Investigation of the Biological Effects of Rosemary (*Rosmarinus Officinalis L.)* Extract in Human Lung Cancer Cells

Jessy Moore, BSc

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Brock University

St. Catharines, ON.

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Abstract

Cancer cells display enhanced growth rates and a resistance to apoptosis. Lung cancer accounts for the most cancer related deaths and non-small cell lung cancer (NSCLC) represents an aggressive form of lung cancer, accounting for almost 80% of all lung cancer cases. The phytochemical rosemary extract (RE) has been reported to have anticancer effects *in vitro* and *in vivo* however, limited evidence exists regarding the effects of RE and its polyphenolic constituents carnosic acid (CA) and rosmarinic acid (RA) in lung cancer. The present study shows RE, CA and RA inhibit lung cancer cell proliferation and survival in various NSCLC cell lines and that CA and RA interact synergistically to inhibit cell proliferation. Moreover RE, CA and RA are capable of altering activation and/or expression of Akt, ERK and AMPK, signaling molecules which regulate cell proliferation and survival. RE shows potential as an anticancer agent and should be further investigated.

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List of Abbreviations

4EBP1: eukaryotic translation initiation factor 4E-binding protein 1

5-FU: fluorouracil

ABD: N-terminal adaptor-binding domain

ACC: acetyl CoA carboxylase

AMPK: 5’ adenosine monophosphate-activated kinase

Apaf-1: apoptotic protease activating factor-1

ATP: adenosine triphosphate

BP: benzopyrene

CA: carnosic acid

CaMKKβ: calcium/calmodulin-dependent protein kinase kinase

CBS: cystathionine beta synthase

CN: carnosol

DD: death domain

DEPTOR: DEP domain-containing mTOR-interacting protein

DISC: death inducing signaling complex

DNA: deoxyribonucleic acid

DNA-PK: DNA-dependent protein kinase

EGF: epidermal growth factor

EMT: epithelial-mesenchymal transition

ERα: estrogen receptor α

ERK: extracellular signal regulated kinase

FADD: fas associated protein with death domain

Fas: fibroblast associated antigen

FGF: fibroblast growth factor

Fos: FBJ murine osteogenic sarcoma virus

GAP: GTPase-activating proteins

GCNT3: glucosaminyl (N-acetyl) transferase 3

GFR: growth factor receptors

GPCR: G-protein coupled receptors

Grb2: growth factor receptor-bound protein 2

H2O2: hydrogen peroxide

HER1: human EGFR

IAP: inhibitor of apoptosis proteins

IR: ionizing radiation

LKB1: liver kinase B1

MAPK: mitogen activated protein kinases

MAPKK or MEK1/2: mitogen-activated protein kinase kinase

MDM2: murine double minute 2

MLST8: mammalian lethal with SEC13 protein 8

MO25: mouse protein-25

mTOR: mammalian target of rapamycin

mTORC1: mTOR complex 1

NSCLC: non-small cell lung cancer

p70S6K: p70S6 kinase

PARP: poly ADP ribose polymerase

PDK1: phosphoinositide-dependent kinase

PH: pleckstrin homology

PI: phosphatidyl inositol

PI3K: phosphatidylinositol 3-kinase

PIP: phosphatidylinositol-4-phosphate

PIP2: phosphatidylinositol-4,5-bisphosphate

PIP3: phosphatidylinositol-3,4,5-trisphosphate

PKB: protein kinase B

PRAS40: proline rich Akt substrate 40

PTEN: phosphatase and tensin homologue deleted on chromosome 10

RA: rosmarinic acid

RAPTOR: regulatory-associated protein of mTOR

Ras: rat sarcoma

RE: rosemary extract

SH2: Src-homology 2

SH3: Src homology 3

Smac/DIABLO: second mitochondrial activator of caspase

SOS: sons of sevenless

STRAD: STe20 related adaptor

TK: tyrosine kinase

TK1: thymidine kinase 1

TNFα: tumor necrosis factor

TRADD: TNF receptor type 1-assocaited death domain protein

TSC2: tuberous sclerosis complex 2

TSP-1: thrombospondin-1

TYMS: thymidylate synthase

UV: ultraviolet

VDA: vitamin D analogue

VEGF-A: vascular endothelial growth factor-A

Chapter 1: Literature Review

1.1 Cancer

Cancer originates within the nucleus of the cell, the body's basic unit of life. Arguably the most fundamental traits of cancer cells are their enhanced proliferative and decreased apoptotic capacities (Figure 1) [1]. Normal cells tightly control the production and release of growth factors which regulate cell growth/proliferation, thereby ensuring cellular homeostasis and maintenance of normal tissue architecture. In cancer cells these signals are deregulated and thus, homeostasis within the cell is disrupted. Proliferation of cancer cells may be enhanced in a number of ways. Cancer cells may produce growth factors, to which they can respond via the expression of cognate receptors. The level of receptor proteins displayed on the surface of cancer cells can also be elevated, rendering these cells hyperresponsive to growth factors; the same outcome can result from structural alterations to the receptor molecules that facilitate activation of downstream signaling pathways independent of growth factor binding [1]. Alternatively cancer cells can signal normal neighbouring cells resulting in mutation/alterations in signaling pathways. These alterations stimulate the release of growth factors which are supplied back to the cancer cells, enhancing their proliferation [2,3].

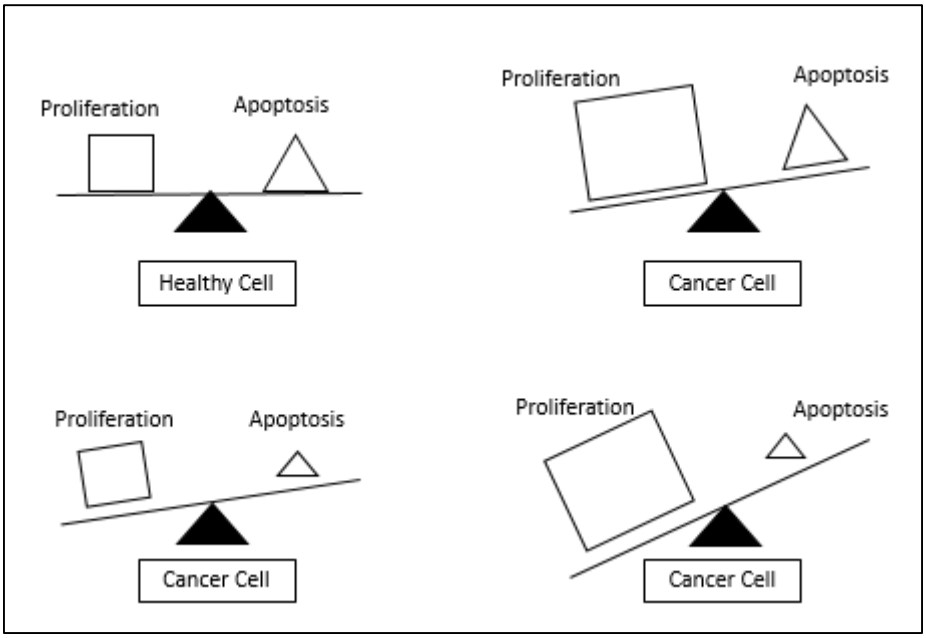

Figure 1: Disruption of proliferation and apoptosis in cancer. Enhanced proliferation and/or inhibition of apoptosis are the fundamental traits of cancer cells compared to their healthy counterparts, which proliferate and undergo apoptosis in a controlled fashion, maintaining homeostasis.

Growth factor receptors (GFR) are plasma membrane proteins with intrinsic tyrosine kinase (TK) activity. Growth factor binding enhances the tyrosine kinase activity of the receptor causing receptor autophosphorylation. The phosphorylated tyrosine residues of the receptor act as docking sites for intracellular proteins containing Src-homology 2 (SH2) domains, leading to stimulation of intracellular signaling cascades that result in enhanced proliferation and/or inhibition of apoptosis (Figure 2). Epidermal growth factor (EGF) signaling is established to play a major role in cancer and for this reason information focused on the EGF receptor and its signaling will be presented in the following sections. In addition, the phosphatidylinositol 3-kinase (PI3K-Akt), the Ras-mitogen activated protein kinase (Ras-MAPK) and the 5’ adenosine monophosphate-activated kinase (AMPK) will be presented because of their significant role in cancer.

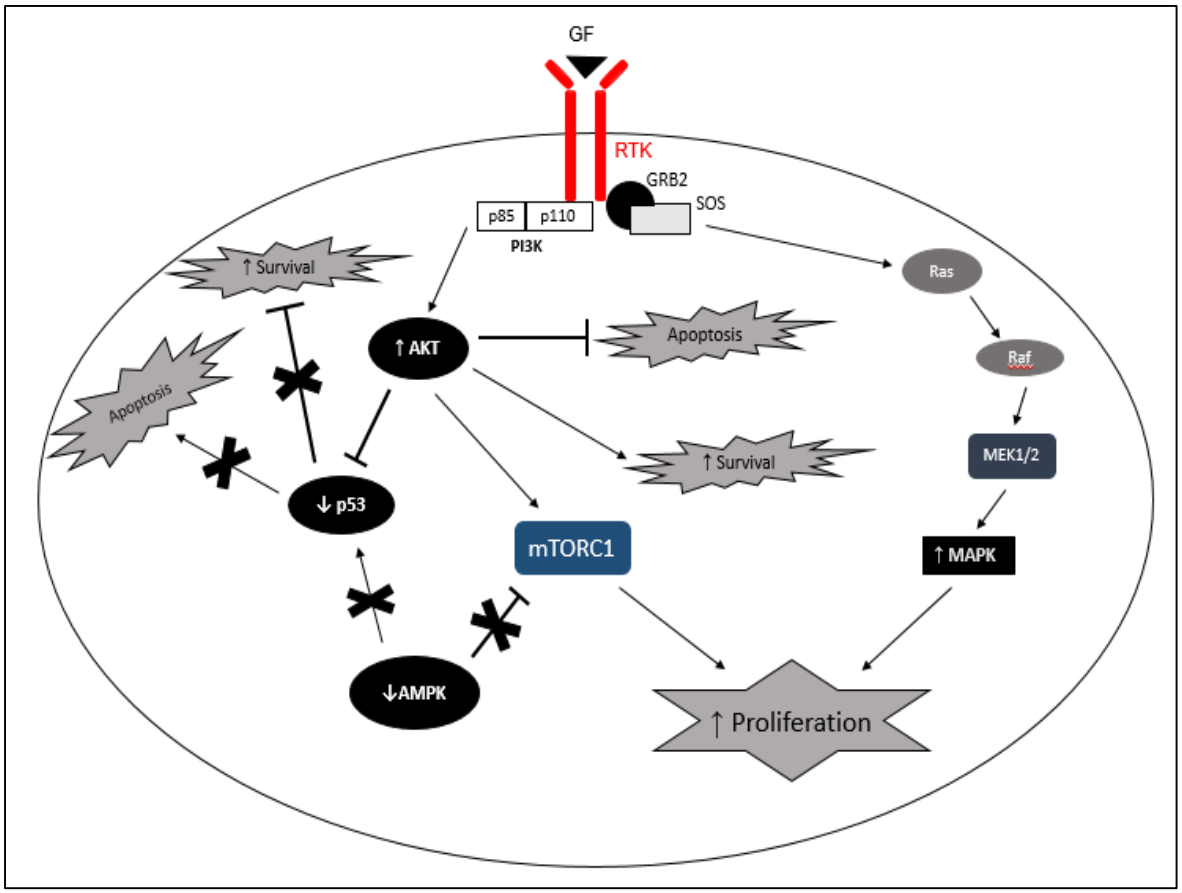

Figure 2: Cell proliferation and survival pathways. The PI3K-Akt, Ras-MAPK and AMPK signaling pathways play a significant role in cancer and are involved in regulating cell proliferation, survival and apoptosis.

1.2 EGFR signaling

The epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors in humans includes human EGFR (HER1) or (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) [4,5]. EGFR (HER1/ErbB1) is a 170 kD transmembrane protein consisting of an N-terminus extracellular ligand-binding site, a hydrophobic transmembrane domain and a C-terminus intracellular region with tyrosine kinase activity [6,7].

The four members of the HER family are capable of forming homo- and heterodimers upon ligand binding. Receptor dimerization is required for receptor activation under normal physiologic conditions. However, these receptors can be additionally activated due to increase in receptor gene copy number, receptor over-expression, and activating mutations [8], including deletion of exon 19 and a single-point substitution in exon 21 which are both found in the tyrosine kinase domain of the receptor and constitute about 90% of all EGFR activating mutations [9].

EGFR is overexpressed as a result of gene amplification in many cancers [10]. Overexpression or mutation of EGFR is common in tumors of epithelial origin, and has been shown in head and neck [11], breast [12], and non-small cell lung [13] cancer cells, among others. Activating mutations (mentioned above) in the EGFR gene lead to increased signaling through associated downstream pathways. Patients whose tumors express high levels of EGFR have a poor prognosis [14] and this makes EGFR an attractive target in cancer therapy; and its inhibition a strategy for augmentation of the efficacy of chemo- and radiotherapy [15]. Activation of EGFR leads to downstream activation of the PI3K/Akt and Ras/MAPK signaling cascades.

1.3 PI3K-Akt/PKB axis

The phosphatidylinositol 3-kinase (PI3K) family are lipid kinases that phosphorylate phosphatidylinositol (PI) substrates, leading to subsequent activation of downstream proteins and cell survival and proliferation. Typically substrates include PI, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP2) and the corresponding products are phosphatidylinositol-3-phosphate (PIP) phosphatidylinositol-3,4-bisphosphate (PIP2) and phosphatidylinositol-3,4,5-

trisphosphate (PIP3). The most predominant product in vivo is PIP3, produced following phosphorylation at the 3'-OH position of the inositol ring in PI-4,5-P (PIP2) by PI3K. The PI3K molecule contains a regulatory (p85) subunit and a catalytic (p110) subunit [16,17]. The p110 subunit contains 5 binding domains, including an N-terminal adaptor-binding domain (ABD) which binds to p85, a Ras-binding domain, a putative membrane-binding domain (C2), a helical domain and a kinase catalytic domain. The p85 regulatory subunit contains an SH2 domain which interacts directly with the tyrosine phosphate motifs of the activated tyrosine kinase receptor, recruiting the PI3K complex to the cell plasma membrane. The SH2 is a structurally conserved protein domain which allows docking to phosphorylated tyrosine residues on other proteins [18]. This binding removes inhibition of the p110 catalytic subunit which proceeds to phosphorylate membrane phospholipids. PIP3 serves as a docking site in the plasma membrane for proteins containing pleckstrin homology (PH) domains, such as Akt.

There are three subclasses of PI3K (Class I-III) based on structure, regulation and substrate specificity. Class II and III vary from Class I in structure and function, both containing a single catalytic subunit, without a regulatory subunit. Class II preferentially use phosphatidylinositol (PI) or phosphatidylinositol-4-phosphate (PIP) as substrates, while Class III use only PI as a substrate. Class I can use PI, PIP and PI-4,5-P (PIP2) as a substrate, with PIP2 being the preferential substrate in vivo [17]. Class I can be further divided into class IA which can be activated by receptor tyrosine kinases, G-protein coupled receptors (GPCR) and oncogenes, and class IB which can be activated only by GPCRs [19].

The PIK3CA gene encodes the catalytic subunit (p110α) of PI3K and was discovered to be somatically mutated in a significant fraction of diverse tumor types [20–22]. This mutation results in a constitutively activated PI3K and associated downstream molecules such as Akt leading to enhanced cell survival capabilities. Mutations in exon 9 or 20 of the PIK3CA gene have been associated with lower relapse-free survival in patients with stage II or III colorectal cancer [23] and progression-free survival with cetuximab (EGFR inhibitor)-based therapy in patients with colon cancer was significantly lower when tumors carried mutations in the PIK3CA gene [24]. The PI3K-Akt signaling pathway can be seen in Figure 3.

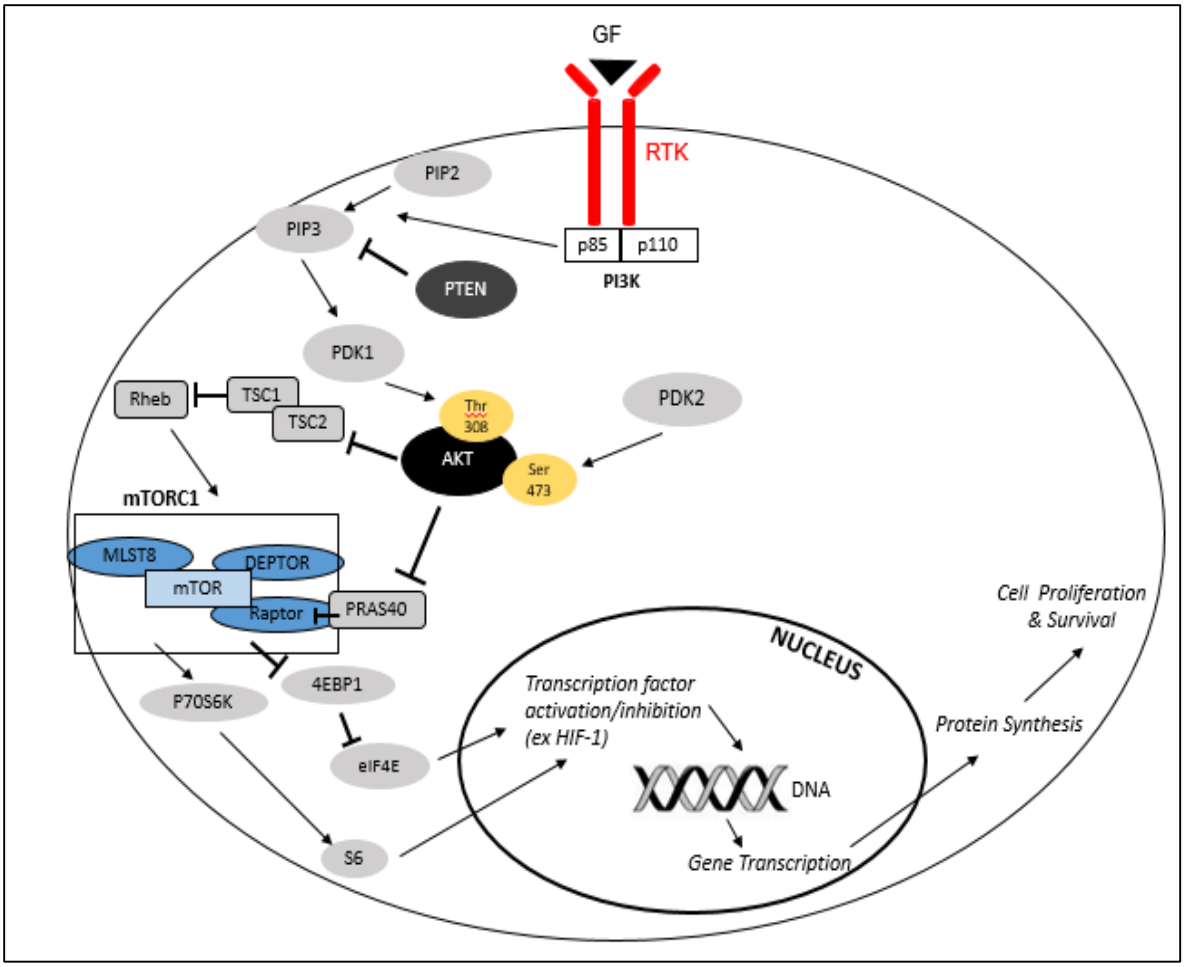

Figure 3: The PI3K/Akt cell signaling pathway is involved in regulating cell survival and proliferation pathways which are often deregulated in cancer cells.

Akt, also known as protein kinase B (PKB) is a serine/threonine- specific protein kinase located downstream of PI3K. The mammalian genome contains 3 Akt genes, encoding the isoforms Akt1, Akt2 and Akt3. Akt2 and Akt3 have 81 and 83% amino acid sequence homology with Akt1 respectively [25]. Each gene encodes a protein containing a pleckstrin-homology (PH) domain in the N-terminus, a central kinase domain, and a C-terminal regulatory domain [26]. All 3 mammalian Akt genes are widely expressed in various tissues, but Akt1 is most abundant in brain, heart and lung, whereas Akt2 is predominantly expressed in skeletal muscle and embryonic brown fat, and Akt3 is

predominantly expressed in brain, kidney and embryonic heart [27–30]. Akt, through its (PH) domain, binds to PIP3 and once at the membrane, Akt is phosphorylated at two sites; Thr308 by phosphoinositide-dependent kinase (PDK1) and Ser473 by PDK2 [26], resulting in full activation of its kinase activity and downstream phosphorylation of numerous protein targets [20]. A constitutively active Akt has been functionally linked with a poor prognosis in many human cancers [17,31–35]. Additionally, Akt activity promotes resistance to chemo- and radiation therapy [36–38].

The levels of membrane-bound PIP3 are tightly regulated by phosphatases such as the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [39] which acts to reduce PIP3 levels. Loss of PTEN increases the activity of the PI3K/Akt cascade leading to enhanced proliferation and survival, and cancer [21,22].

1.3.1 mTOR

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase located downstream of Akt in the cell survival pathway (Figure 3). Activation of mTOR requires the recruitment of multiple molecules to form a complex. mTOR complex 1 (mTORC1) consists of mTOR, regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (MLST8) and DEP domain-containing mTOR-interacting protein (DEPTOR) [40]. Akt activates mTOR through (i) phosphorylation and inhibition of tuberous sclerosis complex 2 (TSC2), which allows the mTOR-activating GTPase Rheb to remain in its GTP-bound (active) state and/or (ii) phosphorylation and removal of proline rich Akt substrate 40 (PRAS40), a Raptor inhibitor [41]. Active mTORC1 promotes gene expression and translation through phosphorylation-mediated activation of p70S6 kinase (p70S6K) and phosphorylation-mediated inhibition of eukaryotic

translation initiation factor 4E-binding protein 1 (4EBP1) [41,42]. Enhanced activation of the mTOR signaling pathway has been reported in a variety of human cancers including malignant melanoma [43] and head and neck cancers [44]. Recent evidence has shown that two different single amino acid mutations in mTOR, S2215Y and R2505P, identified in the human cancer genome database, confer activation of mTORC1, and cells expressing the S2215Y mutant mTOR exhibited altered cell cycle profile [45].

1.4 Ras-MAPK axis

The Rat sarcoma (Ras) family of proteins are oncogenic GTPases and function as a molecular switch that cycles between a GDP-bound inactive state and a GTP-bound active state, regulating processes governing cell proliferation, survival and differentiation [46]. The three mammalian Ras genes (H-, K- and N-Ras) encode the four Ras proteins: HRas, KRas4A, KRas4B and NRas and display extraordinary similarities. These proteins are all 21 kDa, are identical over the 85 N-terminal residues and their identity within the following 80 residues is up to 90% [47].

The growth factor receptor-bound protein 2 (Grb2) is a docking protein containing a Src homology 2 (SH2) binding domain and 2 Src homology 3 (SH3) domains. The SH3 domains allow binding of the guanine nucleotide exchange factor sons of sevenless (SOS). Following ligand binding to the GFR and receptor autophosphorylation, the SH2 domain of Grb2 binds to the phosphorylated tyrosine residues on the GFR, and this activates SOS. Activated SOS promotes the removal of GDP from membrane-bound Ras and addition of GTP. The GTP-bound Ras is now in an active state and can proceed to recruit and activate the serine/threonine protein kinases C-

Raf and B-Raf, which form a heterodimer. Activated Raf phosphorylates and activates the tyrosine/threonine mitogen-activated protein kinase kinase (MEK 1/2), which in turn phosphorylates and activates the mitogen activated protein kinases (MAPK). MAPKs are evolutionarily conserved kinase modules that link extracellular signals to the machinery inside the cell that controls fundamental cellular processes such as growth and differentiation. Phosphorylation of MAPK by MEK leads to phosphorylation of downstream targets including transcription factors that lead to cell growth and survival (Figure 4) [47].

Alternatively, activated Ras is capable of activating the p110 catalytic subunit of PI3K, independent of regulatory subunit p85, leading to subsequent activation of Akt and cell survival as described previously (Figure 3) [48].

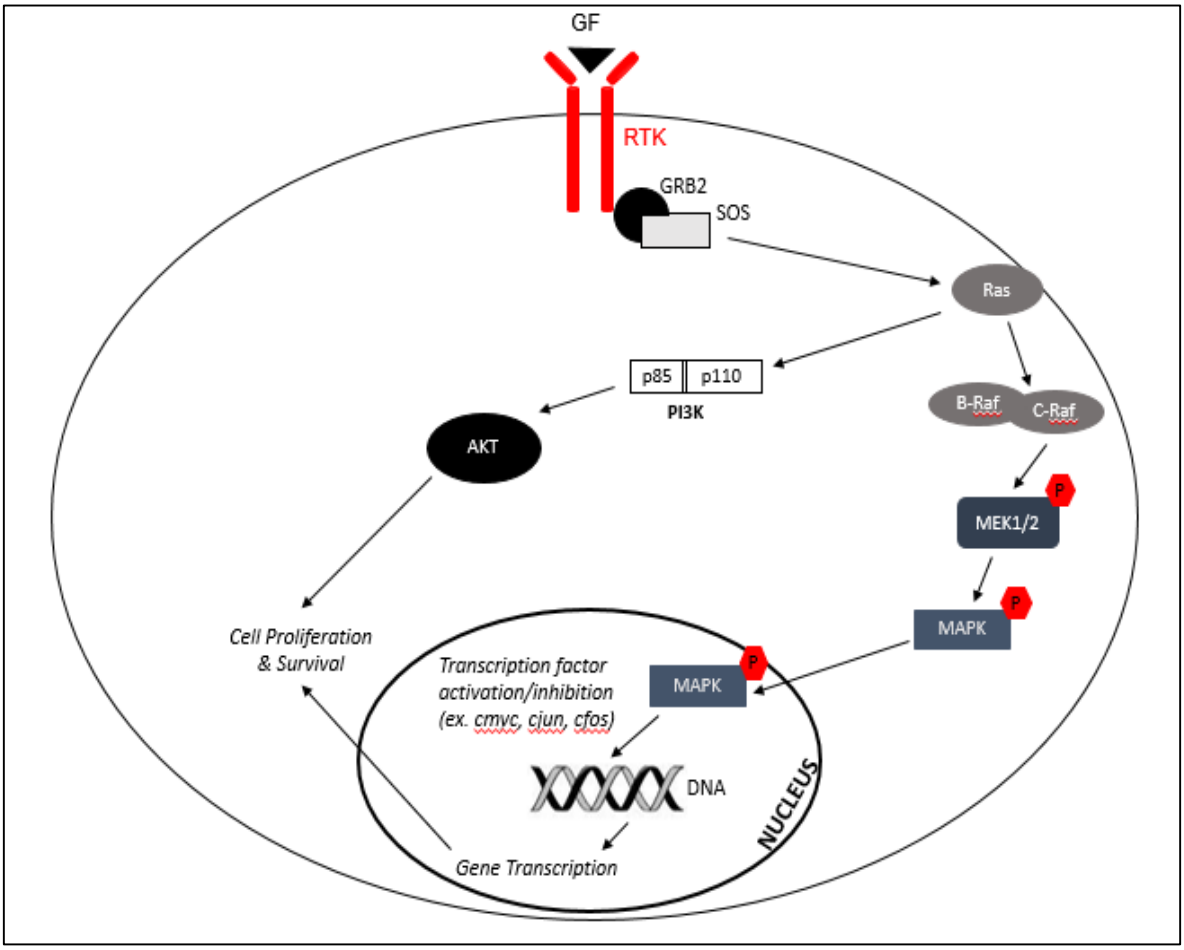

Figure 4: The Ras/MAPK cell signaling pathway is involved in regulating cell growth and proliferation and signaling through this pathway is often deregulated in cancer cells.

