The effects of Akt1/Akt2 selective inhibition on tumorigenic properties of NSCLC cells

by

Ritesh Briah

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Akt is a key signaling kinase that is hyper-activated in many non-small cell lung cancer (NSCLC) cases and is involved in cell survival, proliferation, migration and metabolism. Recent research has shown that the three isoforms of Akt have non-redundant roles in cell signaling and thus this study aims to compare the effects of Akt1 (A-674563), Akt2 (CCT128930), and pan-Akt (MK-2206) inhibition on tumorigenic properties of A549 and NCI-H358 cells. Cell survival curves demonstrated that all inhibitors effectively reduce cell viability, however Akt1 inhibition was the most effective. Additionally, Akt1 inhibition was found to be more effective in reducing cell migration and inducing apoptosis. Interestingly, cell cycle analysis of Akt1 inhibition also indicated a possible G2/M phase block. Taken together, these results suggest that Akt1 inhibition is a more effective therapeutic strategy for human NSCLC cells than either Akt2 inhibition or inhibition of all 3 Akt isoforms.
DEDICATION

I would like to dedicate this thesis to my grandpa, Mr. Harichand Briah, who passed away four and a half months ago after a long fought battle with colon cancer. He was a great inspiration to everyone who had the privilege of meeting him, and continues to motivate me every day. Thank you Papa-ji for all you have taught me.
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DECLARATION OF WORK PERFORMED

I declare that all work reported in this thesis was performed by me.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>7AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>A</td>
<td>Akt 1 inhibitor (A-674563)</td>
</tr>
<tr>
<td>ABAM</td>
<td>Antibiotic-antimycotic</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride</td>
</tr>
<tr>
<td>AGC</td>
<td>cAMP dependent protein kinase family</td>
</tr>
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<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>bFGFR</td>
<td>basic fibroblast growth factor receptor</td>
</tr>
<tr>
<td>BPE</td>
<td>Bovine Pituitary Extract</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Carboplatin</td>
<td><em>cis</em>-Diammine (1, 1-cyclobutanedicarboxylato) platinum</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalytic domain</td>
</tr>
<tr>
<td>CCT</td>
<td>Akt 2 inhibitor (CCT-128930)</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>Cisplatin, cis</td>
<td><em>cis</em>-Diamineplatinum (II) dichloride</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E-64</td>
<td>Protease Inhibitor E-64</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ETOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>EXT</td>
<td>C-terminal extension</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSK3 β</td>
<td>Glycogen Synthase Kinase 3 β</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HM</td>
<td>Hydrophobic motif</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>Insulin-like growth factor binding protein 1</td>
</tr>
<tr>
<td>IGFR1</td>
<td>Insulin-like growth factor receptor 1</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo daltons</td>
</tr>
<tr>
<td>MK</td>
<td>pan Akt inhibitor (MK-2206)</td>
</tr>
<tr>
<td>mTORC 1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NaM</td>
<td>Sodium Molybdate</td>
</tr>
<tr>
<td>NaT</td>
<td>Sodium Tartrate</td>
</tr>
<tr>
<td>NaV</td>
<td>Sodium Orthovanadate</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>NLST</td>
<td>National Lung Screening Trial</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small Cell Lung Cancer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide dependant kinase-1</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PH</td>
<td>Plekstrin Homology domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PHH3</td>
<td>phospho-histone H3</td>
</tr>
<tr>
<td>PHLPP1/2</td>
<td>PH-domain leucine rich repeat containing protein phosphatase 1/2</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline Rich Akt Substrate of 40 kDa</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse Osmosis</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Roswell Park Memorial Institute 1640 Media</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>S473</td>
<td>Serine 473</td>
</tr>
<tr>
<td>S6RP</td>
<td>S6 Ribosomal Protein</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small Cell Lung Cancer</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>T308</td>
<td>Threonine 308</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
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INTRODUCTION AND RATIONALE

Lung cancer is the leading cause of cancer-related mortalities worldwide and is the second most commonly diagnosed cancer (Stewart and Wild, 2014). In 2012, there were approximately 1.8 million new cases worldwide, with an estimated 1.6 million deaths (Torre et al., 2015; Ferlay et al., 2014). Unfortunately current treatment options are not decreasing mortality rates, and thus research today is focused towards identifying molecular targets that have therapeutic potential. One such molecule is Akt, which is hyper-activated in more than 70% of non-small cell lung cancers and is associated with a poor survival outcome (Brognard et al., 2001; Tang et al., 2006). It is involved in regulating cell growth, metabolism, proliferation, survival, and migration (Brazil et al., 2004). Although it was previously believed that all three isoforms of Akt play similar roles within the cell, recent research has shown that these isoforms have non-redundant functions as well. Currently, a pan-Akt inhibitor is undergoing clinical trials to evaluate its effectiveness in treating NSCLC (Yap et al., 2011).

Previously, our lab has generated doxycycline inducible SPC-IGFIR transgenic mice. These mice overexpress the IGFIR in the lungs, which subsequently leads to tumor formation (Linnerth et al., 2009). SPC-IGFIR mice were crossed with Akt1/-/- or Akt2/-/- mice, providing a tool to study the isoform specific effects on tumorigenesis. Compared to SPC-IGFIR wild-type mice, SPC-IGFIR-Akt1/-/- mice developed fewer tumors that were nodular in appearance, while SPC-IGFIR-Akt2/-/- mice developed more invasive tumors. These findings demonstrate the opposing roles of Akt1 and Akt2 in lung tumorigenesis. Due to this reason it may be more beneficial to therapeutically target only Akt1 in treating NSCLC, and thus this study focused on
comparing the effects of Akt1 and Akt2 selective inhibition on tumor suppression properties of NSCLC.
Lung Cancer

Lung cancer is a devastating disease that affects millions of people across the globe. Many treatment options have been explored, yet they all fail to reduce the mortality rate observed with disease progression. This literature review will discuss background information regarding lung cancer, as well as a novel targeted therapy that has emerged within the past decade.

Statistics

Cancer is one of the leading causes of death worldwide, with an estimated 14 million new cases yearly (WHO. Int., 2015). Surprisingly, this number is expected to increase by about 70% within the next few decades (Stewart and Wild, 2014). Among all cancer types, lung cancer is the most commonly diagnosed worldwide with approximately 1.8 million new cases occurring in 2012, and accounting for 13% of total cancer diagnosis (Torre et al., 2015). It has the highest incidence rates among men, and the third highest among women, with 16.7% and 8.8% of all cancer cases respectively (Ferlay et al., 2014). In addition, lung cancer is the leading cause of cancer related deaths worldwide, with an estimated 1.6 million deaths in 2012, greater than the next two leading cancer types combined (Ferlay et al., 2014). It has the highest mortality rates among men with 23.6% of all cancer related deaths, and second highest among women with 13.8% (Torre et al., 2015). Interestingly, lung cancer has a higher incidence rate among men in
less developed countries, however in women the rate is higher in more developed countries (Torre et al., 2015).

Although lung cancer is considered one of the most devastating diseases, the incidence and mortality rates continue to drop yearly. From 2006 to 2010, lung cancer incidence rates in men and women decreased by 1.9% and 1.2% per year respectively. During the same time period death rates also decreased by 2.9% and 1.4% per year in men and women respectively (cancer.org, 2014). This trend is likely due to our understanding of risk factors and improvements in diagnostic technology, allowing us to make healthier lifestyle choices and detect the disease at an earlier stage.

**Risk Factors**

**Smoking**

Cigarette smoking is the number one risk factor for developing lung cancer and is considered to be an independent variable. About 70% of lung cancers worldwide are attributable to smoking, and surprisingly this number is up to 90% in the United States (WHO. Int., 2015). Smokers are 20 times more likely to develop lung cancer than people who have never smoked (Detterbeck et al., 2013). Furthermore, research has shown that the risk increases with quantity and duration of smoking (Cdc.gov, 2014). Tobacco smoke consists of approximately 7000 toxic chemicals, many of which are considered to be carcinogenic. Thus the trends of lung cancer occurrence closely resemble smoking patterns, but rates of occurrence lag smoking patterns by about 20 years (Devesa et al., 1983, Alberg et al., 2007). For example, historical trends indicate that smoking prevalence peaked about twenty years earlier for men than for women. Similarly,
lung cancer rates in men have been steadily decreasing since 1980, while these rates only began decreasing in women in the early 2000s (Torre et al., 2015). Furthermore, lung cancer rates continue to increase in countries where the tobacco epidemic peaked later, such as Hungary and Spain (Torre et al., 2014). Interestingly, smoking cessation has been found to be associated with a decrease in lung cancer mortality rates (Zhou et al., 2006). Thus many researchers claim that lung cancer is one of the most preventable cancers, and emphasize the importance of tobacco control programs to prevent the initiation of smoking or increasing smoking cessation among current smokers. Interestingly, the risks associated with cigar or pipe smoking remain substantial but significantly less than the risks observed for cigarette smoking. This is likely because of the differences in smoking frequency and depth of inhalation (Bethesda, 1998 and Boffetta et al., 1999).

Second-hand Smoking

Inhalation of smoke and toxins excreted when someone else is smoking is referred to as second hand smoking. There is a strong positive correlation between the amount of second hand smoke exposure and developing lung cancer. This association is most evident in non-smokers who reside with regular smokers, with a 20-30% increase is risk of developing lung cancer (Taylor et al., 2007). Second hand smoking has been estimated to cause 21,400 deaths annually from lung cancer (Oberg et al., 2011). Interestingly, the IARC have classified second hand tobacco smoke exposure as a known class A human carcinogen, which means that adequate human data exists and indicates that this substance causes cancer (IARC, 2004).
Air Pollution

Outdoor air contains a number of carcinogens including polycyclic aromatic hydrocarbons and metals such as arsenic, nickel, and chromium (Alberg et al., 2002). The American Cancer Society conducted a study that found that each 10 g/m$^3$ increase in concentration of fine particles carried an increased lung cancer risk of 14% (Pope et al., 2002). Furthermore, compounds such as sulfur dioxide and nitrogen dioxide have been associated with an increased risk as well (Raaschou-Nielsen et al., 2010).

Occupational Exposures

Asbestos is a group of naturally occurring fibrous silicate minerals, and is a well established carcinogen (HEI, 1991). The association of occupational asbestos exposure and lung cancer is strong, with a fivefold increase in risk of developing lung cancer (Doll, 1955). Whether the mechanism is direct or indirect remains to be unknown (Nelson and Kelsey, 2002). Asbestos exposure is considered to be the only independent cause of lung cancer other than smoking. Together, smoking and asbestos exposure work synergistically to elevate the risk.

Occupational exposures to a number of metals, including arsenic, chromium, and nickel, are also causes of lung cancer (Straif et al., 2009). Furthermore, this risk is elevated when the route of exposure is ingestion rather than inhalation. For example, high concentrations of arsenic in drinking water have been associated with increased risk of developing lung cancer (Celik et al., 2009).
Radiation

X-rays, γ-rays, neutrons and radon gas are among some of the types of radiation that increase the risk of developing lung cancer (Detterbeck et al. 2013). Radon, a decay product of uranium, is of particular concern because it is ubiquitously present in homes and buildings in the form of rocks, soil and dirt. Furthermore, it cannot be seen, tasted, or smelled. It poses an even larger threat for individuals who have worked in the mining industry (Lubin et al., 1995). Radon exposure also works synergistically with smoking to greatly elevate the risk of lung cancer. The Environmental Protection Agency estimates that 15,000 to 20,000 lung cancer deaths per year in the U.S. are caused by Radon (UEPA, 1992). Certain steps can be taken to decrease radon exposure, such as sealing and ventilating basements and monitoring radon concentrations in homes.

X-ray radiation, which is substantially less radiation exposure than radon gas, is involved in the screening process, and is useful in detecting lung cancer at earlier stages. However, many studies have shown that x-rays are linked to an increase risk of developing cancer (Berrington and Darby, 2004).

Other Diseases

Certain types of lung disease have been associated with an increased risk of lung cancer. Chronic obstructive pulmonary disease (COPD) is a progressive disease that results in poor airflow into and out of the lungs. Studies have shown that the presence of COPD as well as impaired lung ventilation are positively associated with the occurrence of lung cancer (Tockman, 1994). For example, after controlling for confounding risk factors, one study showed that the risk increase with bronchitis, emphysema, or COPD could be as high 1.9-2.5 fold (Koshiol et al.,
2009). Even asthma could render a person more susceptible to developing lung cancer, specifically adenocarcinoma (Fan et al., 2011).

Another disease that has been extensively researched as a risk factor for lung cancer is the human immunodeficiency virus (HIV). Although many studies fail to account for smoking as a confounding variable, studies that have controlled for smoking suggest that HIV infection increases lung cancer risk by a factor of at least 2.5-fold (Shiels et al., 2009).

**Lifestyle**

Lifestyle factors other than cigarette smoking, such as diet and exercise, have been extensively investigated for a potential role in influencing lung cancer risk (Detterbeck et al. 2013). Fruits, vegetables, and specific antioxidant nutrients present in them appear to be inversely associated with lung cancer risk (WCRF, 2007), although the association is weaker in vegetables than it is in fruits. However, cruciferous vegetable intake, such as broccoli and cauliflower, has been shown to reduce the risk of lung cancer, even when controlling for variables such as smoking (Lam et al., 2010). This evidence has spiked interest in isothiocyanates as potential chemo-preventative agents based on their function to up regulate detoxification enzymes such as glutathione S-transferase (Spitz et al., 2000).

Similarly, moderate to high levels of physical activity has been associated with a 13-30% reduction in lung cancer risk (Tardon et al., 2005). However, this mechanism remains unclear. Many of these studies do not adequately control for smoking as a confounding variable, without which a causal relationship cannot be established (Detterbeck et al., 2013). Therefore it is widely accepted that diet and exercise play minor roles in the prevention of lung cancer.
Types of Lung Cancer

Primary lung cancer can be classified as one of two major types; small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC). About 10-15% of all lung cancer cases are considered to be SCLC, based on the size of the cancer cells seen under a microscope (cancer.org, 2015). These cells also possess characteristics that differ from NSCLC such as poorly defined cell borders, absence of nuclei, and finely granular nuclear chromatin (Travis et al., 2004). More than 90% of SCLC patients are elderly or heavy smokers, which supports the notion that SCLC is usually caused by chronic smoking (Devesa et al., 2005). It is therefore rare to find a patient with SCLC who has not been exposed to constant tobacco smoking (Antony et al., 2010). Due to this reason, the incidence of SCLC has recently begun to decrease in developed countries, reflecting population smoking patterns, however is expected to increase in countries where smoking prevalence remains high (Meerbeeck et al., 2011).

