDG) which is metabolized into mammalian lignans enterodiol (ED) and enterolactone (EL) in the digestive tract. The anti-cancer properties of these lignans have been demonstrated for various cancer types, but have not been studied for lung cancer. In this study we investigated the anti-cancer effects of EL for several non-small cell lung cancer (NSCLC) cell lines of various genetic backgrounds. EL inhibited the growth of A549, H441, and H520 lung cancer cells in concentration- and time-dependent manners. The anti-proliferative effects of EL for lung cancer cells were not due to enhanced cell death, but rather due to G_1-phase cell cycle arrest. Molecular studies revealed that EL- decreased mRNA or protein expression levels of the G_1-phase promoters cyclin D1, cyclin E, cyclin-dependent kinases (CDK)-2, -4, and -6, and p-cdc25A; decreased phosphorylated retinoblastoma (p-pRb) protein levels; and simultaneously increased levels of $p21^{WAF1/CIP1}$, a negative regulator of the G_1-phase. The results suggest that EL inhibits the growth of NSCLC cell lines by down-regulating G_1-phase cyclins and CDKs, and up-regulating $p21^{WAF1/CIP1}$, which leads to G_1-phase cell cycle arrest. Therefore, EL may hold promise as an adjuvant treatment for lung cancer therapy.

Introduction

Lung cancer accounts for about 38% of all cancer-related deaths in the US, which is more than the combined mortality due to breast, colon, and prostate cancers [1]. Conventional lung cancer treatment modalities such as surgery, chemotherapy, and radiotherapy have shown

modest success; however, these treatment options often inflict toxicity to healthy tissues [2]. Recently, bioactive compounds isolated from natural food sources have gained importance in the field of cancer treatment due to their selective action against cancer cells [3].

Lignans are naturally occurring phytoestrogens that exhibit health-promoting effects in several diseases including cancer [4]. Flaxseed has an exceptionally high lignan content and has shown chemopreventive and chemotherapeutic benefits in an observational study of breast cancer [5], human clinical trials [6-8], and animal models of breast [9-11], colon [12, 13], ovarian [14], and prostate [15] cancers. A number of *in vivo* studies have linked the observed anticancer effects of flaxseed to the presence of its primary lignan, secoisolariciresinol diglucoside (SDG) [10-12, 16, 17]. Upon consumption, SDG is metabolized by a consortium of intestinal bacteria into mammalian lignans, ED and EL. The anti-carcinogenic effects of flaxseed and SDG have been attributed to these mammalian lignans in mammary carcinogenesis [18]. Furthermore, *in vitro* studies of breast, colon, and prostate cancers demonstrate that EL has growth-inhibitory properties. Several studies suggest that the anti-proliferative potential of EL may be due to its effects on the cell cycle and induction of cell death [19-21]. The cytotoxic effects of EL appear to be selective for cancer cells as EL (25-75 µM for 24 and 48 h) inhibited the growth of LNCaP human prostate cancer cells, but EL treatment at the same concentrations and time points did not affect the viability of normal prostate epithelial cells (CRL-2221) [19].

Progression through the four sequential phases of the cell cycle (G1, S, G2, and M) is mediated by interactions between cyclins and cyclin dependent kinases (CDKs) and their inhibitors [22]. Cyclin D1 interacts and forms complexes with CDK4 and CDK6 to regulate the G1-phase, while cyclin E forms a complex with CDK2 to regulate the G1-S transition of the cell cycle [22]. These complexes phosphorylate/inactivate retinoblastoma (pRb) protein, leading to

the release of bound transcription factors, such as E2F, and promote expression of genes such as cell cycle division cycle 25 (cdc25), necessary to facilitate the G1-S phase transition [23, 24]. Additionally, CDK inhibitors (CDKIs), such as p21(WAF1/CIP1) and p27(KIP1), act as negative regulators of the cell cycle by binding to and inhibiting cyclin-CDK activity [24]. Disruption in CDKI/CDK/cyclin interactions may interfere with pRb/E2F complex formation, thereby, inhibiting progression of cells from G1 to S-phase of the cell cycle [23]. EL has been shown to down-regulate E2F mRNA expression in LNCaP prostate cancer cells [25]. In addition, EL-treatment has been found to alter CDKI/CDK/cyclin mRNA expressions in MDA-MB-231 breast and in LNCaP prostate cancer cells leading to S-phase cell arrest [21, 25].

There is also evidence that consumption of lignan-rich flaxseed significantly alters the expression of cell cycle regulatory genes in mouse lung tissue [26]. In addition, flaxseed is known to protect lungs against oxidative damage and inflammation in murine models, two major factors contributing towards lung cancer development and progression [27-29]. Therefore, we hypothesized that EL, a flaxseed-derived mammalian lignan, could suppress lung cancer cell growth by inducing cell cycle arrest and cell death. To test this hypothesis, we conducted growth, cytotoxicity, and cell cycle analysis assays for three NSCLC cell lines (A549, H441, and H520) representing unique genetic backgrounds

Materials and Methods

Materials

Purified EL (99.2% pure) was purchased from ChromaDex, Inc. (Irvine, CA). EL was dissolved in 100% dimethysulfoxide (DMSO; Corning Cellgro®; Manassas, VA) at a stock solution concentration of 200 mM. The stock solution was later diluted in PBS (pH 7.2) to result in working solutions of EL (0-100 μM) with a maximum DMSO concentration of 0.2%.

alamarBlue® was obtained from AbD Serotech (Raleigh, NC), and trypan blue was purchased from Amresco (Solon, OH). Propidium iodide (PI) was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Annexin V-FITC/PI apoptosis detection kit was purchased from Thermo Fisher Scientific (Eugene, OR). Antibodies for cyclin D1, p-pRb, CDK2, CDK4, CDK6, p21/Cip1, p27/Kip1, t-cdc25A, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for cyclin E, and p-cdc25A were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX). Anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Labs Inc. (West Grove, PA). (PPLGM) was purchased from Indofine Chemical Company (Catalog#: P-004, 97%, Hillsborough, NJ).

Cell lines and cell culture

The human NSCLC cell lines (A549, H441, and H520) and normal lung fibroblast cell line (Hs888Lu) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The NSCLC cell lines were maintained in RPMI-1640 medium (Sigma Aldrich; St. Louis, MO), with 10% (v/v) fetal bovine serum (FBS; Atlanta Biologicals; Flowery Branch, GA). The normal lung cell line was maintained in DMEM medium (Sigma Aldrich) supplemented with 10% (v/v) FBS. All cell lines were incubated at 37˚C in a humidified atmosphere of 95% air and 5% carbon dioxide. Cell lines were sub-cultured by enzymatic digestion with 0.25% trypsin/1mM EDTA solution when they reached approximately 70% confluency.

alamarBlue® cell viability assay

A549, H441, and H520 cells were seeded into individual wells of 96-well plates at a density of $5x10^3$ cells per well. Twenty-four hours later, 10% alamarBlue® (10 µl/well) was added to each well, and the plate was incubated at 37°C for 4 h protected from direct light. After

incubation, absorbance readings were taken at 570 and 600 nm using a plate reader (Microplate XMark^{TM} spectrophotometer; Bio-Rad; Hercules, CA). The cells were then washed with PBS and fresh RPMI-1640 medium supplemented with 10% FBS was added. The cells were then treated with EL (0-100 μM). Every 24 h, 10% alamarBlue® was added, and absorbency readings were taken after 4 h. Cell viability was measured by calculating the percent reduction of alamarBlue® in response to each treatment for a period of 4 days using the formula given below:

$$\%\ \text{reduction in alamarBlue®} = \left(\frac{((117{,}216 * A1) - (80{,}586 * A2))}{((155{,}677 * B1) - (14{,}652 * B2))}\right) * 100$$

In the formula, 117,216 and 80,586 are constants representing the molar extinction coefficients of alamarBlue® at 570 and 600 nm, respectively, in the oxidized form; whereas 155,677 and 14,652 are constants representing the molar extinction coefficients of alamarBlue® at 570 and 600 nm, respectively, in the reduced form. *A1* and *A2* represent absorbance of wells treated with EL at 570 and 600 nm, respectively. *B1* and *B2* represent absorbance of wells treated with vehicle control (0.2%, DMSO) at 570 and 600 nm, respectively. The % cell viability relative to control ± standard deviation in eight replicate wells per treatment for three independent experiments was determined.

Clonogenic survival assay

A549, H441, and H520 cells were seeded into individual wells of 6-well plates for 24 hours, rinsed with PBS, followed by addition of fresh RPMI-1640 media supplemented with variable concentrations of EL (0, 10, and 100 μM). The cells were then left undisturbed and allowed to grow and form colonies. After 14 days, the colonies were fixed with a methanol and acetic acid (3:1) solution and stained with 0.5% crystal violet. The blue-stained colonies were then counted manually. Three independent experiments were performed for each cell line with

two replicates per treatment. The percent cell colonies relative to control ± standard deviation was determined.

Trypan blue exclusion assay

A549, H441, and H520 cells were seeded at a density of 20 x 10^3 cells/well in 24-well plates. The cells were treated with EL (0, 10, 50, and 100 µM) each day for 24, 48, and 72 h. After treatment, the cells were harvested by trypsinization, stained with trypan blue, and the number of live and dead (blue staining) cells were counted on a hemacytometer under a light microscope. The average live/dead cell count from three independent experiments was determined.

In addition, Hs888Lu normal lung fibroblast cells were seeded at a density of 15 x 10^3 cells/well in 6-well plates. The cells were treated with EL (0, 10, and 100 µM) each day for 24 and 48 h. After treatment, the cells were harvested by trypsinization, stained with trypan blue, and the number of live cells were counted on a hemacytometer under a light microscope. The % cell viability relative to control ± standard deviation for two independent experiments was determined.

Apoptosis detection by Annexin V-fluorescein isothiocyanate/Propidium iodide (annexin-V-FITC/PI)

A549 cells were seeded at a density of 2 x 10^5 cells/well in 60 mm dishes. Twenty-four hours later, the cells were treated with EL (0, 10, 50, and 100 µM) each day for 24, 48, and 72 h. After treatment, the cells were harvested by trypsinization, washed with PBS, and re-suspended in 1X binding buffer. The cells were stained with 5 µl of FITC-Annexin V and 5 µl PI (100 µg/ml) for 10 min at room temperature. Finally, 400 µl of 1x binding buffer was added to the cells and data for live and apoptotic percentage of 10,000 cells were collected using a FACScan

flow cytometer. The viable cells are negative for both annexin-V and PI staining; apoptotic cells are positive for annexin-V staining while negative for PI staining, and necrotic cells are positive for both annexin-V and PI staining. Three independent experiments were performed for each treatment group and time-point.

Cell cycle analysis

The cell cycle distribution was assessed by staining the cellular DNA content with propidium iodide (PI). A549, H441, and H520 cells were synchronized by incubating them in serum-deprived medium for 24 h followed by treatment with either the vehicle control (0.2%, DMSO) or EL (10 and 100 μM). The cells were then harvested by trypsinization, centrifuged at 200 rpm for 5 min, washed with PBS (pH 7.2), and centrifuged again at 200 rpm for 5 min. The resultant cell pellet was then re-suspended in cold 70% ethanol and fixed overnight at 4˚C. Next day, the cells were collected by centrifugation at 200 rpm for 5 min, and the resultant cell pellet was re-suspended in 500 μl of PBS (pH 7.2) containing 100 μg/ml RNase and PI (40 μg/ml) for 30 min at 37°C protected from light. After staining with PI, 10,000 cells per sample were run through a FACScan flow cytometer equipped with a 488 nm argon laser, and the data were analyzed using FlowJo software. The average percentage of cells in each phase of the cell cycle per treatment for three independent experiments was determineddetermining cell survival,

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from EL-treated (100 μM; 24 h) A549, H441, and H520 cells (1 X 10^6 cells) using the Fisher SurePrep Kit (Waltham, MA) as per the manufacturer's instructions. The 260/280 and 260/230 OD ratios were measured using NanoDrop1000 spectrophotometer to assess the purity of RNA samples with respect to protein contamination and residual organic solvent, respectively. RNA samples with 260/280 and 260/230 OD ratios

between 1.8 and 2 were used for RT-qPCR. Fifty nanograms of RNA were reverse transcribed into cDNA using the qScript cDNA synthesis kit (Quanta Biosciences; Gaithersburg, MD). Primers against *cyclin D1 (*5'-TGCGCTGCTACCGTTGACT-3' and 5'-AGCGATGTGAATATTTCCAAACC-3'), *CDK2 (*5'-GGTCCTCCACCGAGACCTTAA-3' and 5'-CAGGGACTCCAAAAGCTCTGG-3'), *CDK4 (*5'-CAGTGTACAAGGCCCGTGATC-3' and 5'-ACGAACTGTGCTGATGGGAAG-3'), c*dc25A (*5'-CCCCAAAGGAACCATTGAGA-3' and 5'-CTGATGTTTCCCAGCAACTG-3'), $p21^{WAF1/CIP1}$ *(*5'-GGACAGCAGAGGAAGACCATGT-3' and 5'-GCCGTTTTCGACCCTGAGA-3'), $p27^{KIP1}$ *(*5'-CTATCTGCTGCGCGGTTACC-3' and 5'-CCTGACAAGCCACGCAGTAG-3'), and 18S rRNA (5'- GGCCCTGTAATTGGAATGAGTC-3' and 5'-CCAAGATCCAACTACGAGCTT-3') and were designed using Primer Express software (version 2.0, Applied Biosystems), and synthesized by Integrated DNA Technologies (Coralville, IA). Steady-state mRNA levels for the cell cycle-related genes were evaluated by real-time PCR using PerfeCTa SYBR Green FastMix (Quanta Biosciences). The cycling parameters were: 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 60°C for 1 min, and a dissociation program that included 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s ramping up at 0.2 °C/s. One distinct peak was observed for each primer set, suggesting target specificity. Duplicate wells were run for each experiment and the experiment was performed in triplicate. The relative change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method using housekeeping gene 18S rRNA as internal control.

Western blotting analysis

A549, H441, and H520 cells ($1x10^6$) were treated with EL (100 μM) for variable times. Following treatment (0-24 h), the cells were collected by trypsinization, washed with PBS, and lysed using an SDS lysis buffer (Cell Signaling Technologies; Danvers, MA) containing protease

and phosphatase inhibitors (Roche; Indianapolis, IN). The cell pellets were briefly sonicated to dissociate cell membranes. Thirty micrograms of total protein isolated from these cells were electrophoresed on 7.5% SDS-polyacrylamide gels at 100 V for 1 h. Proteins were then transferred to PVDF membranes at 100 V for 70 min at 4°C. The blots were then probed overnight at 4 °C with primary antibodies (1:1000) for cyclin D1, cyclin E, p-pRb, p-cdc25A, t-cdc25A, CDK2, CDK4, CDK6, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and GAPDH. The next day, the blots were rinsed with 1X TBS-tween (0.1%) and probed with anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. The western blots were analyzed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific; Rockford, IL) and the images were captured using the MultiImage™ Light Cabinet (Alpha Innotech; San Leandro, CA). Target protein expression levels were normalized to GAPDH expression levels except for p-cdc25A protein expression levels, which were normalized to t-cdc25A protein levels. Western blotting was performed in triplicate. The densitometry results were obtained using ImageJ software.

Statistical analysis

Data are presented as means ± standard deviation for at least 3 independent experiments. The statistical significance of difference between the control and treatment groups was determined by paired t-test or two-way ANOVA. $p \leq 0.05$ were considered statistically significant.

Results

EL inhibits short-term proliferation of lung cancer cells in a concentration- and time-dependent manner but does not affect proliferation of normal cells

We investigated the anti-proliferative effects of EL for three human NSCLC cells: A549, H441, and H520. The short-term growth-inhibitory effects of EL on NSCLC cells were examined using an alamarBlue® cell viability assay. We found that EL-treatment led to a concentration- and time-dependent inhibition of proliferation of all lung cancer cell lines tested. We observed that EL ≥50 μM significantly ($p \leq 0.05$) inhibited the growth of A549 and H520 cells by day 2, while, similar growth inhibition was observed in H441 cells treated with 100 μM EL. By day 4, A549, H441, and H520 cells treated with EL (100 μM) showed a significant ($p \leq 0.05$) growth inhibition by 47%, 31%, and 30%, respectively (Fig.3A).

In addition, we investigated if EL affected proliferation of Hs888Lu, normal lung fibroblast cells. Hs888Lu cells were treated EL (0, 10, and 100 μM) for 24, and 48 h and cell viability was determined using trypan blue exclusion assay. EL-treatment (10 μM) showed no inhibition in cell proliferation after 24 h and a slight 3% inhibition of cell viability was observed after 48 h (Fig. 3B). Similarly, EL-treatment (100 μM) showed a minimal 8 and 7% inhibition of cell proliferation after 24 and 48 h, respectively (Fig. 3B). This indicates that EL has little or no effect on the proliferation of normal lung cells.

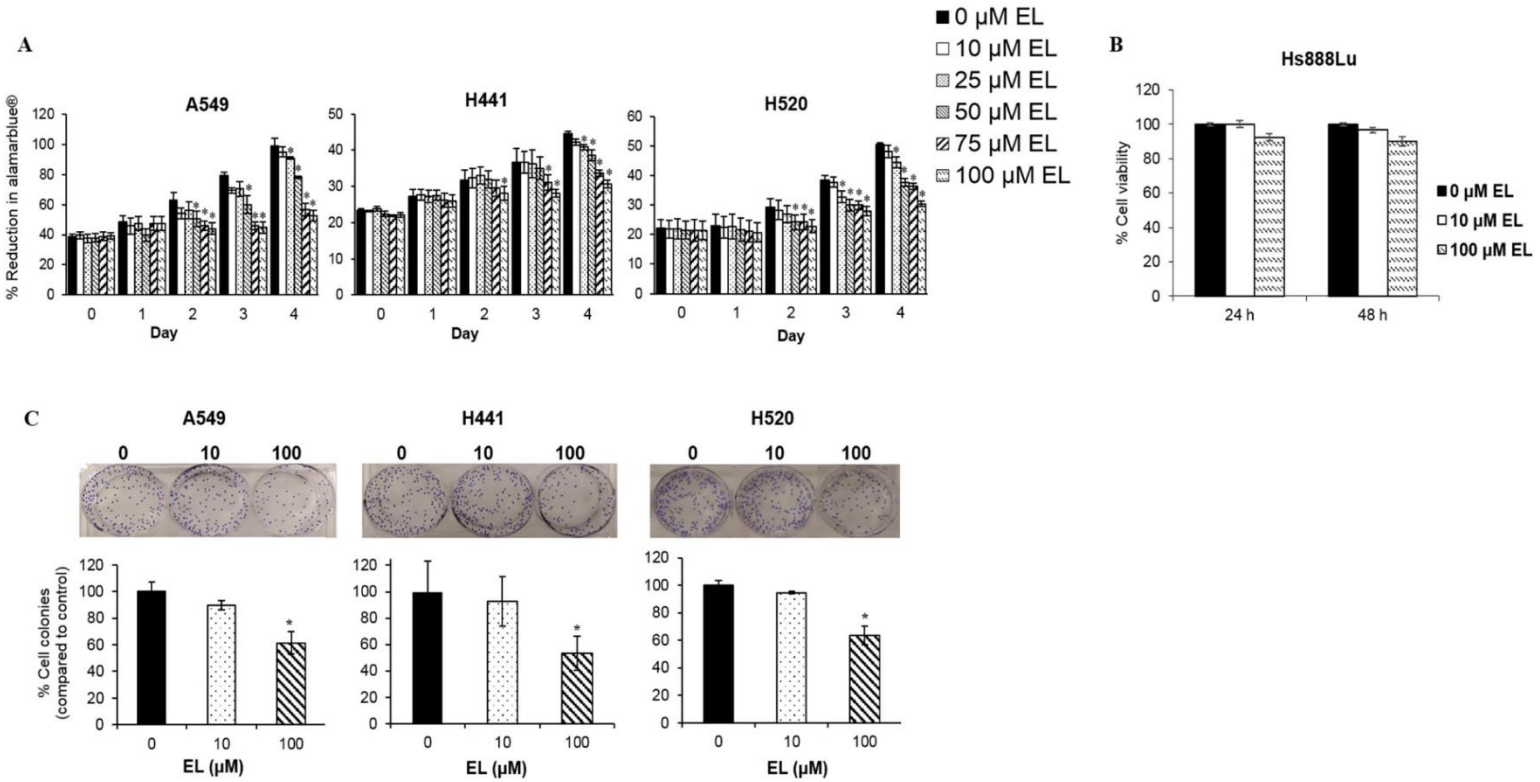

Figure 3. EL inhibits growth of lung cancer cell lines. (A) The short-term growth of A549, H441, and H520 was measured by the percentage of alamarBlue® reduction in the presence of EL (0-100 µM) over a period of 4 days. The data represent the average ± standard deviation of eight replicate wells for three independent experiments for every cell line. In A549 and H520 cells, EL-treatment (≥50 µM) and in H441 cells, EL (100 µM) resulted in a significant inhibition of growth in a time-dependent manner. (B) Cell viability of Hs888Lu cells was examined by a trypan blue exclusion assay following EL (0, 10, and 100 µM) treatment for 24 and 48 h. The bar graph represents the average cell count for two independent experiments. The error bars represent standard deviation from two independent experiments. (C) The long-term growth of lung cancer cells in the presence of EL (10 and 100 µM) was determined by a clonogenic survival assay. The fraction of cells surviving EL treatment was examined after 14 days. The image shown is representative of one typical experiment. The data shown in the bar graph represent the average ± standard deviation for the fraction of surviving cells for three independent experiments for each cell line. (* designates $p \leq 0.05$ as compared to control).

