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Applying MCI-062, A Novel Pan-RAS Inhibitor, To Treat KRAS-Mutant Lung Cancer

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THE UNIVERSITY OF SOUTH ALABAMA
COLLEGE OF ALLIED HEALTH PROFESSIONS

APPLYING MCI-062, A NOVEL PAN-RAS INHIBITOR, TO TREAT *KRAS*-MUTANT LUNG CANCER

BY

Richard Fu

A Thesis

Submitted to the Honors College of the
University of South Alabama
in partial fulfillment of the
requirements for the degree of

Bachelor of Science

in

Biomedical Sciences

May 2022

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LIST OF ABBREVIATIONS

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ERK: extracellular signal-related kinase

FBS RPMI: fetal bovine serum supplemented Roswell Park Memorial Institute media

GAP: GTPase-activating protein

GEF: guanine nucleotide exchange factor

GDP: guanosine-5'-diphosphate

GTP: guanosine-5'-triphosphate

KRAS: Kirsten rat sarcoma virus

PD-1: programmed cell death protein 1

PD-L1: programmed death-ligand 1

RAF: rapidly accelerated fibrosarcoma

RAS: rat sarcoma virus

MAPK: mitogen-activated protein kinase

MAPK/ERK pathway: RAS-RAF-MEK-ERK pathway

MEK: mitogen-activated extracellular signal-related kinase

NSCLC: non-small cell lung cancer

PI3K/AKT/mTOR pathway: phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB, or AKT)/mammalian target of rapamycin (mTOR) pathway

ABSTRACT

Fu, Richard, B. S., University of South Alabama, May 2022. Applying MCI-062, a Novel Pan-RAS Inhibitor, to Treat *KRAS*-Mutant Lung Cancer. Chair of Thesis Committee: Gary Piazza, Ph.D.

RAS is a prevalent oncogene that is mutated in 27% of human cancers. Gain-of-function *RAS* mutations activate multiple downstream pathways, including the RAS-RAF-MEK-ERK and PI3K/AKT/mTOR pathways, which are critical in tumorigenesis and cancer cell proliferation. RAS proteins such as KRAS, a member of the RAS protein family, and their downstream effectors are attractive targets for cancer therapy since their mutations act as frequent drivers in lung, colorectal, and pancreatic cancers. However, RAS proteins have relatively smooth surfaces that lack traditional binding pockets, making inhibitors specific to RAS difficult to create. Recently, a novel small molecule pan-RAS inhibitor named MCI-062 was developed in Dr. Gary Piazza's Drug Discovery Research Center at the Mitchell Cancer Institute. As a potential pan-RAS inhibitor, MCI-062 is hypothesized to serve as a targeted therapy for *RAS*-mutant cancers regardless of mutation isoform, including all types of *KRAS*-mutant lung cancers. The inhibitory effects of MCI-062 were tested on the growth and proliferation of two non-small cell lung cancer cell lines, A549 and H358, using colony formation assays. The cells were plated onto 12-well plates, treated with varying concentrations of MCI-062, and then digitally imaged and analyzed. A549 cells have a $KRAS^{G13D}$ mutation, while H358 cells have a $KRAS^{G12C}$ mutation. The results indicate that MCI-062 effectively suppresses the growth and proliferation of both A549 and H358 cells despite their differing mutation isoforms, suggesting that MCI-062 successfully functions as a pan-RAS inhibitor.

CHAPTER I

INTRODUCTION

1.1 Cancer Statistics

As the second leading cause of death in the United States (US), cancer is a major public health issue. Each year, the American Cancer Society (ACS) reports on the most recent facts about cancer in the US (1, 2). The cancer death rate hit its peak in 1991, with 215 out of every 100,000 people dying from cancer. Since then, the death rate from cancer in the US has declined, as from 1991 to 2018 the cancer death rate fell by 31% (1-3). In regard to cancer-related mortality, lung cancer resulted in the highest mortality rate across all types of cancer worldwide, accounting for one-quarter of all cancer-related deaths for cancer patients (1).

Cancer is typically labeled in stages from I to IV, with IV being the most serious (1, 3, 4). Stage I cancer is a small, one-area disease that is also called early-stage cancer. In stages II and III, tumors are characterized as larger, reaching out to nearby tissues or lymph nodes. In stage IV, the cancer has spread to other parts of the patient's body, which is called an advanced or metastatic cancer (1, 3, 4). The types of treatment administered depend on the type and the stage of cancer (4). An early-stage cancer patient may only need one treatment, such as surgery, while an advanced-stage cancer may need a combination of

treatments, such as surgery combined with chemotherapy and/or radiation therapy (1, 4, 5). Immunotherapy, targeted therapy, or hormone therapy are recently-developed treatments that may be used for a special population of cancer patients (6-8).