NSCLC accounts for more than 85% of all lung cancer cases (Ettinger et al., 2012). It can be further characterized as adenocarcinoma, squamous cell carcinoma, or large cell carcinoma depending on the histological appearance. These subtypes differ in cellular characteristics, but are grouped together due to similar prognosis and treatment approach. Squamous cell carcinoma originates in the cells lining the bronchus of the lungs, and is usually associated with heavy smoking. Large cell carcinoma, the least common of NSCLCs, can appear in any part of the lung and tends to become more invasive than the others. Adenocarcinomas originate from the peripheral airways and are the most common type of lung cancer as well as the most frequently occurring cancer type in nonsmokers (Ettinger et al., 2012)
Diagnosis and Staging

The goal of screening is to detect the disease at an early stage when treatment is most effective. Unfortunately, lung cancer patients are typically asymptomatic during early stages of the disease, and thus the majority of cases are diagnosed at later stages (Mulshine, 2003). This is also partially attributable to symptoms that are common to other diseases such as cough, chest pain or dyspnea (Ettinger et al., 2012). Most patients tend to seek help when presented with symptoms of late stage lung cancer; weight loss, bone pain, headaches, anemia, and paraneoplastic syndromes (Spiro et al., 2007).

A preliminary diagnosis is usually established using a combination of symptoms, signs, and laboratory tests, and is aided by imaging (Silvestri et al., 2007). However, due to the high rate of false positives, imaging is only recommended for high risk individuals (Crosswell et al., 2010). Previously, before the advent of Computed Tomography (CT) scans, X-rays were widely used to diagnose the presence of tumours. While a standard chest X-ray produces a single image of the whole chest, adapted versions of the CT scan, such as low dose helical CT, use multiple X-rays to obtain image stacks of the entire chest, preventing the overlapping of anatomical structures (De Wever et al., 2007). The National Lung Screening Trial (NLST) compared the mortality rates of high risk individuals screened annually with either low dose helical CT or standard chest X-ray. They concluded that low dose helical CT was more accurate at diagnosing early stage lung cancer and that it was associated with a 15-20% reduction in lung cancer mortality rates among high risk individuals (Aberle et al., 2011). Thus low dose helical CT is currently accepted as the gold standard for lung cancer screening.

Positron Emission Tomography is another imaging tool that usually involves monitoring of radiolabelled molecules such as glucose. Since PET scans identify tumour physiology (such as
blood supply and intake) as opposed to anatomy, they are considered to be more accurate at staging NSCLC than CT scans (Pieteran et al., 2000). However, current screening protocols suggest the use of low dose helical CT to assess initial suspicions of lung cancer and incorporate the combined use of PET/CT to evaluate newly emerging or recurrent tumours during follow-up (Ettinger et al., 2012).

Once lung cancer is detected, pathological evaluations are performed to classify the histological type, the extent of invasion, and whether the lung cancer was primary or spread from another organ. These evaluations usually include a combination of bronchial brushings, bronchial washings, fine-needle aspiration biopsy, core needle biopsy, endobronchial biopsy, transbronchial biopsy, and possibly mediastinoscopy (Ettinger et al., 2012). Additionally, phlegm may be analyzed through sputum cytology (Palmisano et al., 2000). Once cell samples are collected immune-histochemical and molecular analysis are performed to detect gene mutations or biomarker status, which can play an important role in determining treatment options.

Staging of lung cancer follows the tumour-node-metastasis (TNM) system (Rami-Porta et al., 2009). The T descriptor defines the extent of the primary tumor, the N descriptor defines the involvement of regional lymph nodes, and the M descriptor defines the extent of spread to distant sites. These factors are then compiled and classified further into a stage (I-IV). Stage I lung cancers are mostly confined to the surrounding tissue with no lymph node involvement. Stage II cancers have increased local tumour invasion including lymph nodes. Stage III consists of extensive lymph node and neighboring tissue invasion, and at Stage IV the cancer has spread to other distant organs (Rusch et al., 2007). Interestingly, recent revisions in lung cancer staging have adopted sub-staging to further specify the cancer and its severity (Detterbeck et al., 2009).
Management & Survival

Treatment options for lung cancer largely depend on the type of cancer. SCLC is considered to be more invasive (unresectable) and is found to respond better to chemotherapeutics (Travis et al., 2004). Surgical resection may be an option for NSCLC patients if the disease is detected at an early enough stage. Unfortunately, the majority of cases are diagnosed at late stages when the cancer has already spread to distant sites. In this case patients usually undergo systemic treatment with a combination of chemotherapeutic drugs, novel targeted therapy and radiation (Socinski et al., 2007). Combination of two or more chemotherapy drugs have shown to increase short term survival in lung cancer patients (Ohe et al., 2007 and Fossela et al., 2003). Some of these combinations include carboplatin/paclitaxel, cisplatin/paclitaxel, gemcitabine/cisplatin, cisplatin/pemetrexed, and docetaxel/cisplatin (Ettinger et al., 2012). The FDA has also approved the use of select molecular inhibitors for treatment of lung cancer. Currently, Gefitinib, an inhibitor of the Epidermal Growth Factor receptor (EGFR), is recommended for patients with EGFR mutations, while Bevacizumab, which blocks the vascular endothelial growth factor (VEGF) is recommended for those who are negative for the EGFR mutation (Giaccone et al., 2005 and Sandler et al., 2006). These inhibitors are usually combined with chemotherapeutics to increase survival. For example, Bevacizumab is usually combined with paclitaxel or carboplatin (Sandler et al., 2006). The molecular kinase Akt is another potential target that is currently being investigated in clinical trials for lung cancer treatment (see section on MK-2206 below).

Despite the wide range of treatments available for lung cancer, the survival rates remain low. The overall 5-year survival for lung cancer is approximately 17%, and that rate drops dramatically to 4.2% for Stage IV patients (Seer.cancer.gov, 2015). From this, it is evident that
methods for better treatment options are urgently needed. Since molecular target inhibitors can provide patient specific therapy, they may become a more popular treatment option in the future.

Akt Structure & Pathway of Activation

Akt, also known as Protein Kinase B (PKB), is a serine/threonine kinase that is part of the cAMP dependant protein kinase A/G/C (AGC) superfamily (Yang et al., 2002). It plays a pivotal role in regulating cellular functions such as growth, metabolism, apoptosis and migration (Brazil et al., 2004). Under normal physiological conditions Akt activity is tightly controlled, however, altered capabilities of Akt are usually associated with human diseases such as cancer (Nicholson and Anderson, 2002). In lung cancer, Akt is found to be hyper activated in many NSCLC cases, and is associated with a poor prognosis (Tang et al., 2006). Akt activity is regulated in multiple ways through its multiple regulatory phosphorylation sites, as well as dependency on many upstream proteins. Akt is a promiscuous kinase which is known to act on hundreds of downstream targets (Manning and Cantley, 2007). Due to its regulatory potential, Akt has become an interesting therapeutic target for cancer research.

General Structure

Akt/PKB was originally identified by three independent groups based on its homology to PKA, PKC, and the cellular homologue to the viral oncogene v-Akt (Song et al., 2005). It is a 60
kDa protein that consists of three major domains that are relatively well conserved in the AGC kinase family. These domains include an N-terminal pleckstrin homology (PH) domain, a central kinase catalytic (CAT) domain and a C-terminal extension (EXT) containing a regulatory hydrophobic motif (HM) (Figure 1) (Hanada et al., 2004). There is also a linker region (LINK) that connects the PH domain to the central CAT domain. This LINK region does not share significant homology with the other members of the AGC family, however, the PH region of Akt displays approximately 30% homology to PH domains of other proteins in the family (Kumar and Madison, 2005). Surprisingly, the CAT domain is closely related to the other members of the AGC family, and the first 30-40 amino acid residues in EXT are homologues in the AKT, S6, SGK, PKA and c-GMP kinase families (Kumar and Madison, 2005). This is the F-X-X-F/Y-S/T-Y/F HM (where X is any amino acid) that is characteristic of the AGC family (Peterson and Schreiber, 1999).

Among the 480 or so amino acid residues of Akt, there are two major residues that are essential in activating the molecule. These sites include T308 in the T-loop of the CAT domain and S473 in the HM of the EXT domain. It is thought that phosphorylation of T308 increases Akt activity 100 fold, and subsequent phosphorylation of S473 increases its function 10 fold more (Alessi et al., 1996). Interestingly, phosphorylation of S473 alone has little effect on Akt/PKB activity (Alessi et al., 1996).
Akt Isoforms

In humans, three Akt/PKB genes have been identified, termed PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3, located on chromosomes 14q32, 19q13, and 1q44, respectively (Song et al., 2005). Among the Akt isoforms, the PH, CAT, and EXT domains are approximately 80%, 90%, and 70% conserved respectively (Kumar and Madison, 2005). Each Akt isoform requires phosphorylation of a threonine and serine amino acid residue, however these amino acids differ slightly in position. These amino acids are T308 and S473 in AKT1, T309 and S474 in AKT2, and T305 and S472 in AKT3. The tissue distribution of Akt isoforms was previously evaluated using quantitative RT-PCR (Yang et al., 2003). Akt1 and Akt2 were ubiquitously expressed in all mouse tissues whereas Akt3 was only highly expressed in the brain and testes. Thus structural studies aimed towards identifying conformational changes during Akt activation have focused on Akt1 and Akt2 (Auguin et al., 2004, Milburn et al., 2003). Interestingly, recent studies examining the subcellular localization of Akt isoforms in human cells propose that Akt1 is
localized in the cytoplasm, while Akt2 and Akt3 are found near the mitochondrial and nuclear membranes, respectively (Santi and Lee, 2010).

Pathway of Activation

The pathway of Akt activation has been extensively researched (Song et al., 2005) (Figure 2). Akt activation is initiated by the stimulation of a cell surface receptor tyrosine kinase (RTK), such as platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), basic fibroblast growth factor receptor (bFGFR) and insulin-like growth factor receptor 1 (IGFR1) (Hanada et al., 2004). This initiates assembly of the phosphatidylinositol 3-kinase (PI3K) complex. PI3K in turn, phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which can then be recognized by the PH domain of Akt. It is speculated that the formation of PIP3 ligand allows for the recruitment of Akt to the membrane (Andjelkovic et al., 1997). PIP3 levels are regulated by the tumour suppressor molecule phosphatase and tensin homologue (PTEN), which removes a phosphate on the 3-OH position on PIP3 converting it back to PIP2 (Simpson and Parsons, 2001). The interaction of PIP3 and the PH domain of Akt results in a conformational change that shifts the PH domain, exposing the T308 residue, and allowing for subsequent phosphorylation by phosphoinositide dependant kinase-1 (PDK1). The PH domain of Akt is suspected to mask the Thr308 residue as studies with PH deleted mutant Akt molecules showed constitutive phosphorylation by PDK1 (Biondi et al., 2000). Phosphorylation of Thr308 in the activation loop partially activates Akt, while subsequent phosphorylation of S473 in the hydrophobic motif is required for full activation. The mammalian target of rapamycin complex 2 (mTORC2), which consists of mTOR, Rictor, Sin1, and mLST8 subunits, is widely considered to phosphorylate
S473 (Xie et al., 2011). However, recent studies have provided accumulating evidence for the existence of an mTOR independent kinase involved in Akt S473 phosphorylation (Zhang et al., 2010, Risson et al., 2009). Furthermore, some studies are looking at the importance of tyrosine phosphorylation on Akt activation (Conus et al., 2002). Tyr474 is widely conserved in the AGC kinase family, and phosphorylation of this residue on Akt might also contribute to full activation. Once fully activated, Akt is able to phosphorylate hundreds of downstream targets that carry on the signal to elicit its effects (Manning and Cantley, 2007). The Akt signal is stopped when protein phosphatase 2 (PP2A) dephosphorylates Akt at the T308 residue, and PH-domain leucine rich repeat containing protein phosphatases 1 and 2 (PHLPP1/2) dephosphorylates the S473 residue (Brognard et al., 2007).

Figure 2: Pathway of activation of the Akt molecule, outlining some of the effects of downstream cascades. Adapted from Garcia-Echeverria and Sellers, 2008.
Role of Akt in Tumorigenesis

Akt regulation of cellular processes such as growth, proliferation, apoptosis, metabolism, migration, and angiogenesis is normally tightly regulated within the cell. However, as mentioned above, Akt is usually found to be hyper-activated in many NSCLC cases (Tang et al., 2006). This results in abnormally high Akt activity, and aids cancer cells to become tumorigenic.

Growth

Akt plays a crucial role in increasing cell mass through the mTOR complex 1 (mTORC1) (Carnero, 2010). mTORC1 is a critical regulator of transcription, ribosome biogenesis and translation initiation, and is evolutionarily conserved in many species (Wullschleger et al., 2006). It is composed of several subunits including mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (MLST8) and the recently identified proline rich Akt substrate (PRAS40) and DEP domain containing mTOR interacting protein (DEPTOR). The mechanism by which Akt regulates mTORC1 involves many molecules. First Akt phosphorylates the tuberous sclerosis protein heterodimer (TSC1/TSC2), and inactivates its GTPase activating protein (GAP) ability. It does so by promoting dissociation of the TSC1/TSC2 complex (Duran et al., 2012). This complex is then unable to convert the active Rheb-GTP complex to the inactive Rheb-GDP complex (Li et al., 2004). The Rheb-GTP complex then binds to the kinase domain of mTOR directly and activates mTORC1 (Long et al., 2005). It is also suggested that Akt regulates mTORC1 activity through regulation of cellular ATP levels (Hahn Windgassen et al., 2005).
Recently, PRAS 40 was identified as a novel downstream target of Akt (Wang et al., 2012). PRAS40 inhibits the activity of mTORC1 by preventing its binding to ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1). Phosphorylation of T246 on PRAS40 by Akt facilitates subsequent phosphorylation of S183 by mTORC1, which dissociates PRAS40 from mTOR and relieves the inhibitory constraint on mTORC1 (Nascimento et al., 2010). This allows mTORC1 to activate its downstream targets and carry on the signal. One important downstream target is the ribosomal protein S6 (S6RP), which is a component of the 40s ribosomal subunit. mTORC1 phosphorylates T389 on S6K1 which then phosphorylates and activates S6RP, resulting in increased formation of ribosomal complexes as well as translation of mRNA (Wullschleger et al., 2006).