Activating mutations in KRAS (gene encoding Ras) are common in cancer and stimulate downstream cascades [49]. Most oncogenic forms of Ras impair their intrinsic GTPase activity, preventing GTP hydrolysis and locking Ras in a GTP-bound activated state. Point mutations in the Ras protein can occur at codon 12, 13 or 61, but are most common (97% of the time) at codons 12 and 13, which can be found in the amino acid loop that interacts with the phosphate group of GTP with the help of GTPase-activating proteins (GAP) [46].

Mutations in KRAS account for 90% of Ras mutations in lung adenocarcinomas. It is the most commonly mutated oncogene in non-small cell lung cancer (NSCLC), with mutations detected in about 25% of all tumors. The presence of KRAS mutations has been shown to portend a poor response to monotherapy and combination therapy using EGFR inhibitors, such as cetuximab or panitumumab, in primary colorectal cancer [50–52]. Similarly, patients with KRAS-mutant tumors treated with cetuximab in any combination, had lower progression-free survival compared to those with wild type KRAS tumors [24,53,54] suggesting that KRAS mutation may bypass aberrant EGFR signalling. This evidence linking KRAS mutations with poor response to EGFR inhibitors now influences treatment decisions in the clinic, and highlights the importance of examining the tumor mutation profile.

1.5 AMPK

5'-adenosine monophosphate-activated protein kinase (AMPK) is an energy sensing protein kinase that is important in regulating cellular homeostasis and protecting cells under conditions of metabolic stress. AMPK is a highly conserved heterotrimeric kinase complex composed of a catalytic α subunit and two regulatory (β and γ) subunits. The α and β subunits can exist as 2 different isoforms (α1, α2; β1, β2) while the γ subunit can exist as 3 isoforms (γ1, γ2, γ3). The most common isoforms expressed in most cells are the α1, β1 and γ1 isoforms however, the other isoforms have been found to be expressed in cardiac and skeletal muscle [55]. Modulation of AMPK activation is shown in Figure 5.

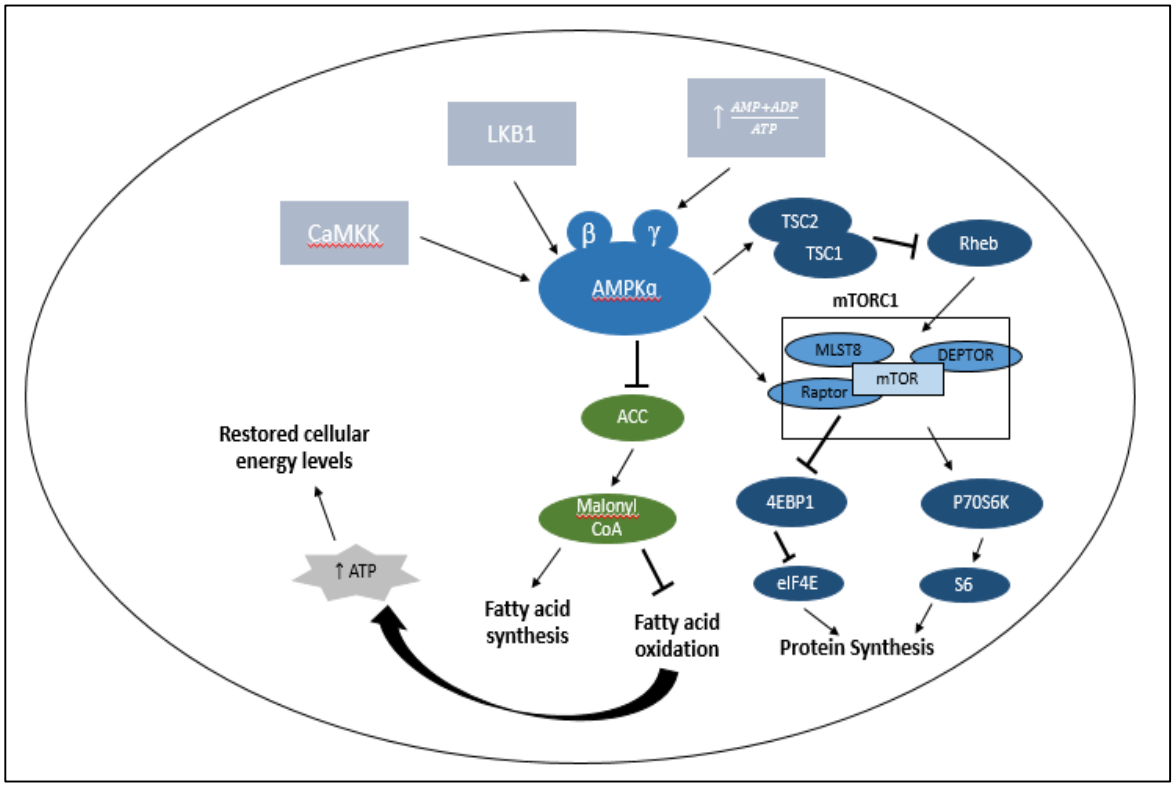

Figure 5: The AMPK signaling pathway. AMPK becomes activated under conditions of cellular stress and low ATP. Increases in glycolytic rate and mutations in LKB1 often result in decreased AMPK activity in cancer.

Activation of AMPK occurs when there is an increased concentration of AMP and a fall in ATP within the cell, through phosphorylation by tumor suppressor liver kinase B1 (LKB1) or by the calcium/calmodulin-dependent protein kinase kinase (CaMKKβ). When ATP levels in the cell drop, resulting in increased AMP, two AMP molecules bind the 2 Bateman domains of AMPK which are found in the γ subunit. Each Bateman domain is a pair of cystathionine beta synthase (CBS) protein domains. AMP binding results in a conformational change of the γ subunit, exposing the threonine-172 (Thr-172) site on the catalytic α subunit. The serine/threonine kinase LKB1 is a tumor suppressor mutated in Peutz-Jeghers syndrome [56] that leads to tumor growth by phosphorylating and thus activating the exposed activating loop on Thr-172 in the α subunit. When

complexed with STe20 Related Adaptor (STRAD) and Mouse protein-25 (MO25), LKB1 shows constitutive activation and localization in the cytosol [57]. The increased levels of AMP within the cell also act to prevent dephosphorylation of the Thr-172 residue, rendering AMPK a better substrate for LKB1 phosphorylation and activation [55,58]. Alternatively, AMPK can be phosphorylated and activated on its Thr-172 residue by CaMKKβ when intracellular calcium levels increase such as during exercise [59]. Somatic inactivation mutations and genetic loss of the LKB1 gene are found in a significant number of lung and cervical cancers, greatly reducing phosphorylation of AMPK at Thr-172 and thus its activity within these cells [60].

Activation of AMPK increases cellular energy levels by inhibiting anabolic energy consuming pathways and stimulating energy producing catabolic pathways. Once activated, AMPK inhibits anabolic processes and protein synthesis by inhibiting mTORC1 through (i) Ser1387 phosphorylation and activation of TSC2, leading to inhibition of Rheb GTPase activity and thus mTOR inhibition, or (ii) direct phosphorylation and inhibition of mTOR-binding partner Raptor [61]. AMPK phosphorylates and inhibits acetyl CoA carboxylase (ACC), a key regulator of fatty acid and glycerol lipid syntheses. Two isoforms of ACC exist, and AMPK inhibits this molecule by phosphorylating serine 79, 1200 and 1215 residues on ACC1 and serine 218 on ACC2. ACC is the rate limiting enzyme for malonyl-CoA synthesis, which leads to inhibition of fatty acid oxidation. Thus, following activation of AMPK and subsequent inhibition of ACC, malonyl-CoA synthesis is decreased leading to enhanced fatty acid oxidation and ATP production, restoring cellular energy levels.

It has been observed that the activation of AMPK in cancer cells leads to suppression of cell growth through inhibition of protein synthesis and up-regulation of the p53 pathway leading to induction of p21 and p27, cyclin-dependent kinase inhibitors, and subsequent cell cycle arrest [62–64]. Activating AMPK using therapeutic drugs such as metformin, a mitochondrial complex I inhibitor, may be able to overcome breast cancer resistance to HER2 inhibitors while decreasing risk of cardiotoxicity [65] as well as inhibit growth and enhance radio-sensitivity of lung cancer cells [65,66].

1.6 Tumor suppressor p53

The tumor suppressor protein p53 is encoded by the gene tumor protein p53 (TP53) and in its active form is a tetramer of four identical subunits. Each subunit contains an N-terminal transactivation domain, a proline-rich region, a DNA-binding core domain, a regulatory domain contained within the C-terminal, a tetramerization domain and an unstructured basic domain [67]. Under healthy conditions, p53 levels are kept low within the cell and are regulated, through continuous degradation, by murine double minute 2 (MDM2) homologue. In a negative feedback loop, p53 increases MDM2 levels while MDM2 suppresses p53 activity (Figure 6) [68]. p53, expressed in all normal cells, acts to induce apoptosis (programmed cell death), modulate the cell cycle and aid in DNA repair. It is required in all cells for the maintenance of homeostasis and to signal the death of unhealthy or damaged cells.

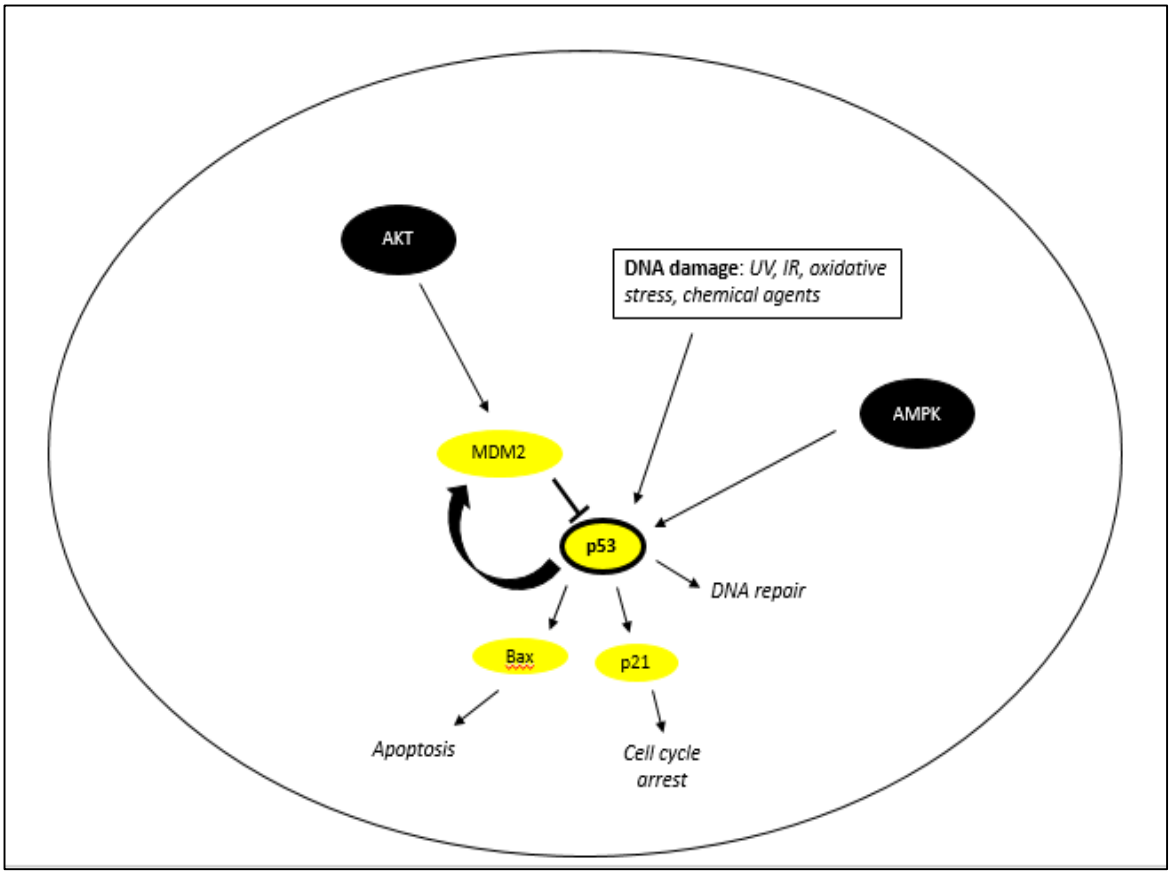

Figure 6: Tumor Suppressor p53. Activation of tumor suppressor p53 leads to increased apoptosis however, loss of p53 function often occurs in cancer.

The p53 protein becomes activated by phosphorylation in response to stresses including DNA damage by ultraviolet (UV) or ionizing radiation (IR), chemical agents, oxidative stress or deregulated oncogene expression [67]. Phosphorylation of p53 occurs by DNA damage response elements, such as DNA-dependent protein kinase (DNA-PK) which phosphorylates p53 at serines 15 and 37 and impairs the ability of MDM2 to inhibit p53 resulting in increased, activated p53 levels within the cell [68]. Activated p53 binds DNA, initiating transcription of genes such as p21 and Bax, which induce cell cycle arrest to allow either repair and survival of the cell, or apoptosis. Tumor-associated mutations to the TP53 gene, typically single nucleotide substitutions in the coding sequence, occur often in cancers leading to dramatic defects in p53 function, reduced

tumor suppression and enhanced survival abilities of the cancer cells [69]. Mutant p53 proteins are often not able to induce MDM2 and thus accumulate at high levels. Alternatively, in some tumors a loss of one or both p53 alleles (through a large chromosomal defect or a localized deletion) reduces the levels of wild-type p53 tetramers [70]. Tumors with enhanced Akt signaling show decreased apoptotic activity because Akt phosphorylates MDM2, allowing it to translocate into the nucleus and inhibit p53 levels [71]. AMPK is able to phosphorylate p53, activating it and thus, low levels of AMPK activity which are common in cancer cells result in decreased apoptosis (Figure 6) [72].

Functional p53 has been shown to be necessary for DNA-damage induced apoptosis and sensitivity of cancer cells to ionizing radiation and the chemotherapy agent cisplatin [73]. Furthermore, ionizing radiation, which can induce G1 and G2 cell cycle arrest in proliferating cells, has been shown to be dependent on wild-type p53 function in various cancer cell lines [74]. Many new cancer treatments aim to restore endogenous wild-type p53 levels within the cell and enhance the function of existing wild-type p53 thus resulting in apoptosis of cancerous cells and/or sensitization of cancer cells to cytotoxic therapies such as ionizing radiation.

1.7 Hallmarks of carcinogenesis

Cancer cells are unique in their ability to mutate and grow uncontrollably by excessive proliferation and inhibition of apoptosis, as mentioned in previous chapters. The pathways mentioned in chapters 1.2 through 1.6 are highly implicated in cancer cell proliferation and survival and will be examined in this study however, there are other important hallmarks of cancer cells which contribute to the complexity of the disease and will be mentioned in this chapter. As normal cells transform into cancer cells, they

acquire a succession of these hallmark capabilities leading to a more aggressive and uncontrolled disease.

1.7.1 Resisting cell death: extrinsic and intrinsic apoptotic mechanisms

Enhanced oncogene signaling resulting in excessive proliferation and DNA damage can lead to programmed cell death called apoptosis as mentioned in Chapter 1.6. There are several mechanisms by which a cell can trigger apoptosis including activation of tumor suppressor p53, as previously mentioned, or by intrinsic and extrinsic caspase signaling pathways (Figure 7). Caspases are cysteine aspartate-specific proteases, which can be rapidly activated. Initially synthesized as inactive pro-caspases, these molecules consist of a small subunit, large subunit and a long prodomain which enables them to interact with other molecules that regulate their activation. Following activation, the majority of the prodomain is cleaved, allowing them to proceed to activate effector caspases and initiate a response.

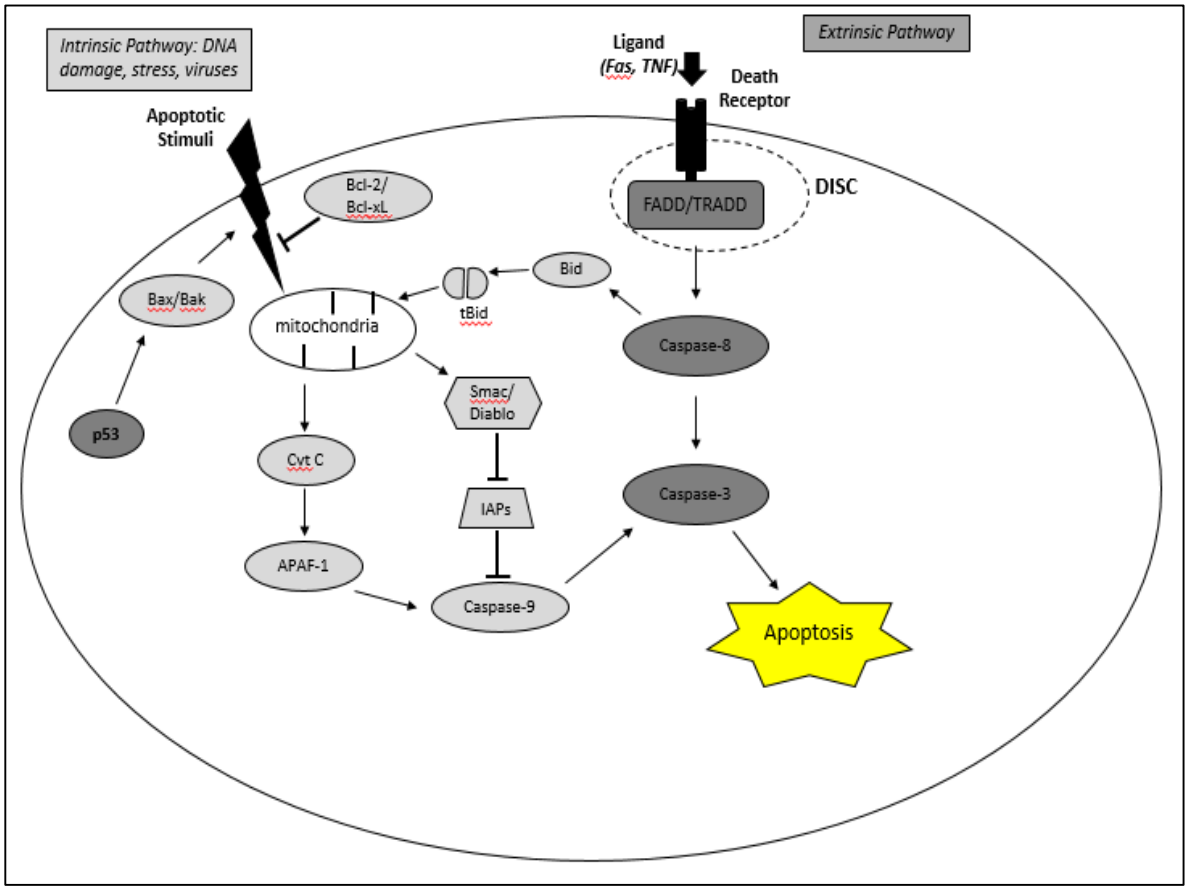

Figure 7: Intrinsic and extrinsic caspase cell signaling pathways are involved in regulating apoptosis and represent a different mechanism of programmed cell death from activation of tumor suppressor p53.

The intrinsic apoptotic pathway (Figure 7) does not involve receptors, but instead is activated in response to stimulation of the cell by a stressor such as DNA damage, ionizing radiation or a virus. Apoptosis through this pathway relies on the insertion of pro-apoptotic members of the Bcl-2 family, Bax/Bak, into the mitochondrial membrane, resulting in release of cytochrome c into the cytosol. Anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL, are able to prevent cytochrome c release by binding and inhibiting Bax and Bak [75]. Cytochrome c released via the intrinsic pathway binds apoptotic protease activating factor-1 (Apaf-1) and together with deoxyadenosine triphosphate (dATP) recruits pro-caspase 9 to the complex. This multi-protein complex is

called the apoptosome and causes cleavage of pro-caspase 9 and thus activation of caspase 9, which activates caspase 3 and executes apoptosis [76]. In addition to cytochrome c, mitochondria release other peptides such as second mitochondrial activator of caspase (Smac/DIABLO) which promotes caspase 9 activation by neutralizing the inhibitory effects of inhibitor of apoptosis proteins (IAPs).

The extrinsic apoptotic pathway (Figure 7) involves membrane bound death receptors, typically fibroblast associated antigen (Fas) receptor or tumor necrosis factor (TNF) receptor, which contain a cytosolic death domain (DD). Upon activation by their respective extracellular ligands, the receptors form a death inducing signaling complex (DISC) whereby the DD of the receptor binds an adaptor protein, either Fas associated protein with death domain (FADD) or TNF receptor type 1-associated death domain protein (TRADD) respectively [77,78]. Caspase 8 is then recruited by the DISC, activated, and proteolytically activates downstream effector caspase 3 (by directly cleaving procaspase-3) which executes apoptosis [79]. Caspase 8 is also capable of cleaving Bid to its' truncated form (tBid) which moves into the mitochondria and induces the release of cytochrome c. Once cytochrome c has entered into the cytosol it binds Apaf-1, activating caspase 9 which in turn activates caspase 3 which executes apoptosis [80]. Caspases execute apoptosis by cleaving their target proteins, thereby disabling important cellular processes and breaking down structural components of the cell [81].

In cancer, tumors evolve a variety of strategies to try and limit or eliminate apoptosis, most commonly the loss of tumor suppressor p53. Alternatively, tumors may increase expression of antiapoptotic regulators (Bcl-2 or Bcl-xL), decrease expression of

proapoptotic factors (Bax or Bak) or inhibit the extrinsic ligand-induced death pathway [1].

1.7.2 Resisting cell death: autophagy and necrosis

Autophagy is a process which leads to the degradation of cellular proteins and cytoplasmic organelles to provide energy to the cell under conditions of stress or when the availability of external nutrient sources is limited. Many studies highlight the importance of autophagy in cancer however, it remains unclear whether autophagy suppresses tumorigenesis or provides cancer cells with a rescue mechanism in unfavourable conditions. Stress, resulting from oxidative damage or hypoxic conditions can damage cellular proteins and organelles. This damage triggers autophagy and elimination of the damaged components. Thus, in healthy cells autophagy acts as a homeostatic and survival-promoting mechanism [82].

In cancer, some cells experience elevated metabolic stress from nutrient, oxygen and growth factor deprivation caused by inadequate blood supply resulting from deficient blood vessel formation (angiogenesis). This stress, compounded with the increased metabolic demand to drive cell proliferation, promotes autophagy and tumor survival. Tumor cells can progressively eat themselves (through autophagy) under prolonged stress, becoming less than one third their normal size and suppressing cell division and motility. These cells are considered dormant and inactive, but retain the capacity to return to their normal size and resume cell proliferation following restoration of normal growth factor conditions [83,84]. Thus, autophagy may act to promote survival of cancer cells.

Alternatively, loss of autophagy can occur in cancer cells with certain mutations. Constitutive activation of the PI3K-Akt pathway (Chapter 1.3) causes unrelenting cell growth signals regardless of nutrient and growth factor availability. This leads to mTOR activation and subsequent inhibition of autophagy. Although constitutive growth signals drive cancer cell proliferation, these cancer cells are also rendered less able to induce autophagy in response to stressors and this can lead to metabolic fragility and increased likelihood of cell death [84]. Thus regulation of autophagy varies in each cancer cell and while it can act to promote cancer cell survival, it may also promote cancer cell fragility and represent an important therapeutic target.

Many cancer treatments aim to reduce levels of autophagy to basal levels or induce apoptosis [83,85,86]. Tumor masses have heterogeneous areas of vessels and nutrient supply, and cancer cells residing in hypoxic tumor regions and undergoing autophagy are the cancer cells that resist chemo and radiotherapy[82]. This subpopulation of resistant cancer cells provides a potential target to improve cancer therapy. Alternatively, some cancer treatments such as nutrient starvation, radiotherapy and some cytotoxic drugs can induce elevated levels of autophagy which are thought to be protective for cancer cells, impairing rather than accentuating the stresses these anticancer treatments exert on cancer cells [82,83,87,88]. This represents an important issue with current cancer therapies and highlights the need for further investigation and development of new therapies.

Necrosis is a form of cell injury that results in the premature death of cells in living tissue. It is caused by factors external to the cell or tissue such as toxins, infection or trauma and is most often detrimental to the organism. Necrosis is often up-regulated

under conditions of stress however, unlike apoptosis it is not programmed, and it results in the release of the cell contents into surrounding tissue. Necrotic cell death results in the release of pro-inflammatory signals, recruiting immune inflammatory cells which, under normal conditions, remove associated necrotic debris. In cancer, evidence indicates that necrotic cells can be tumor promoting, causing increased inflammatory signals that can foster angiogenesis, cancer cell proliferation and invasiveness. These necrotic cells are also capable of releasing regulatory factors such as interleukins which can bind membrane receptors of viable neighbouring cells, stimulating proliferation and potentially facilitating neoplastic progression [89].

1.7.3 Inducing angiogenesis

Tumors, like normal tissues, require nutrients, oxygen and the ability to evacuate metabolic wastes and carbon dioxide. They do so by developing their own vasculature through a process known as angiogenesis, the sprouting of new vessels from existing ones. During tumor progression, an “angiogenic switch” is almost always activated and remains on, causing normally quiescent vasculature to sprout new vessels that help sustain expanding neoplastic growths [90]. Some regulators of angiogenesis, including stimulatory vascular endothelial growth factor-A (VEGF-A) and inhibitory thrombospondin-1 (TSP-1) are signaling proteins which bind to cell-surface receptors displaced by vascular endothelial cells [91,92]. In addition, members of the fibroblast growth factor (FGF) family have been implicated as proangiogenic factors when their expression is chronically up-regulated [91].

The VEGF gene encodes ligands involved in orchestrating new blood vessel growth during embryonic and postnatal development, and then in homeostatic survival of

epithelial cells in adults. VEGF signals through 3 receptor tyrosine kinases (VEGF-1-3) and its gene expression can be up-regulated by hypoxia and oncogenic signals [93,94]. Additionally, VEGF ligands can be sequestered in the extracellular matrix in latent forms that are subject to release and activation by extracellular matrix-degrading proteases such as MMP-9 [95]. TSP-1, a key counterbalance in the angiogenic switch, also binds transmembrane receptors on endothelial cells and evokes suppressive signals to counteract proangiogenic stimuli [96]. Historically, angiogenesis was envisioned to be important only when rapidly growing large tumors had formed, but more recent data indicate that angiogenesis also contributes to the microscopic premalignant phase of neoplastic progression [1].