1.2 Carcinogenesis

Carcinogenesis, also called tumorigenesis or oncogenesis, is the formation of a cancer, when normal cells are transformed into cancer cells (1, 9). The process is characterized by changes at the cellular, genetic, and/or epigenetic levels that drive abnormal cell division (9). Universally recognized characteristics of cancer are unlimited growth, evasion of immune surveillance, nonfunctional growth suppressors, sustained angiogenesis, and the possibilities of tissue invasion and metastasis (1, 9).

Carcinogenesis is a complex and dynamic process consisting of three stages: initiation, progression, and metastasis (9, 10). According to the clonal theory of carcinogenesis, tumors start from a single mutated cell (11). After the initiation of cancer, this mutated cell begins to divide and spread throughout the organ in which it manifested, as mutated cells ignore growth inhibition and apoptotic signals. Finally, once the cancer has grown large enough, individual tumor cells can break away from the main tumor and enter the bloodstream or lymphatic system, and these cells eventually reach different parts of the body and cause cancer metastasis.

Multiple studies have demonstrated that at a molecular level, carcinogenesis is initiated by the activation of oncogenes and inhibition of tumor suppressor genes (9, 10).

Oncogenes are regulatory factors of carcinogenesis that, once mutated, drive the growth and proliferation of cancer cells (11-13). Chromosomal translocation, point mutations, and gene amplification lead to oncogene activation (11). Furthermore, tumor-suppressor genes block the growth of cancer and contribute to the normal development of cells. The loss of function of tumor suppressor genes leads to inhibition of apoptosis, or programmed cell death, and thus to cell immortality, which in turn promotes carcinogenesis (11). Angiogenic factors, such as VEGF, play a key role in solid tumor formation and development by regulating the formation of new tumor blood supply (angiogenesis) (11). Since carcinogenesis cannot be initiated by a single mutation, oncogene and tumor suppressor gene mutations work together synergistically to induce carcinogenesis. Understanding these signaling pathways may provide a basis for better anticancer approaches.

The protein products of oncogenes typically represent components of signal transduction pathways, including growth factors, growth factors receptors, GTPases, protein kinases, or transcription factors (12). Oncogenic activation leads to carcinogenesis by regulating fundamental cellular processes, such as growth control, metabolism, proliferation, and differentiation, through constitutive activation of mitogenic signaling pathways (12). Finally, cancer is formed by uncontrolled growth of malignant cancer cells.

1.3 Development of Cancer Treatments

Recent cancer research has led to significant advances in therapies used to inhibit and treat cancer cell growth (1, 3, 4, 6-8). Cancer survival has improved since the mid-1970s, which partially reflects lead time bias because of changes in early diagnosis and treatment protocols, including the discovery of targeted therapies (3, 7).

The most commonly-used treatments for cancer are surgery, chemotherapy, and radiation therapy (1-3). Other treatment options include targeted therapy, immunotherapy, and hormonal therapy (6, 7). In recent years, biotechnological investigations have identified complex and unique biological features associated with carcinogenesis (6, 7). Tumor cell profiling and immune marker analyses are used to identify and optimize anticancer therapies in individual cancer patients (6, 7). These targeted cancer therapies are also known as precision medicines, which are a form of treatment that uses the individual profile of a person's genes and proteins (14). The rapidly expanding knowledge on the roles of genomics and the immune system in cancer development has enabled the development of therapies targeted to specific molecular alterations and immune suppression (14). Targeted cancer therapies differ from standard chemotherapy and radiation therapy in several ways (15). For example, while most traditional cytotoxic chemotherapy agents act on all rapidly-dividing cells in the body, targeted therapies aim to act only on the specific molecular targets associated with cancer cells (9, 14).

Certain mutant genes and proteins contribute to cancer growth and survival both in the actual tumor cells, such as *RAS* genes, and in cells related to cancer growth, such as

the *VEGF* gene for blood vessel cells (15, 16, 17, 18). Doctors often use targeted therapy in combination with chemotherapy and other treatments (4, 8). The United States Food and Drug Administration (FDA) has approved targeted therapies for many types of cancer (6, 7). As shown in **Table 1**, there are two types of targeted therapies: 1) monoclonal antibodies, which block a specific target protein on the outside of cancer cells and/or protein in the tumor microenvironment to directly or indirectly inhibit the growth of cancer cells; and 2) small-molecule drugs, which act intra cellularly on the molecule controlling cancer cells' growth and spread (6, 7, 11).