EL inhibits long-term proliferation of lung cancer cells in a concentration-dependent manner

Next, we examined the long-term growth-inhibitory effects of EL on these cells using a clonogenic survival assay. Clonogenic survival assays are used to determine the ability of single cells to survive and form colonies after exposure to radiation, a chemotherapeutic agent or a combination of the two. After 14 days, EL treatment (100 μM) significantly reduced the size and the number of A549, H441, and H520 cell colonies by 61%, 53%, and 63% ($p \leq 0.05$), respectively, compared to untreated cells (Fig. 3C). As with the short-term proliferation studies, a low concentration of EL (10 μM) failed to influence proliferation.

EL-mediated inhibition of lung cancer cell proliferation is not caused by enhanced cell death

We carried out a trypan blue exclusion assay to determine whether inhibition of lung cancer cell proliferation was a result of enhanced cell death, as indicated by increased cell membrane permeability for trypan blue. A549, H441, and H520 cells were treated EL (0, 10, 50, and 100 μM) for 24, 48, and 72 h. These concentrations were chosen to show a range of effects (low-moderate-high) for EL-mediated lung cancer cell death. We did not observe significant cell death, as measured by the number of trypan blue stained cells, for EL-treated cells at any time point or concentration (Fig. 4A). A slight, but statistically insignificant increase in H520 cell death was noted with increasing concentrations of EL after 48 h

To confirm the results from trypan blue exclusion assay we carried out an Annexin V-FITC/PI apoptosis assay. A549 cells were treated EL (0, 10, 50, and 100 μM) for 24, 48, and 72 h. Compared to the control, we did not observe any increase in cell death in A549 cells, as measured by the percentage of Annexin V positive/PI positive stained cells, in response to EL-treatment at any time point or concentration (Fig. 4B).

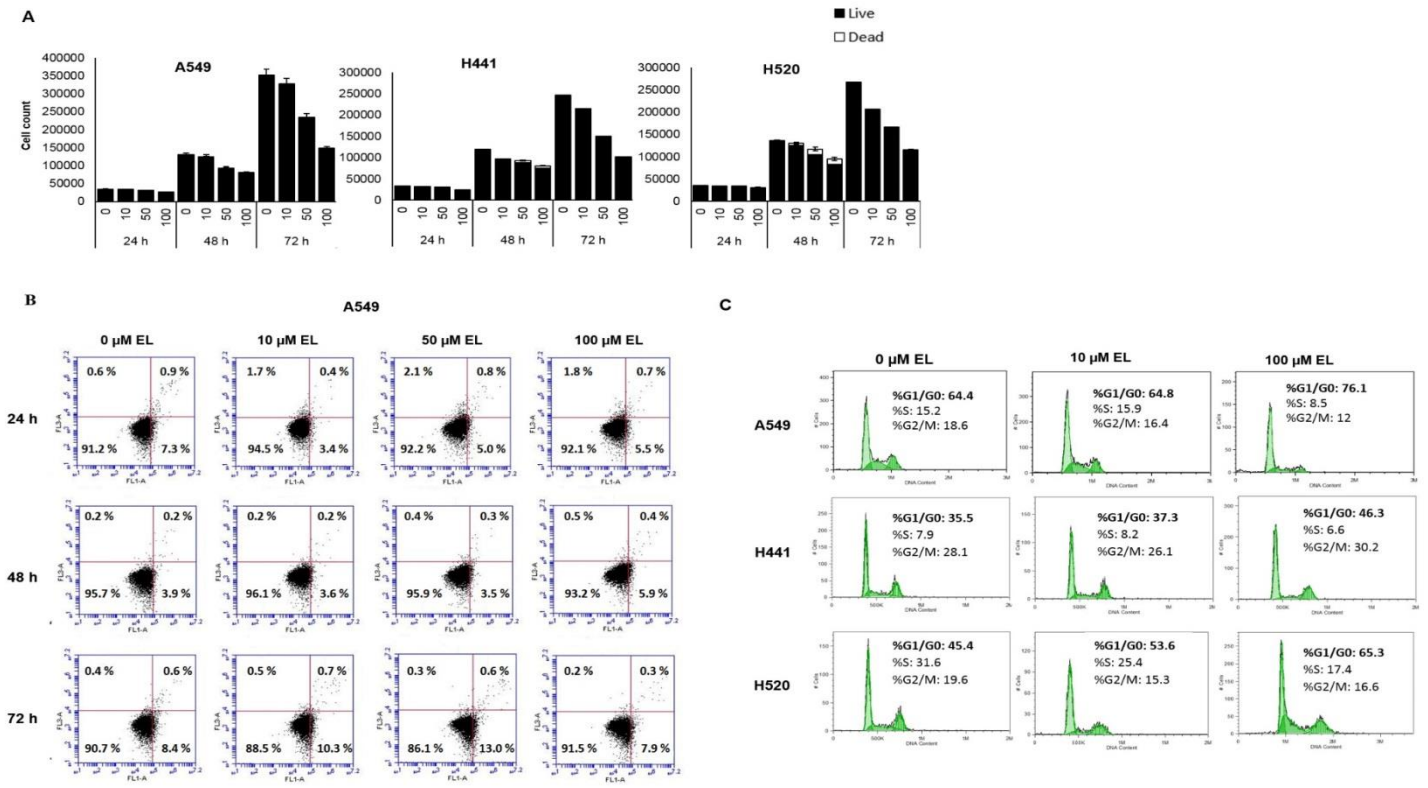

Figure 4. EL exposure results in G_1-phase cell cycle arrest, but not cell death. (A) Cell viability of A549, H441, and H520 cells was examined by a trypan blue exclusion assay following EL (0-100 μM) treatment for 24, 48, and 72 h. The bar graph represents the average total cell count, inclusive of live (black) and dead (white) cells, for three independent experiments for each cell line. The error bars represent standard deviation of the total number of dead cells from three independent experiments for each cell line. (B) An Annexin V-FITC/PI apoptosis assay was performed to further assess the potential cytotoxic effects of EL. A549 cells were treated with EL (0, 10, 50, and 100 μM) for 24, 48, and 72 h. The treated cells were then stained with Annexin V/PI and flow cytometric analysis was performed. The representative data from three independent experiments are shown in dot plots. (C) EL-induced G1-phase cell cycle arrest in lung cancer cells was examined by flow cytometry. The histograms show the cell cycle distribution for A549, H441, and H520 treated with the vehicle control or EL (10 and 100 μM) for 48 h. The data represent the average percentage of cells in each phase of the cell cycle for three independent experiments for each cell line.

EL suppresses lung cancer cell proliferation through G_1-phase cell cycle arrest

An alternative mechanism for reduced cell proliferation is through arrest of the cell cycle. Therefore, we investigated the effect of EL on cell cycle progression in NSCLC cells. A549, H441, and H520 cells that were synchronized by serum starvation for 24 h were treated with EL (10 and 100 μM) for 48 h. Cell cycle analysis was carried out using PI staining. EL-treatment led to an increase in the percentage of G_1-phase cells from 64% to 76% for A549 ($p \leq 0.05$), from 35% to 46% for H441 ($p \leq 0.05$), and from 45% to 65% for H520 ($p \leq 0.05$) cells (Fig. 4C). Simultaneously, the percentage of the cells in the S phase decreased.

EL modulates G_1-phase cell cycle regulatory genes in lung cancer cells

To explain how EL might arrest lung cancer cells in the G1-phase of the cell cycle, we used RT-qPCR to measure mRNA transcripts levels for cell cycle regulatory genes. A549, H441, and H520 lung cancer cells treated with 100 μM EL for 24 h, and the mRNA expression values for cyclin D1, CDK2 and CDK4, cdc25A, p21WAF1/CIP1 and p27KIP1 genes were determined. We observed significant changes in mRNA expression levels of CDK2 ($p \leq 0.05$) and p21WAF1/CIP1 ($p \leq 0.05$) in A549 cells; cyclin D1 ($p \leq 0.05$), CDK4 ($p \leq 0.05$), cdc25A ($p \leq 0.05$) in H441 cells; and cyclin D1 ($p < 0.05$), CDK2 ($p \leq 0.05$), CDK4 ($p \leq 0.05$), cdc25A ($p \leq 0.05$) and p21WAF1/CIP1 ($p \leq 0.05$) in H520 cells. mRNA expression of p27KIP1 showed differential responses in the three cell lines with a slight, but not significant increase in A549 cells, and a significant decrease in H441($p < 0.05$), and H520 ($p \leq 0.05$) cells (Fig. 5).

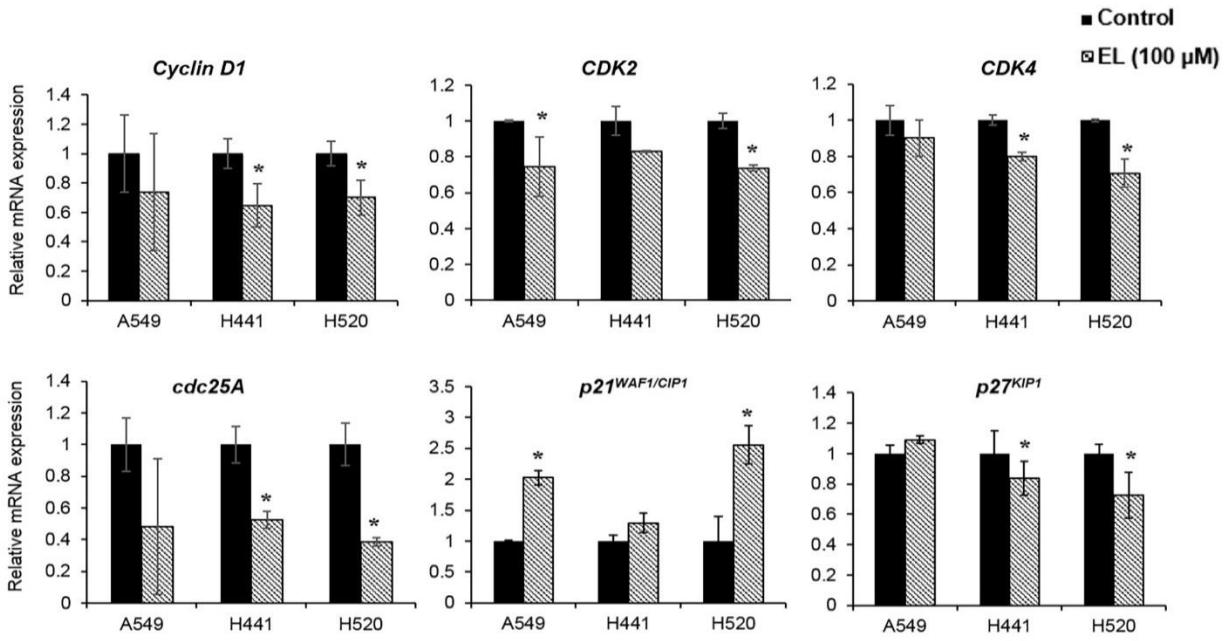

Figure 5. EL regulates the expression of G1-phase related genes. A549, H441, and H520 cells were treated with EL (100 µM) for 24 h. Relative mRNA expression levels for cyclin D1, CDK2, CDK4, cdc25A, p21WAF1/CIP1, and p27KIP1 were determined by quantitative PCR. The relative mRNA levels for all genes were normalized to 18S rRNA. The data shown in the bar graphs represent the average ± standard deviation of relative mRNA expression for each gene for three independent experiments for each cell line. (* designates $p \leq 0.05$ as compared to control).

EL alters the expression of G_1-phase cell cycle regulatory proteins in lung cancer cells

Next, we used western blotting to determine if EL-mediated changes in mRNA transcripts of G_1-phase regulating cyclins, CDKs, and CDKI correlated with changes at their protein levels. EL treatment (100 µM, 0-24 h) led to reduced expression of p-pRb protein in the three lung cancer cell lines, with the most notable decreases in H441 and H520 cells (Fig. 6A and B). Further, EL-treatment affected the expression levels of cell cycle-promoting proteins, such as cyclins, CDKs, and p-cdc25A proteins and cell cycle inhibitory protein, $p21^{WAF1/CIP1}$ in lung cancer cell lines. In A549, EL treatment led to a time-dependent decrease in cyclin D1, cyclin E1 (*p≤0.05,* 6, 12 and 24 h), p-cdc25A (*p≤0.05,* 24 h), CDK2 *(p≤0.05,* 24 h), CDK4 (*p≤0.05,* 6, 12, and 24 h), CDK6, and an increase in $p21^{WAF1/CIP1}$ (*p≤0.05,* 12 and 24 h), and

$p27^{KIP1}$ protein levels (Fig. 6C and 6D). Similarly in H441, EL treatment led to a time-dependent decrease in cyclin E (*p≤0.05,* 6, 12, and 24 h), p-cdc25A (*p≤0.05,* 6, 12, 24 h), CDK2 (*p≤0.05,* 6 h), CDK4 (*p≤0.05,* 6, 12, and 24 h), CDK6 (*p≤0.05,* 12 and 24 h), and a significant increase in $p21^{WAF1/CIP1}$ (*p≤0.05,* 6 h), and decrease in $p27^{KIP1}$ (*p≤0.05,* 24 h) protein levels (Fig. 6E and 6F). While in H520, EL treatment led to a time-dependent decrease in cyclin D1 (*p≤0.05,* 12 h), cyclin E1 (*p≤0.05,* 12 h), p-cdc25A, CDK2 (*p≤0.05,* 24 h), CDK4 (*p≤0.05,* 12 and 24 h), and CDK6 (*p≤0.05,* 12 and 24 h) (Fig. 6 G and 6H).

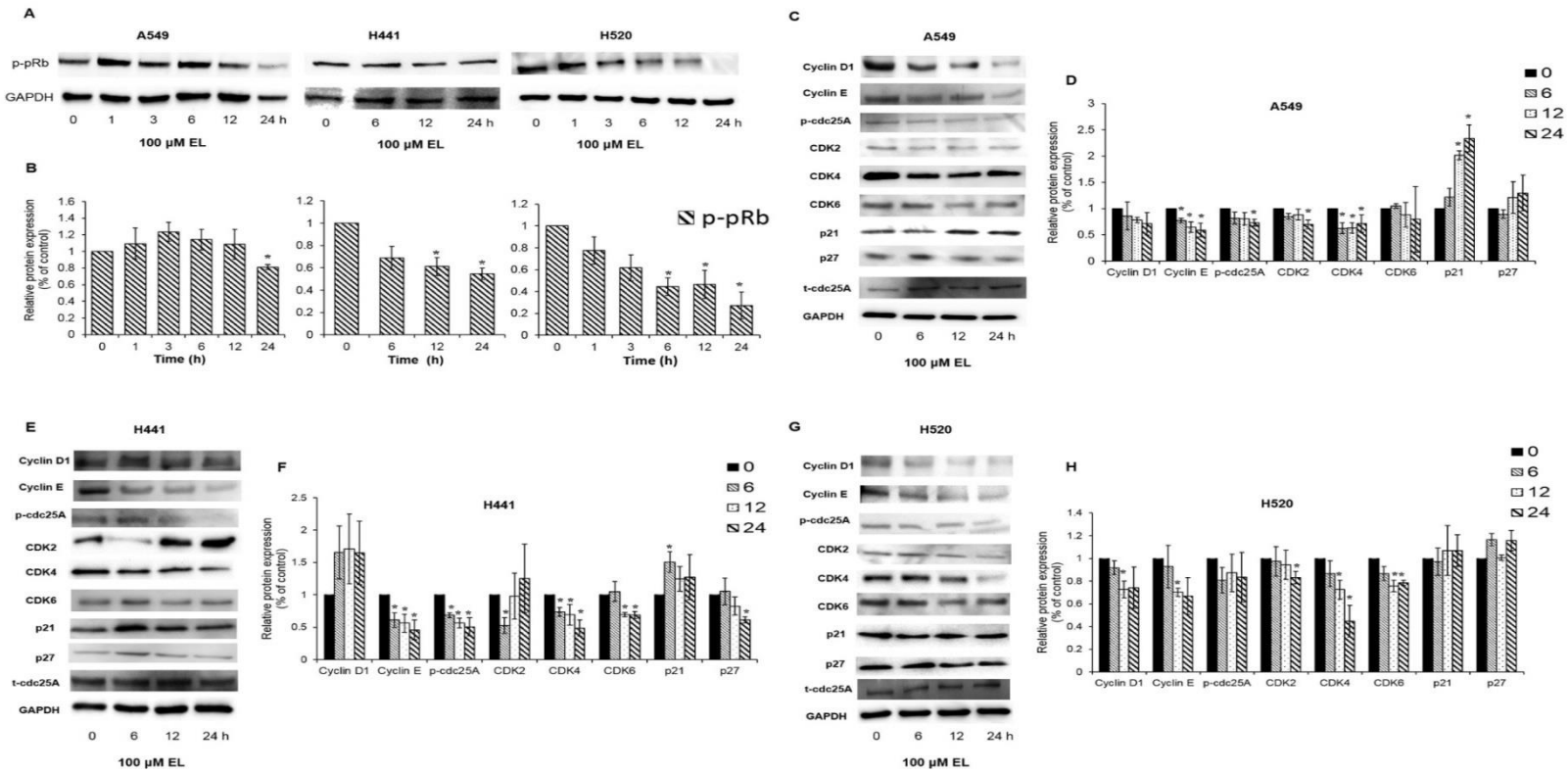

Figure 6. EL induces cell cycle arrest by modulating the expression of G_1-phase related proteins. **(**A) A549 and H520 cells were treated with EL (100 μM) for 0, 1, 3, 6, 12, and 24 h. H441 cells were treated with EL (100 μM) for 0, 6, 12, and 24 h. The cells were lysed to collect proteins for western blotting. 30 μg protein were loaded on the gels, and blots were probed with p-pRb. (B) Quantification of western blotting results was done using ImageJ. The data shown are the average of three independent experiments and are represented as mean ± S.E.M. A549 (C), H441 (E), and H520 (G) cells were treated with EL (100 μM) for 0, 6, 12, and 24 h. The cells were then lysed to collect proteins for western blotting. 30 μg protein were loaded on the gels, and blots were probed with cyclin D1, cyclin E, p-cdc25A, CDK2, CDK4, CDK6, p21WAF1/CIP1, and p27KIP1 G1-phase cell cycle regulatory proteins. Total cdc25A (t-cdc25A) protein levels were used to normalize phosphorylated cdc25A (p-cdc25A) protein levels. Quantification of western blotting results for A549 (D), H441 (F), and H520 (H) was done using ImageJ. The data shown are the average of three independent experiments and are represented as mean ± S.E.M. GAPDH was used as a loading control for the remaining western blots. (* designates $p \leq 0.05$ as compared to control).