1.7.4 Activating invasion and metastasis

Local invasion and distant metastasis are reflections of advanced, high grade malignancies which have developed alterations in their shape as well as in their attachment to other cells and to the extracellular matrix. The loss of E-cadherin, a key cell-to-cell adhesion molecule, by cancer cells through down-regulation or mutation is an alteration commonly involved in invasion and metastasis [97,98]. Adhesion molecules which are normally associated with the cell migration that occurs during embryogenesis and inflammation, such as N-cadherin, are often up-regulated in invasive neoplastic environments. Beyond the gain and loss of such cell-cell/matrix attachment proteins, the master regulators of invasion and metastasis are largely unknown [98]. Generally, the process begins with local invasion of surrounding tissues, followed by entry of cancer cells into nearby blood and lymphatic vessels which carry the cells through the body until they escape into the parenchyma of distant tissues and form small nodules of cancer cells

which will grow into metastatic lesions [1]. Epithelial-mesenchymal transition (EMT) has become prominently implicated as a means by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis and to metastasize [99–101]. This EMT program can be activated transiently or stably, and to different degrees, by cancer cells during invasion and metastasis [1]. The process of EMT is orchestrated by a combination of transcription factors which act to evoke loss of adheren junctions, conversion from epithelial to mesenchymal morphology, expression of matrix-degrading enzymes, increased motility and heightened resistance to apoptosis – all traits implicated in the process of invasion and metastasis [1]. Taken together, these cancer hallmarks highlight the diversity of the disease and the importance of finding new therapies and prevention strategies which target deregulated cancer cell signaling.

1.8 Natural compounds in cancer therapy

Many pharmaceutical agents have been discovered by screening natural products from plants. Some of these drugs such as the chemotherapeutics etoposide, isolated from the mandrake plant and Queen Anne's lace, and paclitaxel and docetaxel, isolated from the wood and bark of the Nyssaceae tree, are currently successfully employed in cancer treatment [102]. The exploration into natural products offers great opportunity to evaluate new chemical classes of anticancer agents as well as novel and potentially relevant mechanisms of action. Many labs, including ours have shown metformin, a drug derived from the lilac, has anticancer properties [66]. In addition, the polyphenol resveratrol, found in high concentrations in wine, has been shown to have anticancer effects *in vitro* [103] and *in vivo* [104]. Rosemary extract, which is rich in polyphenols, has been shown in our lab to increase glucose uptake in rat muscle cells and act on signaling pathways

which are highly implicated in cancer. Thus, further investigation of rosemary's potential anticancer effects are warranted.

1.9 Anticancer effects of rosemary extract (Rosmarinus Officinalis L.)

The plant *Rosmarinus Officinalis L.* a member of the mint family *Lamiaceae,* is native to the Mediterranean region and has many culinary and medicinal uses. The rosemary plant grows quite easily and in a variety of climates as long as sunlight and proper drainage is present, and temperatures are above freezing. The main polyphenols found in rosemary extract include the diterpenes carnosic acid (CA), rosmarinic acid (RA) and carnosol (CN) [105]. Rosemary extract and some of its polyphenol components have recently been explored and found to exert potent anticancer effects summarized in the following sections. Findings are sorted by cancer cell type and are in chronological order (earliest→most recent).

1.9.1 Anticancer effects of rosemary extract: *in vitro* studies

Table 1: Anticancer effects of RE *in vitro*: colon cancer

Cancer Cell	Dose/Duration	Findings	Mechanism	Reference
CaCo-2	0.1-30µg/mL (3-24h)	↓ cell colony formation. Long and short term antioxidant effects.	↓ H2O2-induced DNA strand breaks & oxidative damage. ↓ visible light-induced oxidative damage	[106]
SW480	31.25-500 µg/mL (48h)	↓ cell proliferation. Cytotoxic above 250 µg/mL. IC50 ~ 71.8 µg/mL		[107]
SW620, DLD-1	20-110 µg/mL (24-48h)	↓ cell proliferation IC50 36.4 & 34.6 µg/mL Effect on 5-FU sensitive and resistant cells ↑ apoptosis ↓ cell transformation	Modulates TYMS & TK1. ↑ PARP cleavage.	[108]
SW620, DLD-1	0-120 µg/mL (48h)	↓ cell viability IC50 25µg/mL	↑ PARP cleavage. ↑GCNT3. ↓miR-15b gene expression.	[109]
	CA (10-30% w/w) (48h)	↓ cell viability	↑GCNT3. ↓miR-15b gene expression.	
	CN (1-3.8% w/w) (48h)	↔ cell viability Potentiates CA-induced ↓ cell viability		

H2O2 (hydrogen peroxide), 5-FU (fluorouracil), TYMS (thymidylate synthase), TK1 (thymidine kinase 1), PARP (poly ADP ribose polymerase), GCNT3 (glucosaminyl (N-acetyl) transferase 3), miR-15b (microRNA-15b)

Several *in vitro* studies using colon cancer cell lines have shown rosemary extract (RE) to exhibit anticancer properties. Exposure of CaCo-2 colon cancer cells to RE drastically decreased colony formation at 30 µg/mL (24h) [106]. Yi (2011) examined the anti-tumorigenic effect of several culinary and medicinal herbs on SW480 colon cancer

cells and found RE to significantly decrease cell growth at a concentration of 31.25 µg/mL (48h), with an IC50 of approximately 71.8 µg/mL [107]. Cell growth of SW620 and DLD-1 colon cancer cells was significantly inhibited by RE at 30 µg/mL (48h), with an IC50 as low as 34.6 µg/mL. RE was also able to inhibit colony formation in soft agar and thus may be able to prevent cell transformation. Furthermore, RE enhanced the inhibitory effects of the chemotherapeutic drug 5-fluorouracil (5-FU) on proliferation and sensitized 5-FU resistant cells [108].

In SW620 and DLD-1 colon cancer cells RE was able to inhibit cell viability dose-dependently resulting in significant inhibition at concentrations as low as 20 µg/mL, and an IC50 around 25 µg/mL (48h). This study used 5 different RE's, containing increasing levels of carnosol (CN:1-3.8% w/w) and carnosic acid (CA: 10-30% w/w). Inhibition of cell viability was correlated with increasing CA content. Furthermore, CA alone (at doses found in RE) decreased cell viability and this effect was potentiated by the addition of CN (at doses found in RE). However, the inhibition seen using RE was greater than the response seen with CA or CN alone or in combination [109]. This supports the use of total RE over its individual components. These studies provide evidence for the role of RE as an anticancer agent in colon cancer cells, capable of consistently inhibiting cell growth and viability at relatively low concentrations in the 20-100 µg/mL range.

Table 2: Anticancer effects of RE *in vitro*: pancreatic cancer

Cancer Cell	Dose/Duration	Findings	Mechanism	Reference
RINm5F rat insulinoma	12-100 µg/mL (24-48h)	↓ cell proliferation ↓ cell viability ↑ apoptosis	↑ nitrate accumulation. ↑ TNFα production.	[110]
	CA (12-100 µg/mL) (24-48h)	↓ cell viability		
MIA-PaCa-2, PANC-1	0-120 µg/mL (48h)	↓ cell viability	↑ PARP-cleavage	[109]
	CA (10-30% w/w) (48h)	↓ cell viability		
	CN (1-3.8% w/w) (48h)	↔ cell viability Potentiates CA-induced ↓ cell viability		

TNFα (tumor necrosis factor), PARP (Poly ADP ribose polymerase)

In rat RINm5F insulinoma cells, RE significantly inhibited cell proliferation at 25 µg/mL (24h), viability at 12 µg/mL (24h) and increased apoptosis at 25 µg/mL (24h). To examine the role of the main compound in RE and determine its involvement in these anticancer effects, CA at 50 µg/mL (48 h) was tested and found to significantly decrease cell viability by 75%. The authors suggested that CA could be involved in RE's mechanism of cell viability inhibition [110]. However, 50 µg/mL (150µM) of carnosic acid is a very high concentration, not comparable to the amount that would be found in RE. At the highest concentration of RE used in this study of 100 µg/mL, and given that the content of CA was measured to be 177.3 ± 9.71mg/g dry extract, the maximum concentration of CA (MW 332.4) would be only 17.7 µg/mL (53 µM). Thus, the statement that CA could be involved in RE's mechanism should be taken with caution.

Exposure of pancreatic cancer cells PANC-1 and MIA-PaCa-2 to RE containing increasing concentrations of carnosol (CN: 1-3.8% w/w) and carnosic acid (CA: 10-30% w/w) resulted in significant inhibition of cell viability with an IC50 of 50 µg/mL (48h) and 30 µg/mL (48h) respectively. The RE containing 25.66% w/w CA (sub-max) caused maximal inhibition compared to other RE's in PANC-1 cells, significantly inhibiting cell viability to approximately 60% at 40 µg/mL (48h) [109]. Taken together, these studies suggest a role for RE to inhibit pancreatic cancer cell viability and proliferation, and induce apoptosis at relatively low concentrations in the 10-100 µg/mL range. They also suggest that CA may play a role in RE's anticancer mechanism however, further research needs to be done investigating RE's polyphenol components.

Table 3: Anticancer effects of RE *in vitro*: breast cancer

Cancer Cell	Dose/Duration	Findings	Mechanism	Reference
MCF-7 *(ER+)*	40mg RE powder filter (inserted into cigarette) (2h)		↓ BP levels & associated DNA adduct formation.	[111]
MCF-7 *(ER+)*, MDA-MB-468 *(TN)*	0.1-20% (5-120h)	IC50 ~90 µg/mL & 26.8 µg/mL		[112]
MCF-7 *(ER+)*, MDA-MB-231 *(TN)*	6.25-50 µg/mL (48h)	↓ cell viability IC50 ~20.42 µg/mL		[113]
	CA 6.25-50 µg/mL (48h)	↓ cell viability		
	RA 6.25-50 µg/mL (48h)	↑ cell viability		
SK-BR-3 *(HER2+)*, UACC-812 *(HER2+)*, T-47D *(ER+)*, MCF-7 *(ER+)*, MDA-MB-231 *(TN)*	10-120 µg/mL (48h)	↓ cell viability Enhanced effect of chemotherapeutics ↑ apoptosis ↓ cell transformation	↑ FOS levels ↑ PARP cleavage ↓ HER2 ↓ ERBB2 ↓ ERα receptor	[114]

BP (benzopyrene), Fos (FBJ murine osteogenic sarcoma virus). PARP (poly ADP ribose polymerase), HER2 (human epidermal growth factor receptor 2), ERBB2 (HER2/neu gene), ERα (estrogen receptor α)

Breast cancer can be classified under three subtypes based on the sensitivity of the tumors to chemotherapeutic agents. The subtypes are i) estrogen receptor positive (ER+), which express ERα and therefore respond to estrogens; ii) human epidermal growth factor receptor 2 positive (HER2+) which overexpress HER2 and can be either ER+ or ER-; iii) triple negative (TN) which lack expression of ERα, progesterone receptor and HER2. One study used MCF-7 (ER+) breast cancer cells and a cigarette smoke solution (in PBS) collected from a cigarette with or without 40mg RE added to the filter. The control used in this experiment was cells stimulated with 2.5μM benzopyrene for 12-18 hours and exposed to 1:19 v/v cigarette smoke solution for 2 hours without an RE filter. In the experimental group, the presence of RE in the filter lead to considerably reduced benzopyrene levels and associated DNA adduct formation [111].

RE inhibited cell proliferation in breast cancer cells with an IC50 of 90 μg/mL and 26.8 μg/mL in MCF-7 (ER+) and MDA-MB-468 (TN) cell lines respectively [112]. In a similar study, dose-dependent inhibition of cell viability by 6.25-50 μg/mL (48h) RE was seen in MDA-MB-231(TN) and MCF-7 (ER+) breast cancer cells and MCF-7 cells had an IC50 of ~24.02 μg/mL. There is a large discrepancy between the IC50s of the MCF-7 cell line in these studies, which may be attributed to the different extraction methods used; supercritical CO2 [113] and ethanol extraction [112]. Carnosic acid (CA) and rosmarinic acid (RA) were also examined in breast cancer cells. CA decreased cell viability dose-dependently from 6.25-50 μg/mL in both cell lines, while RA resulted in enhanced cell viability [113].

The effects of RE at 0-120 μg/mL (48h) were explored in all three breast cancer subtypes, ER+, HER2+ and TN. RE caused dose-dependent inhibition of cell viability in

all subtypes of breast cancer cells. Furthermore RE enhanced the effectiveness of the monoclonal antibody (mAb) trastusumab and the chemotherapeutic drugs tamoxifen and paclitaxel, used in the treatment of breast cancer [114]. Thus, although it has been shown that RE as well as its main component CA are capable of reducing cell proliferation and viability within the 10-100 µg/mL range in all breast cancer subtypes, the mechanisms by which it does so remain unclear and require further exploration.

Table 4: Anticancer effects of RE *in vitro*: leukemia

Cancer Cell	Dose/Duration	Findings	Mechanism	Reference
WEHI-3B D murine & HL60, U937 human myeloid leukemia	RE (10µM equivalent of CA) (48-96h)	Potentiated following effects of VDA: ↓ cell proliferation ↑ cell cycle arrest ↑ cell differentiation ↑ apoptosis	↑ G1 phase	[115]
	CA 10µM (48-96h)	Potentiated following effects of VDA: ↓ cell proliferation ↑ cell differentiation ↑ apoptosis ↑ cell cycle arrest	↑ G1 phase	
Mouse RAW 264.7 macrophages & HL60, K562 human leukemia	0.1-20% (5-120h)	↓ cell proliferation IC50 ~ 18.76 µg/mL & 33.5 µg/mL ↑ cell differentiation ↓ LPS-stimulated (LS) antioxidant activity	↓ (LS) NO ↑ antiox. activity ↔ basal TNFα, IL-1β, iNOS or COX2 ↓ (LS) IL-1β & COX2	[112]
WEHI-3B D murine myeloid leukemia	RE (10µM equivalent of CA) (48-96h)	Potentiated following effects of VDA: ↑ cell differentiation ↓ cell viability ↓ cell proliferation	↓ ROS ↑ antiox. ↑ NADP(H)-quinone reductase	[116]
	CA 10µM (48-96h)	Potentiated following effects of VDA: ↑ cell differentiation ↓ cell viability ↓ cell proliferation	↓ ROS ↑ antiox. ↑ NADP(H)-quinone reductase	
K-562	6.25-50 µg/mL (48h)	↓ cell viability IC50 ~12.50 µg/mL		[113]
	CA 6.25-50 µg/mL (48h)	↓ cell viability		
	RA 6.25-50 µg/mL (48h)	↑ cell viability		
K-562, U937	50 µg/mL (0-96h)	↓ cell proliferation	↓ AKT1 ↑ Rb2 ↔ ERK2	[117]
	0.2mM RA (48h)	Not tested on proliferation	↔ AKT1 ↔ ERK2	

VDA (vitamin D analogue), LPS (lipopolysaccharide), NO (nitric oxide), TNFα (tumor necrosis factor α) , IL-1β (interleukin 1β) , iNOS (inducible nitric oxide synthase), COX2 (cyclooxygenase 2), ROS (reactive oxygen species), NADP(nicotinamide adenine dinucleotide phosphate), Rb2(retinoblastoma-related gene 2)

Vitamin D analogues (VDA) are commonly used in clinical differentiation therapy of acute myeloid leukemia (AML) to attempt to restore a defect in the capacity of myeloid progenitor cells to mature into non-replicating adult cells. However, pharmacologically relevant doses have been found to result in many adverse events such as hypercalcemia and attempts to circumvent these adverse events have been unsuccessful. RE containing 10µM equivalent of CA, or 10 µM CA alone (96h) potentiated the ability of vitamin D derivatives to inhibit cell viability and proliferation, and increase differentiation of WEHI-3BD murine leukemic and human HL-60 leukemic cells [115,116]. This RE (48-96h) was also found to potentiate the increased apoptosis and cell cycle arrest seen by VDAs [115]. A study examining the human leukemia HL-60 and K-562 cell lines and the murine RAW264.7 macrophage/monocyte cell line found significant inhibition of proliferation (48-96h) and the IC50 of the human HL-60 and K-562 cell lines to be 0.14% (18.76 µg/mL) and 0.25% (33.5 µg/mL) respectively. At 0.1% (13.4 µg/mL;72h) RE significantly increased differentiation in the HL-60 cell line [112]. RE inhibited viability at 50 µg/mL (48h) in K-562 leukemia cells. CA at concentrations of 6.25-50 µg/mL (48h) significantly inhibited K-562 cell viability while RA was ineffective [113]. Similar effects of RE (50 µg/mL; 24h) were reported by others that lead to decreased cell proliferation in K-562 cells [117].

Table 5: Anticancer effects of RE *in vitro*: prostate cancer

Cancer Cell	Dose/Duration	Findings	Mechanism	Reference
DU145, PC3	6.25-50 µg/mL (48h)	↓ cell viability IC50 ~8.82 µg/mL		[113]
	CA 6.25-50 µg/mL (48h)	↓ cell viability		
	RA 6.25-50 µg/mL (48h)	↔ cell viability		
LNCaP, 22RV1	10-50 µg/mL (24-48h) *RE standardized to 40% CA	↓ cell proliferation ↑ cell cycle arrest ↑ apoptosis modulates endoplasmic reticulum stress proteins.	↑CHOP ↓ PSA production ↑ Bax ↑ cleaved-caspase 3 ↓androgen receptor expression	[118]

CHOP (C/EBP homologous protein), PSA (prostate specific antigen), Bax (Bcl-2 associated X protein)

RE (6.25-50 µg/mL; 48h) inhibited viability of DU145 prostate cancer cells. Furthermore, CA at 6.25 µg/mL(48h) inhibited cell viability of both DU145 and PC3 cells, and RA had no effect on cell viability [113]. In agreement with these data, significant inhibition of LNCaP and 22RV1 prostate cancer cell proliferation and viability, and an induction of apoptosis were seen with RE (50 µg/mL standardized to 40% CA; 24-48h). RE was able to combat the enhanced prostate specific antigen (PSA) levels measured in cell culture media, indicative of prostate cancer, inhibiting levels to less than a fifth of what was seen in the control group. Correspondingly, levels of the androgen receptor, to which PSA binds, were significantly decreased by 50 µg/mL RE [118]. Thus, RE is capable of inhibiting cell viability and proliferation, and inducing apoptosis in both androgen sensitive and insensitive cell lines, making it an attractive potential chemotherapeutic with diverse applications.

Table 6: Anticancer effects of RE *in vitro*: ovarian cancer

Cancer Cell	Dose/Duration	Findings	Mechanism	Reference
A2780, A2780CP70 (cisplatin-resistant)	0.05 - 0.25% (24-48h)	↓ cell proliferation Enhanced sensitivity of cisplatin - resistant cell lines. ↑apoptosis ↑ cell cycle arrest Modulates expression of apoptotic genes.	↓ P-glyco protein ↑ cyt c gene ↑ hsp70 gene	[119]
	CA 2.5-10 µg/mL (48h) (7.5-30µM)	↓ cell proliferation Enhanced sensitivity of cisplatin-resistant cells		
	RA 2.5-10 µg/mL (48h)	↓ cell proliferation Enhanced sensitivity of cisplatin-resistant cells		
	CN 2.5-10 µg/mL (48h)	↓ cell proliferation Enhanced sensitivity of cisplatin-resistant cells		

cyt c (cytochrome C), hsp70 (heat shock protein 70)

Table 7: Anticancer effects of RE *in vitro*: liver cancer

Cancer Cell	Dose/Duration	Findings	Mechanism	Reference
Hep-3B	6.25-50 µg/mL (48h)	↓ cell viability IC50 ~22.88 µg/mL		[113]
	CA 6.25-50 µg/mL (48h)	↓ cell viability		
	RA 6.25-50 µg/mL (48h)	↔ cell viability		

Table 8: Anticancer effects of RE *in vitro*: lung cancer

Cell	Dose/Duration	Findings	Mechanism	Reference
NCI-H82 (small cell)	6.25-50 µg/mL (48h)	↓ cell viability IC50 ~24.08		[113]
	CA 6.25-50 µg/mL (48h)	↓ cell viability		
	RA 6.25-50 µg/mL (48h)	↔ cell viability		
V79 hamster normal lung fibroblasts	0.1-30 µg/mL (3-24h)	Cytotoxic to cells at 30 µg/mL (24h) Long and short term antioxidant effects	↓ H2O2-induced DNA strand breaks & oxidative damage. ↓ visible-light induced oxidative damage	[106]

Exposure of A2780 ovarian cancer cells to 0.08% (48h) RE containing media resulted in significant inhibition of proliferation and induction of apoptosis and cell cycle arrest. However, it should be noted that 0.08% RE corresponds to 800 µg/mL, a concentration far greater than those used in any other cancer cell lines. Cisplatin is a chemotherapeutic agent used often in cancer treatment however, as with many chemotherapeutics, patients often develop a resistance. At 0.08% RE enhanced the sensitivity of A2780 and cisplatin-resistant A2780CP70 cell lines to growth inhibition by cisplatin treatment, suggesting that RE may be of use in combination with cisplatin or potentially other chemotherapeutic drugs in patients who have developed an acquired resistance. Furthermore, the individual components of RE: CA, RA and CN at low concentrations (2.5, 5, 5 µg/mL respectively; 48h) alone were able to inhibit cell proliferation and enhance sensitivity of cisplatin-resistant cells to inhibition of

proliferation by cisplatin. [119]. This indicates that the beneficial effects of RE may be due to these polyphenols.

In human liver Hep-3B and small cell lung NCI-H82 carcinoma cells, RE at 6.25-50 µg/mL (48h) dose-dependently decreased cell viability with an IC50 of 22.88 µg/mL and 24.08 µg/mL in each cell line respectively. Additionally, CA at 6.25 µg/mL (48h) inhibited cell viability while RA had no effect [113]. In a V79 normal hamster lung fibroblast cell line RE was cytotoxic at 30 µg/mL (24h) [106]. The cytotoxicity of RE in normal fibroblasts raises questions about its potential as a successful treatment option however, further research is required to fully examine the cytotoxicity issue in normal tissues. All of the above mentioned studies indicate that RE shows promising anticancer effects and this warrants further research to elucidate the mechanisms employed by RE.

1.9.2 Anticancer effects of rosemary extract: *in vivo* animal studies

Table 9: Anticancer effects of RE *in vivo*

Animal Model	Dose/Duration	Findings	Mechanism	Reference
SW620 colon xenograft (nude mice)	1mg/mL in drinking water (32-35d) ad libitum	↓ tumor size	↓ miR-15b in plasma	[109]
22RV1 prostate xenograft (athymic nude mice)	100mg/Kg/day in olive oil, orally (22d)	↓ tumor volume (induces apoptosis)	↓ androgen receptor expression ↓ PSA ↑ CHOP	[118]
Myeloid leukemia inoculated mice	1% RE w/w in food ad libitum (29d)	↓ tumor volume ↓ tumor incidence Potentiated VDA ability to ↓ tumor volume		[115]
Myeloid leukemia inoculated mice	4% w/w in food ad libitum. (15w)	RE alone ↔ median survival time RE+VDA ↑ median survival time	↓ WBC	[116]
DEN-induced liver cancer (F344 rats)	100mg/Kg/day RE intragastrically (5d) Injected i.p with 20mg/Kg DEN on day 4. Fed normal diet until week 3 (underwent partial hepatectomy).	↑ antioxidant activity	↓ GST positive foci	[120]
Swiss mice exposed to γ-IR (liver)	6Gy γ-IR (once) followed by 1000mg/Kg/day RE orally (5d)	Delayed onset of IR-induced mortality Attenuated negative IR effects Protective effect on liver & blood	↓ LPx levels ↑ GSH levels	[121]
DMBA-induced skin cancer (nude mice)	1000 mg/Kg/day RE orally in water or by gavage (15w)	↓ tumor number ↓ tumor incidence ↓ tumor burden ↓ tumor yield ↑ latency period	↓ LPx levels ↑ GSH levels	[122]
DMBA-induced skin cancer (nude mice)	500mg/Kg/day RE orally in water or by gavage (15w)	↓ tumor number ↓ tumor diameter ↓ tumor weight	↓ LPx levels ↑ GSH levels	[123]

miR-15b (microRNA 15b), PSA (prostate specific antigen), CHOP (C/EBP homologous protein), VDA (vitamin D analogue), WBC (white blood cell), GST (glutathione S transferase), IR (ionizing radiation), LPx (lipid peroxidase), GSH (glutathione), DEN (diethylnitrosamine), DMBA (dimethylbenzanthracene)

A limited number of studies have examined the effects of RE administration on tumor growth in animals in vivo. Administration of RE (1mg/mL) in the drinking water ad libitum for 32-35 days resulted in a significant decrease in tumor size in nude mice xenografted with SW620 colon cancer cells [109]. Similarly, a significant reduction in tumor volume was seen in mice xenografted with 22RV1 prostate cancer cells by RE (100mg/Kg/day) which was administered, dissolved in olive oil for 22 days [118].

In WEHI-3BD myeloid leukemia xenografted mice fed 1% w/w RE in their food ad libitum (29d), investigators noted a significant decrease in both tumor volume and incidence. Vitamin D analogues (VDA) are commonly used in the treatment of leukemia, and RE showed an additive effect when combined with VDAs in these mice by further decreasing tumor volume [115]. Similarly, in WEHI-3BD xenografted mice administered RE (4% w/w in food) for up to 15 weeks combined with VDAs, median survival time was significantly increased and white blood cell count in the leukemic mice decreased to levels comparable to those seen in the control group of healthy mice [116].

In a diethylnitrosamine (DEN)-induced liver cancer model in F344 rats, RE at 100mg/Kg/day (5d) was administered intragastrically for 5 days, with an intraperitoneal (i.p) injection of DEN on day 4. From this point, rats were fed a normal diet for 3 weeks until undergoing partial hepatectomy. Examination of liver tissue suggested RE may exert some protective antioxidant effects [120]. In accordance with this, use of Swiss mice exposed to 6 Grays (Gy) ionizing radiation (IR) in their liver once, followed by treatment with 1000mg/Kg RE fed orally, daily for 5 days suggested protective, antioxidant activity by RE. A delayed onset of IR-induced mortality and attenuated increases in glycogen and protein levels were seen in livers of mice exposed to IR and fed

RE, compared to IR-exposed mice not fed RE [121]. Taken together, these studies suggest a role for RE inhibiting chemical- or IR-induced carcinogenesis by exerting protective, antioxidant effects on healthy tissues. Thus, RE may display radioprotective effects which would benefit healthy tissue during radiation treatment. Caution should be taken however, due to the high concentration (1000mg/Kg) used by Soyal (2007) [121] which is at least 10 times greater than what has been found to exert potent anticancer effects in other studies.

Using a 7,12-dimethylbenz(a)anthracene (DMBA)-induced skin cancer nude mouse model, RE (1000 mg/Kg/day; 15w) administered orally in water resulted in a significant decrease in tumor number, incidence and burden, and an increase in latency period compared to control mice treated with DMBA only [122,123]. Furthermore, this group used 500mg/Kg/day RE and again saw a significant decrease in tumor number, diameter and weight compared to DMBA-only treated mice [123]. One group of mice, which were administered RE for 7 days prior to the first application of DMBA, showed a 50% reduction in tumor growth compared to the DMBA-only treated mice [123]. Taken together, although the concentrations used in these studies are high, they provide evidence of RE's antioxidant effects in vivo, and its significant ability to inhibit tumor growth and prevent carcinogenesis by chemicals.