The mTORC2 complex also plays a role in regulating cell growth through organization of the actin cytoskeleton (Jacinto et al., 2004). However, the mechanism is not yet fully understood in mammals, but is thought to involve the small GTPases Rho and Rac as well as PKCα (Sarbassov et al., 2004). Interestingly, as noted earlier, mTORC2 is also a positive modulator of Akt activity as it is able to phosphorylate S473 on Akt. Activated mTOR, the major component of both mTORC1 and mTORC2, stimulates protein and lipid biosynthesis, and cell cycle growth in response to sufficient nutrient and energy conditions, however it is often considered to be constitutively activated in tumorigenesis (Guertin et al., 2007).

**Proliferation**

Akt can also stimulate cell proliferation through target molecules that block cell cycle progression. It is known to directly phosphorylate and inactivate the cyclin-dependent kinase
(CDK) inhibitor p21-Cip1 (also known as p21-WAF1) on T145 (Zhou et al., 2001). It also phosphorylates another CDK inhibitor called p27-Kip1 at T157 (Viglietto et al., 2002). Phosphorylation of p21 and p27 by Akt promotes their cytosolic localization and ultimately prevents their cell cycle inhibitory effects in the nucleus (Zhou, 2002 and Shin, 2002). This allows activation of CDKs (in the presence of cyclin) and progression through the cell cycle phases. The products downstream of tumour suppressor protein p53 (discussed further under the “Apoptosis” subheading) are also known to be cell cycle regulators, and shed light on another mechanism by which Akt is involved in cell growth (Vousden et al., 2002). Furthermore, AKT-dependent phosphorylation of other targets involved in protein synthesis and stabilization, such as TSC2 and PRAS40, is also likely to stimulate cell proliferation as cell growth is necessary for normal proliferation (Carnero, 2010).

Another major cell cycle regulator that is activated by Akt is cyclin D1. In terms of NSCLC, abnormalities in cyclin D1 are detected in more than 90% of cases and are associated with hyperactive Akt (Marchetti et al., 1998). During G1 phase of the cell cycle, cyclin D1 is responsible for inactivating the growth-suppressive function of retinoblastoma protein (Rb), titrating CDK inhibitors such as p27-Kip1 and p21-Cip1, and facilitating the activation of cyclin E-CDK2 and subsequent entry into the S phase (Sherr et al., 1995). Glycogen synthase kinase 3β (GSK3β) can phosphorylate cyclin D1 at T286, leading to its nuclear export and subsequent degradation. Akt is able to inhibit the activity of GSK3β by phosphorylation on serine 9, and prevent the degradation of cyclin D1 (Diehl et al., 1998).
Apoptosis

One of the mechanisms by which hyperactive Akt promotes cell survival and tumorigenesis is by direct inhibition of apoptosis. The Bcl-2 associated death promoter (BAD) is a member of the Bcl-2 family of proteins that binds to Bcl-2 or Bcl-X and inhibits their anti-apoptotic potential. It is also a downstream target of Akt, which phosphorylates BAD on S136. Once phosphorylated, BAD is released from a complex with Bcl-2/Bcl-X that is localized on the mitochondrial membrane, and forms a complex with 14-3-3 proteins in the cytosol, which sequesters it and allows Bcl-2 and Bcl-X to inhibit the apoptosis signal (Datta et al., 1999). In terms of tumorigenesis, phosphorylation of BAD by Akt will dampen the apoptosis signal, and allow cancer cells to survive.

Another direct target of Akt is caspase-9, an initiator of the caspase apoptosis cascade. Phosphorylation of procaspase-9 on S196 by Akt inhibits cytochrome-c induced cleavage and activation, thus preventing it from activating other downstream caspases (Cardone et al., 1998). Interestingly, the Akt phosphorylation motif is not present in caspase-9 of lower species such as mice or rats (Hanada et al., 2004). This suggests that caspase-9 may only be a target of Akt in more evolutionarily developed species.

Besides regulating molecules directly involved in apoptosis, Akt has also been found to alter pathways that affect transcription factors of apoptosis. Akt is able to directly phosphorylate all four isoforms of the forkhead box O (FoxO) protein (Song et al., 2005). This phosphorylation exposes a binding site for 14-3-3 proteins, which results in the translocation of the complex from the nucleus to the cytoplasm (Dijkers et al., 2002). Thus FoxO is unable to up-regulate genes involved in inhibiting cell survival such as the Fas ligand, TNF-related apoptosis-inducing
ligand (TRAIL), and Bcl-2 interacting mediator of cell death (BIM) (Burgering and Medema, 2003).

The nuclear factor κB (NFκB) is another transcription factor that is regulated by Akt. Akt can directly phosphorylate the inhibitor of κB kinase (IKK) on T23 of the IKKα subunit (Bai et al., 2009). The IKK complex then phosphorylates the inhibitor of κB (IκB) protein, which can no longer sequester NFκB in the cytoplasm. NFκB is then free to enter the nucleus and up-regulate the transcription of its pro survival target genes including Bcl-X and caspase inhibitors (Barkett et al., 1999).

Additionally, Akt is known to undermine the p53-mediated pro-apoptotic transcriptional response, by promoting the interaction of p53 with the murine double minute 2 (Mdm2) E3 ubiquitin ligase (Gottlieb et al., 2002). Phosphorylation of Mdm2 by Akt promotes the interaction with p53, and results in increased degradation of p53 as well as increased cell survival.

**Metabolism**

In response to growth factors, Akt signaling regulates nutrient uptake and metabolism. One of the most important roles of Akt is to promote glucose uptake in response to insulin. The receptors of the insulin ligand are RTKs that activate formation of PI3K and stimulate Akt activation. Akt has been found to associate with glucose transporter-4 (GLUT4) containing vesicles and recruit them to the plasma membrane upon insulin stimulation (Kohn et al., 1996). This results in an increase of glucose uptake and metabolism. Moreover, phosphorylation of
GSK3β by Akt prevents inhibition of glycogen synthase activity, and results in further uptake and storage of glucose (Hemmings and Rustoccia, 2010).

mTORC1, as a messenger of Akt, is also involved in the regulation of glucose/fat metabolism through S6K1. Mice that express hyper-active S6K1 mutants display reduced adipose tissue and a decrease in fat accumulation due to enhanced β oxidation (Um et al., 2004). This mechanism may partially involve activation of certain downstream transcription factors such as hypoxia inducible factor 1 (HIF1), even under normoxic conditions (Cairns et al., 2011). HIF1 is one of the major transcription factors involved in changing gene expression during the cellular response to low oxygen conditions. It can be activated by both S6K1 and 4E-BP1, downstream of mTORC1, and can amplify the transcription of genes encoding glucose transporters and glycolytic enzymes, increasing the capacity of the cells to carry out glycolysis (Semenza, 2010). This is linked to the Warburg effect, which encompasses that cancer cells shift to anaerobic means of glucose metabolism and increase glucose uptake to meet the demands of continuous growth and proliferation (Warburg, 1956).

**Angiogenesis**

It is often vital for tumorigenic cells to continue to grow and proliferate in nutrient depleted environments. They rely on angiogenesis, the formation of new blood vessels to deliver more nutrients. Akt plays an important role in endothelial cells to promote angiogenesis. Vascular endothelial growth factor receptor (VEGFR) is upstream of Akt, and activation of VEGFR is associated with cell growth, proliferation and migration (Olsen et al., 2006). Akt is also known to activate endothelial nitric oxide synthase (eNOS) by phosphorylation on S1117,
which is responsible for the production of nitric oxide, a potent vasodilator (Alderton et al., 2001). These interactions, along with the metabolic properties of Akt, demonstrate the role of Akt in angiogenesis.

Migration

Cell migration can either be promoted through rearrangement of the cytoskeleton, or increased expression of genes that promote epithelial to mesenchymal transition (EMT). There are many novel regulators of migration that are being linked to Akt. One recent study evaluated the role of T-LAK cell originated protein kinase (TOPK) in lung cancer metastasis (Shih et al., 2011). Although TOPK is usually associated with proliferation and cytokinesis, it may also have a role in promoting cell migration and invasion. Numerous NSCLC cell lines were evaluated for TOPK expression and invasion capabilities through Matrigel coated trans-well migration experiments. The cell lines that had the highest expression of TOPK, also had the highest invasive properties, as well as the lowest expression of PTEN. These results indicate that TOPK may enhance PI3K/Akt dependant migration through inactivation of PTEN.

Several other direct and indirect targets of Akt have also been associated with cell migration and invasion capabilities. However, many of these studies are in the preliminary stages. Some of the molecules involved in Akt dependant migration include GSK3β, vimentin, palladin, twist and kank (Xue and Hemmings, 2013). Furthermore, many of these proteins have differential regulatory sites that are associated with Akt, suggesting that Akt regulation of migration may be isoform dependant. For example, palladin, an actin binding protein that regulates cell motility through tethering with F-actin bundles, is directly inhibited by Akt1 (Chin
and Toker, 2010a). However it is also associated with increased motility through interaction with Akt2 (Chin and Toker, 2010b). Since metastasis is one of the main factors in determining prognosis, it is extremely important to understand these mechanisms and how newly identified members contribute to cell migration.

**Isoform Specific Functions**

Since all three isoforms of Akt are closely related and highly conserved, it was previously thought that they had similar roles in regulating cellular processes. Although redundancies in functional roles are likely to exist, several recent findings point towards isoform specific differences concerning proliferation, apoptosis and migration (Grabinski et al., 2011).

**Akt1**

Akt1 is suspected to be primarily involved in cell growth and motility. Studies evaluating overall effects of Akt isoform specific inhibition on mice have demonstrated that ablation of Akt1 alone results in mice with impaired growth (Cho et al., 2001, Yang et al., 2003). This growth retardation is suspected to begin in the embryonic stages and continue throughout adulthood. These Akt1 knockout mice are smaller than their wild-type counterparts, but are otherwise considered normal. Furthermore, tumour cells in these mice display a higher rate of apoptosis (Chen et al., 2001). In NSCLC mouse models specifically, deletion of Akt1 was shown
to significantly delay onset of lung tumour growth through inhibition of proliferation and increase in apoptosis (Linnerth Petrik et al., 2014). Additionally, silencing Akt1 but not Akt2, in non-transformed mammalian cells results in a reduction of cyclin A and D levels as well as entry into S-phase (Heron-Milhavet et al., 2006).

Analysis of double Akt isoform knockout mice reveals some overlap in function among isoforms, but also supports the notion of distinct roles. Dual deletion of Akt1 and Akt2 in mice results in death shortly after birth, while ablation of Akt1 and Akt3 leads to direct embryonic lethality. (Peng et al., 2003, Yang et al., 2005). These studies indicate the importance of Akt1 in embryonic development and continued growth. However, other studies have shown that mice with only a single functional allele of Akt1, but no Akt2 or Akt3, are viable despite reduced body weight and insulin and glucose intolerance (Dummler et al., 2006). Regardless, all of these studies are consistent in identifying Akt1 as an important regulator of cell growth and survival.

In regard to the invasive properties of Akt1, there seem to be opposing roles based on tissue specific cancer types. In mammary cancer cells, down regulation of Akt1 resulted in increased motility of cells as well promotion of EMT (Wyszomierski et al., 2005, Liu et al., 2006, and Irie et al., 2005). However, Akt1 ablation/inhibition was shown to decrease cell migration in many other cancer types including thyroid, colorectal, pancreatic, and ovarian cancer (Saji et al., 2011, Ericson et al., 2010, Tanno et al., 2001, and Ki et al., 2011). These results indicate that Akt1 could be activating certain migratory pathways in some cells, while inhibiting other pathways in other cell types.
Akt2

Akt2 function is usually linked to glucose homeostasis as well as cell migration. However, unlike Akt1, Akt2 is usually found to have pro-invasive stimulation. Up-regulation of Akt2 in an inducible mammary tumour mice model led to increased lung metastasis (Dhillon et al., 2009). Moreover, Akt2 knockdown in a xenograft model of colon cancer lead to decreased liver metastasis (Rychahou et al., 2008). These findings were supported by Akt2 knockdowns in A549 and MEF cells, which are NSCLC and fibroblast cells respectively (Sithanandam et al., 2005). Furthermore, knockdown of Akt2 was shown to reverse the EMT in mammary epithelial cells lines (Irie et al., 2005). Taken together, these results indicate that Akt2 is able to promote cell migration and invasion.

It is also a key regulator of pathways involved in glucose metabolism. For example, mice that are Akt2 null display a type 2 diabetes like phenotype, and cells derived from those mice show impaired glucose uptake and metabolism (Cho et al., 2001b). These findings were validated through a second study that found that Akt2 knockout mice exhibited hyperglycemia, hyperinsulinemia, glucose intolerance, and impaired muscle glucose uptake in both the fed and fasting state (Garofalo et al., 2003). Furthermore, these disruptions to glucose regulation were not compensated by the other two Akt isoforms. These results suggest that Akt2 plays a critical role in insulin mediated signaling of glucose homeostasis.

Akt3

Akt3 is highly expressed in certain tissues such as the testes and brain, but is also sparingly expressed in many other tissue types as well. Due to this reason, studies evaluating the
roles of Akt isoforms in tumorigenesis focus on Akt1 and Akt2. Interestingly, Linnerth-Petrik et al. (2014), reported detecting levels of Akt3 in lung tumour that were comparable to Akt1 and Akt2. However, their findings indicate that ablation of Akt3 in mouse lung tissue did not statistically alter tumour induction or growth compared to the wild-type group.