Discussion

In this study, we evaluated the anti-proliferative effects of EL on NSCLC cells. The selected cell lines harbor different somatic mutations that influence their growth properties and ultimately their response to cancer therapies (Table 1). All the cell lines in this study carry wild-type EGFR. A549 and H441 cells are KRAS mutant, while H520 cells are wild-type KRAS. Further, A549 cells express wild-type p53, while H441 and H520 cells exhibit mutant p53. We found EL to have similar growth-inhibitory effects in all three lung cancer cell lines, which suggests that the growth-inhibitory properties of EL for these cells are independent of their KRAS and p53 gene status. In contrast to the significant growth-inhibitory effects of EL observed in lung cancer cells, no obvious growth-inhibitory effects of EL were detected in normal lung fibroblast cells.

Interestingly, our findings suggest that EL (≥50 μM, 48h) mediates suppression of NSCLC cell growth. Similarly, previous studies have also shown that high concentrations of EL are needed to inhibit in vitro growth of breast, colon, and prostate cancers. Xiong *et al*. (2015) demonstrated that EL with an IC50 of 261.9 ± 10.5 μM inhibited the growth of MDA-MB-231 breast cancer cells after 48 h of treatment [21]. Similarly, Danbara *et al*. (2005) and Chen *et al*. (2007) reported that treatment of COLO 201 colon cancer cells and LNCaP prostate cancer cells with 118.4 and 75 μM EL, respectively, for 72 h significantly inhibited their proliferation by 50% [20, 30]. Furthermore, our results suggest that the *in vitro* inhibition of lung cancer cell growth is not due to induction of cell death. This result is in contrast to *in vitro* studies of colon and prostate cancers that have shown that EL treatment results in a concentration- and time-dependent increase in apoptosis. Danbara *et al.* (2005) demonstrated that EL treatment (118.4 μM, 72 h) in COLO 201 colon cancer cells caused an increase in the percentage of cells in the

sub-G1 phase of the cell cycle which is indicative of apoptosis [20]. They further demonstrated that EL increased the expression of the cleaved form of caspase-3 protein, a marker of apoptosis, and simultaneously decreased the expression of Bcl-2 and PCNA proteins, markers of cell proliferation. Similarly, Chen *et al*. (2007) reported that exposure of LNCaP prostate cancer cells to increasing concentrations of EL (0 - 100 μM, 72 h) led to a gradual increase in sub-G0/G1 DNA content (apoptotic cells) from 2.6% to 56.3% [30]. Further, they showed that treatment of LNCaP cells with EL resulted in an increase in expression of cleaved caspase-3 and PARP proteins that initiate apoptosis. Although we observed slightly more dead cells in H520 cell line with 50 and 100 μM EL treatment (48 h, but not 72 h), the number of dead cells did not make up for the difference in cell count observed between the control and EL treatment, suggesting a cytostatic rather than cytotoxic effect of EL. It is possible that the cytotoxic effects of EL are dependent upon cancer cell type.

We did observe that EL treatment arrested lung cancer cells in the G_1-phase of the cell cycle. Our results are in partial agreement with the study by McCann *et al*. (2013) who observed G_1 and S phase arrest along with an increase in percentage of cells in the sub-G_0/G_1 phase in several prostate cancer cell lines after treatment with EL (20 μM) for 48 h [31]. However, differing results have been reported with regards to the stage-specific cell cycle arrest caused by EL. Studies by Xiong *et al*. (2015) and McCann *et al*. (2008) have shown that the treatment of MDA-MB-231 breast cancer cells with increasing concentrations of EL (50, 100, and 200 μM) and LNCaP prostate cancer cells with 60 μM EL for 48 and 72 h, respectively, induced accumulation of cells in S-phase [21, 32]. These findings may suggest that cell cycle arrest caused by EL is cell-type specific and concentration- and time-dependent.

Progression of cells through the G_1, S, G_2, and M cell cycle phases is positively regulated by a family of proteins called cyclins and cyclin dependent kinases (CDK), and negatively regulated by CDK inhibitors (CDKI). Cyclin D1 interacts with CDK4 and CDK6 to drive progression of cells through G_1 [23, 24]. Therefore, it is not surprising that we noticed down-regulation of cyclin D1 and CDK4 mRNA, which would explain arrest of lung cancer cells in the G_1-phase. Further, the association of cyclin E with CDK2 is active at the G_1-S transition and guides entry of cells into the S phase. We found that EL-treatment down-regulated CDK2 mRNA expression. On the other hand, CDKIs such as p21WAF1/CIP1 and p27KIP1 stop cell cycle progression to S-phase [23, 24]. They inhibit kinase activity of cyclins-CDKs complexes by binding to CDKs, and prevent their association with cyclins. We found that EL-treatment resulted in elevated levels of p21WAF1/CIP1 mRNA in lung cancer cell lines. In addition, cdc25A phosphatases, members of the tyrosine phosphatase family, catalyze dephosphorylation and activation of CDKs which play a role in G_1-S phase transition [24]. We found EL reduced cdc25A mRNA expression in lung cancer cell lines. Previous research by Xiong *et al*. (2015) found that EL treatment (0-200 μM, 48 h) in MBA-MD-231 breast cancer cell line significantly decreased mRNA levels of cyclin E1, cyclin A2, cyclin B1, and cyclin B2 which play a role in S and G2/M phases in the cell cycle [21]. They observed no significant change in mRNA levels of cyclin D1, CDK2, and CDK4.

The tumor suppressor protein pRb plays a critical role in the G_0/G_1 and G_1-S phase transitions. In quiescent cells, pRb exists in an activated/hypo-phosphorylated state, and sequesters members of the E2F gene family of transcription factors, which suppress cell-cycle progression [23]. Phosphorylation of pRb leads to the disruption of the pRb-E2F transcription complex, and the release of active E2F, which promotes cell-cycle progression [23]. We

observed that EL-treatment caused a significant reduction in phosphorylated p-pRb protein in H520 and H441 cell lines. Phosphorylation of pRb is normally initiated by the cyclin D1-CDK4/6 complex. As expected, we found EL treatment resulted in reduced expression of CDK4 and CDK6 protein in lung cancer cell lines. At the time of G_1-S phase transition cyclin E is actively synthesized and accumulated to activate CDK2, leading to further pRb phosphorylation resulting in E2F activation and subsequent entry of the cells into S-phase [23]. We observed that EL-treatment led to a significant reduction in cyclin E protein expression in lung cancer cell lines, as was CDK2 at certain time points.

The findings from this study suggest that exposure to high concentrations of EL ($\geq$ 50 µM) is required to inhibit in vitro growth of lung cancer cells. Achieving such a high plasma concentration of EL from flaxseed consumption may prove difficult given that 25-50 g of flaxseed per day, which is equivalent to consumption of 500 mg/day, results in no more than 2 µM EL in the plasma [33, 34]. Consumption of purified SDG may be more effective at achieving high plasma EL concentrations than consumption of flaxseed. Setchell *et al*. (2013) showed that increasing SDG dose (25-173 mg/day) concomitantly increased plasma EL levels [35]. This suggests that consumption of purified SDG may be needed to achieve EL concentrations capable of lung cancer cell growth inhibition. It is not clear what the tolerable dose of SDG is for human consumption or what plasma EL levels are achievable. Further, it is likely that a clinical application of EL would involve combination with other anti-cancer agents. For example, EL combined with tamoxifen led to enhanced inhibition of breast cancer cell adhesion and invasion [36].

Conclusions

In summary, EL inhibits cell proliferation by inducing G_1-phase cell cycle arrest. EL treatment results in down-regulation of cell cycle promoting cyclins and CDKs and up-regulation of their inhibitors, leading to decreased phosphorylation of pRb protein and thereby, decreased expression of genes that promote G_1 to S-phase transition (Fig. 7). Development of cancer therapies that are effective against KRAS and p53 mutant cancers have proven clinically challenging. Therefore, identification of new non-toxic natural chemotherapeutic agents that target downstream effectors of mutant-*KRAS* and *p53* signaling appears to be a novel treatment strategy for NSCLC.

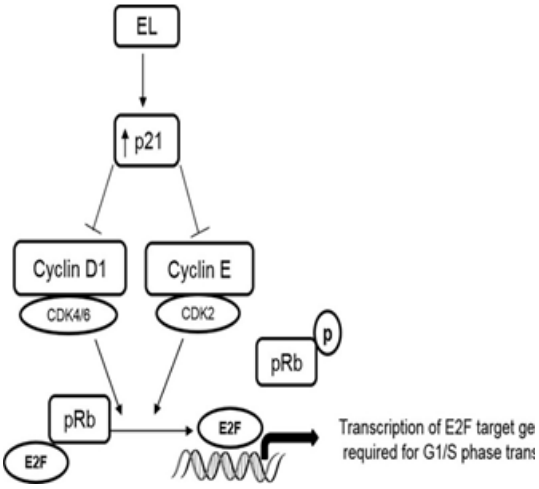

Figure 7. Proposed molecular mechanism of EL-induced G_1-phase cell cycle arrest. EL suppresses expression of cyclin D1-CDK4/6 and cyclinE-CDK2 proteins by up-regulating expression of the cell cycle inhibitor p21WAF1/CIP1. EL-mediated down-regulation of cyclin-CDKs complexes suppresses phosphorylation of pRb. Hypo-phosphorylated pRb binds to transcription factor E2F, and represses its transcriptional activity and the activation of genes required for the G1-S phase transition, such as cdc25A. This leads to G1-phase cell cycle arrest and growth inhibition.

Acknowledgements

We would also like to thank Dr. Devendra Chikara for editing the paper.

Funding

This work was supported by grants from National Science Foundation (No. HRD-0811239) and National Institute of Health (No P30 GM103332-01) from the National Center for Research Resources.

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CHAPTER 3. ENTEROLACTONE ALTERS FAK-SRC SIGNALING AND SUPPRESSES MIGRATION AND INVASION OF LUNG CANCER CELL LINES

Abstract

Systemic toxicity of chemotherapeutic agents and the challenges associated with targeting metastatic tumors are limiting factors for current lung cancer therapeutic approaches. To address these issues, plant-derived bioactive components have been investigated for their anti-cancer properties because many of these agents are non-toxic to healthy tissues. Enterolactone (EL) is a flaxseed-derived mammalian lignan that has demonstrated anti-migratory properties for various cancers, but EL has not been investigated in the context of lung cancer, and its anticancer mechanisms are ill-defined. We hypothesized that EL could inhibit lung cancer cell motility by affecting the FAK-Src signaling pathway. Non-toxic concentrations of EL were identified for A549 and H460 human lung cancer cells by conducting 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Dephenyltetrazolium Bromide (MTT) assays. The anti-migratory and anti-invasive potential of EL for lung cancer cell lines was determined by scratch wound healing and Matrigel® invasion assays. Changes in filamentous actin (F-actin) fiber density and length in EL-treated cells were determined using phalloidin-conjugated rhodamine dye and fluorescent microscopy. Vinculin expression in focal adhesions upon EL treatment was determined by immunocytochemistry. Gene and protein expression levels of FAK-Src signaling molecules in EL-treated lung cancer cells were determined using PCR arrays, qRT-PCR, and western blotting. Non-toxic concentrations of EL inhibited lung cancer cell migration and invasion in a concentration- and time-dependent manner. EL treatment reduced the density and number of F-actin fibers in lung cancer cell lines, and reduced the number and size of focal adhesions. EL

decreased phosphorylation of FAK and its downstream targets, Src, paxillin, and decreased mRNA expression of cell motility-related genes, RhoA, Rac1, and Cdc42 in lung cancer cells. Our data suggest that EL suppresses lung cancer cell motility and invasion by altering FAK activity and subsequent activation of downstream proteins needed for focal adhesion formation and cytoskeletal rearrangement. Therefore, administration of EL may serve as a safe and complementary approach for inhibiting lung tumor cell motility, invasion, and metastasis.

Introduction

Enterolactone (EL) is a mammalian lignan derived from the plant lignan secoisolariciresinol diglucoside (SDG) that shows anti-migratory effects for breast, colon, and prostate cancer cells with limited or no toxicity to healthy cells [1-6]. EL suppressed adhesion, motility, and invasion of breast cancer cells by remodeling the actin cytoskeleton, downregulated gene expression of matrix metalloproteinases (MMP-2, -9, and -14), and inhibited FAK signaling [4, 5]. However, it is not clear what effect EL has on lung cancer cell motility. Further, the anti-cancer mechanisms for EL have not been clearly established for lung cancer.

Support for the use of lignans such as SDG or EL in lung cancer therapy comes from research showing their protective effects for healthy lung tissues in lung injury models. SDG reduced murine lung inflammation and oxidative damage inflicted by radiation, a standard treatment for metastatic lung cancer [7-10]. Given the protective effects of lignans for healthy lung tissue, and their anti-migratory effects for other cancer types, we hypothesized that EL would inhibit lung cancer cell migration.

Focal adhesion kinase (FAK) and steroid receptor coactivator (c-Src) are signaling proteins that regulate cytoskeletal dynamics and cell motility by influencing actin polymerization and focal adhesion turn-over [11, 12]. FAK and Src expression are elevated in non-small cell

lung cancer (NSCLC) tissues as compared to normal lung tissue, and positively correlate with advanced stages of disease [13-15]. Preclinical studies have shown that FAK and Src inhibitors effectively suppress lung cancer metastases; however, these agents induce cytotoxicity in healthy tissues [16, 17]. Therefore, there is a need for less toxic agents that target FAK-Src signaling and inhibit lung cancer cell motility.

The objective for this research was to identify EL as a less toxic agent to inhibit lung cancer cell motility, and to determine its anti-migratory mechanisms by focusing on FAK-Src signaling and down-stream effects. Our central hypothesis was that EL inhibits lung cancer cell motility by altering focal adhesion formation and F-actin structure by decreasing FAK-Src signaling. To address this hypothesis, we investigated the anti-migratory and anti-invasive effects of EL on NSCLC cells (A549 and H460), and determined the impact on focal adhesion formation, actin filaments, and the expression of mRNA and proteins associated with cell motility. Our findings suggest that EL or its parent lignan compound SDG could be used to inhibit NSCLC cell motility by influencing FAK-Src signaling.

Materials and Methods

Materials

Purified EL (99.2% pure) was purchased from ChromaDex, Inc (Santa Ana, CA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Dephenyltetrazolium Bromide (MTT) was obtained from AbD Serotech (Raleigh, NC). Matrigel® invasion chambers with 8 μm pore size inserts were obtained from BD Biosciences (Bedford, MA). Crystal violet was obtained from Alfa Aesar (Ward Hill, MA). Rhodamine phalloidin was purchased from Thermo Fisher Scientific (Waltham, MA). Antibodies for p-FAK^{Tyr397}, t-FAK, p-paxillin^{Tyr118}, t-paxillin, p-Src ^{Tyr416}, p-Src ^{Tyr527}, t-Src, RhoA, p-Rac/Cdc42, t-Rac/Cdc42, GAPDH, and anti-rabbit HRP-conjugated secondary antibody

were purchased from Cell Signaling Technology (Danvers, MA). Antibody for vinculin was purchased from EMD Millipore (Billerica, MA). Ki-67 and anti-mouse Alexa 633 fluorophore-conjugated antibodies were purchased from Abcam (Cambridge, MA). DAPI was obtained from Biotium (Fremont, CA). Coverslips were obtained from Carl Zeiss (Ontario, Canada).

Cell culture and drug treatment

The human NSCLC cell lines A549 and H460 were purchased from ATCC (Manassas, VA). Cells were maintained in RPMI-1640 medium (Sigma Aldrich; St. Louis, MO), with 10% (v/v) fetal bovine serum (FBS; Atlanta Biologicals; Flowery Branch, GA) and 1% penicillin/streptomycin (Thermo Fisher Scientific). The cell lines were incubated at 37˚C in a humidified atmosphere of 95% air and 5% carbon dioxide. They were sub-cultured by enzymatic digestion with 0.25% trypsin/1mM EDTA solution (Hyclone; Logan, UT) when they reached approximately 70% confluency.

EL was dissolved in 100% dimethysulfoxide (DMSO; Corning Cellgro®; Corning, NY) at a stock solution concentration of 200 mM. The stock solution was freshly diluted in PBS (pH 7.2) to desired concentrations for every experiment. The vehicle control was 0.2% DMSO.

MTT assay

The effect of EL on the viability of A549 and H460 cells lines was studied using the MTT assay. Briefly, 3000 cells/well were plated in a 96-well plate. After 24 h of incubation, medium was removed and the cells were washed with PBS. Fresh RPMI-1640 medium was added, and the cells were treated with vehicle (DMSO) or various concentrations of EL (0, 10, 25, 50, 75, and 100 μM) for 24 and 48 h. Next, 10 μl MTT (0.5 mg/ml) was added to each well, and the plate was incubated for 4 h at 37 °C. Later, the medium was removed and the cells were washed with PBS. The resulting formazan crystals were dissolved in 100 μl of DMSO, and the

absorbance reading of each well was taken at 570 nm using a plate reader (Microplate XMark^{TM} spectrophotometer; Bio-Rad; Hercules, CA). The percentage of cell survival was calculated using the background-corrected absorbance as shown in the following formula:

$$\text{Cell survival (\%)} = (\text{Absorbance}_{\text{Treatment}} / \text{Absorbance}_{\text{Control}}) \text{ X } 100$$

The data shown represent the mean and standard deviation from 8 replicate wells for each treatment for 3 independent experiments.

Migration assay

The ability of EL to inhibit migration of A549 and H460 cells was investigated using a wound healing assay. Cells were seeded into 6-well dishes and grown to 80-90% confluency. A sterilized 10 μl pipette tip was used to generate a wound across the cell monolayer. The cellular debris was washed with PBS, serum-free RPMI-1640 medium was added to each well, and the cells were treated with fresh vehicle (DMSO) or EL (10, 50, and 100 μM) every 24 h. The open gap was photographed microscopically after 24 and 48 h. The migration ability of the cells was determined by measuring the width of the monolayer wound for three fields per treatments at 24 and 48 h after scraping, and the migration index was calculated using the following formula, where 'T' stands for time (24 or 48 h).

$$\text{Migration index} = \left(\frac{\text{"0" h scratch width} - \text{"T" h scratch width)}}{\text{0 h scratch width}}\right) \text{X } 10$$

The data shown represent the mean and standard deviation of 3 independent experiments.