The large variability in concentrations of RE used in these in vivo animal studies highlights the need for systematic investigation regarding effective concentrations of RE. Moreover, further research on RE's mechanism *in vitro* and *in vivo* is necessary before conclusions can be made on its' potential as an effective anticancer agent.

1.10 Mechanisms of anticancer effects of rosemary extract: *in vitro* studies

Many studies have examined the anti-proliferative and colony forming abilities of RE *in vitro* in colon [106–108], pancreas [110], breast [112,114], leukemia [112,115–117], prostate [118] and ovarian [119] cancer cell lines however, little is known concerning the underlying mechanism. RE was shown to have an inhibitory effect on ATK1 mRNA and protein expression, a protein involved in the PI3K/Akt survival signaling pathway, in a leukemic cell line [117] however, no measure of Akt activity was mentioned. No effect on ERK2 protein levels, involved in cell proliferation and differentiation, were seen in these cells. Cell cycle arrest prevents further division by proliferating cells and RE was shown to induce cell cycle arrest in a number of cancer cell lines by increasing the proportion of cells arrested in the G1 phase [115] and increasing retinoblastoma-related gene 2 (Rb2) [117] which regulates entry into cell division.

The viability of various cancer cell lines was shown to be significantly inhibited by treatment with RE which many studies attributed to enhanced apoptosis and cell death. Increased poly ADP ribose polymerase (PARP) cleavage, which is an established indicator of enhanced apoptosis, was seen in colon [108,109], pancreas [109] and breast [114] cancer cell lines following treatments with RE. Alternatively, RE enhanced nitrate accumulation (ie increased nitric oxide production) and TNFα production in pancreatic cancer cells [110], indicative of enhanced cell death capabilities and nitric oxide-induced apoptosis. In ovarian cancer cells [119] enhanced apoptosis was associated with increased gene expression of mitochondrial-regulated apoptosis proteins cytochrome c, involved in the electron transport chain, and heat shock protein 70 (hsp70) which is involved in

protein folding and protecting the cell from heat stress and toxic chemicals. Other mechanisms of apoptosis by RE include enhanced protein expression of pro-apoptotic Bax and cleaved caspase-3, increased expression of binding immunoglobulin protein (BiP) and CCAAT/-enhancer-binding protein homologous protein (CHOP) proteins which induce endoplasmic reticular stress, and the unfolded protein response leading to reduced androgen receptor expression and apoptosis in prostate cancer cells [118]. Interestingly, in normal prostate epithelial cells RE treatment resulted in a decrease in endoplasmic reticulum stress related protein PRKR-like endoplasmic reticulum kinase (PERK), suggesting RE selectively induces endoplasmic reticular stress in prostate cancer cells but spares normal prostate cells [118]. Similarly, in breast cancer cells [114] RE decreased expression of estrogen receptor α (ERα) in the ER+ subtype and human epidermal growth factor receptor 2 (HER2) in the HER2+ subtype, and it was suggested the decreased receptor expression was correlated with enhanced apoptosis in these cell subtypes. Correspondingly, increased levels of Fos, an oncogenic transcription factor, were detected in ER+ and HER2+ cell lines, and this event is thought to precede apoptosis and correspond to the PARP-cleavage seen in these cells. Although RE was also capable of inducing anticancer effects in triple negative (TN) breast cancer cells, its mechanism has yet to be elucidated [114]. Thus, it can be concluded that apoptosis is an important mechanism of RE in cancer however, not all cell lines may rely on the same method of apoptosis.

Antioxidants are molecules which scavenge harmful free radicals, protecting cells from oxidative DNA damage and potentially death. RE has been shown to exert antioxidant effects in colon [106], breast [111], and leukemia [112,116] cell lines. Colon

cancer cells pretreated with RE followed by treatment with hydrogen peroxide, often used in cell culture to induce oxidative DNA damage, showed reduced DNA double-strand breaks and oxidative damage compared to control cells treated with hydrogen peroxide only. Similarly, RE reduced oxidative damage induced by methylene blue (oxidizes purines) in these cells [106]. RE treatment resulted in increased levels of antioxidants and NAPD(H)-quinone reductase (oxidoreductase involved in the transfer of electrons from a reduced molecule to an oxidized molecule) which decreased reactive oxygen species (ROS) levels, and inhibited lipopolysaccharide (LPS)-stimulated production of the free radical nitric oxide (NO) in leukemia cell lines [112,116]. In an *in vitro* model of cigarette smoking, the use of an RE containing cigarette filter considerably reduced benzopyrene (carcinogen) levels and associated DNA adduct formation in breast cancer cells [111]. The use of RE for its antioxidant properties is beneficial as a preventative measure in cancer therapy. Antioxidants work to restore damaged DNA back to normal and protect the cell from further damage thus, preventing the potential mutation into a cancer cell and subsequent tumor formation.

In addition to the antiproliferative, apoptotic and antioxidant mechanisms noted above, some evidence indicates that RE may i) exert anti-inflammatory effects [112] through inhibition of interleukin-1 (IL-1) and cyclooxygenase 2 (COX2) molecules, ii) aid in the reversal of acquired drug resistance [119] by inhibiting P-glycoprotein levels (correlated with drug resistance), and iii) alter metabolic-related genes [109] such as glycosyltransferase (GCNT3) which forms glycosidic linkages in a variety of macromolecules and its potential epigenetic regulator microRNA-15b. Although many studies suggest that induction of apoptosis is an important mechanism of RE, there is

little evidence to support the antiproliferative mechanism of RE. As well, the effects of RE in aggressive cancers such as liver and lung cancer have yet to be explored.

1.11 Mechanisms of anticancer effects of rosemary extract: in vivo animal studies

Limited evidence exists regarding RE's mechanism *in vivo* however, few studies list potential antioxidant effects and serum biomarkers for RE's anticancer effects. Increases in glutathione (GSH), an antioxidant, and reductions in lipid peroxidase (LPx), an oxidizing agent resulting in free radical production and cell damage, have been recorded in IR-induced mouse liver [121] and DMBA-induced mouse skin cancer [122,123] models treated with RE. Similarly, RE decreased glutathione-S transferase (GST) positive foci, which are associated with oxidative damage from the reduction of GSH, in a rat DEN-induced liver cancer model however, results were not significant and should be taken with caution [120]. The presented evidence suggests a protective antioxidant effect by RE *in vivo* in animals.

Serum samples from mice xenografted with prostate cancer cells and fed RE in their diet showed a decrease in prostate-specific antigen (PSA) levels (high levels would be suggestive of prostate cancer) and examination of tissue samples showed decreased androgen receptor and CHOP expression, indicative of an induction of apoptosis associated with endoplasmic reticular stress [118]. In colon cancer xenografted mice, a significant decrease in microRNA-15b (miR-15b) plasma levels after administration of RE suggested circulating miR-15b levels may act as a minimally invasive method to monitor the antitumor effects of RE in vivo [109]. Sufficient evidence exists to support the potential use of RE in chemotherapeutics however, before clinical trials can begin

more detail on RE's mechanism and its use across all cancer cell types *in vitro* and *in vivo* needs to be investigated further. There still remain discrepancies in the effective doses of RE and whether the anticancer effects seen are attributable to individual polyphenols (such as CA, RA or CN) or to all the components of RE combined.

Chapter 2: Rationale, Objectives and Hypotheses of the Present Study

2.1 Rationale

It is well established that cancer cells harbor the ability to grow and divide uncontrollably compared to non-cancerous cells. This ability comes from various mutations and deletions to key proteins involved in cell signaling such as the PI3K/Akt/mTOR, Ras/MAPK and AMPK pathways as discussed in Chapter 1. Many drugs and therapies have been developed which target these signaling pathways. Unfortunately, tumor cell populations often develop a resistance to both chemo- and radio-therapies. Discovery of novel compounds/chemicals is necessary to expand options for therapy, overcome resistance and improve patient outcome.

Lung cancer is the leading cause of cancer mortality worldwide in both men and women [124]. Despite advances in treatment such as chemo-and radiation therapy, survival has improved little over the past few decades [125] and the 5 year survival rate for all stages is less than 15% [126]. Non-small cell lung cancer (NSCLC) accounts for almost 80% of all lung cancer cases [127] and represents the most aggressive form of the disease, with a 1-year survival rate of 16% [128]. This highlights the necessity to develop new therapeutic strategies for lung cancer patients and thus, cell lines of this histology

will be used in this study. Approximately 50-80% of NSCLCs are associated with EGFR overexpression; with 65% of them are also found to have an increased EGFR gene copy number [129–131]. Furthermore, high levels of EGFR expression has been associated with a poor prognosis in lung cancer patients [132–134], warranting exploration of potential chemotherapeutics targeting EGFR-associated signaling pathways such as PI3K/Akt, Ras/MAPK and the cell energy sensing pathway involving AMPK.

The Mediterranean diet has long been attributed to preventing or delaying the onset of cardiovascular disease, diabetes and various cancers. One component of the Mediterranean diet which may contribute to these effects is rosemary, a commonly used herb in the Mediterranean. Rosemary extract has been shown to exert anticancer properties including decreased cell growth and proliferation, and enhanced apoptotic activity *in vitro* and *in vivo* in colon [108,109], breast [112,114], prostate [118] and leukemic [115,116] cancers, among others. Limited data exists regarding the effects of rosemary extract in lung cancer and little is known about the signaling mechanisms of its antiproliferative properties.

Historically, many plants and food components have been the basis for isolation of compounds that are used in the treatment of various maladies. Acetylsalicylic acid/salicylate (Aspirin), commonly used to treat minor pain and reduce inflammation, was isolated from the willow tree; and morphine, used to treat pain, is an opioid isolated from the opium poppy. Recently the polyphenol resveratrol, found in wine has been shown to have potent anticancer properties. Thus, further investigation of compounds found in plants such as rosemary may lead to the discovery of chemicals with potent anticancer properties.

The main polyphenolic compounds present in rosemary extract, carnosic acid and rosmarinic acid will also be explored to examine if the anticancer properties of rosemary are attributable to any of these individual compounds or if any synergistic or additive interactions exist between these compounds.

2.2 Objectives

The objectives of the study are to:

1) Examine the effects of rosemary extract on proliferation and clonogenic survival in various human lung cancer cells and normal lung fibroblasts;

2) Examine the cell signaling pathways which may be implicated in rosemary extracts' mechanism of action involving cell survival and proliferation in various human lung cancer cell lines;

3) Examine the anticancer effects of the main polyphenolic compounds found in rosemary extract, rosmarinic acid, and carnosic acid on various human lung cancer cell lines and normal lung fibroblasts;

4) Examine cell signaling pathways which may be implicated in rosmarinic acid and carnosic acids mechanism of action involving cell survival and proliferation in various human lung cancer cell lines;

5) Determine if the main polyphenolic components found in rosemary extract, rosmarinic acid and carnosic acid act synergistically to exert anticancer effects in various human lung cancer cell lines.

2.3 Hypotheses

In the present study it is hypothesized that:

1) Rosemary extract decreases proliferative capacity and clonogenic survival in NSCLC cells;

2) The main polyphenols found in rosemary extract, rosmarinic acid and carnosic acid decrease proliferative capacity and clonogenic survival in NSCLC cells;

3) Rosemary extract and its main polyphenolic components, rosmarinic acid and carnosic acid, alter key signaling pathways involved in cell proliferation and survival in NSCLC cells;

a) Rosemary extract and its main polyphenolic components, rosmarinic acid and carnosic acid, inhibit the PI3K/Akt and Ras/MAPK (ERK) pathways;

b) Rosemary extract and its main polyphenolic components, rosmarinic acid and carnosic acid, activate the energy sensing molecule AMPK;

4) The main polyphenols found in rosemary extract, rosmarinic acid and carnosic acid work synergistically to exert anticancer effects.

Chapter 3: Methodology

3.1 Cell lines

Cells in culture were used to investigate the anticancer effects of rosemary extract and its main polyphenolic components. These cells resemble the primary tumor population but offer a homogenous population of cells with unlimited replicative capacity. The use of cultured cells to investigate the physiology and biochemistry of cells and intracellular signaling pathways is well-established. Immortalized or continuous cell lines are capable of evading cellular senescence and divide continuously. These cells can be used for experiments *in vitro* and environmental factors can be manipulated. The cell lines used in this study were non-small cell lung cancer (NSCLC) cells with varying histologies and mutations which represent the variation that is seen in cancer diagnoses in humans, and a normal lung fibroblasts cell line for comparison.

MRC-5 are normal long fibroblasts which do not differentiate. This cell line was derived from the normal lung tissue of a 14-week-old Caucasian male fetus by J.P. Jacobs in 1966. [135].

A549 are epithelial NSCLC adenocarcinoma cells. This cell line was derived from lung carcinoma tissue of a 58-year-old Caucasian male by D.J. Giard in 1972 [136].

H1299 are epithelial NSCLC adenocarcinoma cells. This cell line was derived from a lymph node metastasis of the lung from a 43-year-old Caucasian male who had received previous radiation therapy [137].

H460 are epithelial large cell lung carcinoma cells. This cell line was derived from the pleural fluid of a male with large cell cancer of the lung by A.F. Gazdar and associates in 1982 [138].

The histology and the known mutations of the cell lines used are summarized in Table 10.

Table 10: Lung cancer cell line properties

Cell Line	Histology	Mutations
MRC-5	Lung fibroblast	*No known mutations*
A549	NSCLC adenocarcinoma	*KRAS mutant; LKB1 null; express wt p53; M CDKN2A[p16]; M CDKN2a[p14]*
H1299	NSCLC adenocarcinoma	*express LKB1; p53 null; NRAS mutant*
H460	NSCLC large cell carcinoma	*KRAS mutant; LKB1 null; express easily detectable wt p53; PIK3CA mutant*

3.2 Materials

Cell culture materials including Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic solution and trypsin were purchased from GIBCO Life Technologies (Burlington, ON). Cell culture materials including 75cm^2 flasks, 6 well plates, 96 well plates, conical tubes, Eppendorf tubes, pipette tips and syringe filters were purchased from VWR International (Mississauga, ON). Non-small cell lung cancer (NSCLC) cell lines A549, H1299, H460 and normal lung fibroblasts MRC-5 were purchased from American Type Culture Collection (ATCC) (Virginia, USA). Chemicals

including carnosic acid (>91% purity), rosmarinic acid (>98% purity), *trans*-resveratrol (>99% purity), metformin, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), formalin, crystal violet, methylene blue and buffer salts were purchased form Sigma Aldrich (Oakville, ON). Western blotting materials including glass plates, polyvinylidene fluoride (PVDF) membrane, running and transfer apparatus, sodium dodecyl sulfate (SDS), Tris-base, glycine, 2-mercaptoethanol, bromophenol blue, TEMED, Tween, ammonium persulfate (APS), phenylmethanesulfonyl fluoride (PMSF), acrylamide/Bis solution, molecular weight protein standards, protein assay dye reagent, luminol/enhancer and peroxide solution were purchased from Bio-Rad (Mississauga, ON). Total and phospho-specific antibodies including Akt, ERK (p44/42 MAPK), AMPK and beta actin, as well as IgG HRP-linked anti-rabbit secondary antibody were purchased from New England Biolabs (Mississauga, ON).

3.3 Buffers and solutions

The buffers and solutions required for each experiment and their compositions are provided below:

Cell Lysis

Phosphate Buffered Saline (PBS) Solution- Wash Buffer

137mM NaCl; 2.7mM KCl; 1.5mM KH_2PO_4; 8.1mM Na_2HPO_4; 0.68mM $CaCl_2$; 0.49mM $MgCl_2$; Add distilled water to achieve final volume, adjust pH to 7.4 and sterilize (autoclave) before use.

Cell Lysis Buffer

20mM Tris-HCl solution (pH 7.5); 150mM NaCl; 1mM EDTA; 1mM EGTA; 1% Triton X-100; 2.5mM $Na_4P_2O_7$; 1mM glycerol 2-phosphate; 1mM Na_3VO_4; 1µg/mL leupeptin; add 1mM PMSF before use and chill on ice.

3X SDS Sample Buffer

187.5mM Tris-HCl solution (pH 6.8); 6% w/v SDS; 30% glycerol, 0.03% bromophenol blue; deionized water; add 0.15% β-mercaptoethanol before use.

Western blotting solutions

1.5M Tris-HCl (pH 8.8)

27.23g Tris base (18.15g/100mL); 80mL deionized water; adjust pH to 8.8 with 6N HCl and bring total volume to 150mL with deionized water

0.5M Tris-HCl (pH 6.8)

6g Tris base (6g/100mL); 60mL deionized water; adjust pH to 6.8 with 6N HCl and bring total volume to 100mL with deionized water.

Resolving Gel Buffer (10%) – for 4 1.5mm gels

12.3mL deionized water; 9.9mL 30% acrylamide/Bis solution; 7.5mL 1.5M Tris-HCl (pH 8.8); 0.3mL 10% w/v SDS. Right before pouring gel add 150µL 10% APS (0.01g into 100µL deionized water) made fresh daily and 30µL TEMED and swirl.

Stacking Gel Buffer (4%) – for 4 1.5mm gels18.3mL deionized water; 3.9mL 30% acrylamide/Bis solution; 7.5mL 0.5M Tris-HCl (pH 6.8); 0.3mL 10% w/v SDS. Right before pouring gel add 150µL 10% APS (0.01g into 100µL deionized water) made fresh daily and 30µL TEMED and swirl.

10x Tris-Buffered Saline (TBS)

24.2g Tris base; 80g NaCl; fill to total volume of 1L with deionized water; adjust pH to 7.6 with HCl. Use as 1x TBS/T (50mL 10x TBS into 450mL deionized water; then add 500µL Tween).

10x Electrode Running Buffer

15.15g Tris base; 72g glycine; 5g SDS; dissolve and bring volume to 500mL with deionized water. Do not adjust pH. Before use dilute to 1x (50mL 10x stock into 450mL deionized water).

Transfer Buffer

25mM Tris base (3.03g); 0.2M glycine (15.01g); 20% methanol (200mL/800mL deionized water); adjust final volume to 1L.

Blocking Buffer

15mL 10xTBS; 135mL deionized water; 7.5g nonfat dry milk (5%w/v); 0.15mL Tween; make fresh each time.

Primary Antibody Dilution Buffer

2mL 10xTBS; 18mL deionized water; 1.0g BSA; 20µL Tween (add after first 3 ingredients are mixed); add 10µL primary antibody before use.

Secondary Antibody Buffer

10mL blocking buffer; 5µL secondary antibody; make fresh each time.

Crystal violet assay solutions

Crystal Violet Stain

0.5% w/v crystal violet stain in 25% v/v methanol; keep solution covered from light

Solubilizer Solution

0.05M NaH_2PO_4 (MW 119.98g/mol) in 50% v/v ethanol; keep solution covered from light

3.4 Cell culture

A549, H1299 and H460 NSCLC cells were cultured in RPMI 1640 medium containing 2.05mM L-glutamine and supplemented with 1% (v/v) antibiotic-antimycotic (100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin) and 10% (v/v) FBS. MRC-5 normal lung fibroblasts were cultured in DMEM media containing high glucose and supplemented with 1% (v/v) antibiotic-antimycotic (100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin) and 10% (v/v) FBS. Media was changed every 48 -72 hours and cells were treated with trypsin and passaged once 70-80% confluency was reached. Cells were maintained in an incubator at 37º C and 5.0% carbon dioxide (CO_2) – 95% air. Concentrations and time course for each experiment are listed under each figure. Cell handling was performed in a Forma Class II, A2 Biological Safety Cabinet (BSC).

3.5 Rosemary extract preparation

A methanol extract of rosemary (RE) was prepared as previously described [139]. Whole dried rosemary (*Rosmarinus Officinalis L.)* leaves (purchased from Compliments/Sobey's, Mississauga, ON, Canada- product of Turkey) were ground and passed through a mesh sieve and extracted following protocols established by the National Cancer Institute of the U.S. The ground plant (5g) was steeped overnight (16 hours) in dichloromethane-methanol (1:1) (30mL). The filtrate was collected under slight vacuum followed by methanol (30mL) extraction for 30 minutes. The solvent was removed by rotary evaporation. Aliquots were prepared in dimethyl sulfoxide (DMSO) (100mg/mL) and stored at -20 degrees Celsius.

3.6 Crystal violet assay

The crystal violet assay was used to assess cell proliferation (growth). The crystal violet stain binds DNA and thus measures the ability of cells to grow and divide. Cells were seeded (800-1000; counted using haemocytometer) in triplicate in 100µL supplemented media in 96 well plates and allowed to adhere overnight. The following day, treatments were added with an extra 100µL supplemented media (final volume per well is 200µL) and cells were maintained in the incubator for 72 hours. At the end of the time course media was removed, cells were fixed with 10% formalin (100µL/well), rinsed using PBS and stained using crystal violet dye (50µL/well). Stain was removed by submerging plates in tap water and plates were allowed to dry overnight. The following day solubilizer solution was added (100µL/well) and absorbance was read at 570nm using the KC4 microplate reader.

3.7 Clonogenic assay

The clonogenic assay was used to assess the colony forming abilities of the cells. Colony formation is characteristic of cancer cells that are highly developed and able to form tumors and become more aggressive. Cells were seeded (1000; counted using haemocytometer) in triplicate in 2mL supplemented media in 6-well plates and allowed to adhere overnight. The following day, media was removed, treatments were administered (2mL per well) and cells were maintained in the incubator for 7 days. At the end of the time course, media was aspirated and cells were washed twice with 1X PBS before being stained with 0.05% w/v methylene blue in distilled water. Cells were stained for 10-15 minutes and left on the counter to dry overnight before counting. Colonies greater than 50 cells were counted under the microscope and results are expressed as the surviving fraction compared to untreated control.

3.8 Cell lysis

Cells were seeded in duplicate in 6 well plates and maintained in the incubator until 90-100% confluency. Cells were treated with the indicated concentrations of the various agents for 24-72 hours. Following treatment, cell lysis was performed using cell lysis buffer with PMSF (150µL/well) on ice and a plastic cell scraper. Lysates were pipetted into 1.5mL Eppendorf tubes and 50µL was removed for protein assay. Lysates were dyed using 3x SDS sample buffer by adding 1:3 ratio of dye to lysate. Samples were then boiled for 5 minutes and stored in -20ºC until use.

3.9 Protein assay

The protein assay was performed to ensure sufficient protein was present in the cell lysate samples and to determine the correct loading volume of each sample to be

used in Western blotting. Dilutions of BSA protein standards were prepared by loading 10µl each 0, 0.1, 0.2, 0.4, 0.6, 0.8, 0.9 and 1.0 mg/mL BSA in distilled water (in triplicate) into a 96 well plate and a standard curve was formulated. Lysate samples were pipetted in triplicate into a 96-well plate (10µl/well) followed by addition of 200µl of diluted Biorad protein assay dye reagent (1:4 dye reagent to distilled water) to each well. Samples were incubated at room temperature for 5-10 minutes and absorbance was measured using KC4 microplate reader at 595nm. Briefly, the acidic dye binds to certain amino acids in the protein causing a spectral shift of the dye from brown to blue, where blue has a maximum absorbance at 595nm. The samples absorbance in comparison to the standards absorbance can then be used to calculate the final protein concentration in the samples.

3.10 Western blotting

Western blotting was used to examine the inhibition/activation of specific signaling cascades by measuring total and phosphorylated (measure of activity) levels of proteins of interest such as Akt, ERK and AMPK. Lysate samples containing 20-25µg protein were loaded alongside the Biorad Kaleidoscope ladder and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels. Samples were then transferred onto PVDF membrane and incubated in blocking buffer for 1 hour at room temperature. The membrane was incubated with primary antibody buffer overnight at 4 ºC and the following day the membrane was incubated with horse radish peroxidase (HRP)-linked IgG anti-rabbit secondary antibody for 1 hour at room temperature before being developed (using LI-COR C-Digit software). Signals were detected using Biorad Clarity Western ECL Solution. Densitometric analysis was

performed using Image J Software. β-actin was used as loading control and band densities of all samples were normalized to β-actin bands before being compared to control.

3.11 Statistical analysis

The results are the mean ± standard error mean of the indicated number of independent experiments. Analysis of Variance (ANOVA) with Tukey post-hoc test or Student's paired t-test was used. Statistical significance was assumed at $P<0.05$. Statistical tests were performed using Graphpad Prism software.

Chapter 4: Results

4.1 Biological effects of rosemary extract (RE) in NSCLC cells and non-cancerous lung fibroblasts

4.1.1 Effects of RE on cell proliferation in NSCLC cells and non-cancerous lung fibroblasts

The antiproliferative effects of RE were evaluated in A549, H460 and H1299 NSCLC cells and in MRC5 non-cancerous lung fibroblasts. RE has previously been shown to inhibit proliferation in other cancer cell lines [107,108,116]. All cell lines were treated with 2.5, 5, 10, 25, 50, 100, 150 and 200 µg/mL RE for 72 hours (Figure 8). Absorbance values (of crystal violet dye) were measured for each treatment group and compared to control, untreated cells (incubated in 10% FBS containing media alone). An incubation time of 72 hours was chosen based on preliminary data performed in our lab which showed 72 hours resulted in more defined antiproliferative effects compared to 48 hours. In A549 cells, RE showed significant inhibition of proliferation at the lowest dose

of 2.5µg/mL (84±7.1%, $p<0.05$). The lowest concentration of RE to result in significant inhibition of proliferation in H460 and H1299 cells was 25µg/mL (50.4±4.8%, $p<0.001$) and 2.5µg/mL (88.4±4.8%, $p<0.05$) respectively. The maximum inhibitory concentration of RE was 50 (21.9±2.6%, $p<0.001$), 50 (30.7±3.0%, $p<0.001$) and 25 µg/mL (23.3±1.3, $p<0.001$) in A549, H460 and H1299 cells respectively. In MRC5 cells, significant inhibition of proliferation was seen at doses above 25µg/mL (71.0±1.1, $p<0.05$). Inhibition of this non-cancerous lung cell line, although significant, was much less compared to the cancerous NSCLC cell lines tested. The IC50 of RE was 15.9µg/mL in A549, 57.2µg/mL in H460, 19µg/mL in H1299 and 103.5µg/mL in MRC5. Microscopic examination of NSCLC cell morphology revealed some cell rounding at doses above 100µg/mL however, no cells had lifted off the plate. No changes to cell morphology were seen at doses of or below 100µg/mL. No changes in cell morphology were seen at any dose in the MRC5 cell line. The RE used for all studies was dissolved in dimethylsulfoxide (DMSO). Cells were never exposed to more than 0.1% DMSO in any treatment. As well, DMSO at 0.1% was tested in parallel with RE experiments (Appendix-Figure s1) and no inhibition of proliferation was seen, confirming that the inhibitory effects seen were due to RE alone. These results indicate RE inhibits cell proliferation in NSCLC cells with varying histologies and mutations.