Thus the majority of Akt3 knockout studies in mice don’t affect tumorigenesis but show an overall effect on brain development. Tshopp et al. (2005) report that Akt3 null mice display a reduction in brain size due to reduced size of cells as well as number of neurons. Interestingly, these mice seem to capably metabolize glucose and are considered to be physiologically normal. These findings indicate the importance of Akt3 in brain development, but also provide evidence that Akt isoforms are able to differentially regulate certain cellular processes.

**Akt Inhibitors as Therapeutics**

*MK-2206*

MK-2206 is a pan-Akt inhibitor, which means that it inhibits all three isoforms of Akt. It is an allosteric inhibitor that binds to the PH domain in Akt, and initiates conformational change that prevents recruitment of Akt to the plasma membrane. (Bilodeau et al., 2008). Allosteric inhibitors are considered to be more specific than ATP-competitive inhibitors (discussed below), and MK-2206 was found to be over 100-fold more specific to Akt than 256 other kinases (Yan, 2009). MK-2206 interactions with Akt have been shown to inhibit phosphorylation at both
activation sites; S473 and T308 (or the equivalent in Akt2 and Akt3) (Okuzumi et al., 2009). Its IC50 potency is 5nM, 12nM, and 65nM against Akt1, Akt2, and Akt3, respectively (Yan, 2009).

This pan-Akt inhibitor is currently being investigated in phase II clinical trials. The results from the phase I study indicate that the maximum tolerated dose should be 60 mg every other day, with plasma concentrations reaching a peak of less than 100nM (Yap et al., 2011a). The half-life was determined to be between 40-100 hours. Some of the drug related toxicities included skin rash, nausea, hyperglycemia, and diarrhea. Although only minor tumour shrinkage was seen in participants, one patient with pancreatic adenocarcinoma experienced a tumour shrinkage of 23%. Currently, MK-2206 is being evaluated for its effects in combinational therapy of cancer with other molecular inhibitors as well as currently used chemotherapeutics. The National Cancer Institute in the United States is supporting a list of trials with MK-2206 treatment given to patients with lung, pancreatic, colorectal, breast, and prostate cancer (National Cancer Institute, 2015).

In terms of NSCLC, a recent study observed the combinational treatment of MK-2206 with either erlotinib (an epidermal growth factor inhibitor) or lapatinib (a dual epidermal growth factor receptor/human epidermal growth factor receptor 2 inhibitor) in human NSCLC cell lines (Hirai et al., 2010). They found that MK-2206 treatment alone was effective in inhibiting cell growth, but also acted synergistically with both erlotinib and lapatinib. Furthermore, 20 µM concentration of erlotinib alone was not sufficient to induce an increase in activated caspase 3/7 in these cells, but a combinational treatment of 3 µM MK-2206 and 2.5 µM erlotinib was able to induce apoptosis. These results outline the beneficial effects of Akt inhibition in treating a variety of cancers, including NSCLC.
CCT-128930

CCT-128930 is a novel ATP competitive inhibitor of Akt2. Unlike allosteric inhibitors of Akt, ATP competitive inhibitors tend to initially increase phosphorylation of both S473 and T308 activation sites (Han et al., 2007). Still, phosphorylation of downstream targets of Akt, such as GSK3β, PRAS40, and S6RP, has been shown to decrease with CCT-128930 treatment, irrespective of S473 and T308 phosphorylation on Akt (Yap et al., 2011b). Although this mechanism is not yet completely understood, it is hypothesized to be attributable to the inhibitor binding directly to the ATP binding site, which may alter the affinity of these amino acids to get phosphorylated, or due to compensatory but futile feedback loops to increase Akt activity (Okuzumi et al., 2009).

One recent study evaluated the effects of CCT-128930 treatment on HepG2 (liver carcinoma) and A549 (adenocarcinoma) cells (Wang et al., 2014). They noticed a G1/G0 cell cycle arrest, an increase in annexin V (apoptotic marker), and an increase in autophagy markers with CCT-128930 treatment. However, these effects were observed in treatment groups of very high concentrations of the inhibitor. Overall, not many experiments have been conducted with CCT-128930, and further roles of this inhibitor remain to be seen.

A-674563

A-674563 is a recently developed inhibitor of Akt1. Like CCT-128930, it is an ATP competitive inhibitors that leads to hyper-phosphorylation of Akt activation sites, but subsequent inhibition of kinase abilities. It was developed by replacing the indole on A-443654 with a
phenyl moiety to gain oral bioavailability (Luo et al., 2005). It has a $K_i$ value of 11 nM towards Akt1 and is 30 fold more selective for Akt1 than PKC.

In terms of its effects on tumorigenesis, A-674563 demonstrated efficacy enhancement of paclitaxel treatment on a PC-3 (PTEN -deficient human prostate carcinoma) xenograft model (Luo et al., 2005). Since this is a novel drug, not many studies have been published regarding its effects on lung cancer. However, one recent study did determine the IC50 concentration of A-674563 on PC9 NSCLC cells to be 0.9 µM, indicating the potential of this inhibitor to be used towards lung cancer treatment (Kekesi et al., 2013).
HYPOTHESIS & OBJECTIVES

I hypothesize that Akt1 selective inhibition of NSCLC cells will be more effective in suppressing tumorigenic properties than either Akt2 selective or pan-Akt inhibition.

Three objectives will be covered in this study:

1. Investigate the effects of Akt1, Akt2, and pan-Akt inhibition on cell survival, proliferation, apoptosis, cell-cycle and migration.
2. Evaluate the specificity of the Akt1 and Akt2 selective inhibitors.
3. Determine changes to phosphorylation status of downstream targets with each inhibitor.
METHODOLOGY

Cell Lines and Inhibitors

A549 and NCI-H358 human lung cancer cells, LA-4 murine lung cancer cells and NBE-135 normal human cells were purchased from American Type Culture Collection (ATCC). A549 and NCI-H358 cells were cultured in RPMI 1640 media (Life Technologies) containing L-Glutamine and supplemented with 10% FBS (Life Technologies) and 1% ABAM (Life Technologies). LA-4 cells were cultured in Ham’s F-12K (Kaighn’s) media (Life Technologies) containing L-Glutamine and supplemented with 15% FBS and 1% ABAM. NBE-135 cells were cultured in Keratinocyte Serum Free media (Life Technologies) containing L-Glutamine and supplemented with 5 ng/ml human recombinant EGF, 0.05 mg/ml BPE, 0.005 mg/ml insulin (Life Technologies) and 500 ng/ml hydrocortisone (Sigma Aldrich). Cells were incubated and maintained at 37º C and 5% CO₂. After trypsinizing NBE-135 cells, they were collected and centrifuged to remove the trypsin before seeding due to lack of serum in the media.

Akt 1 (A-674563), Akt 2 (CCT-128930) and pan-Akt (MK-2206) inhibitors were purchased from Sellekchem and dissolved in DMSO to create 5 mM (A-674563) and 10 mM (CCT-128930 and MK-2206) stock solutions. Inhibitors were diluted further in DMSO for experimental dosage. Cisplatin was purchased from (Sigma Aldrich) and dissolved in 0.9% saline.
Cell Counting

Cells were washed in sterile PBS and trypsinized at 37°C in either 10X Trypsin EDTA (0.5%) solution (Life Technologies) or 1X (diluted in PBS) for NCI-H358 cells, for 2-5 minutes. Media was then added to dilute the cells. 50 µL of cell solution was mixed with 50 µL of Trypan Blue (Life Technologies) and live cells were counted manually via a hemocytometer. The equation used to determine cell density was: \( x \text{ cells/ml} = \text{average count of cells} \times \text{dilution factor} \times 10^4 \).

WST-1 Cell Viability Assay

Cell viability was determined based on the enzymatic cleavage of the WST-1 tetrazolium salt using reagents from Roche. Cells were seeded and incubated in 96-well tissue culture plates at a density of 1 \( \times \) 10\(^3\) (A549, LA4, NBE-135) or 2 \( \times \) 10\(^3\) (NCI-H358) cells per well in a volume of 99 µL of their respective media. After 24 hours, 1 µL of DMSO or increasing concentrations of inhibitors were added to the wells (DMSO concentration was maintained at 1% in all wells). Media and inhibitors were replaced every 24 hours for a 72 hour treatment period. Next, media was completely removed from wells and replaced with 100 µL of fresh media, and incubated with 10 µL of WST-1 reagent at 37°C for 2 hours. Plates were then agitated for 1 minute and optical density was determined at 450 nm using a Bio- Tek EL800 Universal Microplate Reader (Bio-tek Instruments Inc.). Final calculations were performed after subtracting out the optical density determined in blank wells containing only media. Standard curves were generated relative to DMSO only treatment using CalcuSyn software (Biosoft). IC20, IC50 and IC80 concentrations were determined from these curves for each cell line with each treatment.
Trypan Blue Cell Counting Assay

Cell viability and experimental dosages of Akt inhibitors were validated by manually counting live cells counter stained with Trypan Blue. Cells were seeded and incubated in 24-well tissue culture plates at a density of 3 x 10^3 cells per well in a volume of 500 µL of their respective media. They were incubated at 37⁰ C for 24 hours and allowed to adhere. After which, the IC50 and IC80 concentrations of the inhibitors were added to constitute 0.1% DMSO in each well. Media and inhibitors were replaced every 24 hours. Cells were trypsinized and counted at 24, 48, and 72 hour time points according to the method described above, under the Cell Counting sub-heading.

Immunofluorescence

Cells were seeded onto sterile coverslips in 6-well tissue culture plates at a density of 1 x 10^5 cells per well in a volume of 2 ml of their respective media. After 24 hours, 2 µL of inhibitors were introduced to the wells maintaining a DMSO concentration of 0.1%. Inhibitors and media were replaced every 24 hours. Cells were then washed in cold PBS and fixed in 10% buffered formalin for 15 minutes at room temperature. After which, cells were washed in PBS again, and permeabilized with a 0.2% Triton X-100 in PBS solution for 5 minutes. Next, cells were blocked in a 5% BSA, 0.1% Triton X-100 in PBS solution for 10 minutes and incubated overnight at 4⁰ C in phospho Histone H3 S10 primary antibody (Ab5176, 1:2000) (Abcam). Secondary antibody Alexa-fluor 594 conjugated to Texas Red (1:500) (Life Technologies) was then applied for 1 hour at room temperature in the dark. Cell nuclei were then counterstained with DAPI, and Prolong Gold (Life Technologies) was used to mount the coverslips and
preserve the fluorescent signal. Images were taken with an Olympus BX961 fluorescent microscope equipped with MetaMorph Imaging software (Molecular Devices). Results were then manually quantified using Image J software (National Institute of Health).

**Flow Cytometry**

*Single Staining*

Single staining of phospho-histone H3 was performed to assess changes in percentage of cells positive for the marker. A549 and NCI-H358 cells were seeded and incubated in 6-well tissue culture plates at the same densities described in the Immunofluorescence subheading. After 24 hours, inhibitors or DMSO alone were introduced to the wells (0.1% DMSO, with media and inhibitors being replaced every 24 hours). After 24 or 72 hours of treatment, cells were trypsinized and collected, washed in incubation buffer (Appendix I) and fixed in 4% formaldehyde for 10 minutes at 37°C. Subsequently, cells were chilled on ice for 1 minute, washed, and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Next, cells were incubated in primary phospho Histone H3 antibody (Ab5176, 1:500) (Abcam) for 1 hour at room temperature. Cells were then washed and incubated in secondary antibody Alexa-fluor 488 conjugated to FITC (1:500) (Life Technologies) for 30 minutes at room temperature in the dark. After a final wash cells were resuspended in incubation buffer and analyzed by the Accuri C6 flow cytometer (BD Biosciences).
Annexin V & PI

Apoptosis differences were measured by using the Flow Cytometry Annexin V Apoptosis Detection kit (eBioscience) following the protocol provided with the kit. Briefly, cells were seeded and incubated in 6-well tissue culture plates. After 24 hours, inhibitors or DMSO alone were introduced to the wells (0.1% DMSO, with media and inhibitors being replaced every 24 hours). After 24 or 72 hours of treatment, cells were trypsinized and collected, washed in PBS and then in Binding Buffer solution, and incubated in Annexin V FITC conjugated antibody (1:40) for 10 minutes at room temperature in the dark. Cells were then washed, resuspended in Binding Buffer solution, and incubated with PI (1:20) for 10 minutes. Percentage of cells positive for Annexin V, PI, or both were then measured by a BD Accuri C6 flow cytometer (BD Biosciences). Data analysis and channel compensation was performed using the Accuri C6 software (BD Biosciences) provided with the machine. 50 µM cisplatin was used as the positive control.

BrdU & 7AAD

Cell cycle profile was assessed using the FITC BrdU Flow Kit from BD Bioscience. Briefly, cells were seeded and incubated in 6-well tissue culture plates. After 24 hours, inhibitors or DMSO alone were introduced to the wells (0.1% DMSO, media and inhibitors were replaced every 24 hours). After 72 hours of treatment, A549 cells were incubated in a 10 µM BrdU solution at 37°C for 2 hours. Then they were trypsinized and washed in PBS, and fixed in Cytofix/Cytoperm Buffer for 15 minutes on ice. Subsequently, cells were incubated in 300 µg/ml DNase for 1 hour at 37°C. Following this step, cells were treated with diluted fluorescent
anti-BrdU antibody (1:50) for 20 minutes at room temperature. Cells were then washed again
and resuspended in 1 ml of staining buffer with 20 µL of the 7AAD solution. Fluorescence was
then measured by a BD Accuri C6 flow cytometer (BD Biosciences). Data analysis and channel
compensation was performed using the Accuri C6 software and BrdU staining template provided
with the machine.