TABLE 1 Most frequently targeted proteins for cancer treatment

Target protein	Gene alteration	Cancer
Estrogen receptor	Overexpression	Breast
Androgen receptor	Overexpression	Prostate
HER2	Amplification/ overexpression	Breast, gastro-esophageal
EGFR	Mutation/ overexpression[a]	NSCLC, CRC[a]
CDK 4/6	Overexpression	Breast
PARP1	Overexpression	Breast, ovarian,
ALK	Fusion	NSCLC
BRAF	Mutation	Melanoma, NSCLC
NTRK	Fusion	Multiple (tumors with NTRK fusions)
PD-1/PD-L1	Overexpression	Multiple

NSCLC, non-small cell lung cancer. CRC, colorectal cancer.

Mutant EGFR is targeted in NSCLC. EGFR overexpression is targeted in CRC.

Adapted from (17) Duffy MJ. *Int. J. Cancer*. 2021;148:8–17.

1.4 *RAS* Oncogene and RAS Proteins

The RAS oncogene has been extensively studied for over three decades (12, 23, 24). The function of RAS proteins in cancer involves controlling cellular proliferation and differentiation (18, 25). The three *RAS* oncogenes, *HRAS, KRAS,* and *NRAS,* were the first to be discovered and identified and are the most frequently mutated genes in human cancer (17, 18, 25). The RAS proteins contain a G-domain, C-terminal domain, and C-terminal CAAX box as three major domains (26). The G-domain contains the guanine nucleotide binding site and, through interaction with a guanine nucleotide exchange factor (GEF), is responsible for GDP-GTP exchange acting as a "molecular switch" between the GDP-bound (inactive) and GTP-bound (active) forms. The C-terminal domain acts as a hypervariable region that may play a role in determining the isoform-specific function of RAS proteins by sequence divergence, and the C-terminal CAAX box is involved in a tremendous number of cellular signaling processes and regulatory events, including cell proliferation, differentiation, and apoptosis. *RAS* mutations such as *G12C* and *G13D* are single amino acid substitutions that impair the intrinsic and GAP-stimulated GTP hydrolysis activities of RAS proteins, rendering them persistently GTP-bound in the absence of extracellular stimuli. This persistently GTP-bound state excessively transmits growth signals and activates the RAS-RAF-MEK-ERK and RAS/PI3K/AKT/mTOR signaling cascades, which leads to the dysregulation of cell growth and differentiation resulting in tumorigenesis as shown in **Figure 1**. (17, 18, 25, 27).