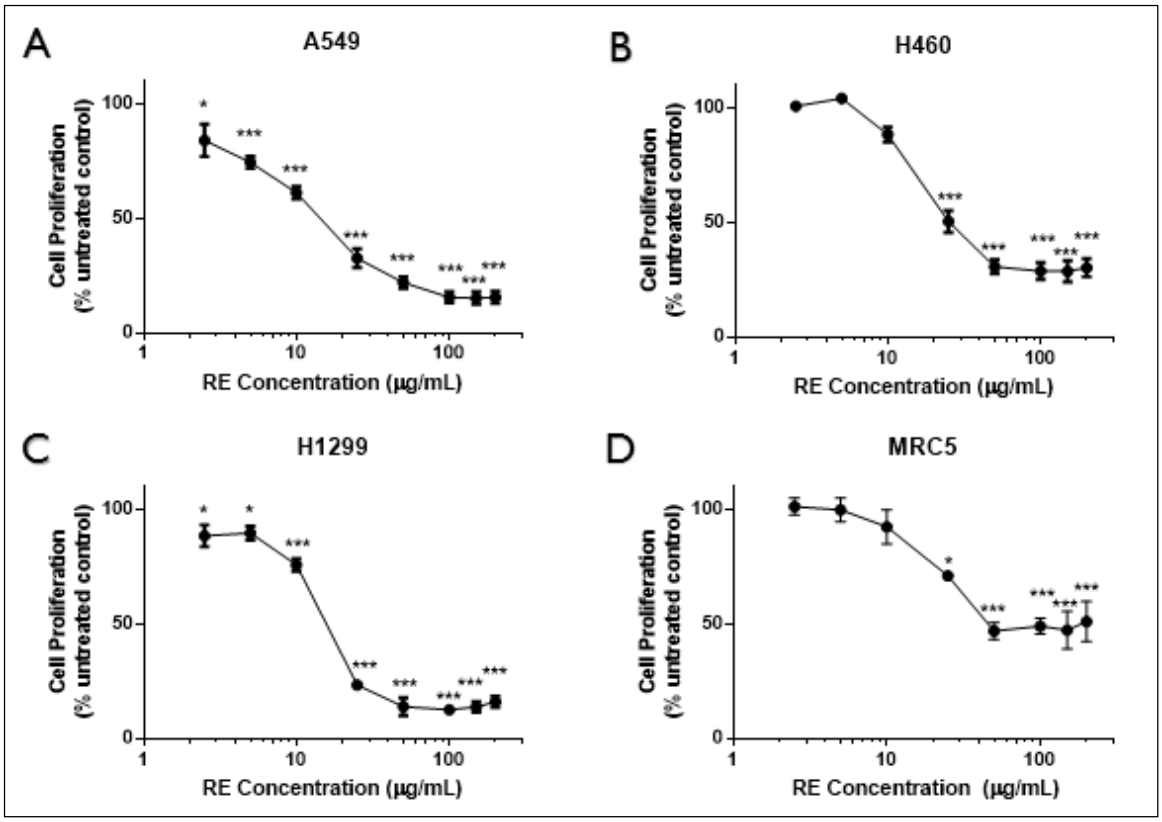

Figure 8: Effect of RE on cell proliferation in NSCLC cells. A) A549 B) H460 C) H1299 and D) MRC5 cells were seeded in triplicate and incubated with the indicated concentrations of RE for 72h followed by fixing and staining with 0.5% crystal violet. Stain was solubilized and absorbance was read at 570nm. Results are expressed as the mean % cell proliferation ± SEM compared to untreated control of 3-4 individual experiments. *p<0.05, **p<0.01, ***p<0.001.

The inhibitory effect of resveratrol, a well-studied plant compound which has been shown to have potent anticancer effects [140,141] on cell proliferation were also examined. At the maximum inhibitory concentration of each cell line (mentioned previously), RE showed a similar effect compared to 100µM resveratrol (22.3±2.5% in A549, p<0.001; 42.6±2.3% in H460, p<0.001; 17.8±2.3% in H1299, p<0.001) (Figure 9). Results suggest RE has potential as an effective anticancer agent.

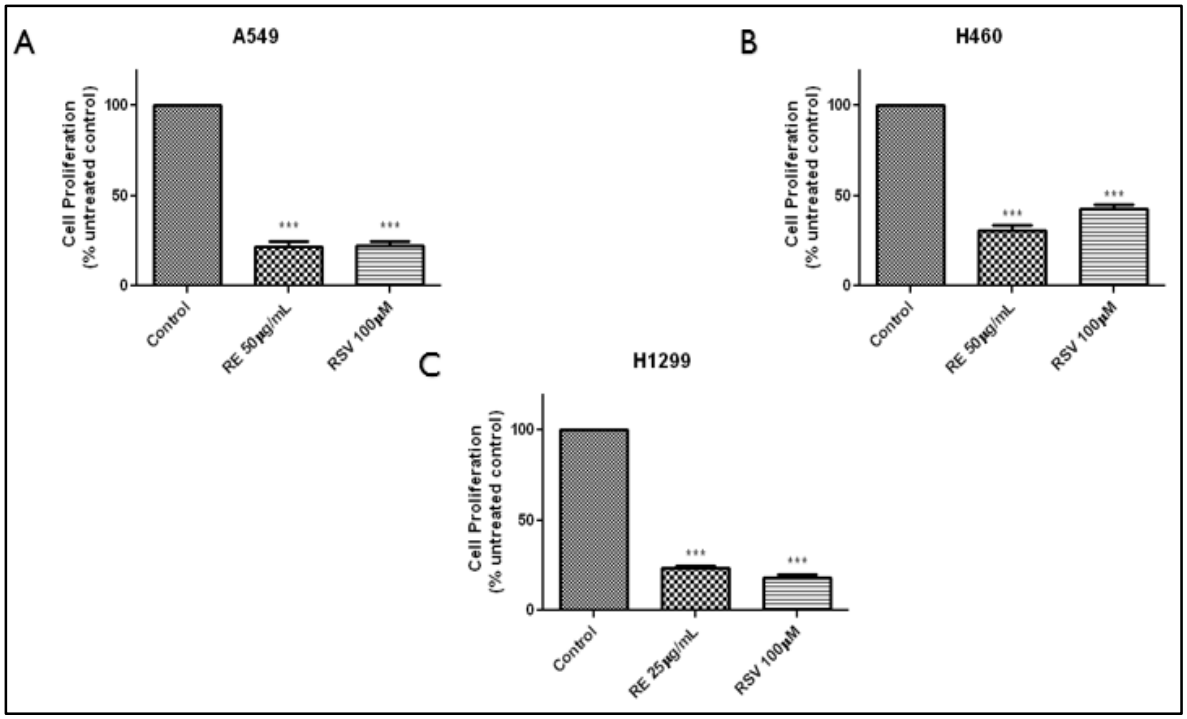

Figure 9: Effect of RE on cell proliferation in NSCLC cells is comparable to RSV. A) A549 B) H460 and C) H1299 cells were seeded in triplicate and incubated with the indicated concentrations of RE or RSV for 72h followed by fixing and staining with 0.5% crystal violet. Stain was solubilized and absorbance was read at 570nm. Results are expressed as the mean % cell proliferation ± SEM compared to untreated control of 3-4 individual experiments. ***p<0.001.

4.1.2 Effects of RE on cell survival in NSCLC cells

The ability of cancer cells to survive and form colonies was assessed using the clonogenic survival assay with varying concentrations of RE (2.5, 5 and 10µg/mL). Following a 7 day incubation with RE, cells were fixed and stained, and colonies with greater than 50 cells were counted and compared to control, untreated cells (incubated in 10% FBS containing media alone). These large colonies are an indication of the cells potential to survive and establish tumor formation *in vivo*. Survival of MRC5 was not tested because this cell line is non-cancerous and does not form colonies. Significant inhibition of survival was seen with 2.5µg/mL RE in all cell lines (39.3±3.1% in A549,

p<0.001; 65.1±7.9% in H460, p<0.01; 42.3±1.9% in H1299, p<0.001) (Figure 10). At 10µg/mL RE caused near complete inhibition of survival in A549 (1.2±3.1%, p<0.001) and H1299 (0.8±0.6%, p<0.001) cells. Inhibition of survival in H460 at 10µg/mL was 25.8±5.9% (p<0.001). Taken together, these results suggest that RE has potent anticancer effects and inhibits cell proliferation and survival *in vitro* in various NSCLC cell lines.

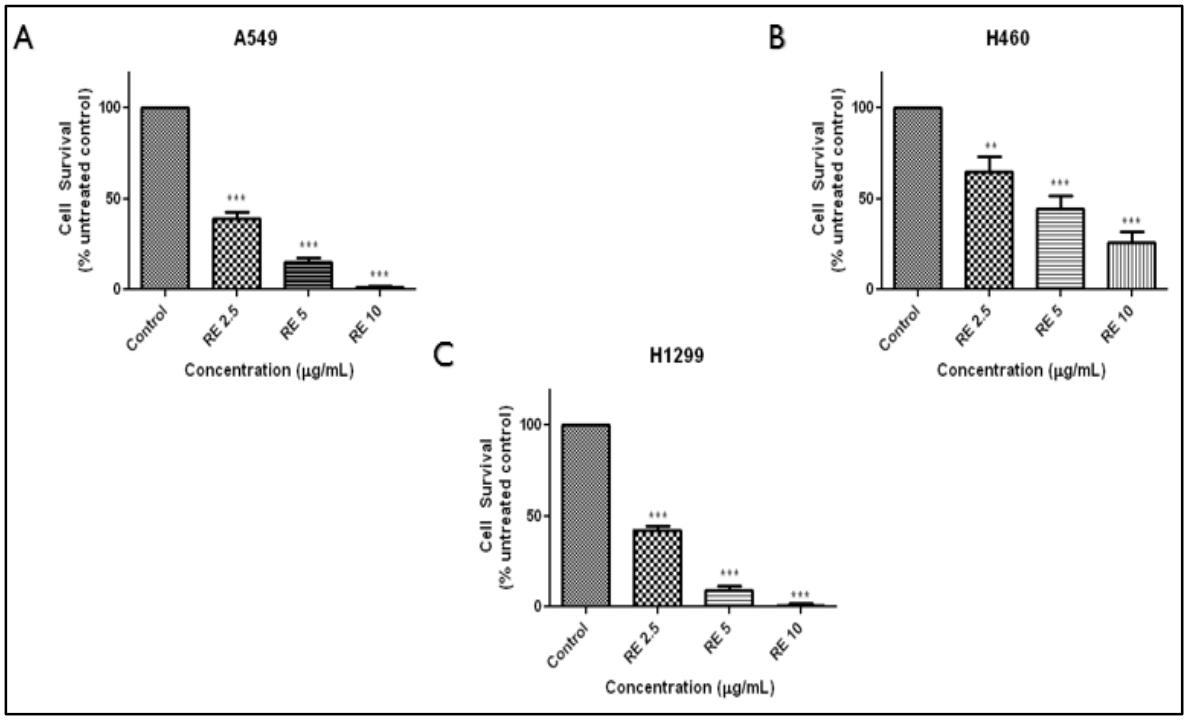

Figure 10: Effect of RE on clonogenic survival in NSCLC cells. A) A549 B) H460 and C) H1299 cells were seeded in triplicate at a low density and incubated with the indicated concentrations of RE for 7 days followed by fixing and staining with 0.05% methylene blue. Colonies of more than 50 cells were counted. Results are expressed as the mean surviving fraction ± SEM compared to untreated control of 3-4 individual experiments. **p<0.01, ***p<0.001.

4.1.3 Effect of RE on Akt signaling in various NSCLC cell lines

Next, we examined the potential underlying mechanisms involved in RE's ability to inhibit cell proliferation and survival in NSCLC cells. The signaling molecule Akt is largely involved in controlling cell proliferation in cancer cells and signaling through this

pathway is often enhanced in cancer, leading to a resistance to radiation and chemotherapy [36]. Thus, Akt is an important molecule to examine when testing potential anticancer agents. Phosphorylation of Akt on the serine 473 residue has been established as an indicator of Akt activity [20] and therefore a phospho-specific antibody against this residue (Ser 473) was used. All NSCLC cell lines were treated without (control) or with RE at 25 and 50µg/mL for 48h and a dose-dependent effect was seen in each cell line when compared to control (Figure 11). RE inhibited Akt phosphorylation in A549 cells at both concentrations (25µg/mL: 57.0±5.04%, $p<0.001$; 50µg/mL: 36.1±4.9%, $p<0.001$) however, the same effect was not seen in H460 or H1299 cells which showed enhanced Akt phosphorylation. Total levels of the Akt protein were significantly inhibited dose-dependently by RE in all cell lines: A549 (25µg/mL:49.8±5.3%; 50µg/mL: 32.4±0.7%) H460 (25µg/mL:74.8±5.2%; 50µg/mL:59.9±6.0%) H1299 (25µg/mL:57.4±2.9%; 50µg/mL:39.8±4.7%). These results suggest that RE is able to alter Akt signaling (and total protein levels) in NSCLC cells.

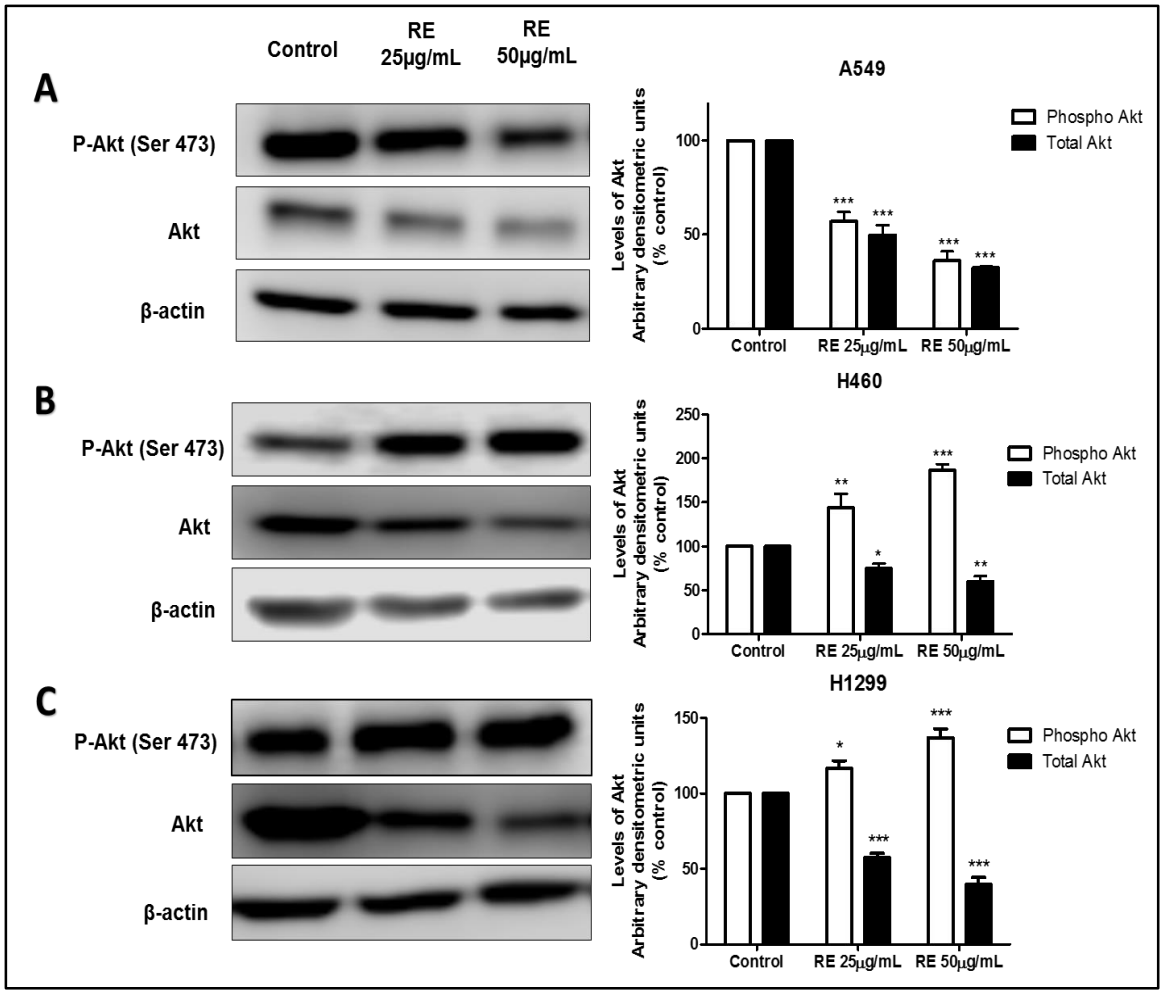

Figure 11: Effect of RE on Akt signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of RE for 48h in 10% FBS containing medium. Cell lysates (20μg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against Akt. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

4.1.4 Effect of RE on ERK signaling in various NSCLC cell lines

The signaling molecule ERK is also largely implicated in cancer and leads to cell growth and proliferation therefore, we wished to examine RE's effects on this protein and

its activation. Phosphorylation of ERK on the threonine 202 and tyrosine 204 (Thr202/Tyr204) residues in the activation loop lead to its activation and thus a phospho-specific antibody against these residues was used. All NSCLC cell lines were treated without (control) or with RE at 25 and 50µg/mL for 48h and a dose-dependent effect was seen in each cell line when compared to control (Figure 12). Phosphorylation of ERK was enhanced in all cell lines: A549 (25µg/mL:148.9±12.8%; 50µg/mL:175.8±10.7%), H460 (25µg/mL:136.0±9.0%; 50µg/mL:158.7±14.7%) with H1299 (25µg/mL:172.9±2.7%; 50µg/mL:211.7±11.8%) being the most sensitive. Similar to the effect seen on Akt, RE inhibited total levels of ERK in all cell lines: A549 (25µg/mL:77.7±3.9%; 50µg/mL:63.5±7.6%), H460 (25µg/mL:82.0±2.8%; 50µg/mL:61.0±1.0%), H1299 (25µg/mL:54.4±4.2%; 50µg/mL:37.8±3.2%). These results suggest RE decreases total protein levels of ERK and enhances its activation in NSCLC.

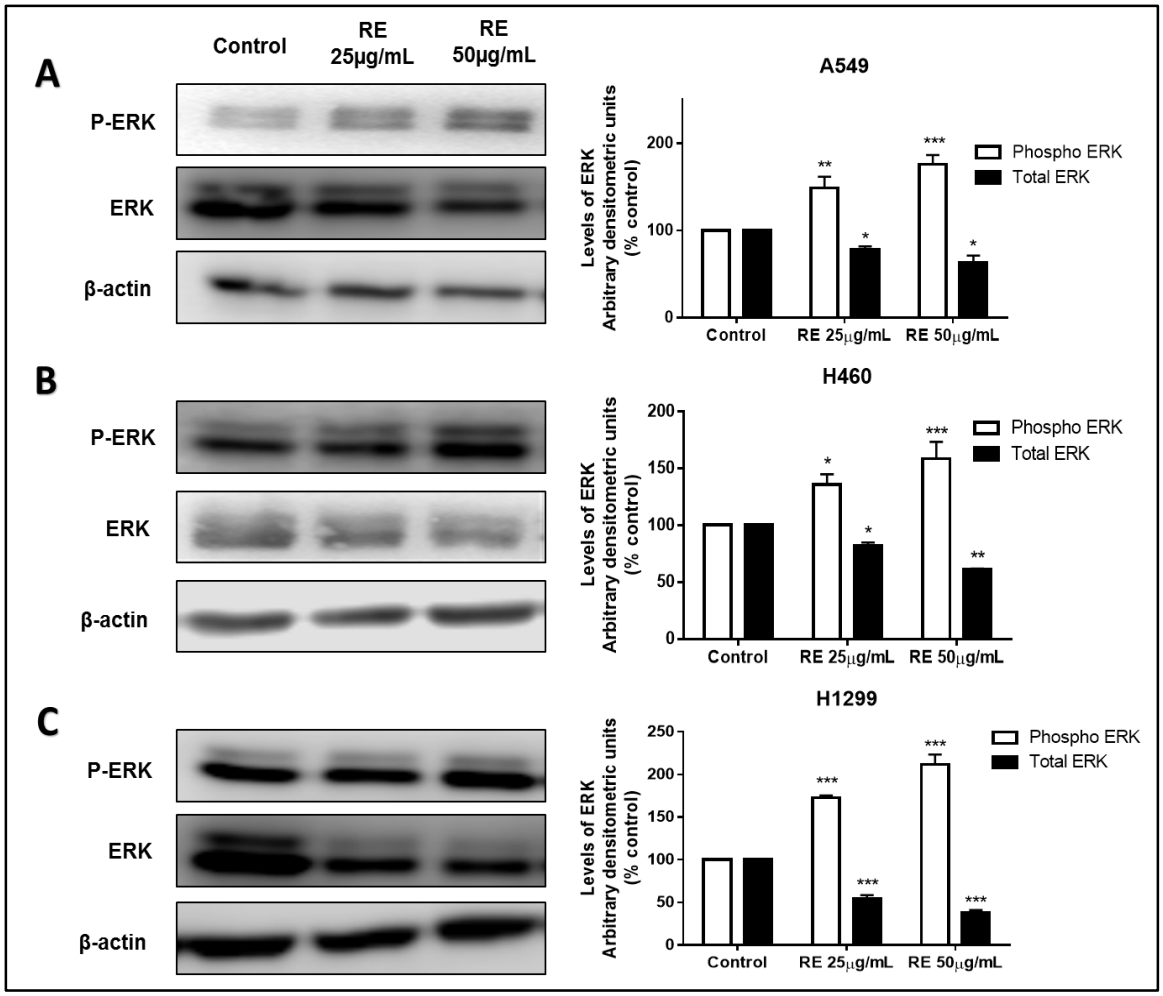

Figure 12: Effect of RE on ERK signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of RE for 48h in 10% FBS containing medium. Cell lysates (20µg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against ERK. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

4.1.5 Effect of RE on AMPK signaling in various NSCLC cell lines

The energy sensing molecule AMPK which helps maintain energy homeostasis within the cell is emerging as an important protein which becomes deregulated in cancer and can lead to cell growth and proliferation. Therefore, we wished to examine RE's

effects on its activation. Phosphorylation of AMPK on the threonine 172 residue of its catalytic alpha subunit by the tumor suppressor LKB1 is required for its activation [59] and thus a phospho-specific antibody against this residue was used. All NSCLC cell lines were treated without (control) or with RE at 25 and 50µg/mL for 48h and a dose-dependent effect was seen in each cell line when compared to control (Figure 13). Phosphorylation of AMPK was enhanced only in H1299 cells (25µg/mL:173.3±13.7%; 50µg/mL:234.6±10.0%) while a small but significant inhibition was seen in A549 (25µg/mL:88.0±4.3%; 50µg/mL:78.7±2.5%) and H460 (25µg/mL:92.6±1.7%; 50µg/mL:80.3±2.1%) cells, when compared to control. Total levels of AMPK were decreased dose-dependently by RE in all cell lines: A549 (25µg/mL:84.6±1.4%; 50µg/mL:73.8±1.4%), H460 (25µg/mL:86.8±3.0%; 50µg/mL:72.1±4.3%), H1299 (25µg/mL:71.7±7.9%; 50µg/mL:62.4±6.9%). These results suggest RE decreases total protein levels of AMPK in NSCLC and alters AMPK activation.

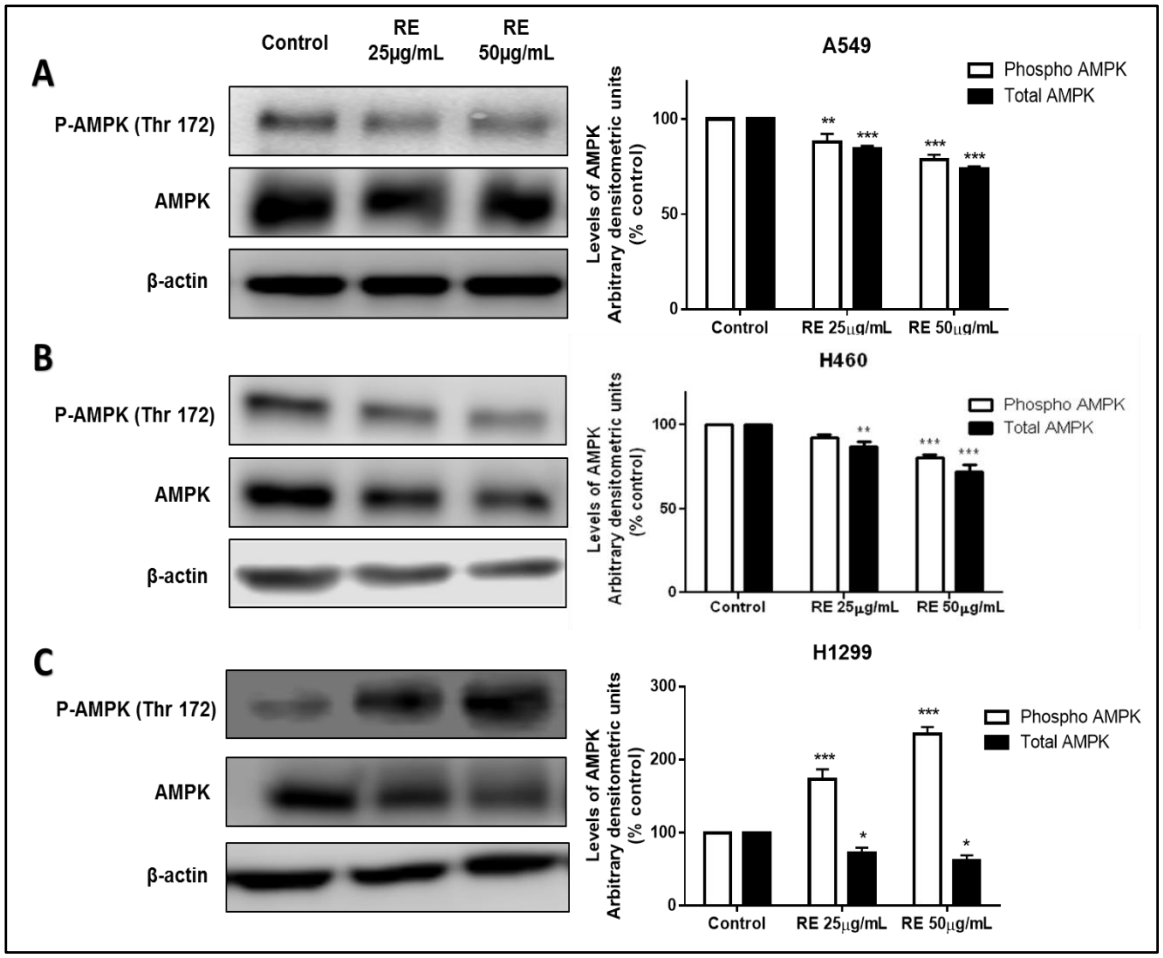

Figure 13: Effect of RE on AMPK signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of RE for 48h in 10% FBS containing medium. Cell lysates (20µg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against AMPK. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Table 11: Summary of the effects of RE on cell signaling in NSCLC cells by mutation status

Mutation Status	Cell Line	Total Protein Levels	Phospho Protein Levels
KRAS mutant	A549	Akt ↓ ERK ↓ AMPK ↓	Akt ↓ ERK ↑ AMPK ↓
	H460	Akt ↓ ERK ↓ AMPK ↓	Akt ↑ ERK ↑ AMPK ↓
	H1299	Akt ↓ ERK ↓ AMPK ↓	Akt ↑ ERK ↑ AMPK ↑
PIK3CA mutant	H460	Akt ↓ ERK ↓ AMPK ↓	Akt ↑ ERK ↑ AMPK ↓
LKB1 null	A549	Akt ↓ ERK ↓ AMPK ↓	Akt ↓ ERK ↑ AMPK ↓
	H460	Akt ↓ ERK ↓ AMPK ↓	Akt ↑ ERK ↑ AMPK ↓
p53 null	H1299	Akt ↓ ERK ↓ AMPK ↓	Akt ↑ ERK ↑ AMPK ↑

4.2 Biological effects of one of RE's major polyphenolic compounds, carnosic acid (CA) in NSCLC cells and non-cancerous lung fibroblasts

4.2.1 Effects of CA on cell proliferation in NSCLC cells and non-cancerous lung fibroblasts

RE showed potent anticancer effects and thus, we examined if these effects may be attributable to CA, one of RE's main polyphenolic compounds. Carnosic acid was tested at concentrations of 0.1, 0.3, 1, 5, 25, 50, 100 and 200μM in A549, H460 and H1299 NSCLC cells and in MRC5 non-cancerous lung fibroblasts for 72 hours (Figure 14). The concentration of 0.3μM was included to represent the concentration of CA

found in 5µg/mL RE, which showed significant inhibition of proliferation in A549 and H1299 cells (Shown previously in Figure 8). All other concentrations were chosen to create a dose-response curve and include those previously shown to have antiproliferative effects in the literature in other cell lines [109,142,143]. Absorbance values (of crystal violet dye) were measured for each treatment group and compared to control, untreated cells (incubated in 10% FBS containing media alone). An incubation time of 72 hours was chosen so results were comparable to those from RE (Section 4.1). The lowest dose of CA to cause significant inhibition of proliferation was 100µM (50.5±7.4%, $p<0.001$) in A549, 25µM (77.9±4.2%, $p<0.05$) in H460 and 50µM (56.6±14%, $p<0.05$) in H1299 cells. No inhibition of proliferation was seen at 0.3µM in any cell line (100.2±4.0% in A549; 95.3±3.2% in H460; 107.7±4.4% in H1299). In MRC5 non-cancerous cells, significant inhibition of proliferation was seen at 50µM (72.7±6.0%, $p<0.05$). The CA used for all studies was dissolved in dimethylsulfoxide (DMSO). Cells were never exposed to more than 0.1% DMSO in any treatment. As well, DMSO at 0.1% was tested in parallel with CA experiments (Appendix-Figure s1) and no inhibition of proliferation was seen, confirming that the inhibitory effects seen were due to CA alone. These results suggest that CA alone, at higher doses, may have anticancer effects in NSCLC however, the antiproliferative effects of RE are not attributable to this compound alone.