**Western Blotting**

*Sample Preparation*

Cells were seeded and incubated in 10 cm tissue culture plates. After 24 hours, inhibitors
or DMSO alone were introduced to the wells (0.1% DMSO) for the duration of treatment (5
minutes, 30 minutes, 6 hours, or 24 hours). Cells were then washed in cold PBS and lysed in 150
µL of RIPA lysis buffer (Appendix I) on ice. Whole cell lysates were collected and incubated on
ice for 1 hour. Next, cells were centrifuged at 12000 g for 20 minutes at 4°C, and supernatant
layers were collected and aliquoted. Protein concentration was quantified using a Bradford assay
(Bio-Rad) as outlined by their protocol (Bradford, 1976). Protein samples (30-40 µg of protein)
were then reduced in reducing buffer (Appendix I) and boiled at 95°C prior to being separated.

*Sample Separation and Imaging*

Samples were separated using 12% sodium dodecyl sulfate (SDS)- polyacrylamide gels
(Appendix I). Separation was achieved using an Xcell II min cell system (Life Technologies) by
applying 127V for 2 hours to the system. Proteins were then transferred onto Hybond ECL
nitrocellulose membranes (Amersham Pharmacia Biotech) by applying 27V for approximately 2 hours using a wet transfer method. The membranes were then blocked in 5% skim milk in Tris-buffered saline containing 0.01% Tween 20 (TBST) at room temperature for 1 hour. Next, blots were incubated in the following primary antibodies diluted in antibody diluting solution (Appendix I) at 4 °C overnight; Akt (#9272, 1:1000), Akt 1 (#2938, 1:1000), Akt 2 (#2964, 1:1000), Akt 3 (#4059, 1:1000), pAkt S473 (#9271, 1:1000), pAkt T308 (#9275, 1:1000), pAkt 1 S473 (#9018, 1:1000), pAkt 2 S474 (#8599, 1:1000), pPRAS40 T246 (#2640, 1:1000), pGSK3 B S9 (#9336, 1:1000), pS6RP S240/244 (#2215, 1:1000), β-actin (#4967, 1:4000) (Cell Signalling), and GAPDH (Santa Cruz Technologies). Blots were then washed three times in TBST for a period of ~10 minutes and incubated in HRP-linked anti-rabbit IgG secondary antibody (#7074, 1:2000) (Cell Signaling Technology) for 1 hour at room temperature. The blots were then thoroughly washed prior to imaging via Clarity Western ECL Substrate (Bio-Rad). Protein detection was imaged on a ChemiDoc™ XRS+ System (Bio-Rad), and densitometry quantification was performed using Image Lab software (Bio-Rad).

**Scratch Wound**

Cells were seeded and incubated in 6-well tissue culture plates at a density of 8.0 x 10^4 cells per well. After 24 hours, inhibitors were introduced, maintaining a DMSO concentration of 0.1% in each well. The wound was performed 24 hours later by scraping the tip of a 200 µl pipette tip across the center of the well. Media and inhibitors were replaced every 24 hours. Images were captured every 24 hours using an inverted Olympus IX71 light microscope equipped with Q Imaging 3.3 software (Q Imaging) until the wound in the control group was
closed. Image J software was used to manually quantify surface area of the wound, and the following formula was used to calculate the percentage of wound closure:

\[
\text{% of wound closure} = \left( \frac{s.a \text{ at } t = 0h - s.a \text{ at } t = \Delta h}{s.a \text{ at } t = 0h} \right) \times 100
\]

“s.a” indicates surface area, “t” indicates time in treatment

Statistics

Statistical analysis was performed using an Analysis of Variance (ANOVA) test to evaluate significance followed by Tukey’s or Dunnet’s post hoc tests to determine where the differences were. P-values <0.05 were considered to be significant.
RESULTS

Baseline expression of Akt isoforms in cell lines

Akt isoform expression was examined in two human lung cancer cell lines (A549 and NCI-H358), a murine lung cancer cell line (LA4) and a normal human lung epithelial cell line (NBE-135) (Figure 3). Western blotting confirmed the presence of Akt1 and Akt2 while Akt3 was only detected at very low levels in the two human lung cancer cell lines. Akt activation, or phosphorylation of the serine 473 residue was also evaluated and shown in Figure 3. Phosphorylated Akt (Ser473) was found in all cell lines suggesting that the Akt signaling axis is active in these cells.

Effects of Akt1, Akt2, and pan-Akt inhibitors on cell survival

Cell survival curves were generated to evaluate the efficacy of the Akt1 (A-674563), Akt2 (CCT-128930), and pan-Akt (MK-2206) inhibitors on cell survival (Figure 4). Cells were treated with increasing doses of each inhibitor for 72 hours, after which a WST-1 assay was performed to assess cell viability. Curves demonstrate that all inhibitors had dose dependent effects on cell survival. Akt1 inhibition was the most potent in decreasing cell survival in A549 and NCI-H358 cells, while Akt2 inhibition was the least potent. Concentrations of 0.2 µM of A-674563 were sufficient to show a significant decrease in cell survival of both A549 and NCI-H358 cells, while higher doses of CCT-128930 (6.25 and 3.13 µM, respectively) were required to show a significant effect on cell survival. MK-2206 also showed a significant reduction in A549 and NCI-H358 cell survival at concentrations higher than 6.25 µM and 1.56 µM,
respectively. Based on the observed rate of cell death for each drug, the Akt1 inhibitor was the most effective at reducing cell survival, while the Akt2 selective inhibitor was the least effective in both human cancer cell lines.

Interestingly, the murine LA4 cancer cells responded most effectively to MK-2206 treatment, while initial exposure of A-674563 or CCT-128930 resulted in increased cell survival compared to DMSO control treatment. The normal human NBE-135 cells followed a trend similar to the human lung cancer cells.

This data was inputted into the CalcuSyn program, from which experimental inhibitory concentrations of IC20, IC50 and IC80 were determined for each cell line (Table 1). These values represent the concentration of inhibitor treatment that reduced cell survival by 20%, 50%, and 80% compared to a DMSO control. In A549 cells, CCT-128930 IC50 values were more than 26 fold greater than A-674563 IC50 values, while MK-2206 IC50 values were approximately 10 times greater. A similar trend was observed in NCI-H358 cells with the IC50 value of CCT-128930 being more than 5 fold greater than the A-674563 IC50 value, while the IC50 for MK-2206 was almost 2 fold greater. These values demonstrate the increased susceptibility of these human lung cancer cells to Akt1 inhibition over Akt2 inhibition. In terms of a comparison between the two human lung cancer cell lines, A549 cells responded more effectively to all three treatments. The IC50 values of CCT-128930 in LA4 and NBE-135 cells were even more elevated, reaching concentrations of 22.5 and 12.8 µM, respectively.

These inhibitory concentration values were validated by manual cell counting with Trypan Blue staining (Figure 5a-c). These treatments resulted in significant decreases in manual cell counts, and by 72 hours of treatment, all treatments were significantly different from the DMSO control and each other. The data was compiled into a summary graph to validate that the
IC50 and IC80 concentration of each inhibitor did result in approximately 50% and 80% reduction in cell count (Figure 5d).

**Evaluation of changes in cellular proliferation**

Histone H3 is part of the eight core histone complex involved in chromatin organization, and phosphorylation of this molecule is highly related to chromosome condensation and cell proliferation. Thus accumulation of phospho-histone H3 in the nucleus is indicative of cells entering mitosis. A549 cells were treated with A-674563 (Akt1), CCT-128930 (Akt2), or MK-2206 (pan-Akt) inhibitors for 24 hours, and changes in phospho-histone H3 detection were measured by flow cytometry (Figure 6a). Compared to DMSO, none of the IC50 concentrations of Akt inhibitors had a significant reduction in phospho-histone H3 detection. Interestingly, only IC50 treatment of A-674563 (118 nM) resulted in a significant increase in phospho-histone H3 detection. Since the IC50 values of all three inhibitors varied greatly, a smaller dose of 0.78 µM was also chosen to assess changes in proliferation when treated with the same concentration of each inhibitor. Although no significant differences were observed compared to the DMSO control, A-674563 treatment of 0.78 µM resulted in approximately 2 fold increase in phospho-histone H3 detection. To evaluate effects of longer term inhibitor exposure on proliferation, A549 cells were also treated for 72 hours, resulting in no changes to phospho-histone H3 detection with any treatment (Figure 6b).

Similar experiments were performed for NCI-H358 cells at 24 and 72 hours, which also resulted in no differences with any treatment compared to the DMSO control (Figure 6c-d). To validate these findings, changes in phospho-histone H3 in A549 cells after 24 hours of treatment
were also measured by immuno-fluorescence imaging and analysis (Figure 6g). There was a significant 2 fold increase with IC80 treatment of A-67563 compared to DMSO treatment. Since the survival curves demonstrated that Akt1 inhibition was the most effective at killing A549 cells, these results show a counter-intuitive increase in A549 proliferation with A-674563 treatment.

**Evaluation of changes in cellular apoptosis**

Annexin V is a well-known marker for cellular apoptosis. Normally phosphatidylserine (PS) residues are hidden within the plasma membrane, however they are shifted to the outer leaflet during early apoptotic events within the cell. Annexin V is then able to bind the extracellular PS residues and detect cells that are undergoing apoptosis. When Annexin V is coupled with propidium iodide, a dye that only binds DNA in dying cells, a profile of early and late apoptotic cells can be established through flow cytometry (Figure 7a). 24 hour treatment of A549 cells with IC50 and 0.78 µM concentrations of Akt inhibitors revealed a significant 50% increase in proportion of cells undergoing apoptosis with IC50 treatment of MK-2006 (1.16 µM) and 0.78 µM treatment of A-674563 compared to DMSO control (Figure 7b). When differentiating between early and late apoptotic populations, the results indicate that MK-2206 treatment may be increasing proportion of late apoptotic cells and A-674563 may be increasing early apoptotic cell proportions (Figure 7c-d). Additionally, 24 hour IC50 treatment of CCT-128930 (3.33 µM) also resulted in a significant increase in proportion of early apoptotic cells. When evaluating changes in apoptosis during longer exposure of Akt inhibitors, only 0.78 µM of A-647563 treatment lead to a significant increase in apoptosis of 2.5 fold (Figure 7e). Again this
increase was only noted in early apoptotic cells (3 fold), along with IC50 treatment of CCT-128930 (~1.8 fold).

The same experiments were performed on NCI-H358 cells, which were less susceptible to apoptotic changes by the cisplatin positive control (50 µM). After 24 hours of Akt inhibitor treatment, the IC50 dose of CCT-128930 (6.62 µM) resulted in a significant 60% reduction in total Annexin V positive cells compared to the DMSO control (Figure 8a). This reduction was present in both early and late apoptotic cells (Figure 8b-c). Furthermore, IC50 treatment of A-67463 (1.26 µM) led to a significant increase in proportion of early apoptotic cells. At 72 hours, none of the Akt inhibitors resulted in differential detection of Annexin V (Figure 8d-f).

**Evaluation of changes in cellular migration**

One method of measuring cellular migration is through performing scratch wound assays. As cells attempt to close the wound, the progression can be evaluated by measuring the area of the wound as it is filled in (Figure 9a). Although IC50 treatment of all three inhibitors demonstrated significantly reduced A549 cell migration compared to DMSO treatment, A-674563 (118 nM) resulted in 25.0% reduced wound closure, while CCT-128930 (3.33 µM) and MK-2206 (1.16 µM) resulted in 15.4% and 13.5% reduction, respectively (Figure 9b). When comparing migration rates of 0.78 µM treatments of each inhibitor to a DMSO control, A-674563 and CCT-128930 resulted in significant decreases of 60.4% and 18.2%, respectively. 0.78 µM treatment of MK-2206 had no effect on A549 wound closure.

In NCI-H358 cells, all IC50 and 0.78 µM treatments resulted in significantly reduced wound closure. IC50 concentrations of A-674563 (1.26 µM), CCT-128930 (6.62 µM), and MK-
2206 (2.02 µM) resulted in 35.9%, 20.9%, and 23.4% reduced wound closure, respectively (Figure 10b). Interestingly, 0.78 µM treatments resulted in similar decreases in cell migration as their IC50 counter parts (Figure 10c). From the results it is evident that Akt1 inhibition was more effective at reducing cell migration in both A549 and NCI-H358 cells, although all treatments had significant reductions at some point during the scratch wound assay.

**Cell cycle analysis**

Bromodeoxyuridine, a synthetic analogue of thymidine, can be used as a marker for cells entering the S phase of the cell cycle. When coupled with 7-AAD DNA dye, cells can be distinguished into G1/G0, S, or G2/M phases (Figure 11a). A549 cells were treated with IC50 concentrations of A-674563, CCT-128930, MK-2206, or just DMSO for 72 hours. Flow cytometry analysis detected changes in proportion of cells in each cell cycle phase. Compared to the DMSO control, IC50 treatment of A-674563 (118 nM) resulted in a small, but significant decrease in proportion of cells in the G1/G0 phase (Figure 11b). Interestingly there was also a slight increase in the S phase for both IC50 treatment of A-674563 and CCT-128930. Changes to cell cycle profile with same concentration treatment of each inhibitor (0.78 µM) were also evaluated (Figure 11b). No changes in G1/G0 phase were detected with A-674563 treatment, however, compared to the DMSO control there was a significant increase in proportion of cells in the G2/M phase from 5.4% to 11.2%. Treatment of A549 cells with MK-2206 also resulted in a slight increase in proportions of cells in S phase compared to the DMSO control.
Inhibitory effects on Akt S473 phosphorylation

The potential of all three Akt inhibitors to inhibit phosphorylation of Akt (S473) was evaluated in A549 cells. Figure 12a demonstrates that MK-2206 was able to inhibit Akt phosphorylation as early as 5 minutes post treatment, which persisted for over 24 hours. Furthermore total Akt levels did not differ from the untreated or DMSO treated controls. To assess A-674563 and CCT-128930 isoform specific inhibition of Akt phosphorylation two novel pAkt1 and pAkt2 antibodies were considered. The specificity of these antibodies was confirmed using Akt1 and Akt2 knockout lung tissue (Figure 12b).