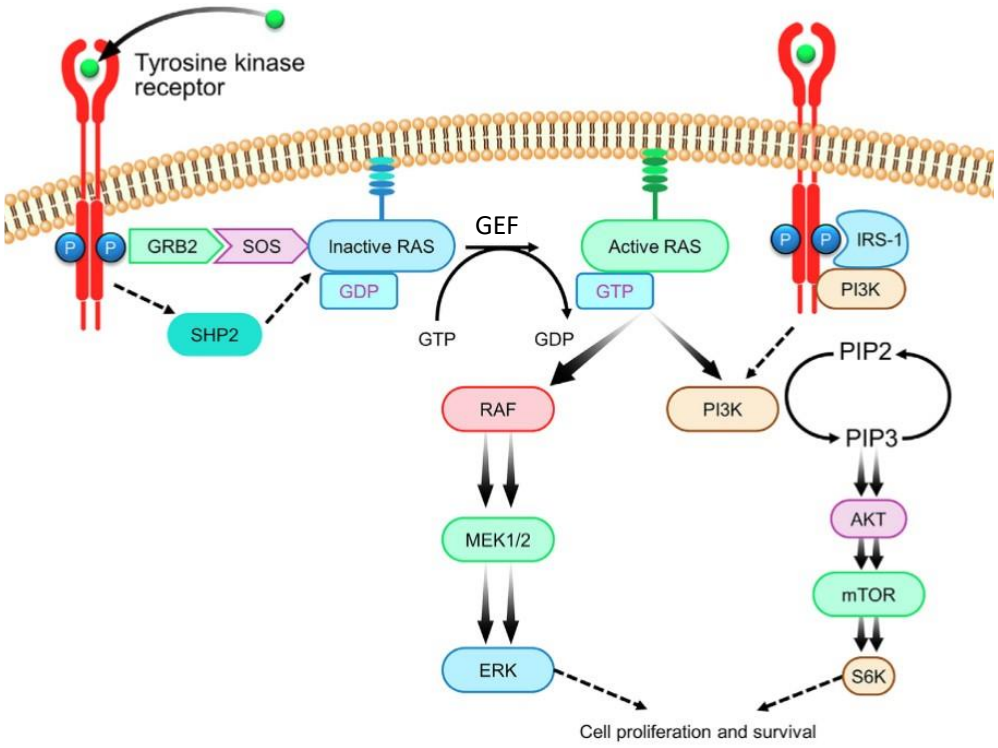


Figure 1. Simplified scheme of Mitogen Activated Protein Kinase activation and signaling cascades, RAS-RAF-MEK-ERK, and RAS-PI3K-AKT-mTOR pathways. Based on upstream signaling, RAS proteins are activated by GEF, which exchanges GDP for GTP. Active RAS then binds to and sends signals down the RAF-MEK-ERK pathway and the PI3K/AKT/mTOR pathway, which induce cell proliferation and survival, respectively.

Adapted from (26) Uprety D, Adjei AA. *Cancer Treat Rev.* 2020. 89:102070

RAS mutations are most common in the top three deadly cancers: pancreatic cancer (95% express *RAS* mutations), colorectal cancer (52% express *RAS* mutations), and lung cancer (31% express *RAS* mutations) (17, 18, 25). The development of

therapeutic strategies to block the activity of RAS proteins has the potential to be a highly effective method of anti-cancer treatment (23). However, the past misconception that all RAS proteins have identical functions has led to unsuccessful attempts to develop anti-RAS drugs (17, 18, 22). In addition, RAS has been deemed "undruggable" for quite some time for three main reasons: 1) all three RAS proteins lack a readily identifiable, accessible deep pocket, 2) RAS proteins have an unusually high affinity for their native ligand (GTP), and 3) all three proteins are located intracellularly on the inner layer of the cell membrane (17, 18, 22, 23). Consequently, they cannot be reached easily with high molecular weight drugs, such as monoclonal antibodies (17). Recently, appreciation of the fact that different RAS proteins have distinct roles has shifted the drug development focus toward KRAS, the RAS isoform most frequently mutated in cancer patients (20, 24, 26, 28). As seen in **Figure 2**, the downstream non-RAS proteins, such as those of the RAF-MEK-ERK and PI3K/AKT/mTOR pathways, have been targeted for drug therapy in the past to varying degrees of success. These treatments hold potential for use in combination therapies with RAS inhibitors to avoid the problem of developing drug resistance to one drug treatment.

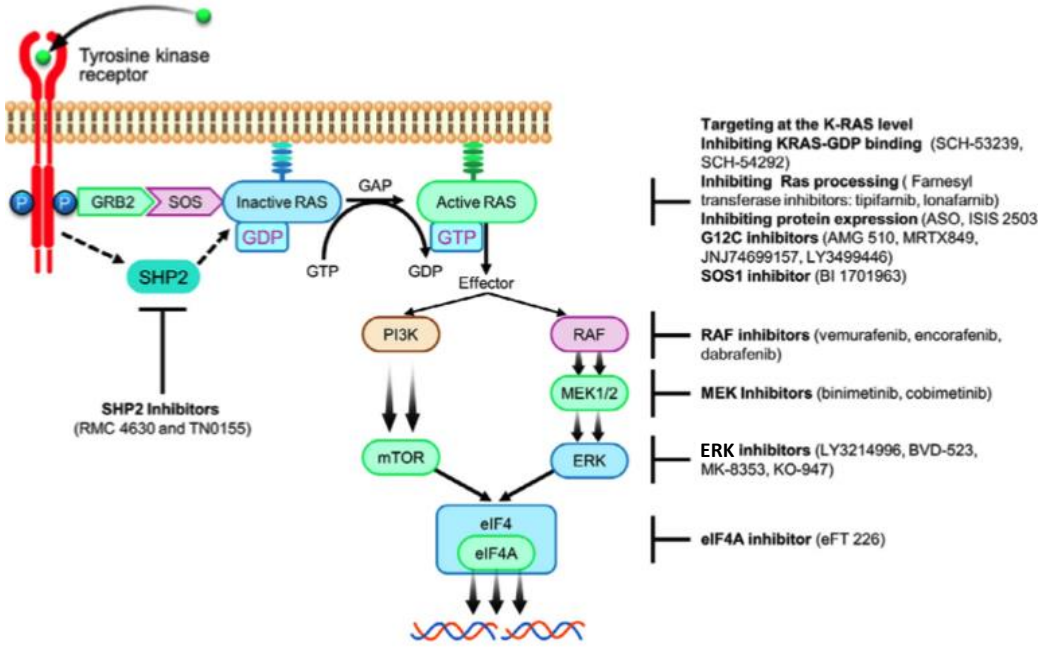


Figure 2. Proteins in the RAS-RAF-MEK-ERK pathway that have been targets of drug therapy. Drugs have been developed for targeting certain mechanisms at the RAS/KRAS level, and other drugs target proteins downstream of RAS, such as RAF, MEK, ERK.