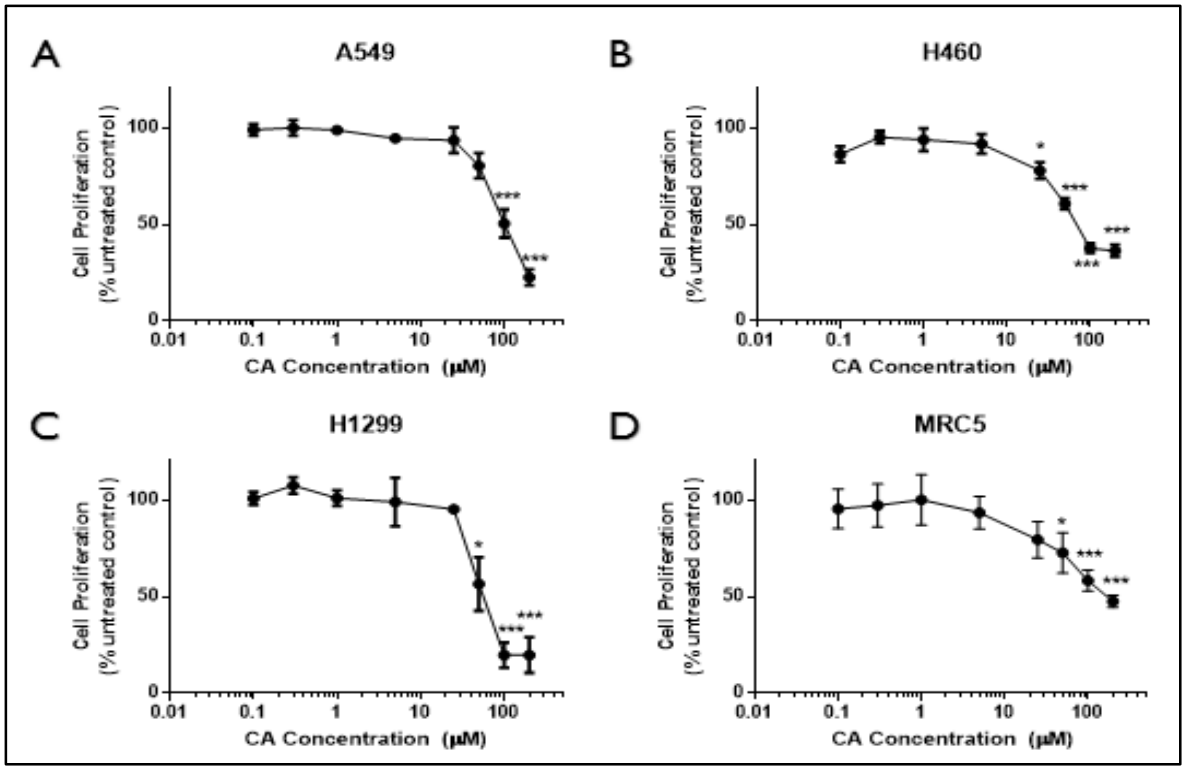

Figure 14: Effect of CA on cell proliferation in NSCLC cells. A) A549 B) H460 C) H1299 and D) MRC5 cells were seeded in triplicate and incubated with the indicated concentrations of CA for 72h followed by fixing and staining with 0.5% crystal violet. Stain was solubilized and absorbance was read at 570nm. Results are expressed as the mean % cell proliferation ± SEM compared to untreated control of 3-4 individual experiments. *p<0.05, ***p<0.001.

4.2.2 Effects of CA on cell survival in NSCLC cells

Next, we examined the effect of CA alone on clonogenic survival of A549, H460 and H1299 NSCLC cells. Significant inhibition of survival was seen with 25μM in all cell lines (48.2±10.9% in A549, $p<0.01$; 36.7±9.7% in H460, $p<0.001$; 7.1±1.0% in H1299, $p<0.001$). The H1299 cell line appear to be most sensitive to the CA treatment (Figure 15).

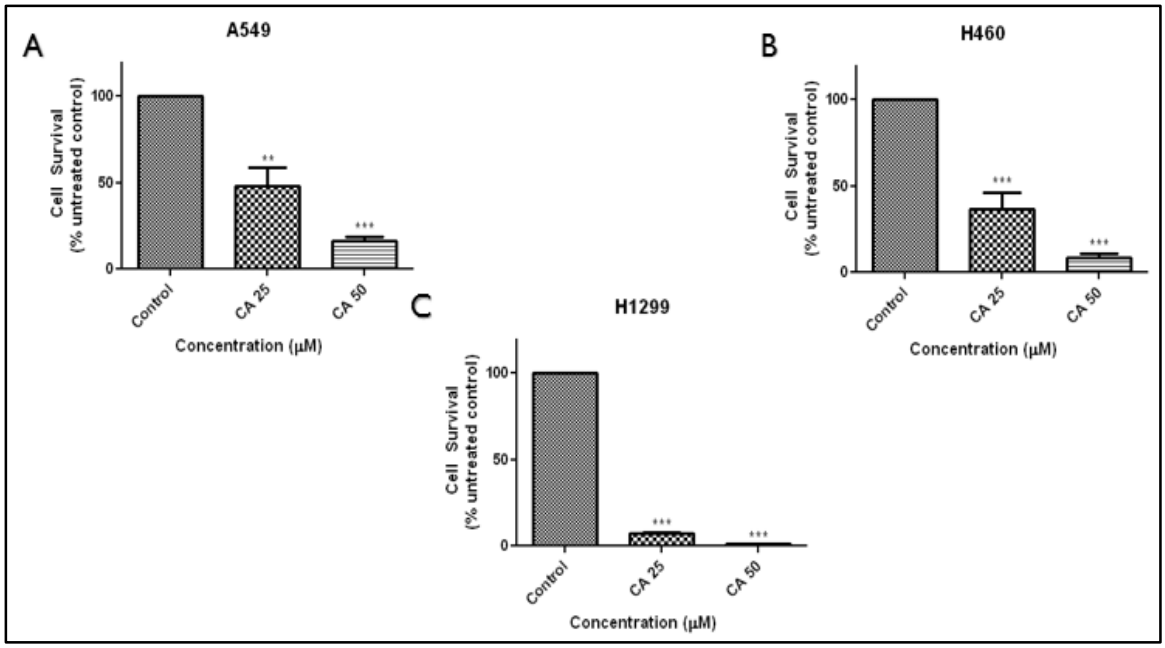

Figure 15: Effect of CA on clonogenic survival in NSCLC cells. A) A549 B) H460 and C) H1299 cells were seeded in triplicate at a low density and incubated with the indicated concentrations of CA for 7 days followed by fixing and staining with 0.05% methylene blue. Colonies of more than 50 cells were counted. Results are expressed as the mean surviving fraction ± SEM compared to untreated control of 3-4 individual experiments. **p<0.01, ***p<0.001.

4.2.3 Effect of CA on Akt signaling in various NSCLC cell lines

As mentioned in section 4.1.3, Akt is an important molecule involved in cancer cell growth and survival. Since RE had a significant effect on altering Akt levels we wanted to examine if this was attributable, at least in part, to CA one of its main polyphenols. Phosphorylation of Akt was inhibited by 50μM CA in A549 (66.9±3.7%) and H1299 (82.0±3.5%) and enhanced in H460 (112.7±3.1%) cells. However, at 3μM the concentration of CA found in 50μg/mL of RE which caused maximum inhibition of proliferation and survival (section 4.1), no significant effects were seen. Total Akt levels were not altered by CA in any cell line.

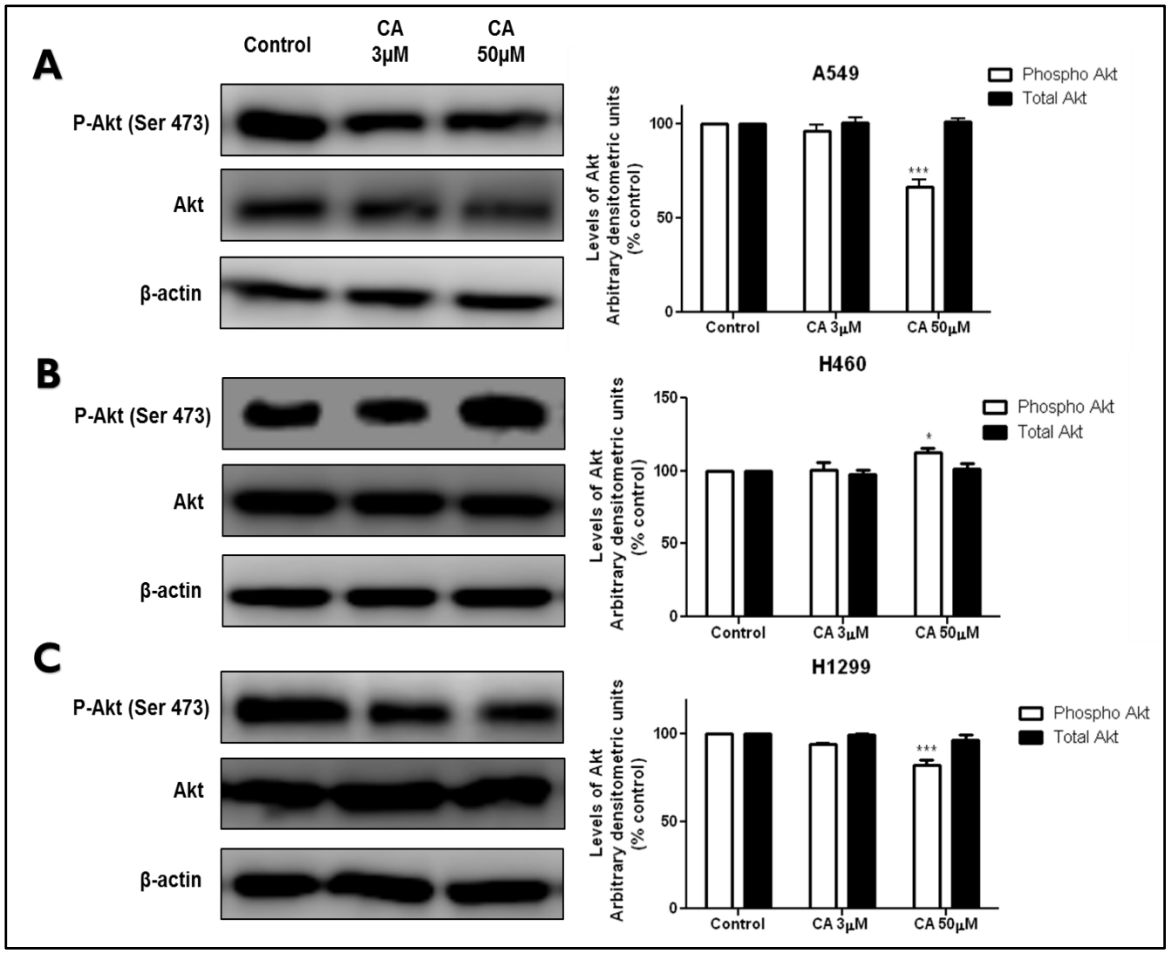

Figure 16: Effect of CA on Akt signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of CA for 48h in 10% FBS containing medium. Cell lysates (20µg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against Akt. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

4.2.4 Effect of CA on ERK signaling in various NSCLC cell lines

Activation of ERK was shown to be enhanced in all NSCLC cell lines by RE (section 4.1) hence, we investigated if CA, one of rosemary's main polyphenols was responsible for this effect. At 50µM CA enhanced phosphorylation of ERK in all cell

lines: A549 (131.6±6.3%), H460 (122.9±7.8%), H1299 (156.8±13.6%). No significant effect was seen by 3μM CA and total levels of ERK were not affected in any cell line (Figure 17).

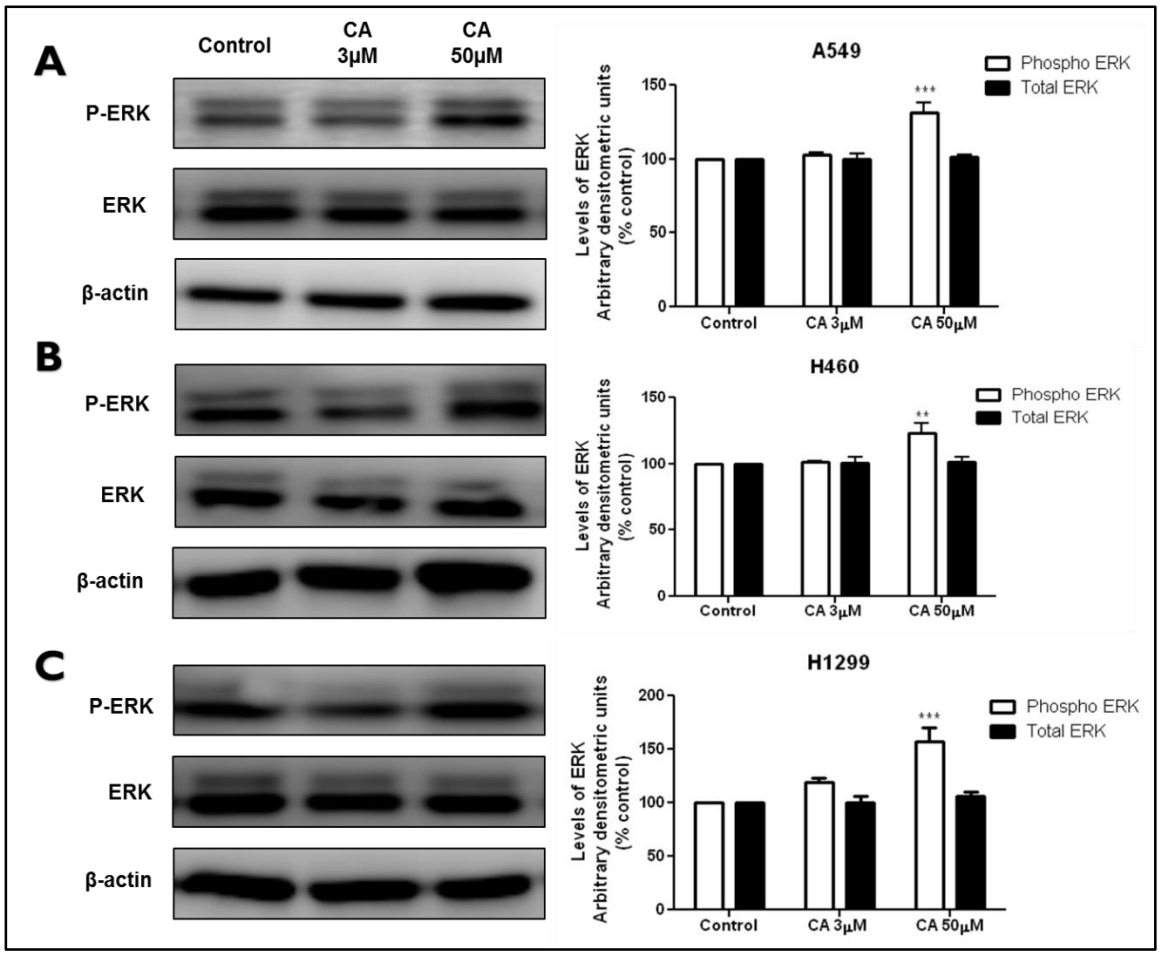

Figure 17: Effect of CA on ERK signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of CA for 48h in 10% FBS containing medium. Cell lysates (20μg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against ERK. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

4.2.5 Effect of CA on AMPK signaling in various NSCLC cell lines

Phosphorylation of AMPK was enhanced by RE in H1299 cells (section 4.1). We investigated if CA, one of rosemary's main polyphenols was responsible, at least in part, for this effect. At 50μM CA enhanced phosphorylation of AMPK in H1299 (169.5±10.4%) cells. No significant effect was seen in A549 cells and a slight inhibition of phosphorylation was seen in H460 (91.8±1.9%) cells compared to control. No significant effect was seen by 3μM CA and total levels of AMPK were not affected in any cell line (Figure 18).

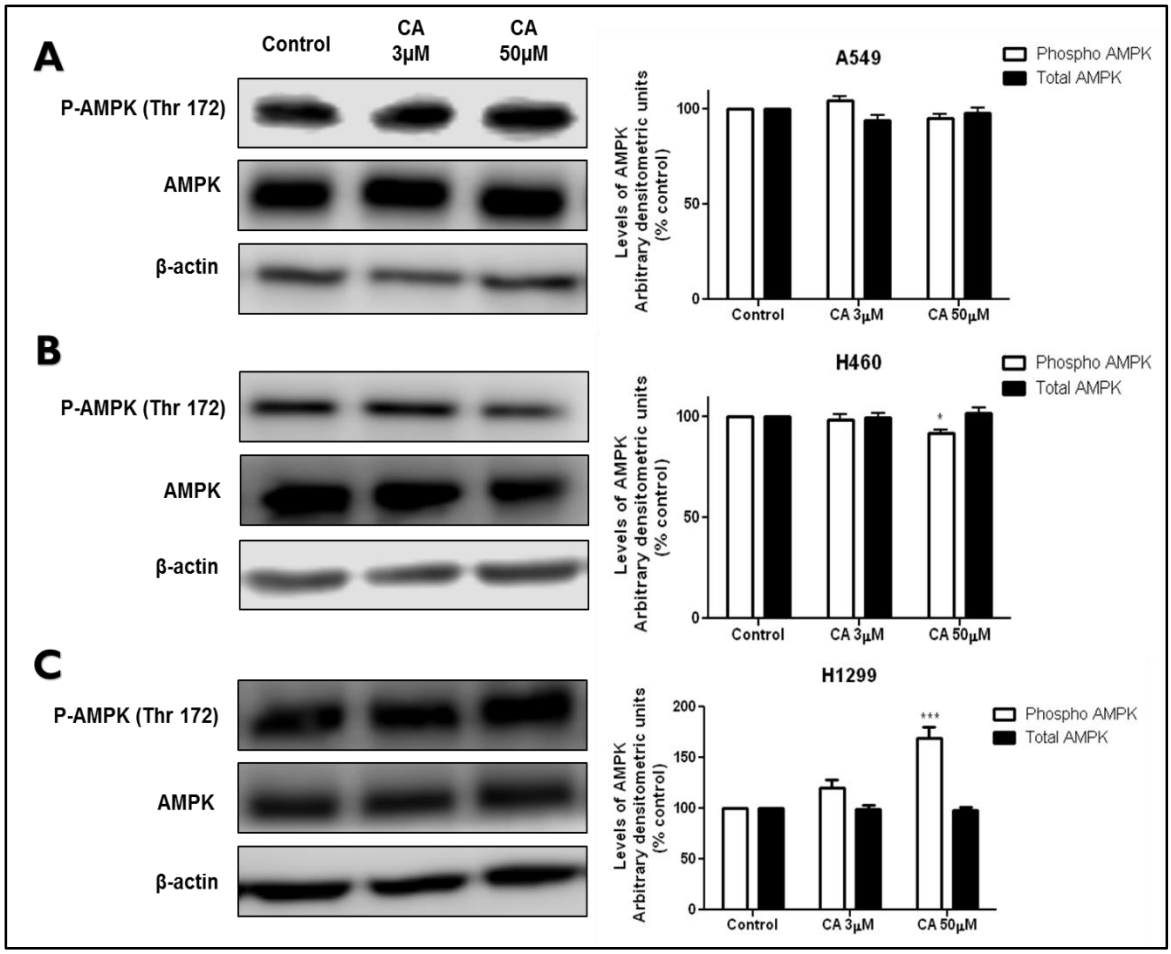

Figure 18: Effect of CA on AMPK signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of CA for 48h in 10% FBS containing medium. Cell lysates (20µg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against AMPK. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Table 12: Summary of the effects of CA on cell signaling in NSCLC by cell line and mutation status

Cell Line	Mutation Status	Total Protein Level	Phospho Protein Level
A549	KRAS mutant LKB1 null	Akt ↔ ERK ↔ AMPK ↔	Akt ↓ ERK ↑ AMPK ↔
H460	KRAS mutant LKB1 null PIK3CA mutant	Akt ↔ ERK ↔ AMPK ↔	Akt ↑ ERK ↑ AMPK ↓
H1299	KRAS mutant p53 null	Akt ↔ ERK ↔ AMPK ↔	Akt ↓ ERK ↑ AMPK ↑

4.3 Biological effects of one of RE's major polyphenolic compounds, rosmarinic acid (RA) in NSCLC cells and non-cancerous lung fibroblasts

4.3.1 Effects of RA on cell proliferation in NSCLC cells and non-cancerous lung fibroblasts

The anticancer effects of RE were found to not be attributable to CA alone however significant anticancer effects were seen by CA at higher concentrations. We next examined the antiproliferative effects of RA, another polyphenolic compound found in RE to determine if it contributed to RE's anticancer effects. Rosmarinic acid was tested at concentrations of 1, 1.86, 10, 25, 50, 100, 200 and 300µM in A549, H460 and H1299 NSCLC cells and in MRC5 non-cancerous lung fibroblasts for 72 hours (Figure 19). The concentration of 1.86µM was included to represent the concentration of RA found in 5µg/mL RE, which showed significant inhibition of proliferation in A549 and H1299 cells (Shown previously in Figure 8). All other concentrations were chosen to create a dose-response curve and include those previously shown to have antiproliferative effects or inhibit viability in the literature in other cell lines [113,144–146]. Absorbance values (of crystal violet dye) were measured for each treatment group and compared to

control, untreated cells (incubated in 10% FBS containing media alone). An incubation time of 72 hours was chosen so results were comparable to those from RE (Section 4.1). The lowest dose of RA to cause significant inhibition of proliferation was 100µM (51.1±8.8%, $p<0.001$) in A549, 25µM (87.3±3.3%, $p<0.05$) in H460 and 100µM (23.5±10.8%, $p<0.001$) in H1299 cells. No inhibition of proliferation was seen at 1.86µM in any cell line (104.7±3.9% in A549; 98.9±4.8% in H460; 99.5±10.3% in H1299). In MRC5 non-cancerous cells, significant inhibition of proliferation was seen at 50µM (70.7±5.0%, $p<0.05$). These results suggest that RA alone, at higher doses, may have anticancer effects in NSCLC however, the antiproliferative effects of RE are not attributable to this compound alone.

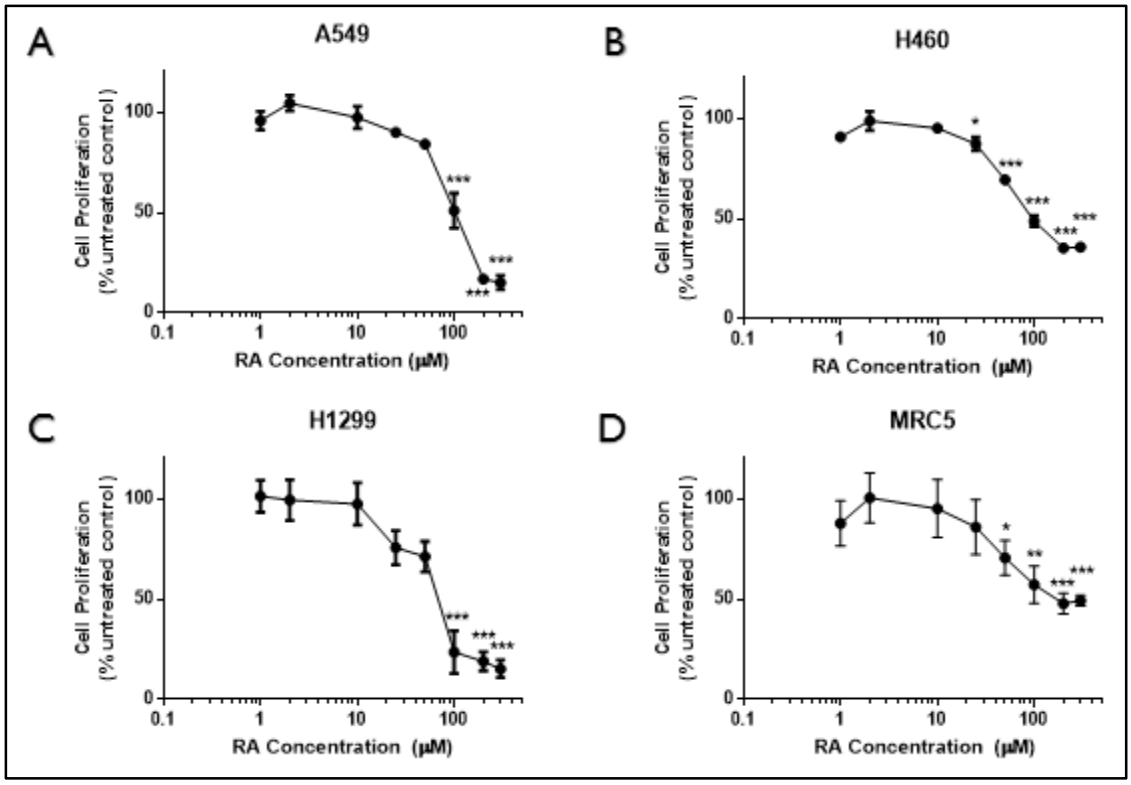

Figure 19: Effect of RA on cell proliferation in NSCLC cells. A) A549 B) H460 C) H1299 and D) MRC5 cells were seeded in triplicate and incubated with the indicated concentrations of RA for 72h followed by fixing and staining with 0.5% crystal violet. Stain was solubilized and absorbance was read at 570nm. Results are expressed as the mean % cell proliferation ± SEM compared to untreated control of 3-4 individual experiments. *p<0.05, **p<0.01, ***p<0.001.