Western blot imaging of A-674563 IC50 (118 nM) and IC80 (490 nM) treated A549 cells revealed a dose dependent increase of pAkt1 at the 0.5 and 6 hour mark, however the signal was decreased after 24 hours (Figure 13a). No changes were observed to total Akt1 levels. Additionally, IC80 treatment of A-674563 revealed an early increase in pAkt2, which was not present after 24 hours of treatment. IC50 (3.33 µM) and IC80 (9.8 µM) treatment of CCT-128930 revealed increases in pAkt2 at the 6 and 24 hour time point compared to the DMSO control, with decreasing total Akt2 levels during these time points (Figure 13b). CCT-128930 treatment also increased pAkt1 signal at 6 and 24 hours compared to the DMSO control. Interestingly, DMSO treatment had a time dependent reduction in both pAkt1 and pAkt2 signal.

Inhibitory effects on Akt downstream targets

The inhibitory effects of A-674563, CCT-128930, and MK-2206 were evaluated on three downstream targets; pGSK3β, pPRAS40, and pS6RP. A549 cells were treated with IC50 and IC80 concentrations of each inhibitor (Figure 14a). Densometric analysis for pGSK3β revealed
no significant changes compared to the DMSO control (Figure 14b). IC50 (3.33 µM) and IC80 (9.8 µM) treatments of CCT-128930 led to a 3.1 and 4.9 fold reduction in pPRAS40, respectively (Figure 14c). IC50 (1.16 µM) and IC80 (1.9 µM) treatments of MK-2206 reduced pPRAS40 signal more drastically by 16.0 and 13.5 fold, respectively. Additionally, only IC50 and IC80 treatment of CCT-128930 led to a significant reduction in pS6RP signal by 10.5 and 30.7 fold, respectively (Figure 14d). Phosphorylation status of these downstream targets were also evaluated with same dose 0.78 µM treatments at 0.5, 6, and 24 hour time points (Figure 15a). Similar to Figure 14, no significant changes were detected with pGSK3β status with any of the inhibitors (Figure 15b). Although no significant changes were revealed for pPRAS40 status, there were notable non-significant decreases in pPRAS40 signal with MK-2206 treatment at 0.5, 6, and 24 hour time points (Figure 15c). Interestingly, 24 hour treatment of A-674563 (0.78 µM) resulted in a significant 2.3 fold reduction in pS6RP signal.
Figure 3: Western blot analysis of whole cell lysates demonstrating baseline levels of total Akt1, Akt2, Akt3, and pAkt (S473) protein detected in untreated NBE, LA4, NCI-H358, and A549 cells. Akt1 and Akt2 were present in all lung cancer cell lines, but Akt3 was detected at only very low levels. Phosphorylation of Akt at S473 was detected in all cell lines. B-actin was used as the loading control. Blots are representative of two independent trials.
Figure 4: Cell survival curves of A: A549, B: NCI-H358, C: LA4, and D: NBE-135 cells treated with the Akt1 selective inhibitor A-674563 (A), Akt2 selective inhibitor CCT-128930 (CCT), or the pan-Akt inhibitor MK-2206 (MK). Cells were treated with increasing doses of inhibitors (0.2 µM to 50 µM) for 72 hours, and incubated with WST-1 reagent for 2 hours before being read on a plate reader. Readings were normalized to DMSO controls and background media readings were accounted for. Line graphs represent mean ± SEM of three independent trials, an (*) indicates difference to DMSO control, p< 0.05.
Table 1: Summary of IC20, IC50, and IC80 values determined from WST1 data from figure 4.

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Curves for A549, NCI-H358, LA4, and NBE-135 cells treated with either A-674563 (A), CCT-128930 (CCT), or MK-2206 (MK) were generated using CalcuSyn software. IC20, IC50 and IC80 values were determined using these curves. Values are presented as µM.
Figure 5: Summary of manual cell counting with Trypan Blue staining. A549 cells were treated with the IC50 and IC80 concentrations of **A**: Akt1 selective inhibitor A-674563 (A), **B**: Akt2 selective inhibitor CCT-128930 (CCT), or **C**: pan-Akt inhibitor MK-2206 (MK) and manually counted at 24, 48, and 72 hours using a hemocytometer. Cell counts are presented as cells/mL. **D**: Validation of IC50 and IC80 values generated from Calcusyn using cell counts from Trypan Blue staining. After 72h of treatment, manual cell counting demonstrated that the IC50 and IC80 concentrations of all three inhibitors resulted in a 50% or 80% reduction in cell counts, respectively. Bars represent mean cell counts ± SEM of three independent trials. Significant difference to DMSO control is represented by an asterisk (*), p<0.05. Dotted lines indicate 50% and 80% cell death.
Figure 6: Changes in proliferation using phospho-histone H3 (PHH3) as a marker. A549 cells were treated with A-674563 (A), CCT-128930 (CCT), or MK-2206 (MK) for A: 24 hours or B: 72 hours. Measurements of PHH3 were taken using an Accuri C6 flow cytometer. The same results were gathered for NCI-H358 cells at C: 24 hours and D: 72 hours. Bars represent mean fold changes in proportion of cells positive for PHH3 relative to the UT control ± SEM of three trials. Immuno-fluorescence microscopy was also used to analyze changes in PHH3. Overlay images of A549 cells treated with MK at E: 20x and F: 40x magnification. DAPI nuclear dye was used as a counter stain (shown in blue), while cells positive for PHH3 are shown in magenta. Images were taken using MetaMorph Imaging software. G: Cells were treated with inhibitors for 24 hours and ratio of positive cells over DAPI stained cells were determined using Image J software. Bars represent the mean percentage of cells positive for PHH3 ± SEM. Significant difference to DMSO control is represented by an asterisk (*), p<0.05.
Figure 7: Flow cytometry analysis of inhibitor dependant apoptotic changes in A549 cells using Annexin V as a marker. **A:** Dot plot of cells sorted into live, early apoptotic, late apoptotic, and necrotic quadrants with the Accuri C6 software. Cells were treated with IC50 and same concentrations of 0.78 \( \mu \text{M} \) of either A-674563 (A), CCT-128930 (CCT), or MK-2206 (MK) inhibitors for **B-D:** 24 hours or **E-G:** 72 hours. Cisplatin (50 \( \mu \text{M} \)) was used as a positive control. Fluorescence of Annexin V and Propidium Iodide markers were detected and measured by an Accuri C6 Flow Cytometer. Cells that were PI-/Annexin+ were considered to be early apoptotic, while cells that were PI+/Annexin+ were considered to be late apoptotic. Proportion of total apoptotic cells were considered to be all cells positive for Annexin V. Bars represent mean ± SEM of three trials. Significant difference to DMSO control is represented by an asterisk (*), \( p<0.05 \).
Figure 8: Flow cytometry analysis of inhibitor dependant apoptotic changes in NCI-H358 cells using Annexin V as a marker. Cells were treated with IC50 and same concentrations of 0.78 µM of either A-674563 (A), CCT-128930 (CCT), or MK-2206 (MK) inhibitors for A-C: 24 hours or D-F: 72 hours. Cisplatin (50 µM) was used as a positive control. Fluorescence of Annexin V and Propidium Iodide markers was detected and measured by an Accuri C6 Flow Cytometer. Cells that were PI-/Annexin+ were considered to be early apoptotic, while cells that were PI+/Annexin+ were considered to be late apoptotic. Proportion of total apoptotic cells were considered to be all cells positive for Annexin V. Bars represent mean ± SEM of three trials. Significant difference to DMSO control is represented by an asterisk (*), p<0.05.
Figure 9: Effects of inhibitor treatment on A549 cell migration through scratch wound assay analysis. A: Images of wounds at 0, 48, and 72 hours. Cells were treated with either A-674563 (A), CCT-128930 (CCT), or MK-2206 (MK) inhibitors, and images were taken using an inverted Olympus IX71 light microscope equipped with Q Imaging 3.3 software. Percentage of wound closure was determined using Image J software. Graphs shown are results of A549 cells treated with B: IC50 concentrations of each inhibitor and C: same concentration of 0.78 μM of each inhibitor. Bars represent mean wound closure percentage ± SEM of three independent trials. Significant difference to DMSO control is represented by an asterisk (*), p<0.05.
Figure 10: Effects of inhibitor treatment on NCI-H358 cell migration through scratch wound assay analysis. A: Images of wounds at 0, 48, and 72 hours. Cells were treated with either A-674563 (A), CCT-128930 (CCT), or MK-2206 (MK) inhibitors, and images were taken using an inverted Olympus IX71 light microscope equipped with Q Imaging 3.3 software. Percentage of wound closure was determined using Image J software. Graphs shown are results of NCI-H358 cells treated with B: IC50 concentrations of each inhibitor and C: same concentration of 0.78 µM of each inhibitor. Bars represent mean wound closure percentage ± SEM of three independent trials. Significant difference to DMSO control is represented by an asterisk (*), p<0.05.
Figure 11: Cell Cycle Profile analysis of BrdU incorporation and 7AAD staining of A549 cells. 
A: dot plot of cells organized into G1/G0 (red), S (green), and G2/M (blue) phases. B: cells were treated with IC50 or same concentrations of 0.78 µM of either A-674563 (A), CCT-128930 (CCT), or MK-2206 (MK) inhibitors for 72 hours. Cells were grouped and measured by an Accuri C6 Flow Cytometer. Bars represent mean ± SEM of three trials. Significant difference to DMSO control is represented by an asterisk (*), p<0.05.
Figure 12: **A**: Western blot of MK-2206 (MK) treated A549 cells evaluating inhibition of pAkt (S473). Cells were treated for 5 minutes, 1 hour, 6 hours, or 24 hours. Antibodies against pAkt (S473) and total Akt were used to detect changes in protein levels. **B**: western blot of WT, Akt1 knockout, or Akt2 knockout lung tissue demonstrating the specificity of the pAkt1 and pAkt2 antibodies. B-actin was used as the loading control, 30 µg of lysates were loaded in each well. Blots are representative of two independent trials. Abbreviations: UT indicates untreated, D indicates DMSO, and WT indicates wild type.
Figure 13: Western blots demonstrating assessment of A-674563 and CCT-128930 specificity. A: A-674563 treated A549 cells. Cells were treated for 30 minutes (early), 6 hours (mid), or 24 hours (late). Antibodies against pAkt1 (S473), total Akt1, pAkt2 (S474), and total Akt2 were used to detect changes in protein levels. B: similar western blot of CCT-128930 treated A549 cells. β-actin was used as the loading control, 30 µg of lysates were loaded in each well. Blots are representative of one trial. Abbreviations: A indicates A-674563, CCT indicates CCT-128930, UT indicates untreated, D indicates DMSO, 50 indicates IC50 concentration, and 80 indicates IC80 concentration.
Figure 14: Western blot analysis of downstream Akt targets of A549 cells treated with IC50 or IC80 concentrations of either A-674563 (A), CCT-128930 (CCT), or MK-2206 (MK). A: cells were treated for 24 hours and antibodies against pGSK3β (S9), pPRAS40 (T246), and pS6RP (S240/244) were used to detect changes in protein levels. Graphs demonstrate densometric analysis for B: pGSK3β, C: pPRAS40, and D: pS6RP. β-actin was used as the loading control and calibrator, 30 µg of lysates were loaded in each well. Bars represent mean ± SEM of three trials. Significant difference to DMSO control for the corresponding time point is represented by an asterisk (*), p<0.05.
Figure 15: Western blot analysis of downstream Akt targets of A549 cells treated with the same concentration (0.78 µM) of either A-674563 (Akt1), CCT-128930 (Akt2), or MK-2206 (pan-Akt inhibitor). A: cells were treated for 30 minutes (early), 6 hours (mid), or 24 hours (late). Antibodies against pGSK3β (S9), pPRAS40 (T246), and pS6RP (S240/244) were used to detect changes in protein levels. Graphs demonstrate densometric analysis for B: pGSK3β, C: pPRAS40, and D: pS6RP. B-actin was used as the loading control and calibrator, 30 µg of lysates were loaded in each well. Bars represent mean ± SEM of three trials. Significant difference to DMSO control for the corresponding time point is represented by an asterisk (*), p<0.05.
Akt is a key modulator of tumorigenesis and cancer promotion due to its involvement in vital cellular processes such as survival, proliferation, growth and metabolism (Brazil et al., 2004; Fayard et al., 2005; Song et al., 2005). It is known to be constitutively active in many NSCLC cases and has been associated with poor survival (Brognard et al., 2001; Tang et al., 2006; David et al., 2004). Many studies have confirmed the involvement of Akt in tumorigenesis, making Akt an attractive target for NSCLC treatment (Nicholson and Anderson, 2002; Lim et al., 2006; Yip et al., 2014). Although current clinical trials are evaluating the treatment potential of MK-2206, a pan-Akt inhibitor, recent evidence is suggesting that isoform specific roles of Akt exist. Understanding these roles and categorizing Akt isoforms corresponding to distinct cellular responses could lead to the development of more effective treatment options. Studies have shown that Akt1 plays more of a role in cell growth and survival, Akt2 is majorly responsible for metabolism, while Akt3 has implications in neuronal cell development (Yang et al., 2003; Cho et al., 2001b; Tshopp et al., 2005). Therefore, in this study we aimed to evaluate NSCLC cellular responses to isoform selective Akt inhibition through the use of three inhibitors; A-674563 (Akt1), CCT-128930 (Akt2), and MK-2206 (pan-Akt). MK-2206 is an allosteric inhibitor of all three isoforms of Akt, and has been shown to have antitumor activities as a single agent as well as in combination with other targeted therapies such as erlotinib (an epidermal growth factor inhibitor) or lapatinib (epidermal growth factor receptor inhibitor) (Lu et al., 2009; Hirai et al., 2010). CCT-128930 and A-674563 are both ATP competitive inhibitors, and function through a mechanism different from MK-2206 that is not yet completely understood. Nonetheless, CCT-128930 treatment has also been shown to be effective
in A549 human NSCLC cells causing a G1/G0 cell cycle arrest as well as increases in apoptotic and autophagy markers (Wang et al., 2014). A-674563, on the other hand, is a relatively novel inhibitor and not many studies have been conducted regarding its effects on NSCLC. However, Kekesi et al. (2013) did determine the IC50 concentration of A-674563 on PC9 NSCLC cells to be 0.9 µM, indicating the potential of this inhibitor to be used towards lung cancer treatment. In this study we did not evaluate Akt3 inhibition. Akt3 is highly expressed in certain tissues such as the brain and testes, but like Akt1 and Akt2, it is also detected in fat, lungs, and mammary glands (Yang et al., 2003). When evaluating baseline presence of Akt isoforms in our lung derived cell lines, we did not detect large levels of Akt3, and thus Akt3 was excluded from this study.