Adapted from (26) Uprety D, Adjei AA. *Cancer Treat Rev.* 2020. 89:102070

1.5 Lung Cancer and RAS Inhibitors

Lung cancer is the leading cause of cancer-related mortality. Based on histology, there are two main subtypes of lung cancer that have been defined: small cell lung cancer (SCLC, 10%) and non-small cell lung cancer (NSCLC, 90%) (17, 20, 21, 23). *KRAS* is mutated in 31% of NSCLC patients (20, 22). There are several missense mutations in single amino acids in different *KRAS* cancers, including hotspots in codons 12, 13, and

61. The *G12C, G12D,* and *G13D* mutations are the most common focus in recent targeted drug development (18, 20, 21, 26). These mutations are associated with resistance to EGFR tyrosine kinase inhibitors and to chemotherapy (25, 27, 32).

KRAS^{G12C} is particularly prevalent in NSCLC, with approximately 13% of Americans with NSCLC expressing the *KRAS*^{G12C} mutation (20, 24, 26). There are about 23,000 new cases of NSCLC with *KRAS*^{G12C} mutation every year (24). *KRAS*^{G12C} is also found in 1-3% of colorectal and pancreatic cancer patients (20, 24, 26). The most successful approach to inhibiting RAS oncogenic kinases is *KRAS*^{G12C} inhibition. This is achieved by taking advantage of the substitution of the unreactive glycine residue with the reactive mutant cysteine residue present on mutant KRAS to covalently bind the inhibitor to (32). *KRAS*^{G12C} inhibitors are designed to target a specific pocket, a recently-discovered allosteric binding pocket/Switch-II pocket (S-IIP) that can be used to selectively target *KRAS*^{G12C} mutant cells while sparing normal cells (32). At this point in time, three KRAS inhibitors are being studied in clinical trials: AMG 510 (Amgen), MRTX849 (Mirati Theapeutics and Novaratis), and GDC-6036 (Roche) (32). On June 3, 2019, Amgen announced that its AMG 510 drug would be the first FDA-granted novel small-molecule inhibitor for *KRAS*^{G12C} to reach the clinical stage of testing after more than three decades of RAS research (24). AMG 510 was designed by targeting the cysteine residue present only on the mutant KRAS^{G12C} protein, which explains why AMG 510 does not show activity against other KRAS mutation isoforms (24, 26, 32). The data from its phase I clinical trial demonstrated that AMG 510 leads to a disease control rate of 90%, with partial response and stable disease in NSCLC patients when

orally administered as a monotherapy with mild adverse effects (24, 26). On May 28, 2021, AMG 510 (sotorasib/Lumakras™, Amgen, Inc.) was formally approved for clinical use to treat advanced NSCLC under the name of Lumakras (20). The success of AMG 510 has encouraged the RAS research community to explore more selective KRAS inhibitors.

1.6 Study of MCI-062

From an extensive screening, Dr. Piazza's Drug Discovery Center Lab at the Mitchell Cancer Institute (MCI) synthesized a novel small molecule inhibitor called MCI-062 that potently and selectively inhibits the growth of tumor cell lines harboring constitutively activated KRAS (29). Unlike the $KRAS^{G12C}$ inhibitors, MCI-062 is not limited by *RAS* mutation type (30). As such, it promises to offer a significant advantage as a cancer therapy due to its potential to benefit a broader base of patients. In previous studies, Piazza and Keeton demonstrated that MCI-062 inhibits *KRAS*-driven pancreatic and colorectal tumor cell growth in an isoform-independent manner by blocking GTP loading of KRAS (32). The research project reported here focused on a preliminary investigation of a novel small molecular pan-RAS inhibitor MCI-062 as a novel therapy for *KRAS*-driven cancer patients, a population in desperate need of more effective treatment options. Although the exact mechanisms of MCI-062 are still being studied and outside of the scope of this project, it explored the viability of the pan-RAS inhibitor MCI-062 for the treatment of the *KRAS*-mutant lung cancer cells.