4.3.2 Effects of RA on cell survival in NSCLC cells

Next, we examined the effect of RA alone on clonogenic survival of A549, H460 and H1299 NSCLC cells. Significant inhibition of survival was seen with 50μM in A549 (62.5±4.1%, p<0.001) and H1299 (22.4±0.8%, p<0.001) cells. Significant inhibition was only seen in H460 cells at a concentration of 100μM (34.9±17.8%, p<0.05). Similar to the clonogenic survival using CA alone, the H1299 cell line appears to be the most sensitive to RA treatments (Figure 20).

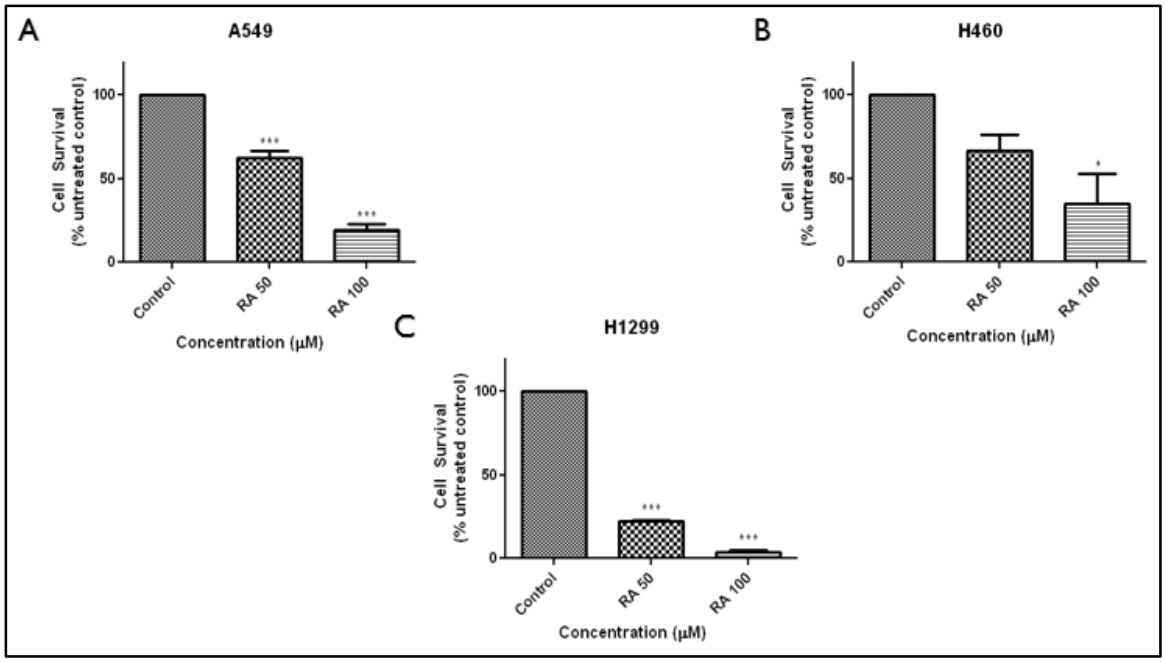

Figure 20: Effect of RA on clonogenic survival in NSCLC cells. A) A549 B) H460 and C) H1299 cells were seeded in triplicate at a low density and incubated with the indicated concentrations of RA for 7 days followed by fixing and staining with 0.05% methylene blue. Colonies of more than 50 cells were counted. Results are expressed as the mean surviving fraction ± SEM compared to untreated control of 3-4 individual experiments. *p<0.05, ***p<0.001.

4.3.3 Effect of RA on Akt signaling in various NSCLC cell lines

As mentioned in section 4.1.3, Akt is an important molecule involved in cancer cell growth and survival. Since RE had a significant effect on altering Akt levels we wanted to examine if this was attributable, at least in part, to RA one of its main polyphenols. Phosphorylation of Akt was inhibited by 50μM RA in A549 (88.4±0.9%) and H1299 (74.4±5.2%) and increased in H460 (111.3±2.3%) cells however at 18μM, the concentration of RA found in 50μg/mL of the RE which caused maximum inhibition of proliferation and survival (section 4.1), no significant effects were seen. Total Akt levels were not altered by RA in any cell line (Figure 21).

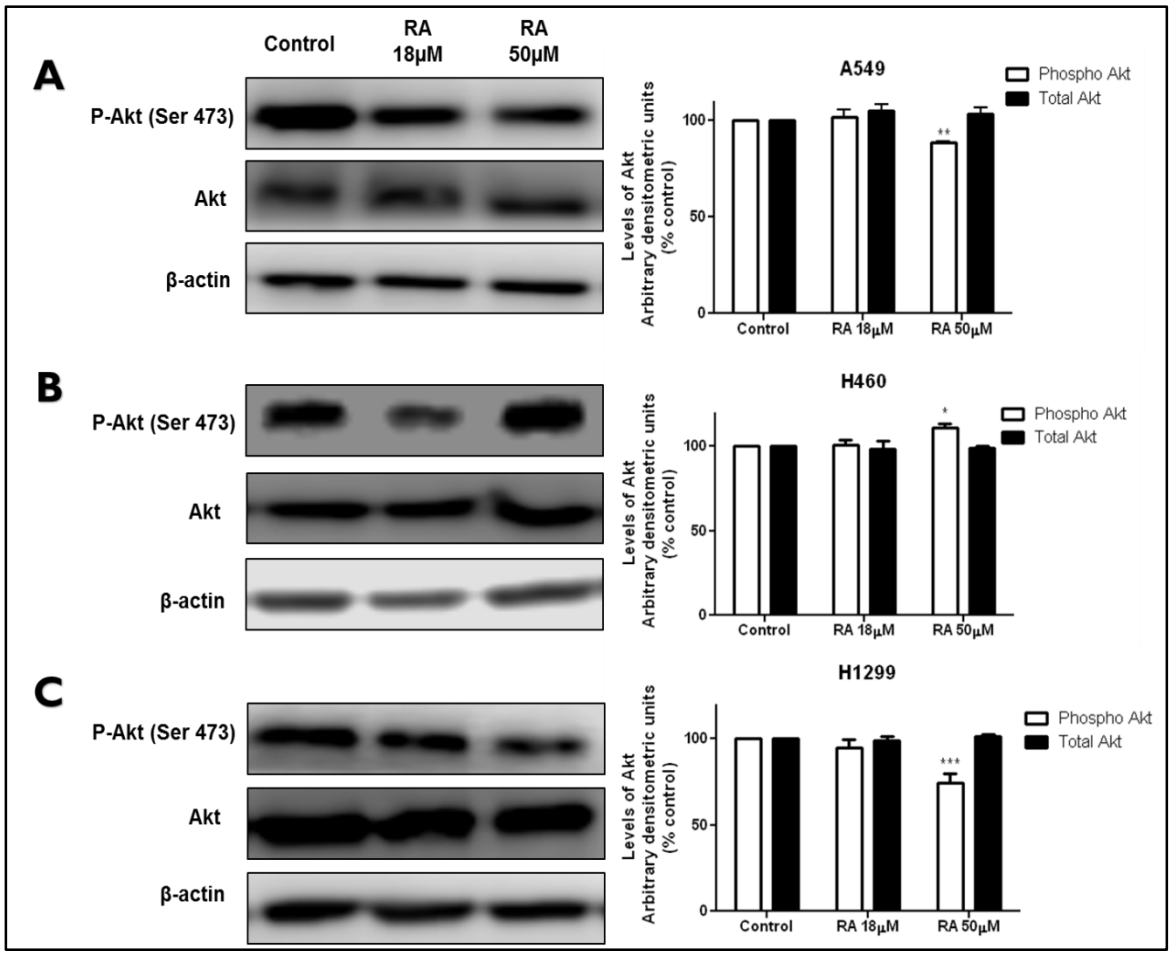

Figure 21: Effect of RA on Akt signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of RA for 48h in 10% FBS containing medium. Cell lysates (20μg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against Akt. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

4.3.4 Effect of RA on ERK signaling in various NSCLC cell lines

Activation of ERK was shown to be enhanced in all NSCLC cell lines by RE (section 4.1) hence, we investigated if RA, one of rosemary's main polyphenols was responsible for this effect. At 50μM RA enhanced phosphorylation of ERK in all cell

lines: A549 (133.4±6.2%), H460 (138.6±6.5%), H1299 (170.6±11.9%). No significant effect was seen by 18μM RA and total levels of ERK were not affected in any cell line (Figure 22).

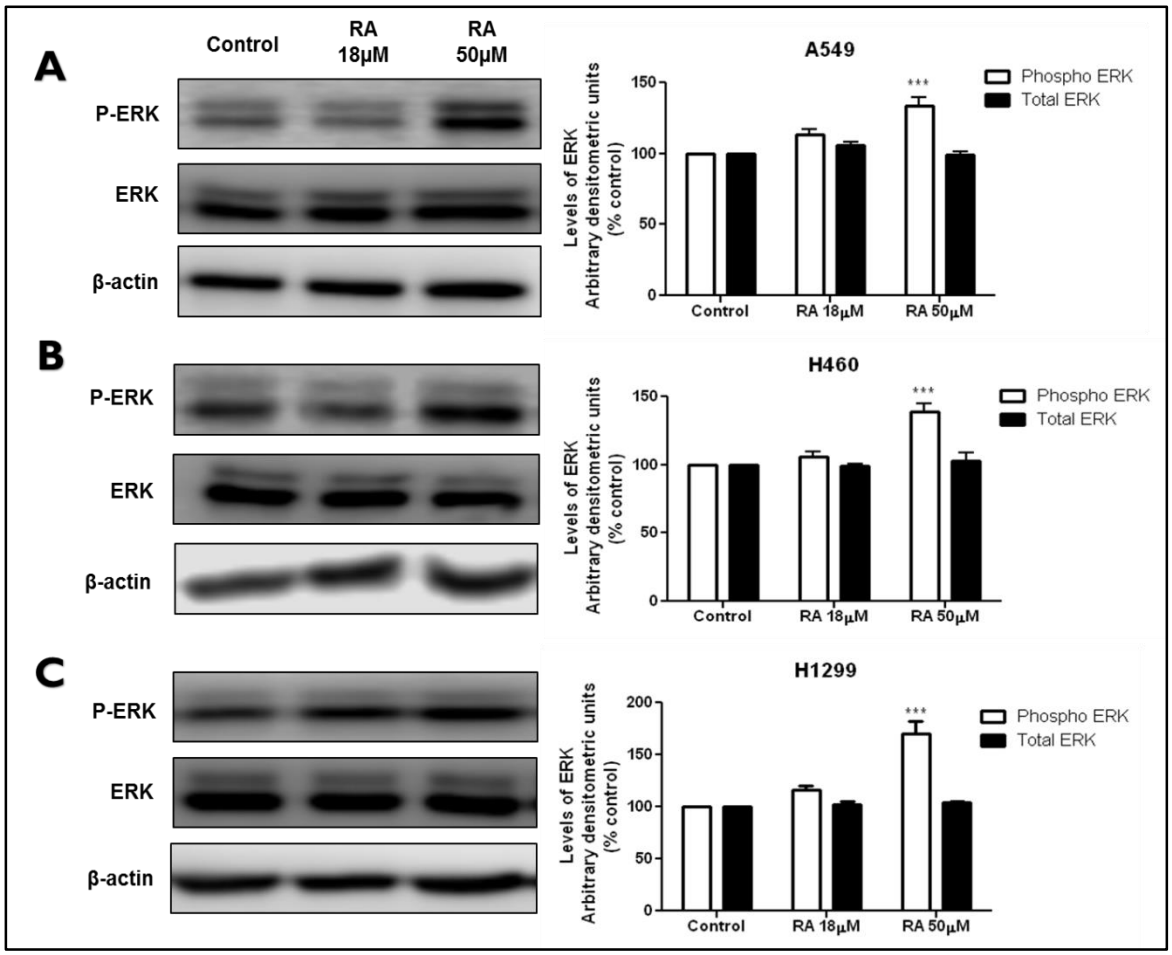

Figure 22: Effect of RA on ERK signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of RA for 48h in 10% FBS containing medium. Cell lysates (20μg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against ERK. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

4.3.5 Effect of RA on AMPK signaling in various NSCLC cell lines

Phosphorylation of AMPK was enhanced by RE in H1299 cells (section 4.1). We investigated if RA, one of rosemary's main polyphenols was responsible, at least in part, for this effect. At 50μM RA enhanced phosphorylation of AMPK in H1299 (138.4±5.8%) cells. No significant effect was seen in A549 or H460 cells compared to control. No significant effect was seen by 18μM RA and total levels of AMPK were not affected in any cell line (Figure 23).

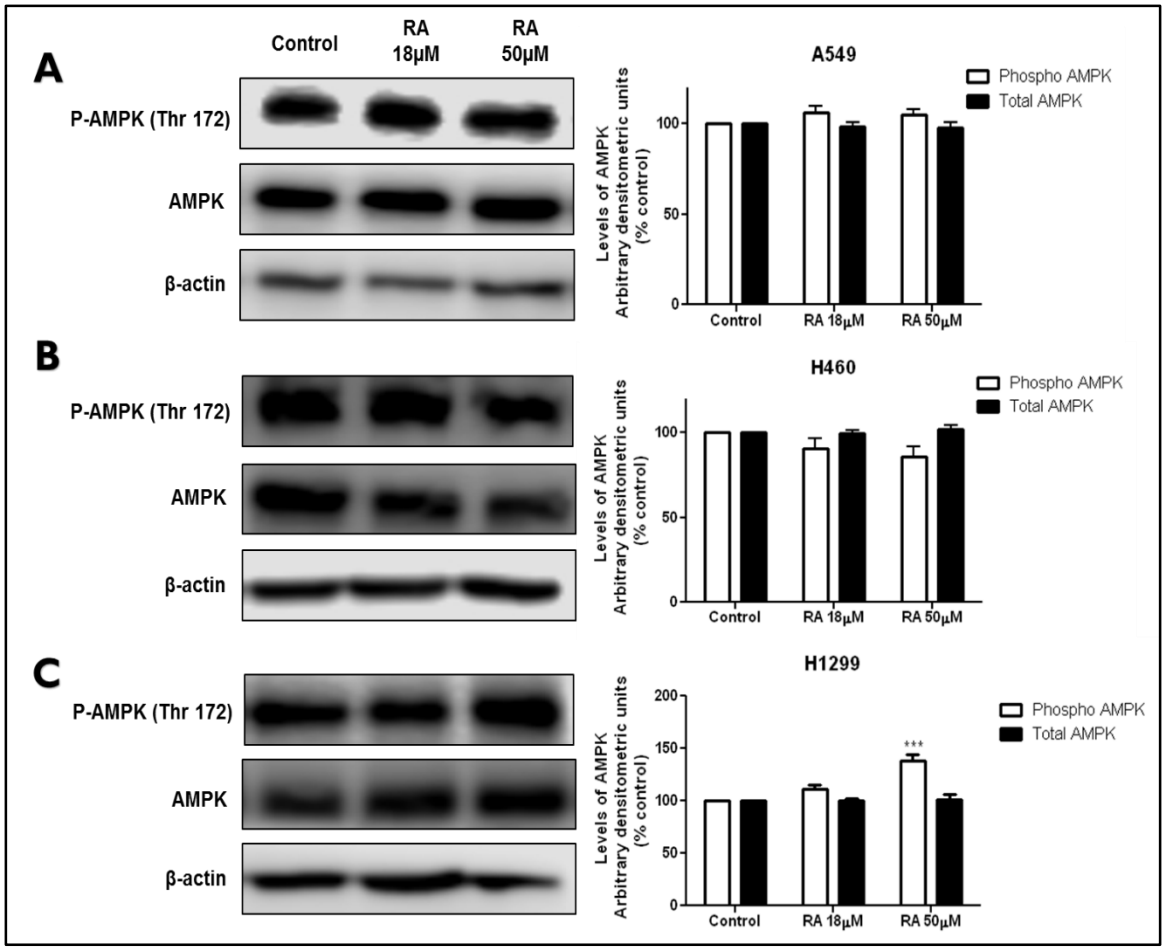

Figure 23: Effect of RA on AMPK signaling in NSCLC cells. Whole cell lysates were prepared from A) A549 B) H460 and C) H1299 cells treated with the indicated concentrations of RA for 48h in 10% FBS containing medium. Cell lysates (20μg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against AMPK. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Table 13: Summary of the effects of RA on cell signaling in NSCLC by cell line and mutation status

Cell Line	Mutation Status	Total Protein Level	Phospho Protein Level
A549	KRAS mutant LKB1 null	Akt ↔ ERK ↔ AMPK ↔	Akt ↓ ERK ↑ AMPK ↔
H460	KRAS mutant LKB1 null PIK3CA mutant	Akt ↔ ERK ↔ AMPK ↔	Akt ↑ ERK ↑ AMPK ↔
H1299	KRAS mutant p53 null	Akt ↔ ERK ↔ AMPK ↔	Akt ↓ ERK ↑ AMPK ↑

4.4 Biological effects of CA and RA in combination in A549 NSCLC cells

The polyphenols CA and RA were found to have potent antiproliferative effects on their own in limited *in vitro* studies [113,144,147] and in the current study, so we wished to explore if the concentration of CA and RA that is present in our RE worked synergistically to induce the antiproliferative effects of RE. We used concentrations of CA and RA to match what is found in 5 (0.32µM CA; 1.86µM RA), 10 (0.64µM CA; 3.72µM RA) and 200 (12.75µM CA; 74.32µM RA) µg/mL of our RE. CA alone showed no inhibition at low concentrations (0.32µM:100.5±1.8%; 0.64µM: 97.3±2.0%), as seen previously (Fig 14A) and a slight inhibition at 12.75µM (93.7±1.0%). RA alone also showed no inhibition at low concentrations (1.86µM:98.4±2.4%; 3.72µM: 97.9±0.8%), as seen previously (Fig 19A) but an inhibition was seen at 74.32µM (72.2±3.2%). In combination, a minimal effect was seen at lower doses (0.32µM CA+1.86µM RA: 94.6±1.6%; 0.64µM CA+ 3.72µM RA: 96.0±0.5%) while a clear effect was seen at the higher concentration (12.75µM CA+ 74.32µM RA: 62.9±4.6%) resulting in a combination index (CI) of 0.43 indicating synergism.

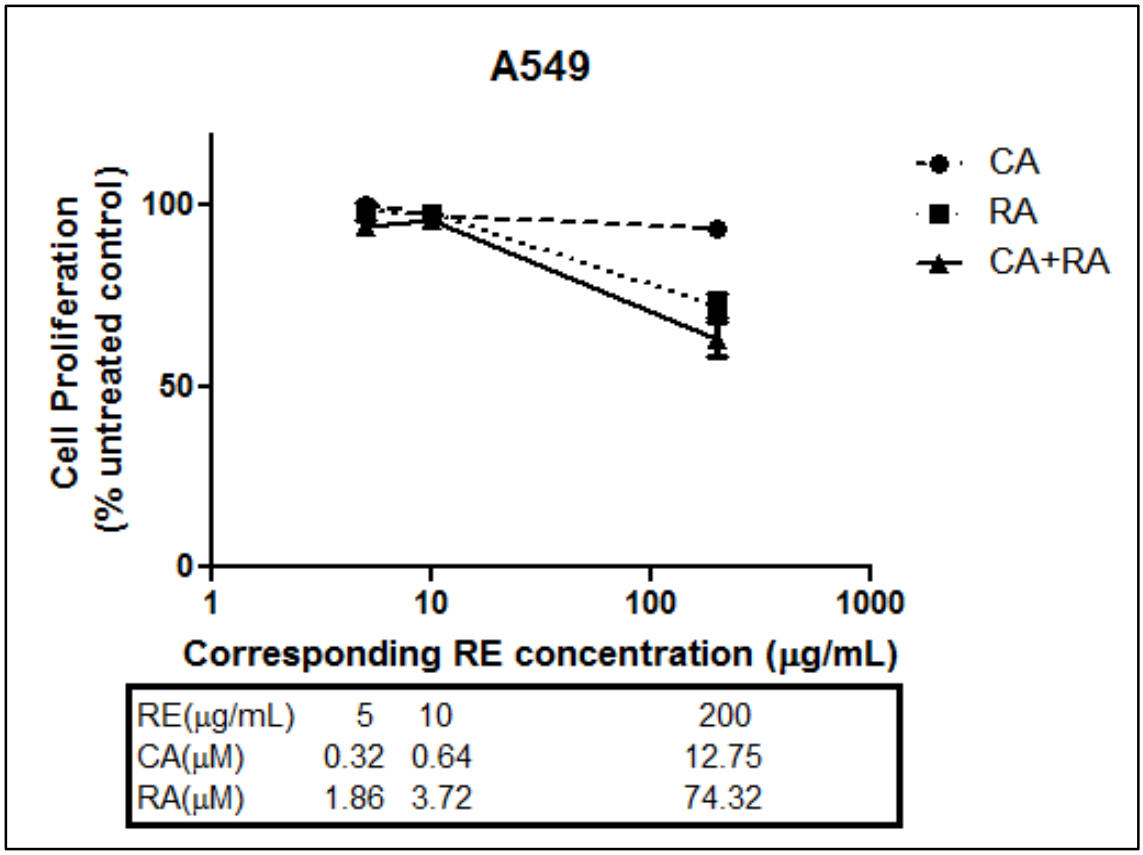

Figure 24: Effect of CA and RA in combination on proliferation in A549 NSCLC cells. Cells were seeded in triplicate and incubated for 72h with concentrations of CA and RA alone and in combination, to match what is found in our RE at 5, 10 and 200µg/mL. Cells were fixed and stained with 0.5% crystal violet. Stain was solubilized and absorbance was read at 570nm. Results are expressed as the mean % cell proliferation ± SEM compared to untreated control of 3-4 individual experiments. CI value=0.432 indicating a synergistic interaction.

Chapter 5: Discussion

Lung cancer is responsible for the most cancer related deaths [124] and importantly, NSCLC accounts for up to 80% of all lung cancer cases [127] and represents the most aggressive form of the disease. Unfortunately fewer than 15% of individuals with NSCLC reach a 5-year survival despite use of aggressive chemo- and radiation

therapy [126]. Patients with NSCLC often develop resistance to these cytotoxic therapies [37], highlighting the need for novel anticancer strategies.

Cancer cells are characterized by their ability to evade homeostasis and grow (proliferate) uncontrollably while avoiding programmed cell death (apoptosis) [1]. They often acquire these characteristics through mutations to key signaling molecules which regulate pathways involved in cell proliferation and survival. Alternatively, there are increased levels of growth factors produced which, through autocrine or paracrine mechanisms, bind to cell surface receptors and activate these cascades. Deregulation of these pathways results in the aggressive growth and potential spread (metastases) of cancer cells [2,3]. Thus, it is important to target these pathways in the development of new chemotherapeutics.

The PI3K-Akt pathway is commonly mutated in cancer leading to enhanced cell proliferation through downstream mTOR-p70S6K activation [41,42] and inhibition of apoptosis (through p53 inhibition) [71]. The presence of a mutated Akt pathway often leads to resistance to chemo- and radiation therapy [36]. Similarly, KRAS mutations are common in aggressive cancers and can lead to enhanced signaling through the Akt or ERK signaling cascades. ERK, is also largely implicated in cancer cells' enhanced proliferation and ability to evade apoptosis [148]. Therefore, targeting these molecules is of importance when considering the potential of new anticancer agents. Activity of the energy sensor AMPK is often decreased in cancer cells, due to loss of expression of upstream tumor suppressor LKB1 [149]. Enhancing AMPK activity in cancer cells leads to inhibition of mTOR-p70S6K and thus decreased cell growth, making this protein a novel target for anticancer therapies. Indeed activation of AMPK, using therapeutic drugs

such as metformin, has been shown to overcome drug resistance in breast cancer cells [65] and enhance radio-sensitivity of lung cancer cells [66].

Many pharmaceutical and chemotherapeutic agents have been discovered by screening natural products from plants. Plants rich in polyphenols have received much attention for their anticancer properties, among other health benefits. The herb rosemary has a high polyphenolic content and has recently emerged as having many health benefits, including potential as an anticancer agent [109,118]. Although there is evidence to support the potential of rosemary as an anticancer agent, limited data exists regarding the effects of rosemary extract in lung cancer and little is known about the signaling mechanisms of its antiproliferative and antiapoptotic properties.

In the present study we used 3 NSCLC cell lines to represent a variety of histologies and mutations which may be found clinically. A normal lung fibroblast cell line MRC5 was used for comparison. Our findings are the first to show the potent antiproliferative properties of RE and its main polyphenolic components CA and RA in NSCLC cells. Additionally, we show the effects of RE, CA and RA on Akt, ERK and AMPK signaling pathways, involved in cell proliferation and survival.

5.1 RE inhibits lung cancer cell proliferation and survival

This study is the first to show inhibition of cell proliferation in NSCLC cells by RE. The A549 and H1299 cells appeared to be more sensitive to RE treatment with IC50 values of 15.9 and 19µg/mL respectively, compared to H460 cells which had an IC50 of 57.2µg/mL. Differences in sensitivity of the cell lines to RE is likely a result of the different mutations present, to be discussed in section 5.2. These results are in agreement

with several previous studies which examined the antiproliferative effects of RE using similar doses in colon [107,108], pancreas [110], leukemia [115–117] and prostate [118] cancer cell lines. Interestingly, at its maximum inhibitory dose in each cell line RE's effects were comparable to that of 100μM resveratrol (Figure 9), an extensively studied polyphenol found to have significant anticancer effects [140,141]. These results demonstrate an antiproliferative effect of RE and support its use as an anticancer agent.