The assessment of cell survival of these inhibitors was performed on two characteristically different human NSCLC cell lines (A549 and NCI-H358), a murine NSCLC cell line (LA4) and a normal human lung cell line (NBE-135), all of which were available in our lab. A549 cells are p53 wild type, while NCI-H358 cells harbour a homozygous deletion of p53 (Lehman et al., 1991; Takahashi et al., 1989). The p53 tumor suppressor protein has an inhibitory influence on upstream regulation of Akt through increasing transcription of PTEN and IGF-BP1 (Feng et al., 2007; Buckbinder et al., 1995). Interestingly it is also indirectly regulated by Akt, as MdM2 E3 ubiquitin ligase acts as a mediator to degrade p53 (Gottlieb et al., 2002). Although Koseoglu et al. (2007) report that Akt knockdown in tumor cells by small interfering RNA was not affected by p53 status, evaluation of both human NSCLC cell lines with differing p53 status allows for assessment of a broader range of tumor profiles, and increases the validity of the findings.

Cell survival curves in Figure 4 demonstrate the increased susceptibility of the human NSCLC cells to the Akt1 selective inhibitor over the other two. The low IC50 values for A-
674563 are comparable to IC50 values found in other NSCLC cell lines (Kekesi et al., 2013). Since the IC50 value of the pan-Akt inhibitor was in between that of the Akt1 and Akt2 selective inhibitors in both cell lines, it is likely that inhibition of Akt1 alone is more effective in supressing human NSCLC cell survival. MK-2206 inhibits all isoforms of Akt but has a higher potency towards Akt1 than Akt2 (Yan, 2009). Due to this reason it is possible that the overall effects observed through MK-2206 are more a result of Akt1 inhibition than Akt2 inhibition. It may also be possible that Akt2 inhibition as a component of MK-2206 pan-Akt inhibition is dampening the effects on cell survival, while the Akt1 inhibition component is augmenting them, resulting in intermediate IC50 values. This is consistent with previous findings in our lab that Akt2 knockout in our inducible SPC-IGFIR transgenic mice led to enhanced tumour formation and invasion, while Akt1 knockout resulted in more nodular like tumor formation and less tumor burden. Similarly, low dose treatment of CCT-128930 on NCI-H358 cells seemed to enhance survival, but contrary to our in-vivo model, higher concentrations of CCT-128930 resulted in significant cell death. The discrepancy in in-vitro and in-vivo findings pertaining to CCT-128930 treatment may be attributable to the environment that these cells were exposed to, as monolayer cell culture is quite different from a physiological micro-tumor environment. For one, not all cells may be exposed to treatment in a micro-tumor setting, whereas almost all cells are constantly exposed to treatment in monolayer cell culture. Moreover, the inhibitors may not be degraded as quickly in cell culture, due to the absence of differentiated cells and organs involved in clearing toxic substances.

Interestingly, the murine LA4 cells responded most effectively to MK-2206 treatment, indicating that targeting all isoforms of Akt may be a more effective strategy in some cell lines. Normal human NBE-135 cells were also significantly affected by all of the Akt inhibitors,
especially A-674563 since the IC50 value was very small (600 nM). However, NBE-135 cells expressed high levels of Akt1, and not nearly as much Akt2 as the other cell lines, which could explain the higher susceptibility to Akt1 inhibition (see Figure 3). Additionally NBE-135 cells showed very high levels of phospho-Akt, and thus it was determined that these cells may not be the best normal control for this study.

Cell survival was evaluated using the WST-1 colorimetric assay, which involves the conversion of a tetrazolium salt to formazan by mitochondrial dehydrogenases (Riss et al., 2015). Although it is widely used as a cell viability assay, it does technically detect metabolically active cells and thus metabolism may influence readings. Due to this reason we sought to validate the IC50 and IC80 concentrations obtained from the CalcuSyn program through Trypan Blue staining and manual counting of live cells. Figure 5 demonstrates that the corresponding IC50 and IC80 concentrations of each inhibitor in A549 cells did indeed result in 50% or 80% cell death through manual counting. Thereby supporting our experimental dosages through two techniques. However, due to the large discrepancy in A-67463 and CCT-128930 IC50 values, we also decided to incorporate same concentration analysis of each inhibitor; 0.78 μM. This concentration was chosen because it was the lowest concentration point in both human NSCLC cell lines where some effect of A-674563 was noticed on cell survival, with minimal effect from CCT-128930. Since all three inhibitors had a significant reduction in cell survival, it was expected that we would see an increase in apoptosis or a decrease in proliferation. For this we selected markers pertaining to proliferation and apoptosis, and evaluated changes with inhibitor treatment through flow cytometry.

Phospho-histone H3 S10 (PHH3) is a well-used marker of proliferation in many assays and has been shown to fluoresce in A549 cells (Duan et al., 2008). Histone H3 is a subunit of the 8
core histone complex and gets phosphorylated at S10 during chromosome condensation and cell division (Preuss et al., 2003). Our analysis of changes to proliferation with inhibitor treatment was not as expected. Treatment of both A549 and NCI-H358 cells with CCT-128930 or MK-2206 did not result in significant changes to percentage of cells positive for PHH3 with any treatment. Even more interesting was that instead of decreasing proliferation, 24 hour treatment of IC50 A-674563 in A549 cells (118 nM) resulted in a significant 1.7 fold increase in PHH3 detection. Furthermore, a higher 0.78 µM concentration of A-674563 resulted in a larger but non-significant increase of 2.1 fold. This increase in PHH3 detection was also confirmed by immunofluorescence (Figure 6g). However, there were no changes to proportions of cells positive for PHH3 at 72 hours of treatment. One explanation to these findings could be that the cells were trying to escape A-674563 treatment by compensating and increasing their rate of proliferation. This increase was only observed at 24 hours of treatment and not at 72 hours, indicating initial attempts at increasing proliferation and an eventual decrease. It is important to note that, the analysis may not be ideal as very low percentages of PHH3 positive cells were detected through both techniques (approximately 2-4%). Chang et al. (2015) reported that most A549 cells exit mitosis within 3 hours, which could explain the low counts. Additionally, we noticed that the PHH3 antibody worked more effectively in the murine LA4 cells reaching positive percentages of up to 15%, indicating that it has decreased sensitivity towards human samples (data not shown). Therefore, PHH3 may not have been the best marker to evaluate proliferation in this case. Another proliferative marker that could have been used is Ki-67, which is actively expressed throughout the cell cycle, but is relocated to the surface of chromosomes during mitosis (Scholzen and Gerdes, 2000). Ki-67 quantification has been used in our lab in the
past, however it is considered to be less specific than PHH3. Perhaps, if time permitted, a combined analysis of both markers could assist in interpretation of these findings.

In terms of changes to apoptosis, the marker that was selected was Annexin V coupled with propidium iodide (PI). Annexin V is a calcium dependent phospholipid binding protein that has affinity for phosphatidylserine (PS). Normally phosphatidylserine (PS) residues are hidden within the plasma membrane, however they are shifted to the outer leaflet during early apoptotic events within the cell. Annexin V is then able to bind the extracellular PS residues, and when labelled with a fluorochrome can be detected (Walton et al., 1997). PI is fluorescent DNA staining dye that cannot permeate live cell membranes. However, the cellular membrane becomes compromised during apoptosis and PI is able to enter and stain these cells. With flow cytometry, cells can be grouped into live (Annexin V- / PI-), early apoptotic (Annexin V+ / PI-), late apoptotic (Annexin V+ / PI+), or dead/necrotic cells (Annexin V- / PI+).

In A549 cells, we observed a significant increase in total apoptosis with 24 hour IC50 treatment of only MK-2206 (1.16 µM). Indicating that MK-2206 may be the most effective at increasing Annexin V detection when using concentrations required to kill 50% of the cells. Since the IC50 concentration of MK-2206 for A549 cells (1.16 µM) was approximately 10 fold greater than the IC50 concentration of A-674563, and no increases were observed with CCT-128930 at any concentration, it is possible that the increase in apoptosis observed with MK-2206 was attributable to Akt1 inhibition as a result of pAkt-inhibition. However, it is important to note that the calculated IC50 values were based on a 72 hour timeline, so the 24 hour IC50 comparison of each inhibitor may not provide much insight on the apoptotic inducing abilities of these inhibitors. More important is the comparison of same concentration (0.78 µM) treatment of each inhibitor, with which only A-674563 resulted in a significant increase at both 24 and 72
hours. Even when comparing IC50 treatment of MK-2206 (1.16 µM) and lower dose 0.78 µM treatment of A-674563, the increases in proportion of apoptotic cells were similar. Taken together, these results indicate that Akt1 inhibition by A-674563 in A549 cells is more effective at increasing A549 cellular apoptosis than either Akt2 or pan-Akt inhibition.

The results from apoptosis assessments in NCI-H358 cells followed a different trend, but still support the same claim. None of the treatments at any time point resulted in significant increases in total apoptosis, however IC50 treatment of CCT-128930 did result in a significant 2 fold decrease at 24 hours and a notable non-significant decrease at 72 hours. This indicates that Akt2 inhibition may have protective effects on NCI-H358 cells by decreasing the proportion of apoptosis, which still supports the notion that Akt1 inhibition is more effective at increasing apoptosis human NSCLC cells than Akt2 inhibition. Contrary to our findings, Lee et al. (2011), reported that siRNA knockdown of Akt2 in NCI-H460 NSCLC cells resulted in increased apoptosis through cleaved caspase 3 expression. However, they used different apoptotic markers for the evaluation of Akt1 knockdown and did not consistently compare the roles of Akt1 and Akt2 in apoptosis. Interestingly, we also tried to evaluate changes in apoptosis through cleaved caspase 3. Unfortunately we could not detect cleaved caspase 3 through immunofluorescence or western blotting with the antibodies available in our lab.

This discussion only interpreted the results of total apoptosis. The analysis for early and late apoptotic cells may not provide much insight on the effects of these inhibitors as the cells were grouped based on unspecific markers, and not markers that can differentiate between cells undergoing apoptosis slowly or quickly. Moreover, many of the late apoptotic, non-adhering cells could have been washed away prior to cell trypsinization and collection. A more appropriate analysis would involve the growth media that may contain many late apoptotic cells.
Cell migration rates were assessed through scratch wound analysis, which is a time and cost efficient method. A scratch is performed across an area of cells, clearing the path for cells to migrate into. As cells compete for space within the culture dish, they move towards the free space and fill in the wound. A549 scratch wound analysis revealed significant reductions in migration with IC50 treatment of all three inhibitors. However, 0.78 µM treatments only resulted in significant reductions with A-674563 and CCT-128930 of approximately 60% and 18%, respectively. In both treatment groups Akt1 inhibition was more effective at decreasing cell migration than Akt2 inhibition. A similar trend was observed with NCI-H358 cells. These results support the notion that both Akt1 and Akt2 isoforms have a role in cell migration. Chin and Toker (2010) describe that many migratory proteins have differential regulatory sites that are associated with Akt, suggesting isoform specific Akt regulation. For example, Akt1 can directly inhibit palladin, an actin binding protein, while Akt2 interaction is associated with increased motility (Chin and Toker, 2010a; Chin and Toker, 2010b). Furthermore, Akt isoforms can inhibit cell migration in one tissue type while promoting it in another (Xue and Hemmings 2013). Down-regulation of Akt1 has been shown to promote EMT and migration in mammary epithelial cells, and inhibit it in colon cancer cells (Irie et al., 2005; Ericson et al., 2010). Therefore, determining the roles of Akt isoforms in migrational changes must involve the assessment of individual tissue types and proteins.

While the scratch would assay is effectively able to measure cell migration across a wounded area, it lacks the capacity to accurately measure cellular invasion. Cell invasion is a complicated process which involves, EMT transition, secretion of multiple proteases and constant re-organization of the extra cellular matrix and adherent proteins. Monolayer cell culture does not provide a comparable environment to which a cell might be exposed to physiologically. If time
permitted, a more accurate assessment of cell invasion could be performed by measuring the movement of cells through semi solid Matrigel in a Boyden chamber. Another issue with the scratch wound assay is that it does not control for cellular proliferation and movement of the cell front through cell division. One way to rectify this issue is to follow the movement of individual cells through live imaging. JuLI™ Br is a live cell movie analyzer developed by NanoEnTek. It is small enough to fit in a standard incubator, and records cell migration under normal cell culture conditions. A recent video posted by their research lab in Korea demonstrated movement of A549 cells across a scratch wound (NanoEnTek 2012). They determined the time of A549 wound closure to be 68 hours, which is similar to our determined time of 72 hours.

Flow cytometry can also be used to group cells into the cell cycle phases when analyzing bromodeoxyuridine (BrdU) coupled with 7AAD. BrdU is a synthetic analogue of thymidine, and is incorporated into the DNA strand during the synthesis phase. Like PI, 7-AAD is a DNA dye that can be used to detect quantity of DNA. However, in this experiment 7-AAD was able to stain all of the cells because they were permeabilized. The flow cytometer is able to sort cells into two distinct populations with either one (non-replicating) or two sets of chromosomes (replicating). Based on BrdU incorporation and chromosome quantity cells can be distinguished into G1/G0, S, or G2/M phases (Figure 11a). Cell cycle analysis of A549 cells treated with corresponding IC50 concentrations of each inhibitor for 72 hours revealed a significant decrease in proportion of cells in the G1/G0 phase with A-674563 and CCT-128930 treatment. Akt is an upregulator of cyclin D activity through the inhibition of GSK3β, and we did not expect to see a decrease in the G1/G0 phase with Akt inhibition. Contrary to our findings, Yun et al. (2009) noticed a G1/G0 block with Akt1 ablation in MEF cells and not with Akt2 ablation, while Santi and Lee (2011) noticed a G1/G0 block with Akt2 siRNA knockdown and not Akt1 knockdown
in MDA-MB231 breast cancer cells. Interestingly, both studies are in disagreement about which Akt isoform is independently able to promote cell cycle progression, demonstrating the inconsistencies in determining isoform specific roles of Akt in cell cycle progression.