CHAPTER II

HYPOTHESIS AND AIMS

The hypothesis of this project was that treatment with pan-RAS inhibitor MCI-062 inhibits proliferation of *KRAS*-mutant A549 and H358 lung cancer cells. A549 and H358 are NSCLC cells that express $KRAS^{G13D}$ and $KRAS^{G12C}$ mutations, respectively. MCI-062 was expected to inhibit the proliferation of lung cancer cells with *KRAS* mutations and to act as a more effective inhibitor of *KRAS*-mutant cancer growth than AMG 510 due to MCI-062's proposed ability to act as a pan-RAS inhibitor and inhibit all mutant isoforms of RAS as opposed to AMG 510's selective inhibition of $KRAS^{G12C}$ mutations. The aims of the project were as follows:

1. To determine whether there are optimal conditions for colony formation in the A549 and H358 cells.
2. To determine whether there is an optimal concentration of MCI-062 for inhibition of colony formation for the A549 and H358 cells.
3. To compare whether the tumor-inhibiting effects of MCI-062 are equal to or better than those of AMG 510 in the A549 and H358 cells.

CHAPTER III

MATERIALS AND METHODS

3.1 Cell Lines and Inhibitors

Cell lines expressing different *RAS* mutants are available at: https://www.cancer.gov/research/key-initiatives/ras/outreach/reference-reagents/cell-lines. Two NSCLC cell lines, H358 and A549, were used in this project.

The A549 and H358 cells lines were obtained from the American Type Culture Collection (ATCC). MCI-062 is synthesized on-site, so it was obtained directly from the Mitchell Cancer Institute (MCI). AMG 510 was purchased from Selleck Chemicals, LLC (Houston, TX) for research purposes.

First, the A549 and H358 cell lines were cultured by growing them in 10 mL cell culture dishes and passaging the cells into new dishes every 3-4 days once the cells reached confluence. Once the materials were prepared for the colony formation assays, the cells were plated into 12-well plates at different cell concentrations (control) or with different dose concentrations of MCI-062 at optimal cell concentrations (in duplicate) in a serial dilution for use in a colony formation assay.

3.2 Colony Formation Assays

As shown in **Figure 3**, the therapeutic effects of MCI-062 in the A549 and H358 lung cancer cell lines were determined using a colony formation assay, which evaluates the proliferation and growth capacity of cancer cells. The H358 and A549 cells were grown in normal growth medium with 10% fetal bovine serum supplemented Roswell Park Memorial Institute (FBS RPMI)-1640 medium in a 12-well plate for 10 days. The plate was incubated with stain (0.5% crystal violet) for one hour to visualize the living cell colonies that had formed. Plates containing the stained colonies were photographed through microscopy, and colony formation was measured using a custom macro written for NIS-Elements image analysis software (Laboratory Imaging) by Dr. Joel F. Andrews, Manager of the Bioimaging Core Facility for the USA College of Medicine and USA Mitchell Cancer Institute. This macro can analyze whole-plate images of 6 or 12-well cell culture plates and reports colony formation as the number of observed colonies as well as area fraction of the whole well. Following is a brief description of the macro's algorithm: after selecting 6 or 12 well plate formats and naming the wells if desired, the user was prompted to place a circular region of interest (ROI) over the first well, allowing for the automated placement of the remaining wells of the plate, and removal of non-well image data from the analysis. Thresholding on the image is then performed to delineate colonies from background, followed by object counting and area measurement for each well. Measured values are then exported to a tab-separated text file or Excel spreadsheet.