MRC5 lung fibroblasts were used to determine the effects of RE on a non-cancerous cell line. A dose-dependent inhibition of cell growth was seen in MRC5 cells but to a lesser extent (IC50 of 103.5μg/mL) than what was seen in the NSCLC cell lines (IC50 ranging from 15-57μg/mL). MRC5 is an embryonic fibroblast cell line [135] and embryonic cell lines have been reported to display properties similar to cancer cells [150]. MRC-5, like cancer cells, rely heavily on glycolysis and exhibit a rather high proliferation rate compared to mature non-cancer cell lines [150]. In a study examining inhibition of cell growth by AICAR, which acts to mimic a low intracellular energy state and inhibit proliferation of cancer cells, MRC5 cells showed a dose-dependent growth inhibition by AICAR comparable to that seen in the various cancer cell lines used. AICAR was also able to induce Akt phosphorylation and increase hypoxia inducible factor (Hif1α - controls cell response to hypoxia and cell survival) content in MRC5 cells which are characteristic factors of cancer cells [150] however, the same effect was not seen in a mature non-cancerous fibroblast-like lung cell line, HLF. Thus, MRC5 cells are not the best model of a non-cancerous cell line in the present study and a more mature lung cell line should be used in future studies to determine if RE has growth inhibitory or apoptotic effects on epithelial cells compared to their cancerous counterparts. The higher

IC50 seen in MRC 5 cells compared to cancer cells together with the fact that MRC5 are cells of embryonic origin suggests that RE would have negligible growth inhibitory effects on healthy epithelial cells which would support a role for RE to preferentially target cancer cells *in vivo* without affecting their surrounding normal cells. This suggestion is supported by one study showing RE enhanced levels of the endoplasmic stress related protein kinase RNA-like endoplasmic reticulum kinase (PERK) in prostate cancer cells however, RE decreased PERK levels in a normal prostate epithelium cell line suggesting RE selectively induces endoplasmic reticular stress in prostate cancer cells but spares normal prostate cells [118].

After confirming that RE had antiproliferative effects in NSCLC we wanted to explore further the anticancer properties of RE. Another characteristic of cancer cells is their augmented ability to survive and form tumors, even in the harsh environments generated by chemo- and radiation therapy. Our findings showed that in all 3 NSCLC cell lines, clonogenic cell survival was inhibited dose-dependently by RE (Figure 10). Significant inhibition of survival was seen at 2.5µg/mL in all cell lines, although again the H460 cell line appeared to be the least sensitive compared to A549 and H1299 cells. Similarly, RE was shown to inhibit colony formation of colon cancer cells [106] and inhibit viability, which is an indicator of survivability, in pancreas [109], breast [113,114], leukemia [113,116], prostate [113], liver [113] and small cell lung [113] cancer cells at doses comparable to those used here. Importantly, the study by Yesil-Celiktas, et al (2010) is the only other study to examine lung cancer however, they used a small cell lung cancer cell line and the only property they explored was cell viability

[113]. They found RE to have an IC50 of 24.08 µg/mL in this cell line, which is comparable to the results, using NSCLC cells, of the present study.

5.2 Exploring signaling molecules involved in RE's anticancer mechanisms

Given the ability of RE to inhibit NSCLC cell proliferation and survival in this study (Section 5.1) and the *in vivo* evidence supporting RE's ability to decrease tumor size and incidence in animal models of colon [109], prostate [118], leukemia [115] and skin [123] cancer, we next wanted to examine potential signaling cascades that were involved in RE's antiproliferative mechanism. Three different NSCLC cell lines were used to represent varying mutations and histologies of the disease and interestingly RE activated/inhibited the Akt, ERK and AMPK molecules differently in each cell line depending on their mutations (Table 11).

It was hypothesized that Akt phosphorylation would be down-regulated and AMPK phosphorylation would be enhanced by RE in NSCLC given their important role in cell survival however surprisingly, Akt phosphorylation was only down-regulated in A549 cells (Figure 11A) and AMPK phosphorylation was only enhanced in H1299 cells (Figure 13C). All cell lines harbor a mutation to the Ras GTPase protein (*KRAS* in A549 and H460 or *NRAS* in H1299) which leads to constitutive activation of Ras-PI3K-Akt signaling and thus, increased Akt phosphorylation. In addition to a *KRAS* mutation, H460 cells have a mutant *PIK3CA* gene which leads in increased PI3K-Akt signaling and thus, increased Akt phosphorylation. Of note, both A549 and H460 cells lack expression of the tumor suppressor LKB1 which is an upstream regulator of AMPK [151]. A significant increase in AMPK activation in H1299 (LKB1 expressing) cells was seen. The fact that

no effect on phosphorylation of AMPK was seen in LKB1 null cells suggests that RE may increase AMPK phosphorylation via LKB1. No previous reports have examined RE's effect on AMPK or associated proteins in this pathway in cancer and this represents a novel finding of this study. Interestingly, RE has been shown to enhance AMPK activity in L6 muscle cells resulting in increased glucose uptake, and the same study also showed RE to have no effect on Akt activation in these cells [139]. Akt has been shown to be a negative regulator of AMPK, leading to a decreased AMP/ATP ratio within the cell and thus inhibition of AMPK activation. Therefore, cells with enhanced Akt activity likely also show decreased AMPK activity [152]. This may explain why H460 cells, which show very high Akt phosphorylation after RE treatment, have decreased AMPK phosphorylation. Previous reports have found that in cancer cells, including NSCLC, treated with 2-deoxyglucose (2-DG), an inhibitor of glycolysis/glucose metabolism and potential chemotherapeutic which is known to lead to increased AMPK phosphorylation, increased apoptosis and inhibition of cell growth, Akt phosphorylation was in fact increased [153,154]. It was established that this mechanism was independent of LKB1/AMPK activation but was dependent on PI3K activity. Akt activation is often associated with cell survival and resistance to cancer therapy and in this context RE-induced Akt activation would have adverse effects in RE-based therapy. When Akt activity was inhibited, 2-DG-mediated growth inhibition was enhanced suggesting that 2-DG would work better as an anticancer agent in combination with PI3K/Akt inhibitory agents such as LY294002 [153]. The same may be true for RE, which holds potential as a natural anticancer compound which may be used in combination with chemotherapy, IR or other anticancer agents.

A recent study discovered that the increased Akt activation seen by 2-DG lead to, and was required for activation of sestrin-2, a member of a family of sestrin molecules which regulate oxidative and genotoxic stress [149]. Sestrin-2 is suggested to directly activate AMPK (forming a complex with Sestrin-2/AMPKα/TSC1/TSC2) leading to inhibition of mTOR and thus protein synthesis and cell growth ,in human embryonic kidney (HEK) and some cancer cells [156]. Sestrin 1 and 2 are target genes of p53 and are a critical link allowing p53 to lead to inhibition of mTOR and thus cell growth [156]. Increase of sestrin-2 is independent of p53 but requires the anti-apoptotic pathway, PI3K/Akt [155]. While sestrin-2 has been found to aid in inhibition of cancer cell growth, recently a cell protective role was discovered whereby it leads to enhanced activation of pro-survival Akt following 2-DG-induced oxidative and genotoxic stress [155]. The role of Sestrin-2 as a pro- or anti-apoptotic protein is still not fully understood however, strong evidence does exist showing sestrin-2 expression inhibits cell growth and proliferation in response to genotoxic stresses [156,157] and it is hypothesized that this signaling molecule may play a role in RE's anticancer mechanism. Unfortunately little has been done to examine any underlying mechanisms involved in RE's antiproliferative activity. In cancer-preventative models, RE has been shown to inhibit carcinogen-induced DNA strand breaks and oxidative stress *in vitro* [106,112,116] and *in vivo* [121–123] however, it is possible that it plays an opposing role as a chemotherapeutic (treatment option).Two studies supporting this hypothesis of RE-associated increases in oxidative stress showed that RE induced endoplasmic reticular stress leading to apoptosis in prostate cancer cells [118] and increased nitric oxide (NO) production leading to NO-induced apoptosis in pancreatic cancer cells [110].

Pro-apoptotic p53 plays a critical role in sensing oxidative and genotoxic stresses and has been shown to be activated in response to these events in cancer cells [158]. p53 can inhibit cell growth by activating AMPK and subsequently inhibiting mTOR [158] or by inhibiting Akt activity through several mechanisms, one of which is enhancing PTEN expression [159,160] (reviewed in [161,162]). This mechanism could explain why Akt activity is not enhanced only in A549 cells, which express wild type p53. While H460 cells also express normal levels of p53 (as opposed to H1299 which are p53 null) and it has been shown that p53 increases PTEN expression in this cell line, but not in H1299 cells [160], the mutation to the *PIK3CA* gene which controls PI3K, located upstream of PTEN, may override the increased PTEN and still be able to phosphorylate Akt. Alternatively, other signaling molecules downstream of PI3K and Akt, such as sestrin-2 and mTOR play a role in its activation [155]. Another possibility is that RE has a direct interaction with Akt and controls its phosphorylation. In support of this hypothesis, the glycolysis-inhibiting compound 2-DG was shown to directly activate Akt in NSCLC and other cancer cell lines [153] but was still capable of inhibiting cell growth and survival. Taken together, these results suggest the RE's principal mechanism is to induce oxidative and genotoxic stress in NSCLC cells leading to apoptosis and inhibition of proliferation however, the mechanism varies greatly between cell lines depending on mutation status.

Our hypothesis that ERK phosphorylation would be inhibited in NSCLC cells was also disproven and surprisingly findings showed a significant dose-dependent increase in ERK phosphorylation in all cell lines (Figure 12). Interestingly, another group has recently shown that RE induces apoptosis in colon cancer cells through a nuclear factor erythroid 2-related factor 2 (Nrf2)/sestrin-2 pathway which requires phosphorylation of

ERK. Of note, the cells (HCT116 and SW480) used in this study contained mutant *RAS* genes which are also found in all NSCLC cells used in the current study and can lead to enhanced Ras-MAPK signaling [143]. This study used comparable RE concentrations (20-60µg/mL) and time-course (24h) to our own. RE increased nuclear localization of Nrf2 (active form, compared to inactive cytoplasmic form) and activation of ERK, which is reportedly required for Nrf2 localization to occur [143]. Nrf2 is known to interact with the antioxidant response element (ARE) which regulates many proteins including sestrin-2, glutathione peroxidase (GPX1), glutathione S-transferase (GSTP1) and superoxide dismutase (SOD1). Interestingly, sestrin-2 was clearly upregulated in both cell lines but no significant effects were seen in any of the other ARE-regulated proteins. This evidence supports a role for sestrin-2 in RE-mediated apoptosis/inhibition of cell growth in NSCLC as mentioned earlier. Additionally, Nrf2 and sestrin-2 expression were both significantly enhanced *in vivo* by RE treatment (100mg/kg/day; 28days) using a mouse xenograft model [143]. Nrf2 has been shown to be a direct substrate of protein kinase RNA-like endoplasmic reticulum kinase (PERK) which is involved in the unfolded protein response, a cellular stress-response mechanism [163]. Increased expression of PERK and phosphorylated PERK was observed [143]. In support of this, members of the same group have previously shown RE induces endoplasmic reticular stress and enhances apoptosis in prostate cancer cells [118]. This study suggested that in addition to the Nrf2 pathway activating sestrin-2, another mechanism may be involved, because silencing of Nrf2 (using siRNA technique) did not inhibit RE-induced sestrin-2 expression. This supports a potential role for the p53/Akt/mTOR/sestrin-2 axis described earlier.

Expression of Akt, ERK and AMPK proteins was inhibited dose-dependently in all NSCLC cells (Figure 11-13). Inhibition of total protein levels are generally an indication that the gene is being transcriptionally regulated and mRNA levels are also inhibited however unfortunately PCR (to detect mRNA levels) was not performed in the current study. Inhibition of total Akt protein and mRNA levels by RE (50µg/mL; 48h and 24h respectively) has previously been reported in K562 leukemia cells [117] which supports that RE may also act transcriptionally in NSCLC to inhibit Akt mRNA expression (phosphorylated Akt levels not examined). Interestingly this study also examined total ERK protein expression but found no inhibition by RE. This may be associated with the different characteristics of each cell line. Given that ERK is involved in regulating differentiation and K562 cells are highly undifferentiated [164] RE may not target this pathway in this cell line. It has been suggested that inhibition of Akt protein expression may be part of a process whereby cells commit to apoptosis by removal of anti-apoptotic proteins [161] and this may hold true for ERK and AMPK expression in NSCLC.

5.3 CA and RA, polyphenols found in RE, inhibit lung cancer cell proliferation and survival

Rosemary extract consists of various polyphenols which have also been shown on their own to have anticancer properties. Present in the largest quantity are CA, RA and carnosol [105]. Since RE was shown to have significant anticancer effects, we wished to explore if these effects were attributable to an individual polyphenol. The quantity of CA and RA present in the extract used for this study were determined by HPLC analysis previously (Madina Naimi, MSc Thesis). Relative concentrations of CA and RA were

found to be 2.12 and 13.39% respectively. These concentrations are in the same range as levels of CA and RA which have been found by previous studies [108,109,143,147].

The present study showed significant inhibition of proliferation by CA at 25µM in H460, 50µM in H1299 NSCLC and 100µM in A549 (Figure 14). Significant inhibition by RA was seen at 25µM in H460 and at 100µM in A549 and H1299 (Figure 19). Interestingly the levels of inhibition were comparable between the two compounds in each cell line. Significant inhibition of clonogenic survival by CA (Figure 15) and RA (Figure 20) was also seen in each cell line. The survival assay (7 days) results showed that NSCLC cells are much more sensitive to CA than RA. This varies from the results of the proliferation assay (3 days) which showed similar sensitivity of the NSCLC to both CA and RA. These assays differ in length (7 days and 3 days) and thus, these results could suggest that CA inhibits cell growth more efficiently over a longer period of time compared to RA. Alternatively the difference could arise from clonogenic survival being a more sensitive assay for detecting cell growth/survival. A study by Yesil-Celiktas et al, 2010 found that while CA had significant inhibitory effects on cell viability at doses of 18-150µM, RA at similar concentrations had no effect and even caused a slight increase in cell viability [113]. A possible reason for the lack of inhibition seen by Yesil-Celiktas et al, 2010 is that the concentrations used in their study are lower than what has been used to show inhibition in the literature. Most studies see significant inhibition using 100-300µM RA [144,147]. Another possibility is the sensitivity of the cell lines used. The NSCLC cells used in the present study appear to be more sensitive, at least to RA compared to other cell lines seen in the literature. Both CA and RA have been shown to inhibit cell proliferation or enhance apoptosis at similar concentrations to those used in

this study in colon [109,165], pancreas [110], breast [113,166] and leukemia [113,145] cancer cells, among others. Importantly, both compounds have also shown anticancer effects *in vivo* by inhibiting tumor growth and formation of preneoplastic lesions, and increasing animal survival time [167–170].

It was hypothesized that CA and RA would work synergistically to induce a greater inhibition of cell growth and results showed this to be true in A549 NSCLC cells. Synergism has been shown previously between CA and RA as well as carnosol, another RE polyphenol, in colon and pancreatic cancer cell lines [109] and suggests that perhaps all of the components of RE, or at least the polyphenols, work synergistically to exert RE's potent anticancer effects. Our study presents strong supporting evidence of this by demonstrating that CA and RA alone, at the concentrations found in RE, show inhibition of cell proliferation and alteration of cell signaling pathways to a lesser extent than RE. Thus use of the complete extract may be more beneficial as a cancer preventive/treatment option rather than CA or RA alone.

Both RA and CA have shown reasonable bioavailability *in vivo* in animal models. The levels of RA in mouse plasma and intestinal tract were found to be 1.1μM and 38nmol/g respectively after chronic (8 week) ingestion of 360 mg/Kg/day RA (~9mg/mouse/day) showing that quantifiable levels of the parent compound are present after ingestion and may help slow colorectal adenoma development [171]. Surprisingly, another study reported an LD50 of 169.9mg/Kg/day and stated doses as low as 1-2mg/Kg/day significantly inhibited tumor growth in mice implanted with Lewis lung carcinoma cells [172]. In agreement with the latter study, two groups found RA capable of reducing DMH-induced DNA damage and aberrant crypt foci (preneoplastic lesions)

in rat models of colon carcinogenesis using low doses of 4-16 mg/Kg/day [169,173]. Although there are still discrepancies regarding effective doses of RA *in vivo,* the literature supports its potential as a chemotherapeutic and shows it can be found in quantifiable levels *in vivo*. A study *in vitro* in Caco-2 cells showed CA had a bioavailability of 19-34% [174]. Similarly, *in vivo* in rats oral administration of 65mg/Kg CA had a bioavailability of 40.1% and resulted in a plasma concentration of 105µM [175]. Thus, it seems reasonable that the concentrations of RE used in most *in vivo* studies (~100mg/Kg) which would be approximately 35mg/Kg of diterpenes would be readily absorbed and achieve concentrations consistent with those of CA and RA used in cell culture in the 10-100µM range [143].

5.4 Exploring signaling molecules involved in CA and RA's anticancer mechanisms

In section 5.2, we showed that RE at 25 and 50µg/mL affected the Akt, ERK and AMPK signaling molecules differently in each NSCLC cell line used depending on their mutation status. Low levels of CA and RA, equivalent to the concentrations found in 50µg/mL RE, caused no significant alteration to Akt, ERK or AMPK signaling. However, at 50µM both CA and RA altered these pathways in a similar manner to RE in each cell line, although to a lesser extent. In A549 cells 50µM CA and RA inhibited Akt activation (Figure 16A, 21A) and enhanced ERK activation (Figure 17A, 22A) however, neither polyphenol significantly affected AMPK activity (Figure 18A, 23A). In H460 cells 50µM CA and RA enhanced Akt (Figure 16B, 21B) and ERK (Figure 17B, 22B) activation as seen with RE but had no effect on AMPK (Figure 18B, 23B). Finally, in H1299 cells 50µM CA and RA inhibited Akt (Figure 16C, 21C) and enhanced ERK

(Figure 17C, 22C) and AMPK (Figure 18C, 23C) activation. As previously stated, the A549 and H460 cell lines lack LKB1 and this may explain the lack of activation of AMPK. CA and RA similar to RE may act via LKB1 to activate AMPK. Increased AMPK activation was seen in H1299 cells that are LKB1 positive. The inhibition of Akt activity in A549 and H1299 cells seen using CA and RA alone differs from what was found using RE (Figure 11-13). This suggests that while CA and RA's anticancer mechanism may involve inhibition of Akt activity at higher doses, the amounts found naturally in RE are not sufficient to induce inhibition of this pathway. This finding may support the use of standardized rosemary extracts which contain greater amounts of CA or RA and have been used previously [109,115,116,118] to show significant inhibition of cancer cell growth. Alternatively, other components present in RE are responsible for the enhanced Akt activity that was seen in H460 and H1299 cells.

CA and RA have been shown to inhibit cell proliferation and induce apoptosis by various mechanisms in different cancer cell lines. Inhibition of Akt phosphorylation by CA has been shown in leukemia [176], hepatoma [177], colon [142] and prostate [178] cancer cells and by RA in colon cancer cells [172], supporting the Akt inhibition seen in A549 and H1299 cells by both compounds. Unfortunately no studies have been performed examining the role of CA or RA on AMPK in any NSCLC or other cancer cell lines. However, a recent study claimed RA had an anti-Warburg effect and suppressed glucose uptake and lactate production and inhibited expression of Hif1α which affects the glycolytic pathway [144] *in vitro* in gastric cancer cells and *in vivo* in a mouse gastric carcinoma xenograft model. AMPK is largely involved in regulating energy levels within the cell, and suppression of glucose uptake would lead to an increased AMP:ATP ratio

thus activating AMPK (in cells expressing LKB1). Therefore this study supports the potential role of AMPK-activation seen in the present study by CA and RA in H1299 cells. Interestingly, CA and RA have been shown to inhibit ERK phosphorylation in colon cancer [142,165,172], leukemia [176] and neuroblastoma [179] cells however both compounds were found to induce activation of ERK in all NSCLC cell lines in our study. It was proposed that RE induces ERK activation through a Nrf2/ARE/Sestrin2 dependent pathway, and strong evidence also supports the role of Nrf2/ARE-regulated apoptosis by CA in rat liver clone 9 cells [180,181], neuroblastoma [179], glioblastoma [182] and leukemia [183] cells, albeit through slightly different mechanisms in each cell line. No studies have explored this mechanism using RA however, CA and RA showed similar effects on NSCLC cell signaling in this study and share similar chemical structures, thus their mechanisms are likely similar. Taken together the results suggest that CA and RA contribute to RE's anticancer effects but are not solely responsible and it is likely the interaction of multiple components within the extract that give RE its potent anticancer effects.

Total protein levels were not affected by CA or RA in any cell line and this is supported by previous reports that RE polyphenols do not alter protein expression [117,172,177,179]. Terpinolene, a non-polyphenolic component of RE, inhibited Akt expression in a leukemic cell line [117] and may contribute to the inhibition of total protein levels seen by RE in the current study. Thus, it is likely the presence of other, non-polyphenolic compounds in RE that lead to its inhibition of protein expression.

5.5 Limitations and future directions

One limitation to this study is that it was performed *in vitro* using a cell culture model and has limited applicability and generalizability physiologically, towards human models of cancer. However, cell culture is a well-established model for exploring the anticancer effects of novel compounds to determine their potential use *in vivo* and to establish toxic doses. The major advantage of using cell culture is the consistency and reproducibility of results and that the cellular environment can be easily manipulated to examine different conditions. Second, RE contains many different compounds besides CA and RA which were not examined in this study. It would be interesting to see if other polyphenols such as carnosol and rosmanol had the same effects in each cell line as CA and RA. As well, as suggested in the discussion components of RE besides the polyphenols may have an effect on protein expression levels in cancer cells and it would have been interesting to explore some of these compounds, such as terpinolene.

We chose to examine Akt, ERK and AMPK signaling molecules as a representation of how each pathway was affected in cancer however, because cancer cell signaling is so complex it would have been beneficial to look at more downstream signaling molecules such as mTOR, p70S6K, 4EBP1 or ACC. As well it would have benefited the strength of the study to include some measure of RE's effects on apoptosis of NSCLC cells.

Future studies should explore these downstream signaling molecules *in vitro* and examine apoptosis using cell cycle analysis or by Western blotting for apoptotic proteins such as p53, PARP or caspases. As well, the involvement of Nrf2 and sestrin2 in RE's

mechanisms in NSCLC should be explored to confirm the hypothesis discussed in section 5.2 (RE signaling mechanisms). The finding that RE inhibited protein expression of Akt, ERK and AMPK in all cell lines was proposed to be a result of RE altering mRNA expression and PCR analysis should be performed to explore this hypothesis. Since the effects of RE on the signaling molecules in NSCLC cells were complex and varied between each cell line it would be interested to use inhibitors or a silencing technique to knockdown expression of Akt, ERK and AMPK and examine how this altered the anticancer effects seen by RE. Combination therapies are useful in treating cancer because they can target different signaling pathways at once and future studies should investigate RE's effect in combination with current chemotherapeutic agents such as cisplatin, or other natural compounds that have shown anticancer activity such as resveratrol and metformin.

Once more is known about RE's anticancer mechanism *in vitro,* studies should be performed using *in vivo* xenograft lung cancer models in mice or rats. These studies could confirm findings from *in vitro* studies and further examine toxic doses and bioavailability of RE to support its use in human clinical trials.

5.6 Summary/Conclusions

Lung cancer is the leading cause of cancer related deaths and NSCLC accounts for over 80% of all lung cancer cases and represents the most aggressive form of the disease. Thus, various NSCLC cell lines were used in this study to represent different histologies and mutations that would be found in patients. Rosemary extract has a high polyphenolic content and is associated with the beneficial effects of a Mediterranean diet

thus, it has recently gained attention for its health effects, including anticancer activity. Two of RE's polyphenols CA and RA have also been shown to exhibit anticancer properties. This study aimed to examine the anticancer effects of RE in NSCLC cells and potential underlying signaling mechanisms. We also wished to determine if the polyphenols CA and RA exhibited anticancer effects in NSCLC cells and if they were responsible, at least in part, for RE's actions.

The present study found RE inhibited NSCLC cell proliferation and survival significantly at low concentrations (2.5-10µg/mL). The signaling proteins Akt, ERK and AMPK were found to be involved however, RE's growth inhibitory mechanisms varied by cell line. These findings supported a role for RE as a potential chemopreventive or chemotherapeutic agent, if used in combination with an Akt inhibitor. Findings also support a novel role of RE to activate ERK as an apoptotic mechanism, through an Nrf2/ARE/Sestrin2 pathway, at least in NSCLC cells. The polyphenols CA and RA did not have significant anticancer activity at low concentrations comparable to those found in effective RE doses however, at higher concentrations both compounds significantly inhibited cell survival and proliferation. Both CA and RA also significantly altered activity of Akt, ERK and AMPK signaling proteins at 50µM in NSCLC. Therefore, while CA and RA may not be responsible in full, they likely play a role in RE's anticancer activity. The current study supports the potential use of RE as an anticancer agent in NSCLC and provides novel findings on RE's growth inhibitory mechanisms which should be explored further using *in vivo* and tumor xenograft animal models.

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Appendix

Supplemental Figures

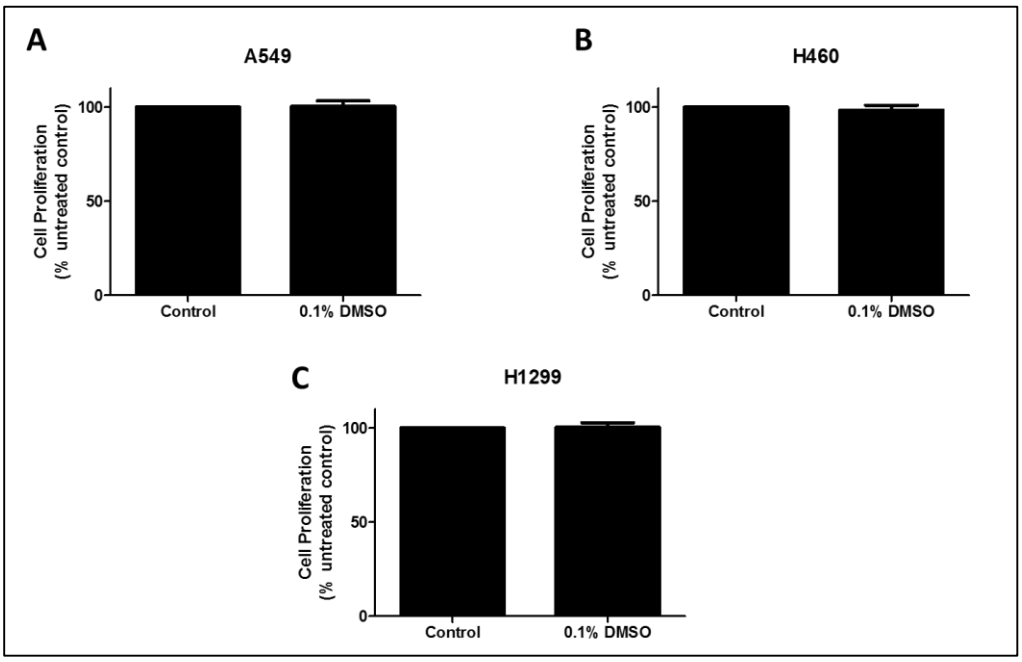

Figure s1: Treatment of NSCLC cells with 0.1% DMSO has no effect on cell proliferation. A) A549 B) H460 and C) H1299 cells were seeded in triplicate and incubated with 0.1% DMSO for 72h followed by fixing and staining with 0.5% crystal violet. Stain was solubilized and absorbance was read at 570nm. Results are expressed as the mean ± SEM compared to untreated control of 3 individual experiments.