Ki-67 Immunocytochemistry

To verify that EL inhibited migration of lung cancer cells independent of its effects on cell proliferation, we stained lung cancer cells with the proliferation marker Ki-67. Cells were seeded into 6-well dishes, and after they reached 80-90% confluency, a wound across the cell

monolayer was created with a sterilized 10 µl pipette. After removal of cellular debris, serum-free RPMI-1640 medium was added, and the cells were treated with vehicle (DMSO) or EL (100 µM) for 24 and 48 h, with fresh treatment added after 24 h. The cells were fixed with 4% formaldehyde solution and permeabilized with 0.1% Triton-X 100. Next, the cells were washed with 1x TBS-tween (0.1%) and blocked with 10% normal goat serum (NGS) for 1 h, washed with 1x TBS-tween (0.1%), and incubated with anti-Ki-67 primary antibody for 3 h. The cells were washed again with 1x TBS-tween (0.1%) and incubated with Alexa Fluor 633 anti-rabbit secondary antibody for 1 h. After DAPI counterstaining, the number of Ki-67 positive cells across the wound were examined using a Zeiss Axio Observer Z1 inverted microscope with LSM700 laser scanning unit and 20x 0.8 objective (Zeiss, Thornwood, NY). The cell proliferation ratio was calculated using ImagePro Premier software (Media Cybernetics, Silver Spring, MD, USA). The data shown represent the mean and standard deviation of 2 random fields per sample for three independent experiments for each cell line.

Invasion assay

The ability of EL to inhibit invasion of A549 and H460 cells was investigated using Matrigel® invasion chambers consisting of invasion inserts (8 µm pore size). A549 and H460 cells (5 x 10^6 cells/well), suspended in serum-free RPMI-1640, were placed in the upper chamber of the transwell inserts and incubated with vehicle (DMSO) or EL (100 µM). RPMI-1640 medium supplemented with 50% FBS was added to the lower chamber. The plates were incubated in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C for 24 and 48 h. Fresh serum-free medium along with EL treatment was added in the upper chamber after 24 h. The non-invasive cells, present on the inside of the upper chamber, were removed by wiping with a cotton swab dipped in PBS, and invasive cells, present on the underside of the upper chamber,

were fixed with 4% formaldehyde in PBS and later stained with 2% crystal violet. Invasive cells were then photographed under a light microscope at 200X.

Cytoskeleton organization analysis

Actin cytoskeleton staining was carried out with phalloidin conjugated-rhodamine dye. A549 and H460 cells, grown on glass coverslips, were treated with vehicle (DMSO) or EL (10, 50, and 100 μM) for 24 h. After treatment, the cells were washed twice with PBS and fixed with 4% formaldehyde in PBS for 15 min at room temperature. The cells were washed twice with PBS and permeabilized with 0.1% Triton-X 100 for 5 min. Following permeabilization, the cells were washed with PBS and blocked with 1% BSA for 1 h. The cells were then incubated with phalloidin conjugated-rhodamine for 20 min at 37 °C. After incubation, the cells were washed with PBS and incubated with DAPI for 3-4 min to label nuclei. Finally, coverslips were mounted onto slides with the help of aqua-poly-mount mounting medium. Confocal images were acquired using an inverted fluorescence microscope with 40X oil immersion lens. At least 5 independent random fields per sample were captured from three independent experiments. The acquired images were converted into binary images for quantification of the density and average length of F-actin fibers using Image Pro Premier software (9.0).

Immunofluorescence and confocal microscopy

To visualize focal adhesions, A549 and H460 cells grown on fibronectin-coated coverslips were treated with vehicle (DMSO) or EL (100 μM) for 24 h. After treatment, the cells were washed with PBS, fixed with 4% formaldehyde solution for 15 min at room temperature, and permeabilized with 0.1% Triton-X 100 for 10 min. Next, the cells were washed with 1x TBS-tween (0.1%),blocked with 10% NGS for 1 h, washed with 1x TBS-tween (0.1%), and incubated with anti-vinculin primary antibody for 3 h. The cells were washed again with 1x

TBS-tween (0.1%) and incubated with Alexa Fluor 633 anti-mouse secondary antibody for 1 h. These cells were then incubated with DAPI for 3-4 min to label nuclei. The coverslips were then mounted onto slides with the help of aqua-poly-mount mounting medium and examined using Zeiss Axio Observer Z1 inverted microscope with LSM700 laser scanning unit and 40x 1.3NA oil objective (Zeiss, Thornwood, NY). The number and size of focal adhesions per cell were examined analyzed using the ImagePro Premier software (Media Cybernetics, Silver Spring, MD, USA) in 10 individual cells for each treatment for three independent experiments.

Microarray analysis for cell-motility related genes

Total RNA was isolated from untreated control and EL-treated (100 μM; 24 h) A549 cells (1 X 10^6 cells) using the Fisher SurePrep kit as per the manufacturer's instructions. cDNA was synthesized using 100 ng of total RNA and the qScript cDNA synthesis kit (Quanta Biosciences; Beverly, MD). Subsequently, qPCR was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences) and the PrimePCR™ Pathway Plates (Bio-Rad; Hercules, CA) to investigate the effects of EL on cell motility-related genes regulating FAK and platelet-derived growth factor (PDGF) signaling. The cycling parameters were: 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 60°C for 1 min, and a dissociation program that included 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s ramping up at 0.2 °C/s. One distinct peak was observed for each primer set, suggesting target specificity. Duplicate wells were run for each experiment, and the experiment was performed in duplicate. The relative change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method using housekeeping genes TBP, GAPDH, and HPRT1 as internal controls.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from untreated control and EL-treated (100 μM; 24 h) A549 and H460 cells (1 X 10^6 cells) using the Fisher SurePrep Kit (Waltham, MA) as per the manufacturer's instructions. cDNA was synthesized using 100 ng of total RNA and the qScript cDNA synthesis kit (Quanta Biosciences). Primers for RhoA, Rac, Cdc42, and 18S rRNA were designed using Primer Express software (version 2.0, Applied Biosystems; Foster City, CA), and were synthesized by Integrated DNA Technologies (Coralville, IA). The primer sequences were as follows: RhoA Forward GAGTTGGCTTTGTGGGACACA, RhoA Reverse ACTATCAGGGCTGTCGATGGA, Rac1 Forward GCTTATGGGATACAGCTGGACAA, Rac1 Reverse AGGACTCACAAGGGAAAAGCAA, Cdc42 Forward GATTACGACCGCTGAGTTATCCA, Cdc42 Reverse CAGGCACCCACTTTTCTTTCAC.

Steady-state mRNA levels for the cell cycle-related genes were evaluated by qPCR using PerfeCTa SYBR Green FastMix (Quanta Biosciences). The cycling parameters were: 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 60°C for 1 min, and a dissociation program that included 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s ramping up at 0.2 °C/s. One distinct peak was observed for each primer set, suggesting target specificity. Duplicate wells were run for each experiment and the experiment was performed in triplicate. The relative change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method using the housekeeping gene 18S rRNA as an internal control.

Western blotting

A549 and H460 lung cancer cells (3 x 10^5 cells/well) were seeded in a 24-well plate. After 24 h of incubation, the cells were treated with vehicle (DMSO) or EL (100 μM) for 0, 1, 3, 6, 12, and 24 h. The cells were then harvested by trypsinization, centrifuged at 300 x g for 10

min. The resulting cell pellet was then lysed by brief sonication in 100 µl of SDS lysis buffer (Cell Signaling Technologies) containing protease and phosphatase inhibitors (Roche; Indianapolis, IN) to dissociate cell membranes. Fifty micrograms of total protein isolated from these cells were electrophoresed on 7.5% SDS-polyacrylamide gels at 100 V for 1 h. Proteins were then transferred to nitrocellulose membranes at 100 V at 4°C for 70 min. The blots were then probed overnight at 4 °C with primary antibodies (1:1000) for p-FAK^{Tyr397}, t-FAK, p-paxillin^{Tyr118}, t-paxillin, p-Src^{Tyr416}, p-Src^{Tyr527}, t-Src, RhoA, p-Rac/Cdc42, t-Rac/Cdc42, and GAPDH. The next day, the blots were rinsed with 1X TBS-tween (0.1%) and probed with anti-rabbit HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. The western blots were analyzed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and the images were captured using the MultiImage™ Light Cabinet (Alpha Innotech; San Leandro, CA). Western blotting was performed in triplicate. The densitometry results were obtained using ImageJ software.

Statistical analysis

Data are presented as means ± standard deviation for at least 3 independent experiments. The statistical significance of difference between the control and treatment groups was determined by paired t-test or two-way ANOVA. $p \leq 0.05$ were considered statistically significant.

Results

EL has minimal effect on growth of lung cancer cells at 24 and 48 h

To investigate the effect the EL on lung cancer cell viability, A549 and H460 cells were treated with different concentrations of EL (0-100 µM) for 24 and 48 h. Viability was assessed using the MTT assay. The results show that EL had no effect on the proliferation of A549 and

H460 cells at 24 h (Fig. 8A and 8B). A minimal concentration-dependent decrease in cell proliferation was observed in response to EL-treatment at 48 h, with a 20% decrease observed in A549 and 15% decrease in H460 treated with the highest concentration (100 µM) of EL (Fig. 8A and 8B).

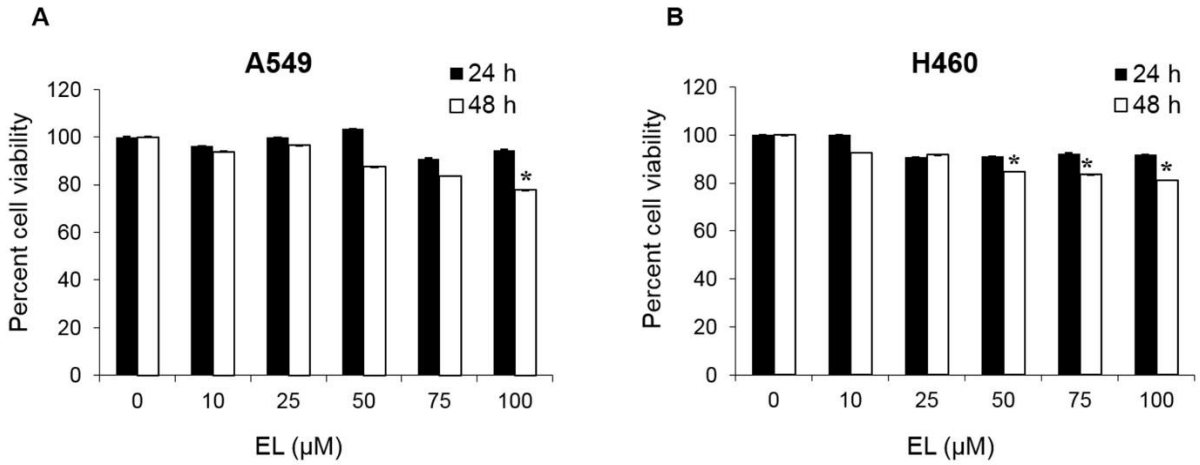

Figure 8. EL has minimal effects on lung cancer cell viability. Lung cancer cell lines (A) A549 and (B) H460 were treated with different concentrations of EL (0, 10, 25, 50, 75, and 100 µM) for 24 and 48 h, and cell viability was measured using an MTT assay. The data represent the average ± standard deviation of eight replicate wells for three independent experiment for each cell line. $p \leq 0.05$ was considered statistically significant when compared with untreated control.

EL inhibits in vitro migration of lung cancer cells

A scratch wound healing assay was used to examine the anti-migratory effects of EL in A549 and H460 cells. Cells were either treated with vehicle control (DMSO) or EL (10, 50, 100 µM) for 24 and 48 h. Control A549 and H460 cells demonstrated their migration potential by resulting in 55 and 40% wound repair after 24 h, and 100 and 90% wound repair after 48 h, respectively (Fig. 9). On the other hand, EL treatment (10, 50, and 100 µM) of A549 cells suppressed wound healing in a concentration- and time- dependent manner, with 42, 22, and 23% wound closure after 24 h, respectively (Fig. 9A and 9B), and 88, 70, and 56% wound

closure after 48 h, respectively (Fig. 9A and 9B). For H460 cells, 10, 50, and 100 µM EL treatment resulted in 35, 28, and 17% wound closure after 24 h (Fig. 9C and 9D), respectively, and 39, 39, and 36% of wound closure after 48 h (Fig. 9C and 9D), respectively.

Ki-67 immunocytochemistry was performed to rule out the possibility that EL inhibited cell proliferation which resulted in reduced cell migration. After creating a scratch wound, A549 and H460 cells were either treated with the vehicle control (DMSO) or EL (100 µM) for 24 and 48 h. The cell proliferation rate, qualitatively measured by the number of Ki-67 positive cells near the wound edge, was similar in control and EL-treated A549 and H460 cells (Fig. 9E and 9G). In A549 cells, on an average, there were 7.9% Ki-67 positive cells in the control group and 8.4% in the treatment group after 24 h, and 9.4% and 9.0%, respectively, after 48 h (Fig. 9F). Similarly, in H460, these numbers were 8.8% and 9.2% after 24 h, and 10.1% and 9.5% after 48 h (Fig. 9H). These results suggest that the anti-migratory effects of EL on lung cancer cells are independent of its effect on cell proliferation.

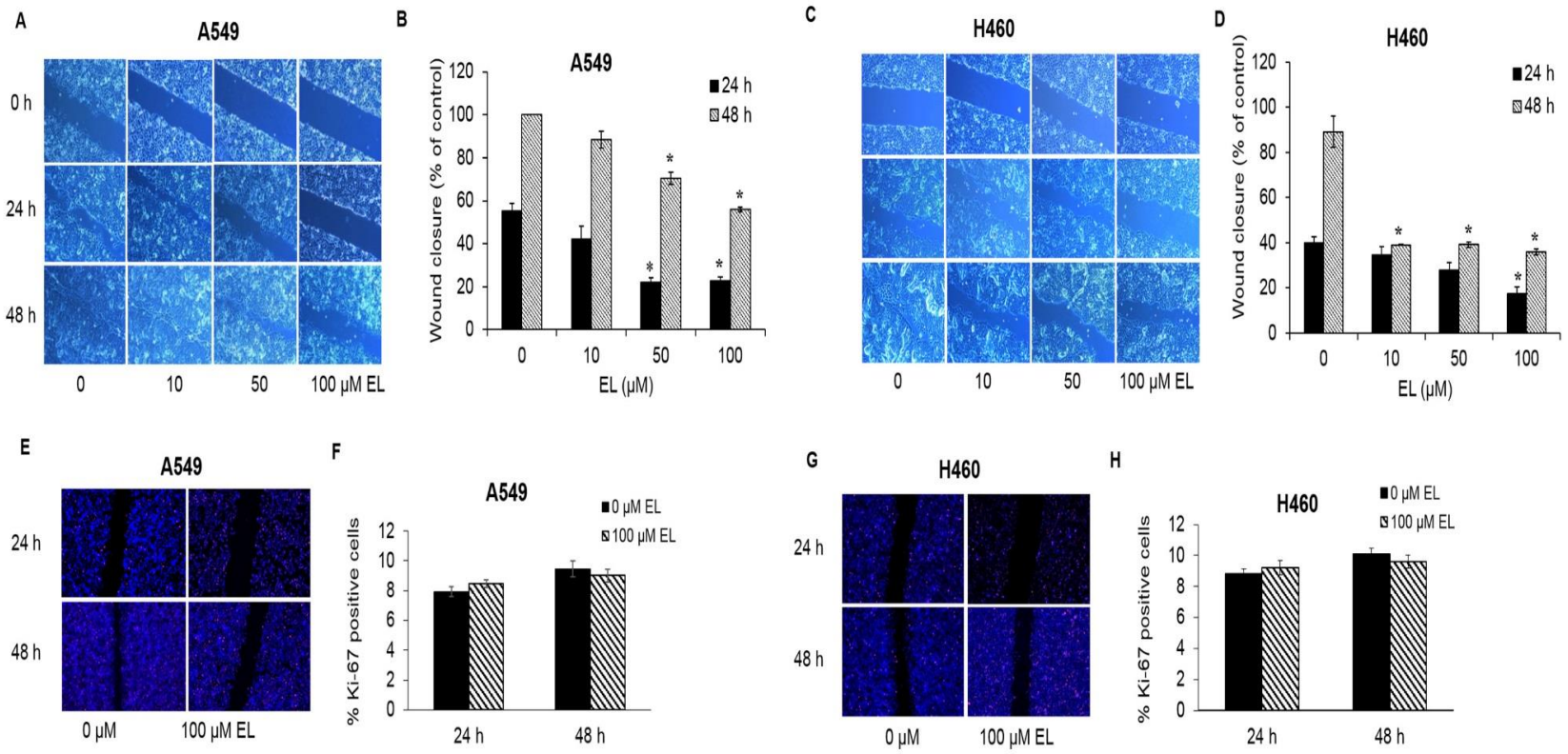

Figure 9. EL impairs *in vitro* migration potential of lung cancer cells independent of cell proliferation. (A) A549 and (C) H460 cells were grown to 90% confluency in cell culture dishes. A scratch/wound was made in each dish. The cells were then treated with 0, 10, 50, and 100 μM EL for 24 or 48 h. Images were taken at each time point for the respective control and treatment groups. The distance across the wound was measured for three replicate experiments for (B) A549 and (D) H460 cells and quantified as the % migration index. Ki-67 staining and quantification of (E and F) A549 and (G and H) H460 cells were performed to identify the % of Ki-67 positive cells near the wound. The data represent the average ± standard deviation % migration index for three fields per treatments for three independent experiments. $p \leq 0.05$ was considered statistically significant when compared with untreated control.

EL inhibits in vitro invasion of lung cancer cells

The effect of EL treatment (100 µM) on cancer cell invasion after 24 and 48 h was studied using Matrigel® invasion chambers. As shown in Figure 10A and 10C, compared to the untreated control, the number of invading cells represented by crystal-violet stain was reduced remarkably in a time-dependent manner in A549 and H460 cells. The inhibitory effects of EL (100 µM) on cancer cell invasion, as measured by the percentage of control, were 47% and 68% in A549 cells, and 20% and 37% in H460 cells, after 24 and 48 h, respectively (Fig. 10B and 10D). These results suggest that EL inhibits the *in vitro* invasive potential of lung cancer cells.