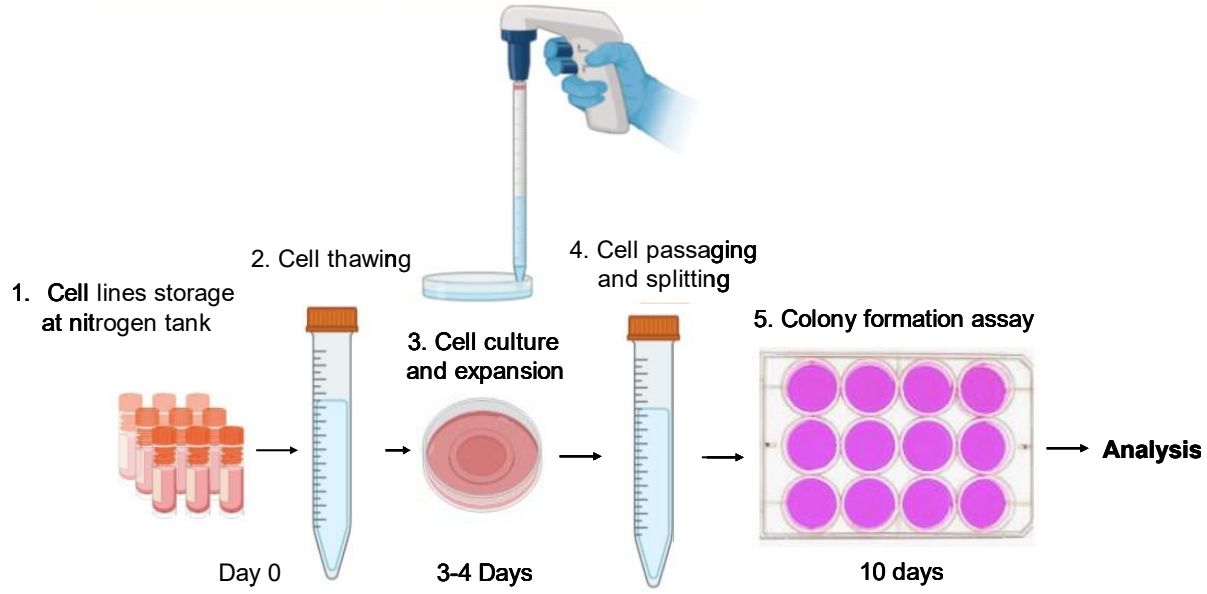


Figure 3. Tissue culture and colony formation assay. Cells were taken from liquid nitrogen storage, thawed, and plated into 10 mL cell culture dishes in liquid 10% FBS RPMI-1640 medium. Once the cells reached confluence every 3-4 days, the cells would be passaged and split into new cell culture dishes with new medium. To begin a colony formation assay, cells would be plated into a 12-well plate and be allowed to grow for ten days. After ten days, the plates were photographed under microscopy, and the digital images were analyzed in NIS-Elements.

CHAPTER IV

RESULTS

4.1 Colony Formation in A Serial Dilution of A549 Cells

As shown in **Figure 4**, to test the inhibitory effects of the pan-RAS inhibitor MCI-062, the best conditions and cell numbers for colony formation were determined using a serial dilution of A549 cells (*KRAS*^{G13D}) starting from 1000 cells/well (**4A**). As indicated in **4B** and **4C**, the best condition to test the therapeutic effect of drug treatment in tumor cell colony formation was found to be between 63 and 125 cells, as these cell concentrations resulted in colony growth that was neither too crowded nor too sparse. Cell overcrowding can lead to an underestimation of colony number due to interference with the software's ability to identify individual colonies, as seen with colony number for the 1000 cells/well concentration in **4C**. 80 cells/well was the concentration of A549 cells used in the subsequent colony formation assays.