Within the similar 0.78 µM treatment of each inhibitor, A-674563 treatment led to an increased proportion of cells in the G2/M phase. The lower dose data of A549 (IC50, 118 nM) indicates that Akt1 inhibition is promoting cells through the G1/G0 phase, while the higher dose data (0.78 µM) indicates either a G2/M block in the cell cycle or an increase in proportion of cells undergoing mitosis. Akt is involved in G2/M cell cycle progression through upregulating activity of cyclin B. Therefore inhibition of Akt, would decrease the levels of cyclin B and subsequent progression through mitosis. Therefore, it is likely that these results indicate a potential G2/M phase arrest with A-674563 treatment. Although conclusions are hard to draw from this data, it is evident that Akt1 and possibly Akt2 play a role in regulating the cell cycle, however more experiments need to be performed to determine if/where isoform specific Akt regulations of the cell cycle exist.

After analysing the anti-tumorigenic properties of the inhibitors, we sought to evaluate the capabilities of these inhibitors to inhibit Akt activity. Phosphorylation of the S473 residue on Akt was used as an indicator of total Akt activity. In order to evaluate full activation of Akt we also attempted to evaluate T308 phosphorylation through western blotting (not shown), but the antibody had a very faint signal. Western blot analysis of MK-2206 treated A549 cells revealed ablation of pAkt (S473) signal within 5 minutes of treatment, which lasted 24 hours post treatment. Figure 12 demonstrates the potent ability of MK-2206 to inhibit phosphorylation of Akt while not affecting total Akt levels. From these findings we sought to determine A-674563 and CCT-128930 specificity on Akt1 and Akt2 inhibition using three time points; 0.5 hour
(early), 6 hour (mid), and 24 hour (late). As described earlier, A-674563 and CCT-128930 are both considered to be ATP competitive inhibitors of Akt (Okuzumi et al., 2009). Although the mechanism of action is not completely understood, multiple studies have reported seeing increases in pAkt status with treatment (Yap et al., 2011b; Zhu et al., 2008). Some researchers believe that this is attributable to the inhibitor binding directly to the ATP binding site, which can alter the affinity of the S473 and T308 amino acids to get phosphorylated, or due to compensatory but futile feedback loops to increase Akt activity. We also noticed an increase in pAkt1 with A-674563 treatment up until 24 hours, but also observed an increase in pAkt2. With CCT-128930 treatment pAkt2 levels remained elevated, however we noticed a similar trend with elevated pAkt1 up until 24 hours of treatment, after which it began to decrease. Interestingly, as seen in figure 13, DMSO might be having a detrimental effect on pAkt1 and pAkt2 signal. If that is the case, then it’s quite possible that both A-674563 and CCT-128930 are countering the dampening of signal by DMSO, and are inhibiting pAkt1/pAkt2 to a larger extent than observed (if an increase in pAkt1 or pAkt2 is reflective of inhibition). Before drawing any conclusions it is important to note that these westerns were only performed once, but it is possible that these Akt isoform selective inhibitors are not completely specific towards their corresponding isoform. Unfortunately no other researchers have validated the specificity of A-674563 towards Akt1 or CCT-128930 towards Akt2. Ultimately, more experiments need to be conducted before drawing any conclusions on the specificity of these inhibitors.

Since it was difficult to evaluate the specificity of the inhibitors, we decided to look at the phosphorylation status of molecules downstream of Akt. Yap et al. (2011) evaluated the effects of CCT-128930 treatment on downstream targets of Akt in U87MG glioblastoma and other human cancer cell lines. They observed reductions in pGSK3β (Ser9), pPRAS40 (T246), and
pS6RP (S240/244) among others, without seeing changes to levels of their total non-phosphorylated counterparts. Similarly, Luo et al. (2005) saw reductions in pGSK3 and pS6RP in MiaPaCa-2 human pancreatic cancer cells with A-674563 treatment, which was consistent with data from Zhu et al. (2011). For these reasons, we sought to evaluate phosphorylation status of these three molecules with our IC50 concentrations. GSK3β is a direct target of Akt and is involved in regulating metabolism through glycogen synthesis and cell cycle progression through cyclin D1 and inhibition of p21-cip (Hemmings and Rustoccia, 2010). PRAS40 is also a direct target of Akt and has inhibitory effects on protein translation (Wang et al., 2010). Downstream of PRAS40 is S6RP (indirect target of Akt), which is responsible for increasing the formation of ribosomes and promoting protein synthesis (Wullschleger et al., 2006). Our evaluation of these targets when treated with IC50 concentrations of each inhibitor only resulted in reductions to pPRAS40 with CCT-128930 and MK-2206 treatment, and pS6RP only with CCT-128930. This discrepancy in findings may largely be due to the concentrations used to treat cells. Yap et al. (2011) treated their cells with 18.2 µM of CCT-128930 while Luo et al. (2005) used A-674563 concentrations greater than 10 µM. Nonetheless, these findings support the functionality of these inhibitors as phosphorylation of multiple downstream targets were decreased.
SUMMARY & CONCLUSIONS

In this study we evaluated the effects of isoform specific inhibition of Akt in two human NSCLC cell lines; A549 and NCI-H358. Tumorigenic properties such as cell survival, proliferation, apoptosis, migration and cell cycle profile were assessed when cells were treated with A-674563 (Akt1 selective inhibitor), CCT-128930 (Akt2 selective inhibitor), or MK-2206 (pan-Akt inhibitor). Results demonstrate that all inhibitors effectively reduced cell survival, however the Akt1 inhibitor was by far the most effective in both cell lines, followed by the pan-Akt inhibitor. Significant increases in A549 apoptosis were observed with 0.78 µM treatment of A-674563 and 1.16 µM MK-2206, while high concentration treatment of CCT-128930 resulted in decreased apoptosis in NCI-H358 cells. Proliferation assessment of A549 cells revealed an increase in PHH3 proliferative marker when treated with A-674563, which may be explained by compensatory mechanisms to combat the increase in apoptosis. Furthermore, cell cycle analysis indicted a possibility of G2/M phase block with A-674563 treatment, which may also help to explain the increases seen in PHH3. Both Akt1 and Akt2 inhibitors effectively reduced migration rates of both cell lines, however A-674563 was more effective at slowing cell motility. Taken together, these results indicate that Akt1 inhibition may be more effective in treating NSCLC than Akt2 or pan-Akt inhibition. Currently MK-2206 is undergoing phase two clinical trials to combat NSCLC, however side effects pertaining specifically to Akt2 and insulin regulation have been reported. Akt1 inhibition may prove to be just as effective as MK-2206 in treating NSCLC, if not more, and may potentially reduce side effects of treatment.
Study Limitations

One of the prominent study limitations is the inability to validate the specificities of the Akt1 and Akt2 selective inhibitors. So far, multiple studies have compared these inhibitors to other kinases, yet no one has evaluated the specificity of these inhibitors towards their corresponding Akt isoforms (Luo et al., 2005; Yap et al., 2011). Without this validation it is difficult to draw conclusions from these data sets. Furthermore, as previously outlined, multiple markers could have been used to confirm the changes to cell proliferation with inhibitor treatment. This coupled with the cell cycle analysis would provide more a more thorough understanding of the effects of Akt1 and Akt2 inhibition in promoting cell division. Also, instead of the scratch wound analysis, other assays could be performed to more accurately measure cell migration and invasion such as the use of a Boyden chamber. The Boyden chamber involves the migration of cells through a semi-solid medium, which more closely resembles an in-vivo environment than monolayer cell culture. Finally, with validation of our cell lines about a month ago, we received some disheartening news that our NCI-H358 cells were actually A549 cells. Therefore all experiments involving the NCI-H358 cells had to be repeated, providing less time for other experiments.

Future Directions

In the future there is a potential for more effective treatment of NSCLC with Akt1 inhibition alone, rather than inhibiting all three isoforms. An interesting project to explore would be to study these inhibitors in an in-vivo model. Currently, our lab is in the early phases of evaluating the effects of A-674563 and MK-2206 on NSCLC tumorigenesis of doxycycline
inducible lung tumor forming mice. Additionally, it would be useful to assess the phosphorylation status of more downstream targets of Akt. For example, since we did not notice a change in pGSK3β, while other studies have, it could be useful to look at cyclin D1 levels. Cyclin D1 is involved in S phase entry, and is directly inhibited by active (un-phosphorylated) GSK3β through down regulation of mRNA and increased degradation (Takahashi et al., 2008). With Akt inhibition we would expect to see a decrease in cyclin D1 levels, which would be indicative of decreased Akt activity. Additionally, through isoform specific inhibition of Akt and studying individual downstream molecules, it would be possible to determine targets that are independently regulated by each isoform. This would be a great tool to study individual Akt isoforms.
REFERENCES


APPENDIX I - RECIPES FOR REAGENTS

Phosphate Buffered Saline (PBS) (2L)
Sodium Chloride 16.0 g
Potassium Chloride 0.4 g
Sodium Potassium Dibasic Anhydrous 2.3 g
Potassium Phosphate Monobasic 0.4 g
pH to 7.4 with HCL

*Immunofluorescence*

**Permeabilizing Solution (50 mL)**
Triton X-100 100 µL
PBS 50 mL

**Blocking Solution (50 mL)**
BSA 2.5 g
Triton X-100 50 µL
PBS 50 mL

**Antibody Diluting Solution (50 mL)**
BSA 0.5 g
Triton X-100 100 µL
PBS 50 mL

*Flow Cytometry (Single Staining)*

**Incubation Buffer (50 mL)**
BSA 0.25 g
PBS 50 mL

**Permeabilizing Solution (50 mL)**
Triton X-100 250 µL
PBS

**Antibody Diluting Solution (50 mL)**

- BSA 0.25 g
- Triton X-100 50 µL
- PBS 50 mL

*Western Blotting*

**RIPA Lysis Buffer (500 mL)**

- 10 mM Tris-HCL pH 7.6 0.788 g
- 5 mM EDTA 0.7306 g
- 50 mM NaCl 1.461 g
- 30 mM Tetrasodium pyrophosphate 3.988 g
- 1% Triton X-100 5 ml
- H2O to 500 ml

*10 µL of each vial listed below was added to each mL of lysis buffer prior to lysing protein*

Phosphatase Inhibitor Cocktail A (Biotool, Burlington, ON) containing: NaF, NaV, NaT, NaM, and Imidazole

Phosphatase Inhibitor Cocktail B (Biotool, Burlington, ON) containing: (-)-p-Bromotetramisole oxalate, Cantharidin, and Microcystin LR, Microcystis aeruginosa

Protease Inhibitor Cocktail (EDTA-free) (Biotool, Burlington, ON) containing: AEBSF, Aprotinin, Bestatin, E-64, Leupeptin, and Pepstatin A

**Reducing Buffer (6X, 10 mL)**

- SDS 1.2 g
- Glycerol 4.7 mL
- 0.5M Tris-HCL pH 6.8 1.2 ml
- Bromophenol Blue 0.006 g
- DTT 0.93 g
**Running Buffer (5X, 1L)**

Tris 15.1 g  
Glycine 72.1 g  
10 % SDS 10 ml  
RO H2O to 1 L  

**Transfer Buffer (1X, 1L)**

Tris 7 g  
Glycine 3.02 g  
Methanol 200 ml  
H2O to 1 L  

**Tris (1.0M, 100 mL)**

Tris Base 12.12 g  
H2O to 100 ml  
pH to 6.8 with HCL  

**Tris (1.5M, 100 mL)**

Tris base 18.16 g  
H2O to 100 ml  
pH to 8.8 with HCL  

**Tris Buffered Saline (TBS) (10X, 1L)**

Tris 24.2 g  
NaCl 80 g  
H2O to 1 L  
pH to 7.4 with HCl
### Tris Buffered Saline-Tween 20 (TBST) (1X, 1L)

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<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBS 10X</td>
<td>100 mL</td>
</tr>
<tr>
<td>RO H2O</td>
<td>899 mL</td>
</tr>
<tr>
<td>Tween-20</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

### Tris-Glycine SDS-Polyacrylamide Gels (12%) for 1 gel

#### Resolving Gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>4 ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% Ammonium Persulfate</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

#### Stacking Gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>2.1 µl</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>500 µl</td>
</tr>
<tr>
<td>1.5 M Tris (pH 6.8)</td>
<td>380 µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>30 µl</td>
</tr>
<tr>
<td>10% Ammonium Persulfate</td>
<td>30 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>3 µl</td>
</tr>
</tbody>
</table>

### Blocking Solution (50 mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaporated Skim Milk</td>
<td>2.5 g</td>
</tr>
<tr>
<td>TBST</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

### Antibody Diluting Solution (50 mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>2.5 g</td>
</tr>
</tbody>
</table>
TBST        50 mL
APPENDIX II - SOURCE OF MATERIALS

Abcam Inc, Cambridge, Massachusetts, USA
Amersham Pharmacia Biotech, Buckinghamshire, UK
BD Biosciences, San Jose, California, USA
Bio-Rad, Mississauga, Ontario, CA
Biosoft, Cambridge, United Kingdom
Bio-tek Instruments Inc., Winooski, Vermont, USA
Biotool, Burlington, Ontario, CA
Cell Signalling Technology, Beverly, Massachusetts, USA
eBioscience San Diego, California, USA
Life Technologies, Burlington, Ontario, CA
Molecular Devices, Sunnyvale, California, USA
National Institute of Health, Maryland, USA
Olympus, Tokyo, JPN
Q Imaging, Surrey, British Columbia, CA
Roche Mississauga, Ontario, CA
Santa Cruz Technologies, Santa Cruz, Massachusetts, USA
Sellekchem Burlington, Ontario, CA
Sigma Aldrich, Oakville, Ontario, CA