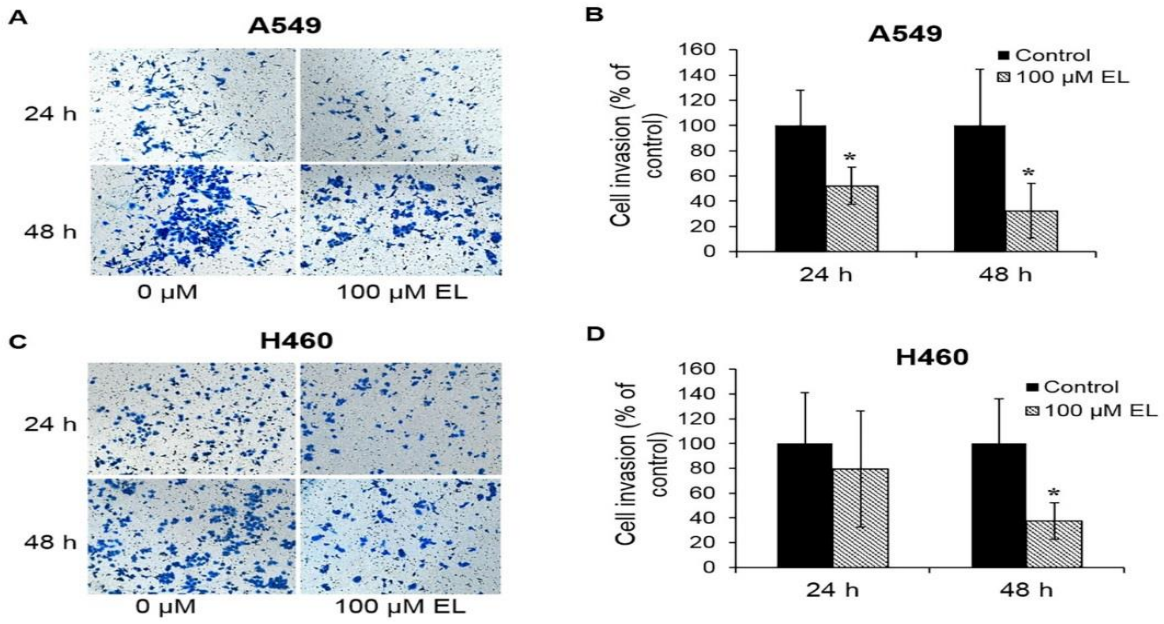

Figure 10. EL suppresses *in vitro* invasive potential of lung cancer cells. (A) A549 and (C) H460 cells were placed in the upper chamber of the Matrigel inserts (8 µm pore size) in serum-free RPMI-1640 medium, and then treated with 0 or 100 µM EL for 24 or 48 h. The number of invaded cells were fixed with 4% formaldehyde and stained with 2% crystal violet. Invasive cells were then photographed under a light microscope at 200x. The number of invasive (B) A549 and (D) H460 cells were counted for three replicate experiments and quantified. $p \leq 0.05$ was considered statistically significant when compared with untreated control.

EL affects the actin cytoskeleton in lung cancer cells

Given the importance of the cytoskeletal structure on cell motility, the effect of EL treatment (100 µM; 24 h) on the distribution of F-actin fibers was visualized with the help of phalloidin-conjugated rhodamine dye. The results from immunofluorescence microscopy indicate that after 24 h, control A549 and H460 cells exhibited dense F-actin fibers, while EL-treated cells showed a loss of F-actin fibers (Fig. 11A and 11D). Further, long F-actin fibers running across the cell body were seen in control cells, while branched and broken actin fibers were observed in EL-treated cells (Fig. 11A and 11D). Compared to the control, EL treatment resulted in a decrease in the percentage of polymerized F-actin fibers from 52.9% to 39.2% in A549 cells and from 50.9% to 32.9% in H460 cells (Fig. 11B and 11E). This difference was statistically significant in A549 cells ($p \leq 0.05$). In addition, compared to the control, EL-treatment decreased the average length of F-actin fibers from 201.4 to 115.5 nm in A549 and 206.5 to 147.3 in H460 cells, with a statistically significant difference observed in A549 cells ($p \leq 0.05$) (Fig. 11C and 11F). These results suggest that EL inhibits lung cancer cell motility by interfering with actin microfilament formation.

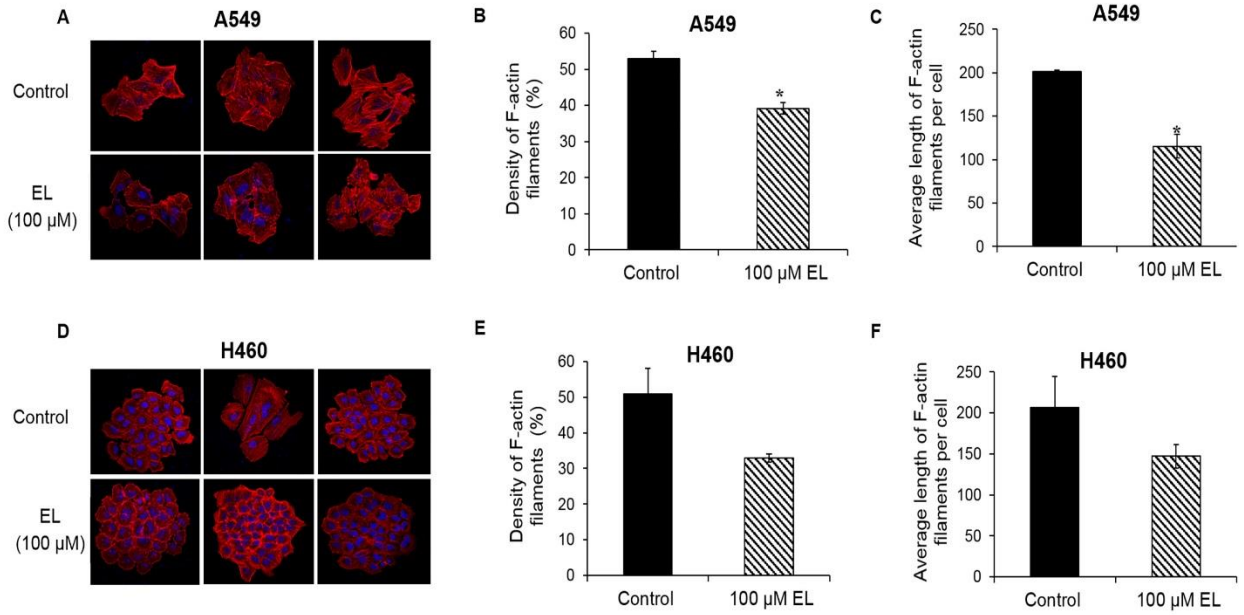

Figure 11. EL effects cytoskeleton organization. (A-C) A549 and (D-F) H460 lung cancer cells grown on glass coverslips were treated with DMSO or EL (100 μM) for 24 h. Immunocytochemistry was conducted using rhodamine-conjugated phalloidin to visualize F-actin fibers. Cells were imaged using an inverted fluorescence microscope (blue fluorescence represents DAPI stained nuclei and red fluorescence indicates rhodamine-conjugated phalloidin-stained F-actin filaments). The acquired images were converted into binary images for quantification on Image Pro Premier software (9.0). The density of F-actin filaments was determined for both (B) A549 and (E) H460 cells treated with or without EL. The average length of the F-actin filaments per cell was determined for (C) A549 and (F) H460 cells. $p \leq 0.05$ was considered statistically significant when compared with untreated control.

EL reduces the number and size of focal adhesions in lung cancer cells

Focal adhesions provide a structural link between the actin cytoskeleton and extracellular matrix (ECM). Therefore, we investigated the effect of EL on focal adhesion number and size in lung cancer cells. A549 and H460 cells were seeded onto fibronectin-coated coverslips, and immunofluorescent staining for expression of vinculin, a membrane-cytoskeletal protein present in focal adhesion plaques, was performed. Control and EL-treated (100 μM; 24 h) A549 and H460 cells displayed prominent focal adhesions at their periphery (Fig. 12A and 12D). However, EL-treatment caused a reduction in the average number of focal adhesions per cell for both A549

and H460 cells. In A549 cells, control cells exhibited an average of 21.6 focal adhesions, while EL-treated cells showed a statistically significant reduction in focal adhesions with an average of only 12.5 focal adhesions per cell ($p \leq 0.05$) (Fig. 12B). Similarly, in H460 cells, the control cells exhibited an average of 22.5 focal adhesions, while EL-treated cells showed a reduction in focal adhesion with an average of 13.1 focal adhesions per cell (Fig. 12E). In addition, EL treatment affected the size of focal adhesions in A549 and H460 cells. In A549 cells, the average size of focal adhesions was 1.33 μm^2/cell in control cells and 1.22 μm^2/cell in EL-treated cells (Fig. 12C). Similarly, in H460 cells, the average size of focal adhesions was 2.02 μm^2/cell while EL-treated cells showed a statistically significant decrease in the size of focal adhesions with the average being 1.38 μm^2/cell ($p \leq 0.05$) (Fig. 12F).

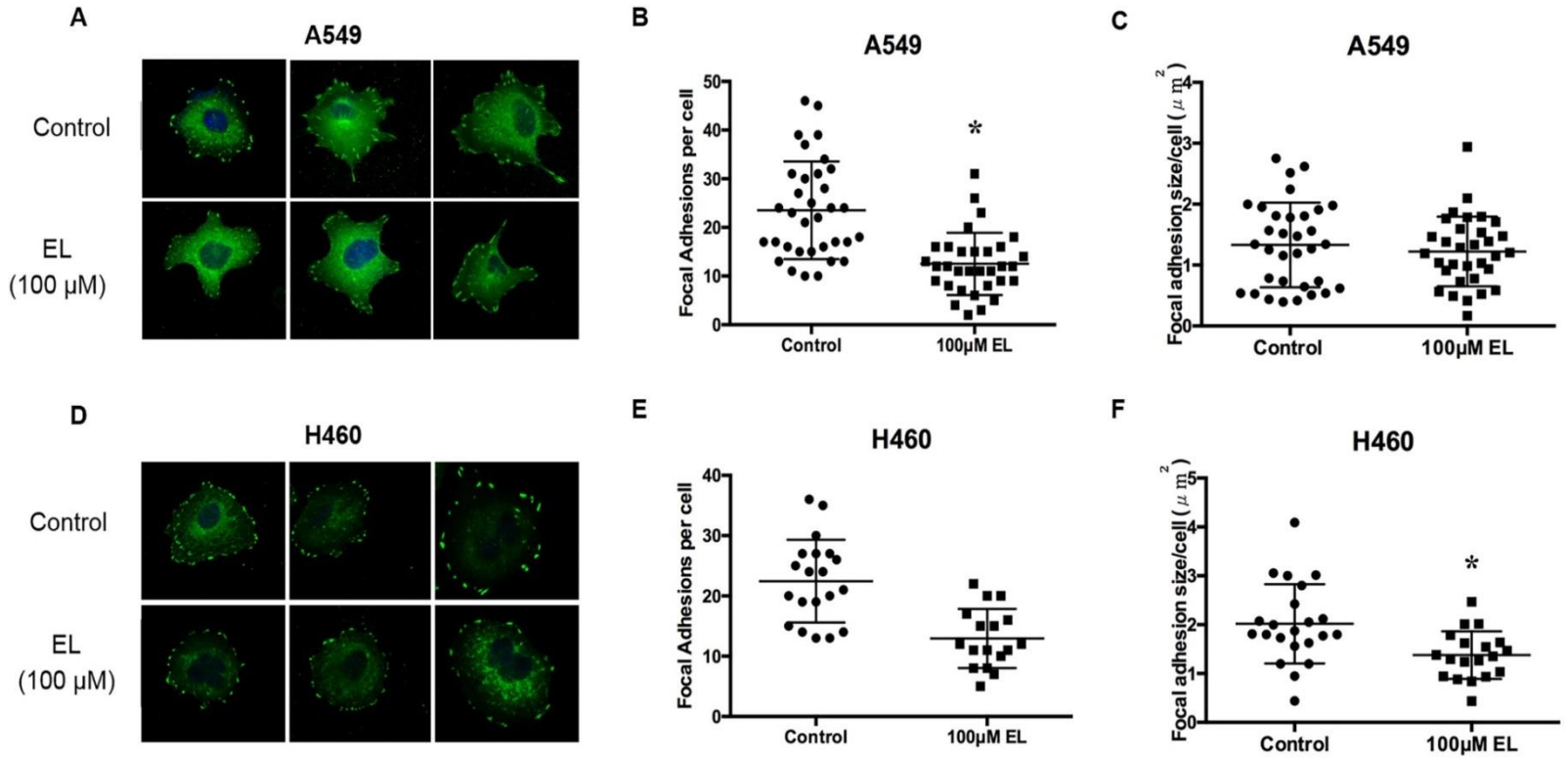

Figure 12. EL reduces the number and size of focal adhesions in lung cancer cells. A549 and H460 lung cancer cells grown on glass coverslips were treated with DMSO or EL (100 μM) for 24 h. Immunocytochemistry for vinculin expression and localization was performed for (A) A549 and (D) H460 cells. Cells were imaged using an inverted fluorescence microscope. Image Pro Premier software (9.0) was used to identify the number of focal adhesions per cell for (B) A549 and (E) H460 cells. The focal adhesion size (μm^2) per cell was also determined for (C) A549 and (F) H460 cells based on pixel count.

EL influences the mRNA expression of cell motility-related genes

We employed PrimePCR^{TM} Pathway microarray plates to investigate the effects of EL on the expression of genes associated with FAK and PDGF signaling pathways. We found that EL-treatment (100 µM; 24 h) led to differential regulation of a number of genes in the two signaling pathways (Supplemental Table 1). Importantly, we observed that *RhoA*, *Rac1*, and *Cdc42* genes that overlapped in the two signaling pathways, were consistently down-regulated in response to EL-treatment. Further, an integrin subunit alpha 2 (*ITGA2*) that helps anchor cells to the ECM was significantly up-regulated. To validate the results obtained by microarray analysis, we performed RT-qPCR. Compared to the control, EL-treatment (100 µM; 24 h) led to down-regulation of *RhoA*, *Rac1*, and *Cdc42* mRNA expression in A549 and H460 cells (Fig. 13). The observed decrease in mRNA expression for all the three genes was statistically significant in A549 cells, while H460 cells showed a significant ($p \leq 0.05$) decrease in *Rac1* and *Cdc42* mRNA expression. These results suggest that EL treatment alters the expression of key transcripts associated with cell motility.

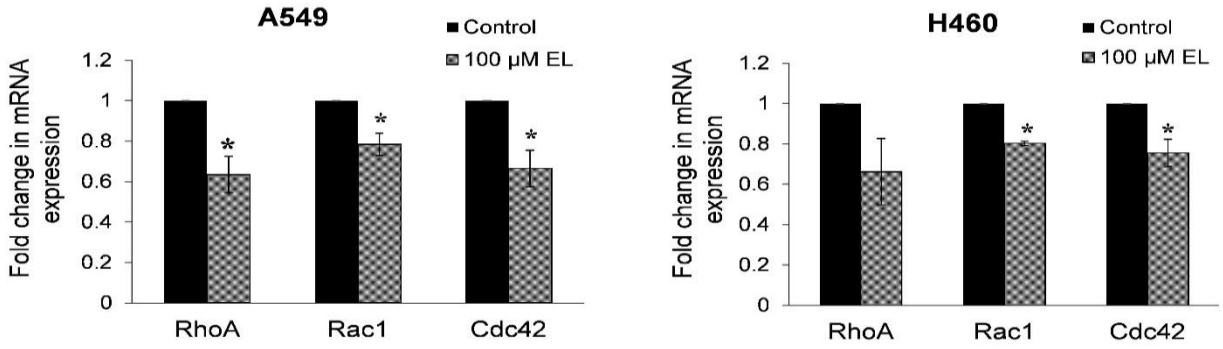

Figure 13. EL treatment of lung cancer cells results in decreased mRNA expression of Rho GTPases. (A) A549 and (B) H460 cells were treated with 0 or 100 µM EL for 24 h. Total RNA was extracted from the cells and subjected to qRT-PCR for RhoA, Rac1, and Cdc42. The experiments were conducted in triplicate. The data were normalized to 18S rRNA expression and represent the average fold change in mRNA expression for EL-treated cells relative to the control.
$p \leq 0.05$ was considered statistically significant when compared with untreated control.

EL modulates FAK-Src signaling in lung cancer cells

De-regulation of the FAK-Src signaling cascade mediates cancer cell migration in lung cancer cells. Therefore, we investigated the effect of EL treatment on the phosphorylation status of FAK and Src proteins. The western blot results show that 100 µM EL decreased phosphorylation of FAK on Y397 (Fig. 14A-D). In addition, in A549 and H460 cell, 100 µM EL decreased phosphorylation of Src on its kinase domain containing an auto-phosphorylation site (Y416) that is necessary for its activation (Fig. 14A-D). Simultaneously, EL increased phosphorylation on the carboxyl terminal domain of Src that contains a regulatory tyrosine (Y527), that upon phosphorylation maintains Src in an inactive conformation (Fig. 14A-D). These results show that EL inhibits FAK-Src activation which may explain the observed changes in cell migration and invasion in A549 and H460 lung cancer cells.

EL inhibits phosphorylation of paxillin and Rho proteins in lung cancer cells

Paxillin is a major substrate of the FAK-Src complex and plays an important role in cell migration and cytoskeletal reorganization. Phosphorylated FAK initiates phosphorylation of paxillin on Y118, a prerequisite for cell migration. Therefore, we investigated if inhibition of FAK-Src signaling pathway by EL as seen in Fig. 15A-D was accompanied by inhibition in paxillin phosphorylation. EL-treatment (100 μM) decreased phosphorylation status of paxillin on the Y118 residue in A549 and H460 cells (Fig. 15E-H).

RhoA, Rac1, and Cdc42 are critical proteins downstream of FAK-Src-paxillin signaling pathway and play important roles in cell migration. Therefore, we next investigated if EL-mediated downregulation in mRNA expression of *RhoA*, *Rac1*, and *Cdc42* co-related with a decrease in their protein expression. As shown in Fig. 14E-H, decreased RhoA protein levels were observed in A549 and H460 cells after EL treatment (100 μM). EL treatment (100 μM) resulted in a slight increase in proteins levels of p-Rac/Cdc42 in A549 cells, and a decrease in p-Rac/Cdc42 protein levels in H460 (Fig. 14E-H). These results suggest that EL may affect stress fiber formation (a role of RhoA), but to a lesser degree filapodia or lamellopodia formation (roles for Rac and Cdc42).

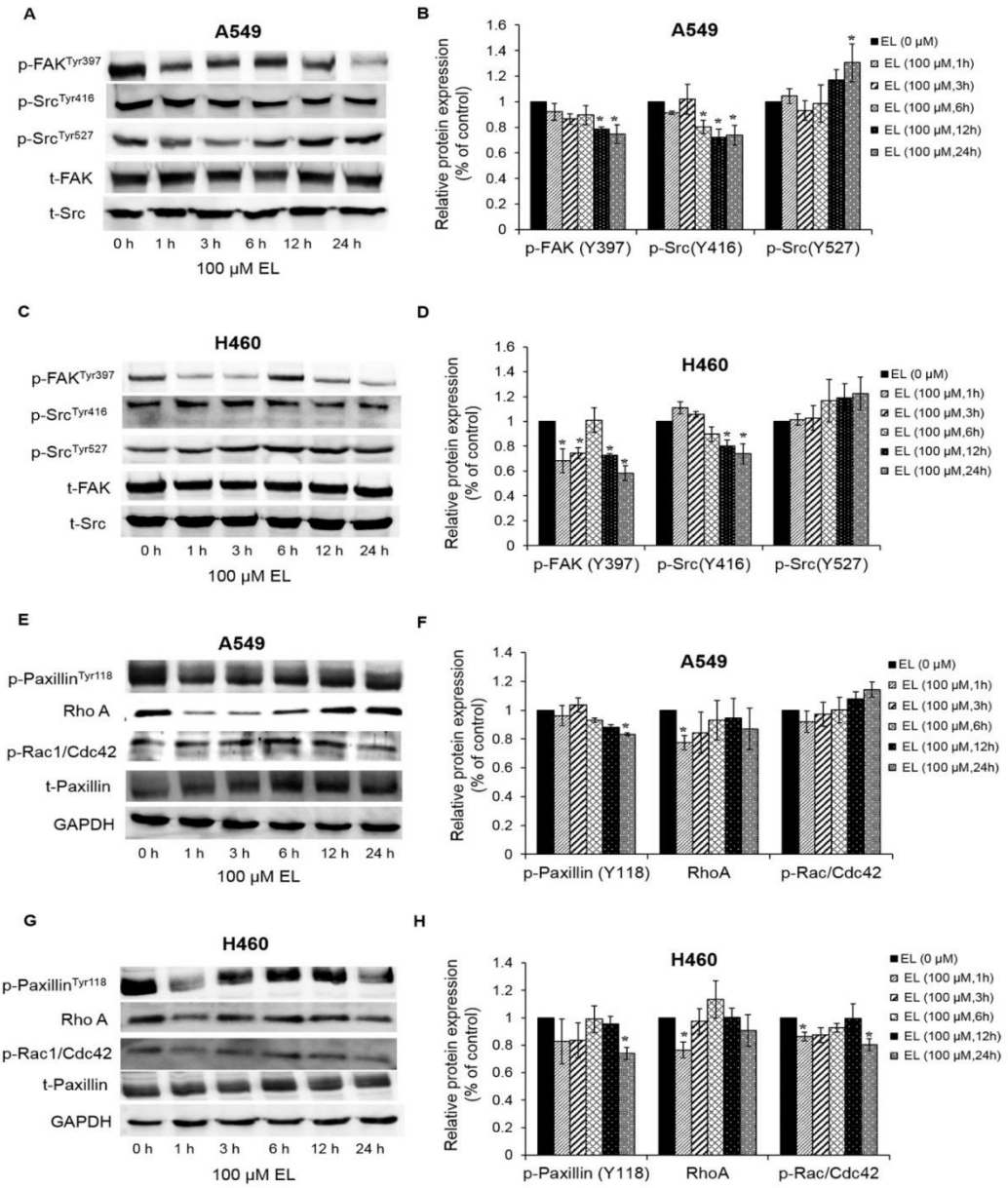

Figure 14. EL treatment of lung cancer cells alters levels of FAK-Src signaling proteins. A549 and H460 cells were treated with 100 μM EL for 0, 1, 3, 6, 12, or 24 h. Western blotting was done to identify changes in total or phosphorylated levels of FAK, Src, paxillin, RhoA, and Rac/Cdc42 proteins. The blots represent one typical result from three independent experiments. Phosphorylated levels of proteins were normalized to their respective total protein levels. Alternatively, GAPDH was used as a loading control. Densitometry was performed using ImageJ software. $p \leq 0.05$ was considered statistically significant when compared with untreated control.

Discussion

Our data support the hypothesis that EL modulates lung cancer cell motility by inhibiting FAK-Src signaling. In this study, we observed that the anti-migration and invasion effects of EL in lung cancer cells were not dependent on its anti-proliferative effect. Here we have shown evidence that EL, at non-toxic concentrations, inhibits A549 and H460 cell invasion and migration by: (i) disrupting F-actin cytoskeleton dynamics, (ii) reducing the number and size of focal adhesions, and (iii) inhibiting the activation of the FAK-Src-paxillin signaling cascade and expression of down-stream motility regulators. These results are significant because inhibition of lung cancer metastasis remains a therapeutic challenge. Natural products, such as EL, that show anti-migratory effects with limited or no toxicity to healthy tissues would be valuable additions to lung cancer treatment regimens.

A number of studies have demonstrated the anti-proliferative effects of EL in cancer cell lines. In PC-3 prostate cancer cells, EL (40 μM; 24 h) inhibited IGF-1-induced cell proliferation by arresting cells at G_0/G_1-phase of the cell cycle and EL (60 μM; 20 h) suppressed migration [2]. Similarly, in MDA-MB-231 breast cancer cells, EL (100 μM) suppressed migration at 24 h and arrested cells at S-phase of the cell cycle after 48 h [5]. We have previously observed that EL ($\geq$ 50 μM) inhibits proliferation of lung cancer cells and induces G_0/G_1- phase cell cycle arrest in lung cancer cells after 48 h (under review). In this study, we observed that non-toxic concentrations of EL (10, 50, and 100 μM) inhibited motility of lung cancer cells after only 24 h. Further, Ki-67 staining results indicate that EL-mediated inhibition of migration was not influenced by inhibition of cell proliferation. Therefore, our data supports previous research

highlighting the cytostatic and anti-migratory potentials of EL and extends the research to lung cancer, which has a high metastatic potential.

The majority of studies involving the anti-migration and invasion potential of EL have been performed using breast cancer cell lines, namely MDA-MB-231 cells [1, 4, 5]. Studies have shown that EL inhibits breast cancer cell adhesion to ECM proteins [1], and inhibits breast cancer cell migration and invasion by reducing MMP-2, -9, and -14 mRNA expression [4]. We did not detect significant changes in MMP mRNA or protein expression in A549 or H460 lung cancer cells treated with EL (data not shown). A likely explanation for differing molecular responses may stem from the fact that EL is derived from a phytoestrogen known as SDG, and may show varied effects on different cancer cell types.

A recent study provided more mechanistic detail for the anti-migratory effects of EL for breast cancer. This study demonstrated that EL inhibited breast cancer cell migration and invasion by decreasing levels of phosphorylated FAK at tyrosine 397 (Y397) and phosphorylated paxillin [5]. Phosphorylation of FAK at Y397 is critical for its activation and recruitment of Src [18]. Similarly, we found that EL reduced FAK Y397 phosphorylation and decreased phosphorylated paxillin in lung cancer cell lines. We also found EL elevated phospho-Src at Y527 and reduced phospho-Src at Y416. Phosphorylation at Y527 maintains Src in an inactive conformation and inhibits its recruitment to FAK, while phosphorylation at Y416 results in its activation [19, 20]. Deregulation of FAK-Src signaling is seen in several tumor types [21-23]. Lung tumors with elevated FAK and Src activity have increased metastatic potential [14, 15]. Therefore, inhibition of FAK-Src signaling by EL may reduce the capacity of lung cancer cells to migrate.

Cell migration is primarily driven by: (i) polymerization of F-actin, and formation of focal adhesion complexes at the leading edge, and (ii) F-actin depolymerization and disassembly of focal adhesion complexes at the rear end of the cell. The activated FAK-Src-paxillin complex contributes to cell migration through the Rho family of small GTPases, such as RhoA, Rac1, and Cdc42 [24]. RhoA affects cell-cell or cell-ECM interaction by inducing cytoskeleton changes; Rac1 plays a role in membrane ruffling by driving actin polymerization; and Cdc42 is involved in the formation of filopodia through initiating F-actin filament assembly. In this study, we have shown that EL decreased the density of F-actin fiber and disrupted the formation of long stress fiber that traverse the cell body. This change in actin cytoskeleton leading to reduced cell migration was associated with down-regulation of *RhoA*, *Rac1*, and *Cdc42* expression, which might impair cell body contraction and retraction at the rear end of the cell, and limit formation of protrusions at the leading edge.

In addition to down-regulating Rho GTPases, we found EL elevated ITGA2 mRNA expression in lung cancer cells. Integrins are transmembrane proteins that connect the extracellular matrix with the actin cytoskeleton. Loss of ITGA2 expression is frequently observed in solid tumors, and the silencing of ITGA2 resulted in enhanced breast cancer migration [25]. Therefore, EL-induced ITGA2 expression may anchor lung cancer cells to the matrix and prevent cell migration.

Conclusions

In summary, EL treatment inhibits lung cancer cell migration by altering F-actin dynamics, suppressing formation of focal adhesion complexes and activating FAK-Src signaling. EL also alters the expression of a number of regulators of the actin cytoskeleton and cell

migration. Additional studies are needed to identify how EL could be used as a complementary approach to currently used chemotherapies for lung cancer. Also, more studies designed to investigate the mechanisms behind EL's anti-cancer effects are needed. Altogether, EL holds promise as an adjuvant treatment to prevent tumor cell motility.

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CHAPTER 4. FLAXSEED CONSUMPTION INHIBITS NNK-INDUCED LUNG TUMORIGENESIS AND MODULATES PHASE II ENZYMES AND INFLAMMATORY CYTOKINES GENE EXPRESSION IN A/J MICE

Abstract

Flaxseed consumption is associated with reduced oxidative stress and inflammation in lung tissue, and has shown anti-cancer effects for breast and prostate tissues. However, the chemopreventive potential of flaxseed remains unexplored for lung cancer. In this study, we investigated the chemopreventive potential of flaxseed in an A/J mouse model of tobacco smoke carcinogen NNK-induced lung tumorigenesis. Mice exposed to NNK were fed a control diet or a 10% flaxseed-supplemented diet for 26 weeks. Flaxseed-fed mice showed reduced lung tumor incidence (78%) and multiplicity, with an average of 2.6 ± 2.4 surface lung tumor nodules and 0.8 ± 1.0 H&E cross-section nodules per lung compared to the control group with 100% tumor incidence and an average of 10.2 ± 5.7 surface nodules and 3.1 ± 2.2 H&E cross-section nodules per lung. Western blotting performed on normal lung tissue showed that flaxseed suppressed phosphorylation (activation) of p-AKT, p-ERK, and p-JNK kinases. RNA-Seq data obtained from normal lung and lung tumors of control and flaxeed-fed mice suggested that flaxseed intake resulted in differential expression of genes involved inflammation-mediated cytokine signaling (IL-1,-6,-8,-9, and -12α), xenobiotic metabolism (several CYPs, GSTs, and UGTs), and signaling pathways (AKT and MAPK) involved in tumor cell proliferation. Our results indicate that dietary flaxseed supplementation may be an effective chemoprevention strategy for chemically-induced lung carcinogenesis.

Introduction

Tobacco smoke contains over 5,000 compounds of which 73 are carcinogenic in laboratory animals and/or humans [1]. Among these, tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most potent pulmonary carcinogens [1]. NNK exposure is closely associated with lung carcinogenesis in smokers and in A/J mice independent of the route of administration [1-3]. Further, the occurrence of early onset DNA lesions such as mutations in KRAS oncogene and molecular processes underlying tumor development are similar in NNK-treated A/J mice and humans [4-6]. Therefore, this mouse model is commonly employed to study the chemoprevention potential of phytochemicals in lung cancer [7].

Several plant-derived phytochemical have shown lung chemopreventive effects. Phytochemicals such as EGCG [8] and polyphenon E (green tea) [9], theaflavins (black tea) [10], PEITC [11, 12] and indole-3-carbinol (cruciferous vegetables) [13, 14], and kavalactones (kava) [15-17] inhibit development of NNK-induced lung tumors in A/J mice. The tumor growth inhibitory properties of these phytochemicals have been attributed to altering expression of phase I cytochrome P450 and phase II detoxifying enzymes. These metabolizing enzymes are involved in NNK activation and detoxification [12, 13, 15]; suppression of NF-κB, Akt, c-Jun and ERK-mediated signaling pathways regulating aberrant cell proliferation; activation of caspase-3 and PARP-mediated apoptosis; and decreased VEGF-mediated angiogenesis [9, 14, 17, 18]. This evidence suggests that plant-derived phytochemicals are beneficial in lung cancer chemoprevention.

Flaxseed is a whole-grain rich in phytochemicals such as omega-3 fatty acid (α-linolenic acid) and lignans (predominantly secoisolariciresinol diglucoside). The chemopreventive properties of flaxseed have been demonstrated in several epidemiological, preclinical, and clinical studies of breast [19-23], colon [24, 25], and prostate cancers [26-28]. Intake of a flaxseed-supplemented diet has been reported to decrease tumor incidence, size, volume, and multiplicity by decreasing cell proliferation [21, 27], inducing cell cycle arrest [25] and apoptosis [23] , and inhibiting angiogenesis [22, 29]. However, the chemopreventive potential of flaxseed has not been investigated in lung.

There is evidence that flaxseed supplementation ameliorates oxidative stress and inflammation, precursors of carcinogenesis, in mouse models of acute and chronic lung injury [30-32]. In addition, consumption of flaxseed has been shown to significantly alter the expression of genes involved in apoptosis, cell proliferation, inflammation-associated cytokine signaling, oxidative stress, and phase I and phase II detoxification pathways in healthy mouse lung tissue [33]. Further, we have previously shown that enterolactone, a flaxseed-derived mammalian lignan, inhibits lung cancer cell proliferation by inducing G1-phase cell cycle arrest [34], and suppressing cell motility and invasion by altering FAK-Src signaling [35].

Therefore, we hypothesized that flaxseed inhibits NNK-induced lung tumorigenesis in A/J mice by modulating genes promoting inflammation and oxidative stress. To test this hypothesis, we studied the effect of 10% flaxseed consumption on inhibition of NNK-induced lung tumor incidence and multiplicity in A/J mice. Further, we performed RNA sequencing (RNA-Seq) to identify differential expression of genes and the associated mechanistic pathways responsible for the observed chemopreventive effects of flaxseed in lung.

Material and Methods

Chemicals and diets

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was purchased from Toronto Chemicals Inc. (TRC, North York, Ontario, Canada) and was dissolved in saline solution immediately before use. Mouse diets in pellet form were obtained from TestDiet® (St. Louis, MO) and were kept under dry and cool (2°C) conditions for long-term storage. The control diet was AIN-93G, while the treatment diet consisted of AIN-93G supplemented with 10% flaxseed. The diets were isocaloric and were prepared according to the composition shown in Supplementary Table S1.

A/J mouse model of lung cancer

Male A/J mice, five-to six-week-old (n=24), were procured from The Jackson Laboratory (Bar Harbor, ME) and housed in the Animal Nutrition and Physiology Center at North Dakota State University. The mice were kept in individual solid-bottomed polycarbonate cages with ALPHA-Dri bedding in a pathogen-free, humidified (50-70%), and temperature-controlled (22-25℃) environment with a 12 h light/dark cycle. The mice were fed ad libitum the control AIN 93G diet for one week in order to acclimate them to the food source and laboratory conditions prior to the start of the experiment. The study was approved by North Dakota State University's Institutional Animal Care and Use Committee (IACUC) and conducted according to the approved protocol.

Treatment of A/J mice with NNK

After 1 week of acclimatization, mice were assigned into one of two groups; a control group and flaxseed group, with 12 mice/group, such that the average weight of mice in each

group was the same. Mice in both groups received intraperitoneal injection (i.p) of NNK (50 mg/kg body weight) once a week over the course of the next 4 weeks. Immediately after the first intraperitoneal (i.p.) injection of NNK, the treatment group mice were switched to AIN 93G diet supplemented with 10% flaxseed, while the control group mice continued feeding on the AIN 93G diet. The experimental design for the study is represented in Fig. 15A. Three mice in the treatment group did not readily accept the 10% flaxseed diet, and were removed from the study. One mouse from the control group was evaluated at 23 weeks to determine if sufficient time was allowed for lung tumor development. The remaining mice, 11 mice in the control group and 9 mice in the treatment group were maintained on their respective diets for 26 weeks after the first i.p. injection of NNK. Mouse cages were cleaned, and fresh bedding and water were provided once a week. Mice were weighed once a month for 6 months. In addition, diet consumption was regularly monitored every two days. The mice were euthanized under anesthesia using ketamine/xylazine followed by cervical dislocation. The lung tissues were harvested, weighed, and imaged using a camera attached to an inverted microscope (Zeiss lumar, V12 stereo motorized microscope). Subsequent to imaging, the left lung from each mouse was snap frozen in liquid nitrogen and stored at – 80 °C for subsequent molecular analysis, and the right lung mouse was perfused with freshly prepared 10% buffered formaldehyde solution until sectioning and slide-preparation.

Quantification of flaxseed-derived mammalian lignans enterodiol (ED) and enterolactone (EL) in lung and blood plasma

The ED and EL levels in lung tissue of control group mice (n=11) and 10% flaxseed-fed mice (n= 9), and their levels in circulating blood plasma in control group mice (n=3) and 10%

flaxseed-fed mice (n=7) were determined by liquid chromatography tandem mass spectrometry as previous described.

Hematoxylin and eosin (H&E) staining

Formaldehyde-fixed paraffin-embedded lung tissue samples were used to prepare 5 μm thick sections on poly-L-lysine-coated slides. Tissue sections were stained with hematoxylin and eosin (H&E) for histopathological observation of the incidence, multiplicity, and size of surface lung tumor nodule in each mouse. Tumor incidence was defined as the number of mice in each group bearing one or more tumor nodules divided by the total number of mice examined. Lung tumor nodules were microscopically examined and quantified by four independent researchers blinded to control and treatment groups.

Tumor morphometry

Quantitative morphometric analysis was performed on H&E stained serial lung sections, with an incremental depth of 20 μm, from control and 10% flaxseed group mice (n=4/group). Image analysis was performed using the Aperio ScanScope SC (Leica Biosystems Inc; Buffalo Grove IL, USA), Aperio ImageScope, and Aperio Genie Histology Pattern Recognition Software. The glass slides with H&E stained-lung sections were scanned and the images created at 20x magnification. Using the Genie software, a unique algorithm was created based on pattern recognition to separate: i) tumor nodules, ii) benign lung tissue, and iii) glass background. The slides were visualized with Aperio ImageScope for quality control and quantitative measurement of the area occupied by tumor in the digital images. Tumor burden was calculated as % tumor area with respect to total lung area.

RNA extraction

RNA from normal lung tissue and lung tumor nodules of three mice from the control and three mice from the 10% flaxseed groups was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA). Briefly, 5 mg of tissue was homogenized in 1 mL of TRIzol. After incubating the homogenate at room temperature for 15 min, 50 µl of BCP was added. The tube was shaken vigorously for 30 s, incubated again at room temperature for 3 min, and then centrifuged at 12,000xg for 15 min at 4°C. The supernatant was transferred to a fresh tubes and total RNA was precipitated by adding 1.5 volumes of 100% ethanol. The precipitated RNA sample was transferred to RNeasy Mini spin column (Qiagen; Valencia, CA) and RNA purification was performed using the manufacturer's protocol.

Western blotting

Five milligrams of normal lung tissue from control group mice (n=9) and 10% flaxseed group mice (n=9) was lysed using SDS lysis buffer (Cell Signaling Technologies, Danvers, MA) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN). Samples were briefly sonicated while on ice. Thirty micrograms of total protein was separated on 10% SDS-polyacrylamide gels at 100 V for 1h. Proteins were transferred to nitrocellulose membrane at 100 V for 70 min at 4°C. The blots were then probed overnight at 4 °C with primary antibodies (1:1000) for p-Akt, t-AKT, p-ERK, t-ERK, p-JNK, and t-JNK. The next day, the blots were rinsed with 1X TBS-tween (0.1%) and probed with anti-rabbit HRP-conjugated secondary antibodies (1:1000) for 1 h at room temperature. The western blots were analyzed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and the images were captured using the MultiImage™ Light Cabinet (Alpha Innotech; San Leandro, CA). The

densitometry results were obtained using ImageJ software. p-AKT, p-ERK, and p-JNK protein levels were normalized to their corresponding total proteins.

RNA-Seq

Three micrograms of total RNA from three mice within each group: normal lung tissue from control group (CN), tumor lung nodule from control group (CT), normal lung tissue from flaxseed group (FN), and tumor lung nodule from flaxseed-fed group (FT) were sent for sequencing to the University of Minnesota Genomics Center. Six barcoded libraries were created and sequenced on Illumina HiSeq 2500. Single-end reads of 50 base pairs (bp) were obtained and mapped to the Mus musculus genome (Build: Mus_musculus.GRCm38) downloaded from Ensembl. The raw fastq were subjected to quality trimming using Sickle, for a minimum length of 50. HISAT2 [36] was used to map the reads on the genome. SAMtools [37] and BAMtools [38] were used to convert and sort the BAM files. HTSeq [39] was used to count the reads.

Regularized log transformed (rlog) data were obtained using the method implemented in the Bioconductor DESeq2 package(v1.14.1; [40]). Fragment per kilobase of exon per million fragments mapped (FPKM) values were calculated for each gene by normalizing the read count data to both the length of the gene and the total number of mapped reads in the sample [41]. Differential gene expression analysis was performed using the DESeq2 package. An adjusted P value (q-value) threshold of ≤ 0.1 using the Benjamini & Hochberg's method and $\log_2$ fold change ≥ 1 were used as the statistical cutoff criteria to further study the differentially expressed genes.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was used to confirm expression changes for select genes found differentially expressed by RNA-Seq. The validation of RNA-Seq results was done using RNA from normal lung tissue of 11 mice in the control group and 9 mice in the flaxseed-fed group, or RNA from lung tumor nodules of 11 mice in the control group and 7 mice in the flaxseed-fed group. Total RNA was isolated from tissues using the Fisher SurePrep Kit (Waltham, MA) as per the manufacturer's instructions. cDNA was synthesized using 50 ng of total RNA and the qScript cDNA synthesis kit (Quanta Biosciences; Beverly, MA). Primers for genes listed in Supplementary Table S2 were designed using Primer Express software (version 2.0, Applied Biosystems; Foster City, CA), and were synthesized by Sigma-Aldrich (St. Louis, MO). Steady-state mRNA levels for the listed genes were evaluated by qPCR using PerfeCTa SYBR Green FastMix (Quanta Biosciences). The cycling parameters were: 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 60°C for 1 min, and a dissociation program that included 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s ramping up at 0.2 °C/s. One distinct peak was observed for each primer set, suggesting target specificity. The relative change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method using housekeeping genes 18S rRNA and β-actin as internal controls.

Results

Flaxseed consumption is associated with reduced body mass in A/J mice

Mice from both diet groups were weighed every 4 weeks over the 6-month period and their body mass was determined. Prior to the first NNK administration, the average body weight of mice in the control and flaxseed-fed groups was 20.0 and 20.1 g, respectively. During the

study, mice on the 10% flaxseed diet showed a slightly reduced average body weights, with statistically significant differences observed at 2, 4, and 6 months of the study (Fig. 15B). At the end of the study, the average body weight of mice in the control group (26.3 ± 0.8 g) was significantly higher than that of the 10% flaxseed group (25.0 ± 1.6 g; $p \leq 0.05$).

Flaxseed-fed mice exhibit reduced lung mass, tumor incidence, and multiplicity

The experiment was terminated 26 weeks after the first NNK administration. The lungs from control-fed mice (n=11) and flaxseed-fed mice (n=9) were weighed on the day of euthanasia. The average lung mass for control group mice was 0.2 g compared to 0.1 g for the flaxseed-fed mice. This difference was statistically significant ($p \leq 0.05$) (Fig. 15C). Even though initially the flaxseed group mice were reluctant to eat the fortified diet, they became accustomed to it and consumed it as well as the control diet during the majority of the study (Fig 15D).The control group mice had 100% incidence of lung adenocarcinoma formation with a range of 2 to 36 surface lung tumor nodules per mouse, and an average of 10.2 ± 5.7 lung tumor nodules per lung. Strikingly, 10% flaxseed treatment reduced adenocarcinoma incidence to 78%, and the number of surface lung tumor nodules per mouse was significantly less ranging from 0 to 15, with an average of 2.6 ± 2.4 ($p \leq 0.05$) lung tumor nodules per lung (Fig. 15E). H&E lung tissue sections showed that the number of lung tumor nodules ranged from 1 to 8, with an average of 3.1 ± 2.3 per lung in control group mice, while flaxseed-fed mice exhibited a significant ($p \leq 0.05$) decrease in the number of lung tumor nodules ranging from 0 to 2, with an average of 0.8 ± 1.0 per lung (Fig. 15 F-G). Tumor morphometry data showed flaxseed-fed mice had significantly decreased tumor burden as compared to control group mice (0.0-5.4 vs. 0.7-10.2 % of total lung area) (Fig. 15H). These findings suggest that 10% flaxseed intake reduces NNK-induced lung

tumor development, multiplicity, and tumor burden in A/J mice. These findings show that 10% flaxseed intake reduces NNK-induced lung tumor development and multiplicity in A/J mice.

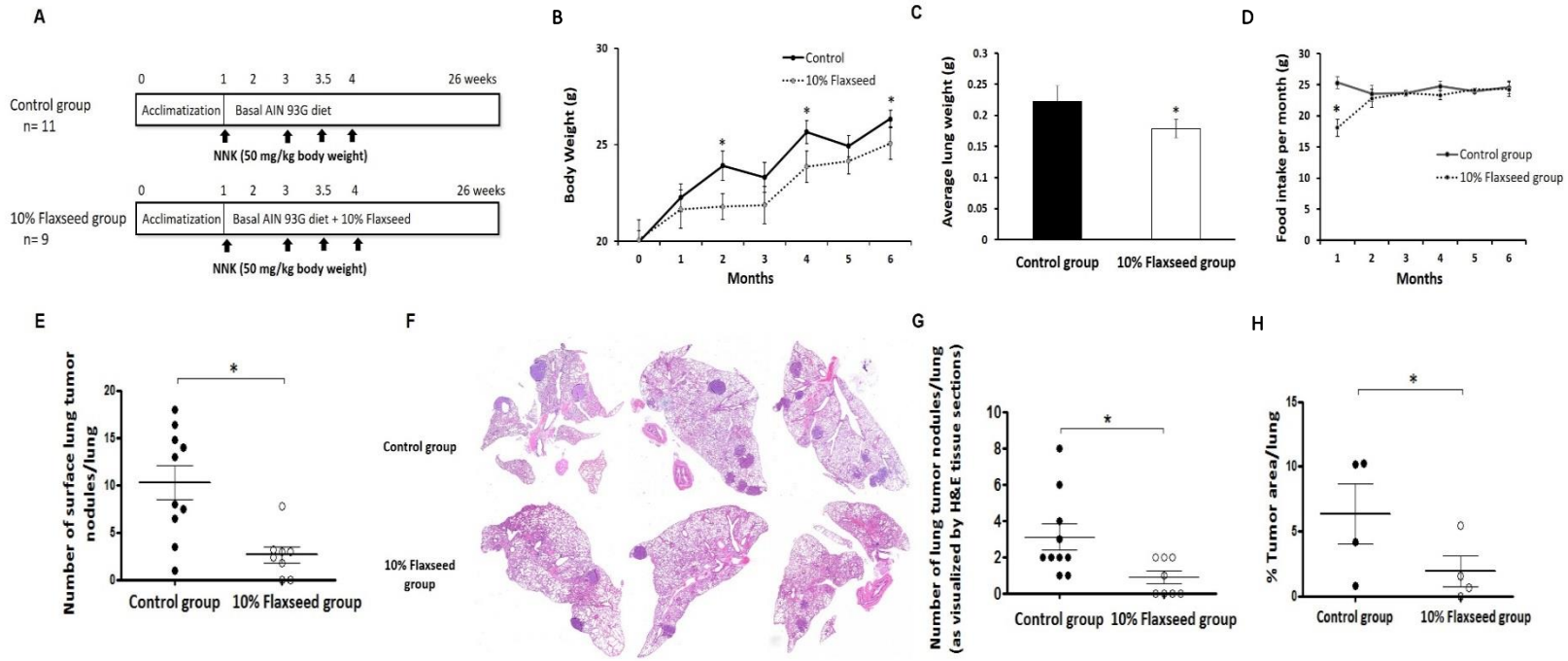

Figure 15. Experimental design and observations. (A) Schematic representation of the experimental design. A/J mice were injected intraperitoneally with NNK (50mg/kg), once a week for 4-weeks. After the first NNK injection, mice (n=12) in control group continued feeding on control diet (basal-AIN-93G) and mice (n=9) in 10% flaxseed group were fed basal diet-supplemented with 10% flaxseed. (B) Effect of 10% flaxseed on body weight gain. (C) The effect of 10% flaxseed on lung weight of mice. (D) Food intake by mice in the group throughout the course of the study. (E) Effect of 10% flaxseed on the number of surface lung tumor nodules per lung. (F) Representative images for lung histology using H&E staining. (G) Quantification of the number of lung tumor nodules per lung from H&E cross-section images. (H) Tumor morphometry data from H&E stained lung tissue sections to analyze tumor burden in control and 10% flaxseed group mice (n=4/group) *, $p \leq 0.05$.

Levels of ED and EL in mouse lung tissue and blood plasma

The amount of flaxseed-derived mammalian lignans ED and EL were quantified in lung tissues of control and 10% flaxseed-fed mice. As expected, ED, the first mammalian lignan to appear after consumption of flaxseed, was not detected in lung tissues of control group mice, while, 5 out of 9 flaxseed-fed mice showed ED levels ranging from 19.5-106 ng/g, with an average of 31.70 ng/g of tissue. Surprisingly, EL was not detected in the lung tissues from either diet group.

Circulating levels of ED and EL were detected in blood plasma of both diet groups. Minor amounts of ED, ranging from 1.8-4.7 ng/ml, with an average of 2.8 ng/ml were detected in control group mice, while levels of ED ranging from 16.9-4,360 ng/ml, with an average of 1,539.6 ng/ml were detected in the 10% flaxseed group mice. EL was not detected in blood plasma of control-fed mice, while it was found to be 0-68.4 ng/ml, with an average of 20.8 ng/ml in blood plasma of 10% flaxseed-fed mice.

Flaxseed-supplementation reduced phosphorylation of AKT, ERK, and JNK in normal lung tissue of NNK-treated mice

Sustained phosphorylation leading to activation of PI3K/Akt signaling [41] and mitogen-activated protein kinase (MAPK) signaling such as ERK [42] and JNK [43], which are downstream substrates of KRAS, have been shown to play a role in NNK-induced lung carcinogenesis in A/J mice. Therefore, we investigated the effect of 10% flaxseed on phosphorylation status of key signaling proteins in normal lung tissue. Reduced phosphorylation of AKT, ERK, and JNK protein kinases was detected in flaxseed-fed mouse lung tissue compared to control-fed mice (Fig. 16A-F). These results suggest that 10% flaxseed reduces the

incidence of lung tumor development by inhibiting aberrant activation of AKT, ERK, and JNK signaling molecules that play a role in cancer cell survival and proliferation.

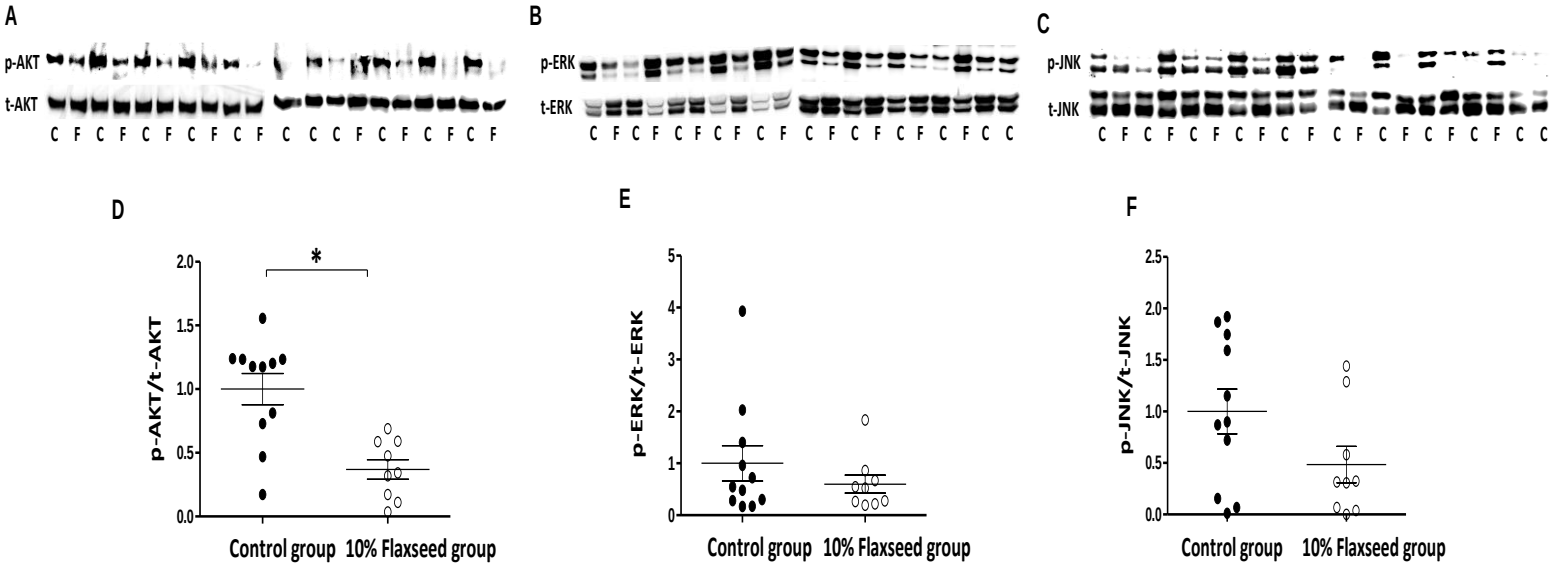

Figure 16. Effect of 10% flaxseed on phosphorylation of AKT, ERK, and JNK protein kinases. (A-C) 50 ug of proteins isolated from normal lung tissue of mice fed control diet (C) and 10% flaxseed diet (F) were subjected to western blotting to analyze expression levels of phosphorylated and total AKT, ERK, and JNK protein kinases. (D-F) Quantification of western blotting results. Densitometry measurements of western blot bands were performed using ImageJ software. *, $p \leq 0.05$.

Identification of differentially expressed transcripts in normal lung tissue and tumor nodules of control and flaxseed-fed mice using RNA-Seq

To investigate the gene expression alterations contributing to the observed chemopreventive effects of 10% flaxseed in NNK-induced lung carcinogenesis in A/J mice, RNA-Seq was performed on the RNA isolated from three mice each from CN, CT, FN, and FT groups (explained in Material and Methods sections). Principal component analysis (PCA) showed that gene expression analysis for CN and CT groups clustered close to one another, showing strong inter-group correlation of gene expression. However, the gene expression analysis from FN and FT groups segregated in two distinct clusters, indicating different inter-group gene expression but a strong intra-group correlation (Fig. 17A). In addition, PCA plot indicated that there is inter-group variation in the gene expression signature between FN vs. CN and FT vs. CT groups.

We compared the transcriptomes of FN with CN group and FT with CT group to identify differentially expressed genes. A total of 16,837 genes were differentially expressed between FN vs CN and FT vs CT groups. A cutoff criterion, mentioned in the Material and Methods section, was used to generate a list of significantly differentially expressed transcripts. Compared to the control-group, 10% flaxseed-supplementation resulted in significant differential expression of 5,951 (35.3%; $q \leq 0.10$) genes in the normal lung tissue, of which 2,094 (35.1%) transcripts had > 1 $\log_2$ fold change (upregulated) while 547 (9.1%) had < 1 $\log_2$ fold change (downregulated). Similarly, in the lung tumor nodules, 10% flaxseed-supplementation resulted in significant differential expression of 3,096 (18.3%; $q \leq 0.10$) genes, of which 1,329 (42.9%) transcripts had > 1 $\log_2$ fold change (upregulated) while 151 (4.8%) had < 1 $\log_2$ fold change (downregulated).

Data analysis using Reactome software (a knowledgebase of diverse biological pathways) revealed that the differentially expressed genes between FN vs CN and FT vs CT groups categorized into diverse molecular pathways. The RNA-Seq data for FN vs CN group identified significantly differentially regulated genes belonging to pathways regulating, (i) Phase I and II xenobiotic metabolism, (ii) cell proliferation, such as MAPK and PI3K/AKT signaling pathways, (iii) immune system via cytokines, (iv) detoxification of reactive oxygen species (ROS), (v) cell death, (vi) cellular response to stress and hypoxia, (vii) cell cycle, and (viii) DNA repair (Table 5). Similarly, Table 6 lists signaling pathways that were significantly differentially regulated between the FT vs CT comparison. The heat map in Fig. 17 B-C, generated using FPKM values, displays genes identified in signaling pathways that were differentially expressed in FN vs CN and in FT vs CT groups. A list of these genes along with their average $\log_2$ fold change for 3 mice in each group has been provided in Supplementary Table S3 and S4.

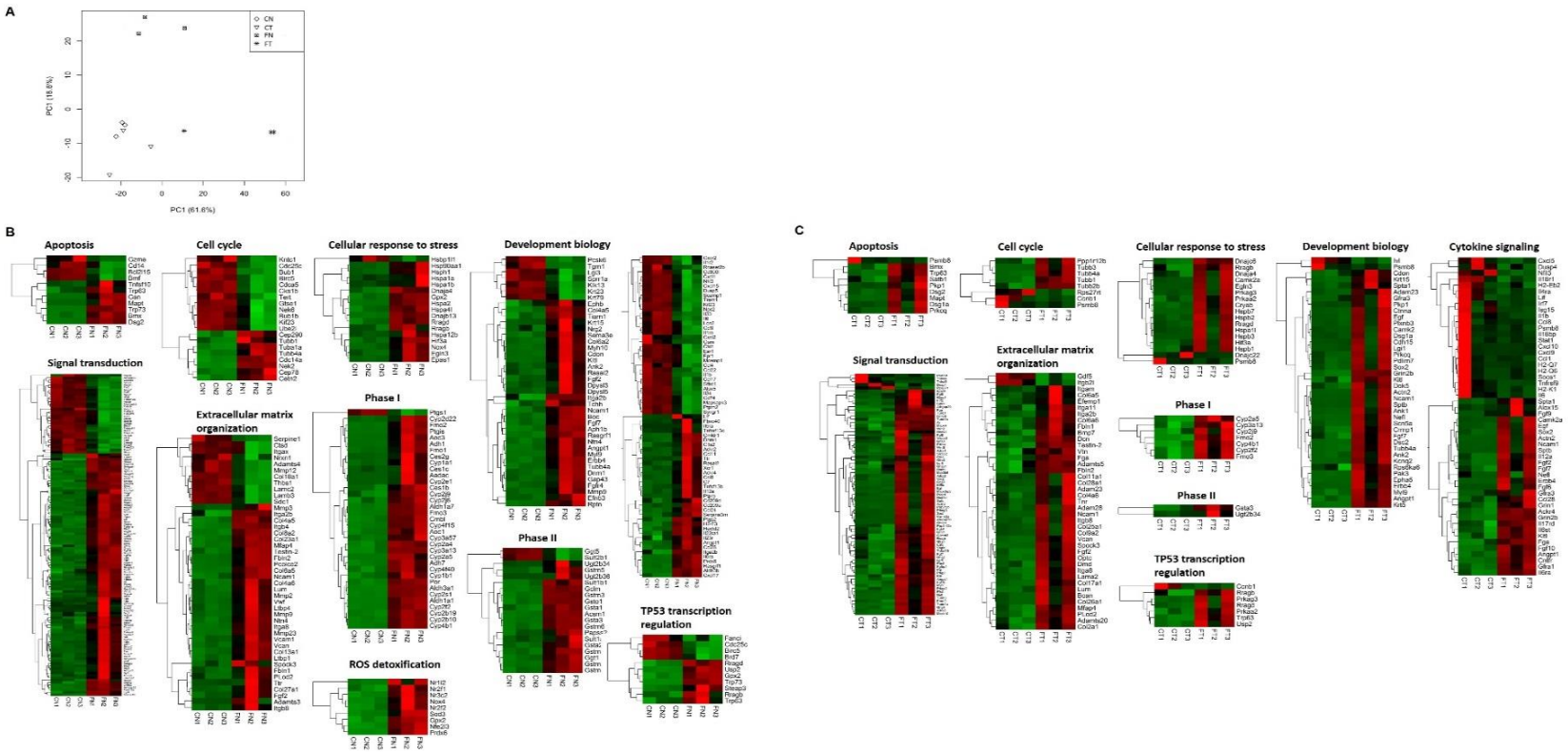

Figure 17. Principal component analysis (PCA) and heat maps for RNA-Seq results. (A) PCA plot shows transcriptome clustering of data from normal lung tissue (CN) and tumor lung nodule (CT) of control-fed mice, and normal lung tissue (FN) and tumor lung nodule (FT) from 10% flaxseed-fed mice. (B) Heat map for gene expression results obtained by RNA-Seq for FN vs CN, and (C) Heat map for gene expression results obtained by RNA-Seq for FT vs. CT. Transcripts were clustered using hierarchical clustering based on expression levels (FPKM). Color gradient represents low (green) to high (red) levels of expression.

Table 4. Pathway categorization of significantly ($q \leq 0.1$) differentially expressed genes in normal lung tissue in flaxseed and control group

Pathway name	Total genes in the pathway	Significant genes in the pathway	% Gene significant
Phase I and II	106	55	51.88
RAF/MAPK signaling	253	43	17.00
SOS-mediated signaling	253	43	17.00
IRS-mediated signaling	329	51	15.50
EGFR signaling pathway	373	53	14.21
VEGF signaling	347	48	13.83
PI3K-AKT signaling	138	18	13.04
FGFR signaling	94	11	11.70
Cytokine signaling in Immune system	835	70	11.26
Development biology	1066	111	10.41
Detoxification of ROS	39	4	10.26
Wnt signaling	296	23	7.77
Apoptosis	174	11	5.75
Cellular response to hypoxia	75	3	4.00
Cellular response to stress	423	17	3.55
Cell cycle	604	20	3.31
Transcription regulation by TP53	364	11	3.02
Cell cycle checkpoints	183	3	1.64
DNA repair	293	4	1.37

Table 5. Pathway categorization of significantly ($q \leq 0.1$) differentially expressed genes in lung tumor nodules in flaxseed and control group

Pathway name	Total genes in the pathway	Significant genes in the pathway	% Gene significant
ERBB signaling	46	6	13.04
MAPK signaling	298	30	10.07
IRS-mediated signaling	302	30	9.93
Development biology	1066	91	8.54
FGFR signaling pathway	94	8	8.51
VEGF signaling	347	29	8.36
PI3K-AKT signaling	135	11	8.15
EGFR signaling pathway	373	30	8.04
Mitochondrial biogenesis	56	4	7.14
Metabolism	2,157	142	6.58
Cytokine signaling in Immune system	835	53	6.34
Wnt signaling	296	16	5.41
Apoptosis	174	9	5.17
JNK Signaling	22	1	4.55
Cellular response to hypoxia	66	3	4.55
Cellular response to stress	423	18	2.84
Transcription regulation by TP53	364	7	1.92
Cell cycle	499	8	1.60

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

To confirm differential expression by RNA-Seq, we used RT-qPCR to validate the differential expression of selected genes which are involved in signal transduction, xenobiotic metabolism, transcriptional regulation, and tumor suppression. In FN vs CN group (Fig. 18A), all genes except *IL-8* and *IL-9*, and in FT vs CT group (Fig. 18B), all genes except *IL-1*, showed similar differential expression as observed by RNA-Seq. The inconsistencies in the amount of $\log_2$ fold differential expression of selected genes between RNA-Seq and RT-qPCR result may be due to the small sample size in our study.

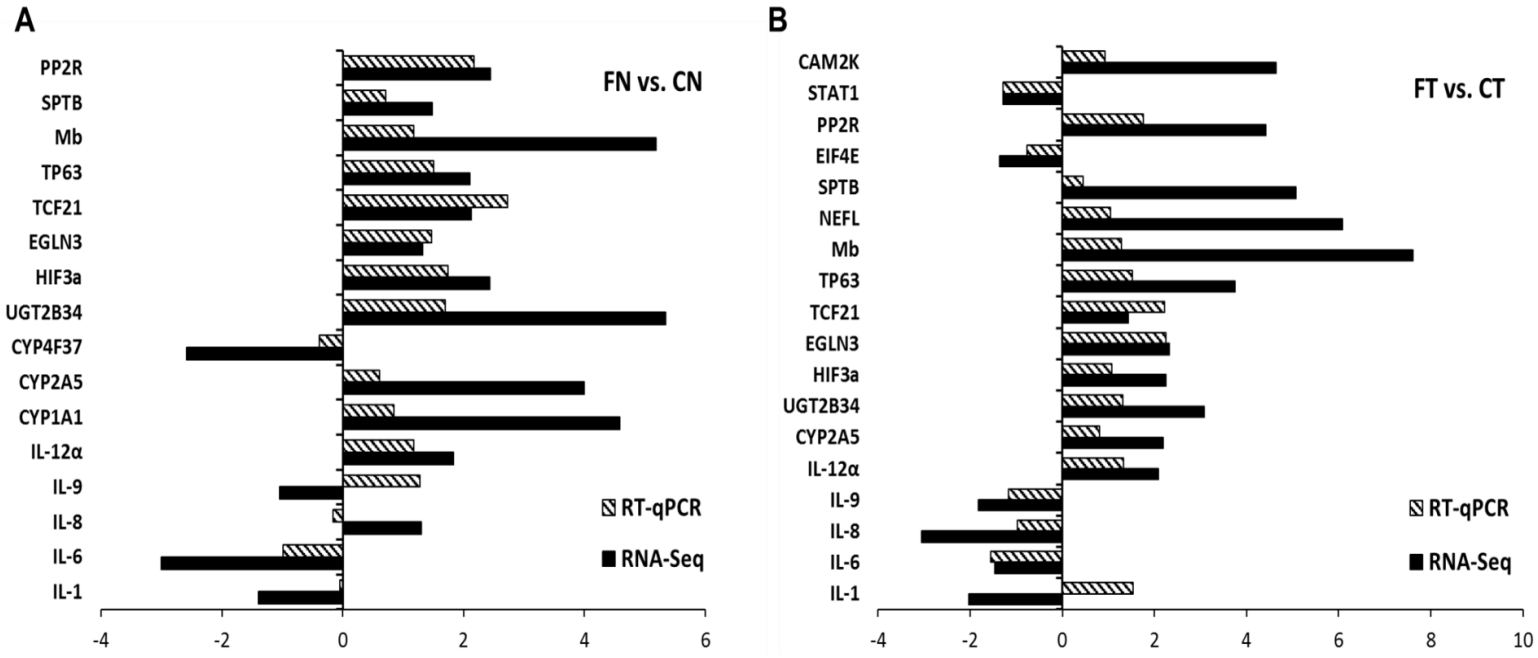

Figure 18. Correlation of differential expression between RNA-Seq and RT-qPCR. RNA-Seq results for selected genes were validated with qRT-PCR using (A) RNA from normal lung tissue of mice in the control and flaxseed group, and (B) RNA from lung tumor nodules of mice in the control and flaxseed group.

Discussion

The vast majority of lung cancers are caused by exposure to tobacco smoke carcinogens. A dietary intervention with potential to protect against chemically-induced lung tumorigenesis would be extremely useful as lung cancer has one of the lowest 5-year survival rates among cancer patients. The present study investigated the effects of flaxseed on the transcriptome in mouse lung tissues exposed to NNK on a control or flaxseed diet to reveal molecular alterations that may contribute to the observed lung chemopreventive properties of flaxseed.

Previous evidence show that flaxseed supplementation ameliorates oxidative stress and inflammation in lung injury models [30-32] and significantly alters gene expression associated with apoptosis, cell proliferation, inflammation-associated cytokine signaling, oxidative stress, and phase I and phase II detoxification pathways in healthy mouse lung tissue [33]. Similarly, we found flaxseed was associated with gene expression changes for signal transduction pathways such as PI3K/AKT and MAPK, apoptosis, cell cycle regulation, cellular response to hypoxia and stress, cytokine response in immune system, development biology, extracellular matrix organization, transcription regulation by TP53, and xenobiotics metabolism. Most transcriptomics studies similar to ours evaluated gene expression changes only in the tumor tissue from control vs treatment animals. Here, we also compared normal tissue from control and flaxseed-fed animals and found that gene expression patterns were similar for FN vs CN and FT vs CT groups. This suggest that the gene transcription changes happening in normal tissues are a good indicator for what is also happening in the tumor tissues.

In our study, the mice fed on flaxseed had lower average body weight at the end of the study which could be attributed to, (i) reduced tumor burden, (ii) reduced fat digestibility [45], and/or (iii) altered adipogenesis-gene regulation [46]. The decrease in average weight of lungs in the flaxseed fed mice as compared to control group mice suggest tumor burden may be a contributing factor. Flaxseeds contain ~ 30% dietary fibers, consumption of which has been further shown to reduce fat digestibility and body weight gain in rats [45]. In addition, flaxseed lignan secoisolariciresinol diglucoside exerts a suppressive effect on body weight gain in mice by altering lipid metabolism [46]. Our result is in line with the effect of flaxseed on body weight gain reported earlier in rodents.

High levels of phosphorylated AKT and ERK have been observed in bronchial preneoplastic lesions [47-50], which are precursors of lung adenoma and adenocarcinoma [9]. In addition, NNK-induced murine lung lesions also exhibit increased activation of AKT [42] and ERK [43]; therefore, inhibition of these signaling pathways may be critical for improved lung cancer treatment. We observed decreased phosphorylation of AKT and ERK protein kinases in normal lung tissue from NNK-treated flaxseed-fed mice. Our findings are in line with other reports showing decreased phosphorylation of AKT and ERK protein kinases after treatment with chemopreventive agents such as, degeulin [51], indole-3-carbinol [14], myoinositol [50], and metformin [52] in NNK-induced mouse models of lung tumorigenesis. In addition to AKT and ERK signaling pathways, de-regulation of another pivotal kinase, JNK, promotes the development of tobacco smoke–induced lung tumors [53]. Genetic ablation of JNK1 alone is associated with reduced tobacco smoke-induced lung tumor multiplicity and size [44]. In this study, we observed that NNK-treated flaxseed-fed mice exhibited decreased phosphorylation of

JNK protein kinase in normal lung tissue. These findings, together with our observations suggest that inhibiting AKT, ERK and JNK activity might be a valuable approach to suppress/delay the effects of NNK-induced lung tumorigenesis.

The protein phosphatase-2A (PP2A) gene encodes a major cellular serine-threonine phosphatase and is a potential tumor suppressor due to its negative regulation on kinase-driven intracellular signaling pathways in several cancers, including lung cancer. Our RNA-Seq data revealed that mRNA expression levels of PP2A were significantly upregulated in response to flaxseed intake in both normal lung tissue and in lung tumor nodules of NNK-treated mice. This suggest that dephosphorylation of AKT, ERK and JNK protein kinases observed in healthy lung tissue of flaxseed-fed mice may be due to increased expression of PP2A. Further, enhanced AKT signaling directly correlates with increased rates of aerobic glycolysis in cancer cells. Inhibition of AKT signaling may inhibit glycolysis and elevate reactive oxygen species, leading to preferential killing of cancer cells through oxidative stress.

NNK is a strong agonist of nicotinic acetylcholine receptors [54], activation of which causes immunosuppression in A/J mice [55]. Suppressed immunologic response to NNK in A/J mice renders them susceptible to lung tumor development [55]. Deregulated production of cytokines promotes a conducive environment for lung tumor growth. A previous study showed that NNK up-regulated pro-inflammatory IL-6 gene expression which was inversely correlated with tumor suppression [56]. NNK lead to activation of PI3K/AKT and ERK signaling pathways which resulted in increased pro-inflammatory IL-8 production and lung tumor-associated inflammation, neovascularization, and increased cell proliferation [57, 58]. Activation of JNK signaling pathway may also play a role in induction of IL-8 transcription [58]. Further, binding

of IL-1α to IL-1 receptor enhanced the production of IL-8 in human airway epithelial cells, indicating that suppression of IL-1α production may reduce IL-8-mediated lung inflammation [59]. Our RNA-Seq data showed a significant decrease in *IL-6* mRNA expression in response to dietary administration of flaxseed. We found a significant decrease in *IL-1α* but an increase in *IL-8* mRNA levels in FN vs CN, and a significant decrease in *IL-1α* and *IL-8* in FT vs CT. NNK has been shown to inhibit the production of the anti-inflammatory cytokine IL-12α by alveolar macrophages which inhibits their ability to activate natural killer cells and increases the risk of developing lung cancer. In our data, *IL-12a* was significantly elevated in FN vs CN and FT vs CT groups, which may have contributed to reduced lung tumor formation in flaxseed-fed animals [60]. Our findings suggest that flaxseed may suppress lung carcinogenesis by mitigating NNK-induced lung immunosuppression by reducing expression of pro-inflammatory cytokines (*IL-1α, IL-6, IL-8,* and *IL-9*) and increasing the expression of anti-inflammatory cytokine (*IL-12α*).

In summary, we found flaxseed consumption protects against NNK-induced lung tumor formation in mice. Flaxseed influenced gene expression in normal lung and lung tumor tissues including up-regulating the expression of phase II enzymes, anti-inflammatory cytokines and tumor suppressors, and down-regulating the expression of pro-inflammatory cytokines which may explain the anti-tumor effects of flaxseed in this model. Further work is needed to identify the potential for flaxseed to impact gene expression in airways of a human population exposed to tobacco smoke carcinogens

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CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The anticancer potential of flaxseed and its derived mammalian lignan EL has been documented in several *in vitro* and *in vivo* studies of breast, colon, and prostate cancers. In addition, flaxseed has been shown to protect lung tissue against oxidative stress and inflammation in murine models of lung injury. However, the anticancer potential of flaxseed and EL remains uninvestigated in lung. We explored the anticancer mechanisms of EL in NSCLC cell lines *in vitro* and the chemopreventive potential of flaxseed in A/J mouse model of NNK-induced lung carcinogenesis. The findings from our studies suggest that EL may serve as a chemotherapeutic agent in the treatment of lung cancer, while flaxseed may serve as a chemopreventive dietary agent in individuals who are at a high risk of lung cancer development.

We observed that EL exerted anti-proliferative effects in NSCLC cells (A549, H441, and H520). These cell lines harbor different somatic mutations that may influence their growth properties and ultimately their response to cancer therapies. This indicates that the growth-inhibitory properties of EL for these cells are independent of their genetic background. In addition, EL showed minimal growth-inhibitory effects in normal lung fibroblast cells (Hs888Lu), which suggest that EL is non-toxic to normal cells. The growth-suppressive property of EL was due to its ability to arrest lung cancer cells in the G_1-phase of the cell cycle. EL down-regulated cell cycle-promoting cyclins such as cyclin D1, cyclin E and CDK-2, -4, and -6, and up-regulated expression of their inhibitor p21. This resulted in decreased phosphorylation of pRb protein and, thereby, decreased cdc25A gene expression that promoted the G_1 to S-phase cell

cycle transition. The anti-proliferative effects of EL we observed in lung cancer cells suggest that EL may hold promise as an adjuvant chemotherapeutic agent for lung cancer therapy.

Given the anti-migratory property of EL in other cancer types, we investigated the *in vitro* anti-migratory effects of EL in NSCLC cells. We observed that EL inhibited A549 and H460 lung cancer cell migration and invasion. This inhibition in cancer cell motility may be due to inhibition of FAK-Src signaling, dysregulation of which has been shown to mediate lung cancer cell migration. EL decreased phosphorylation of FAK and its downstream targets Src and paxillin, and decreased mRNA expression of cell motility-related genes RhoA, Rac1, and Cdc42 in lung cancer cells. In addition, EL treatment inhibited F-actin polymerization and decreased formation of focal adhesion, two important events critical in cell motility. Therefore, our results indicate that EL-mediated inhibition of FAK-Src signaling may be responsible for the reduced migratory capability of lung cancer cells.

Finally, we investigated the chemopreventive potential of flaxseed in an A/J mouse model of tobacco smoke carcinogen NNK-induced lung tumorigenesis. We observed that NNK-treated flaxseed-fed mice had reduced lung tumor incidence and multiplicity as compared to NNK-treated mice fed a control diet. In addition, we observed that in normal lung tissue of NNK-treated mice flaxseed suppressed phosphorylation (activation) of protein kinases such as AKT, ERK, and JNK, aberrant activation of which promotes cancer cell proliferation. To decode the molecular mechanisms by which flaxseed inhibits lung tumor development in our mouse model, we employed an RNA-Seq approach. The RNA-Seq data revealed that flaxseed up-regulated gene expression of phase II enzymes (*GSTs* and *UGTs*), anti-inflammatory cytokines (*IL-12α*), and tumor suppressors (*PP2A*), and down-regulated the expression of pro-

inflammatory cytokines (*IL-1α*, *IL-6*, and *IL*-8) in normal lung and lung tumor tissues. This indicates that flaxseed may exert anti-tumor effects in lungs by enabling detoxification of NNK, alleviating NNK-induced immunosuppression, and/or activating tumor suppressor genes that dephosphorylate aberrantly phosphorylated protein kinases such as AKT. The findings from this study show that flaxseed has a potential to serve as a chemopreventive dietary agent in lung.

Future Directions

The future directions may include:

1) Investigating the anticancer potential of EL in combination with other known lung chemotherapeutic agents such as cisplatin and erlotinib. EL has been shown to enhance the inhibitory effect of tamoxifen on *in vitro* adhesion and invasion of breast cancer cell. Hence, the combinatorial treatment with EL and chemotherapeutic agents may be investigated to uncover their additive and/or synergistic effects on lung cancer cells.
2) Achieving a high plasma concentration of EL from flaxseed consumption may prove difficult given that previous studies have shown that 25-50 g of flaxseed per day, which is equivalent to consumption of 500 mg/day of SDG, results in no more than 2 μM EL in the plasma. Therefore, consumption of purified SDG may be more effective at achieving high plasma EL concentrations than consumption of flaxseed. Hence, further studies of the effects of purified SDG intake in animal models of lung carcinogenesis may be undertaken to find out the optimum dose which may yield a higher concentration of EL in the plasma.
3) We have shown that flaxseed serves as a chemopreventive dietary agent in a mouse model of NNK-induced lung carcinogenesis. Hence, a prospective study in human

volunteers at high risk of lung cancer (smokers and ex-smokers) may be undertaken to evaluate the chemopreventive potential of flaxseed intake. Anti-inflammatory and anti-oxidant biomarkers could be used to determine if flaxseed can reduce NNK-induced inflammation and oxidative stress in smokers.

4) Lignan-rich flaxseed has been shown to decrease tumor cell proliferation and increase apoptosis in diagnosed cases of breast and prostate cancer patients awaiting surgery. Our *in vitro* studies in a panel of NSCLC cells suggest that EL inhibits lung cancer cell proliferation and motility. Therefore, a clinical trial of lignan-rich flaxseed may be undertaken in lung cancer patients awaiting surgery.