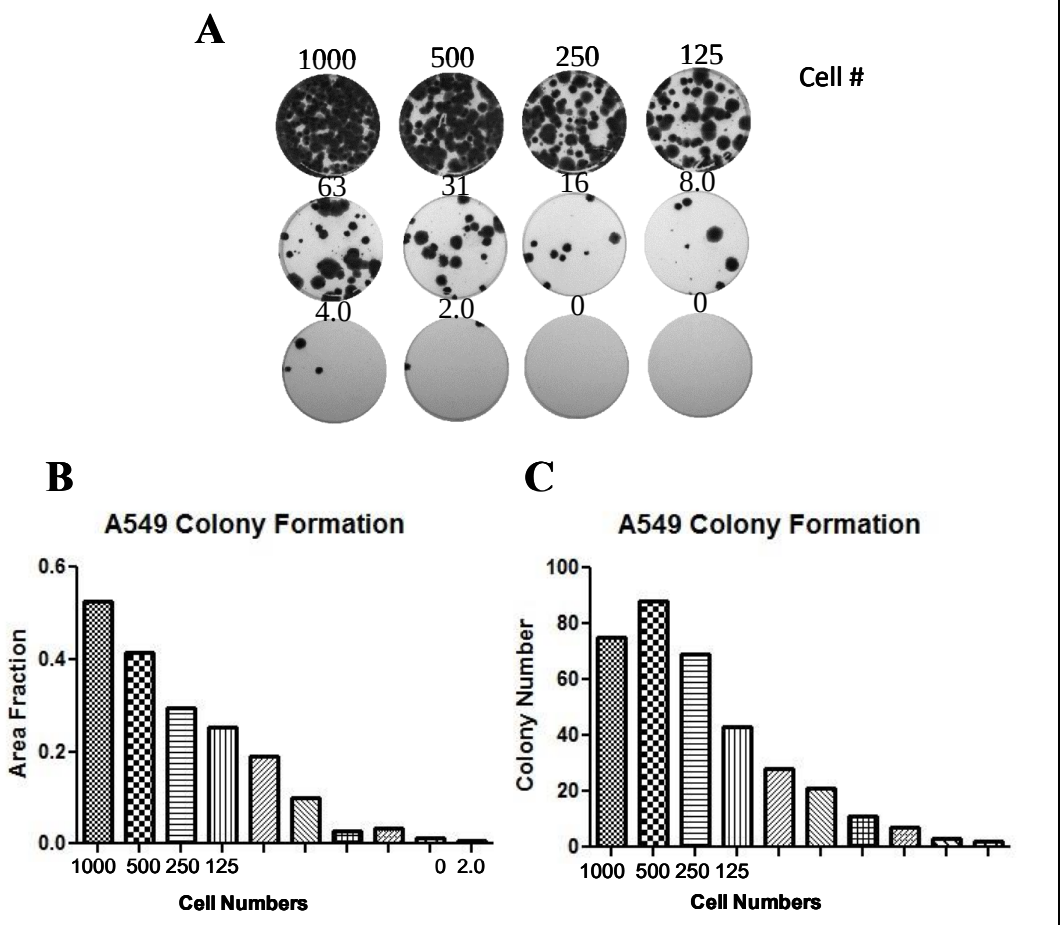


Figure 4. Colony formation assay with A549 lung cancer cells. (A). Cells were cultured in a 12-well plate with a serial dilution starting with 1000 A549 cells per well. Cells were incubated and monitored for 10 days. Colonies were stained with 0.5% crystal violet dye so that the live colonies could be analyzed. The area fraction **(B)** and the colony number **(C)** were calculated using data from NIS-Elements, and the graphs were created using GraphPad Prism 7.

4.2 Quantification of MCI-062 in Suppressing Cell Growth of A549 Cells

From the results in **Figure 4**, an 80-cell initial cell plating density was used to determine the potency of MCI-062 to inhibit the growth of A549 cells. For the first attempt, a concentration range of 0-500 nM MCI-062 was used (**Figure 5**). These results indicated that the A549 lung cancer cells did not survive when the MCI-062 concentration exceeded 20 nM. However, given the relatively wide gaps between experimental conditions, lower concentrations of MCI-062 were used in the next step to determine a more precise concentration at which MCI-062 begins to show signs of cancer cell inhibition.

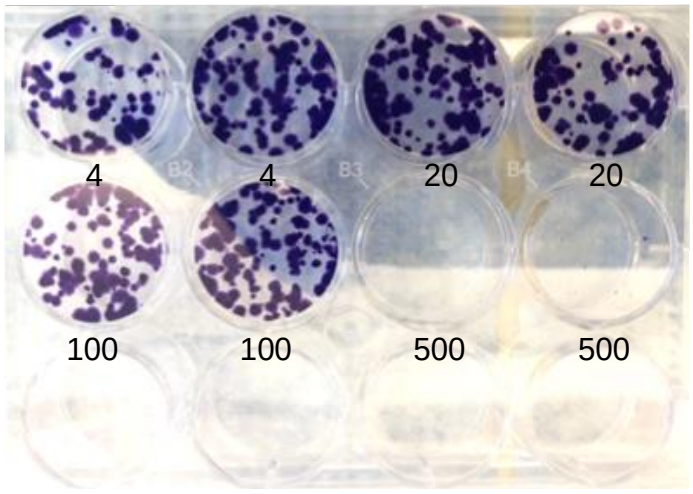


Figure 5. Inhibitory effects of MCI-062 on the growth of A549 lung cancer cells. Colony formation was analyzed in MCI-062 at 0, 0.8, 4, 20, 100, 500 nM using a crystal violet dye.

For the next experiment, the concentrations of MCI-062 used were 0, 1.5, 3.1, 6.13, 12.25 or 25 nM (**Figure 6**). The results demonstrated that MCI-062 concentration that fully inhibited fully inhibited A549 cell growth was somewhere between 3.1 nM and 6.3 nM (**6A**). The half maximal effective concentration (EC_{50}) of MCI-062 was interpolated from an area fraction vs. MCI-062 concentration constructed in GraphPad Prism, which was found to be 3.025 nM (**6B**). This value suggests that MCI-062 inhibits 50% of A549 cell ($KRAS^{G13D}$) growth at 3.025 nM.

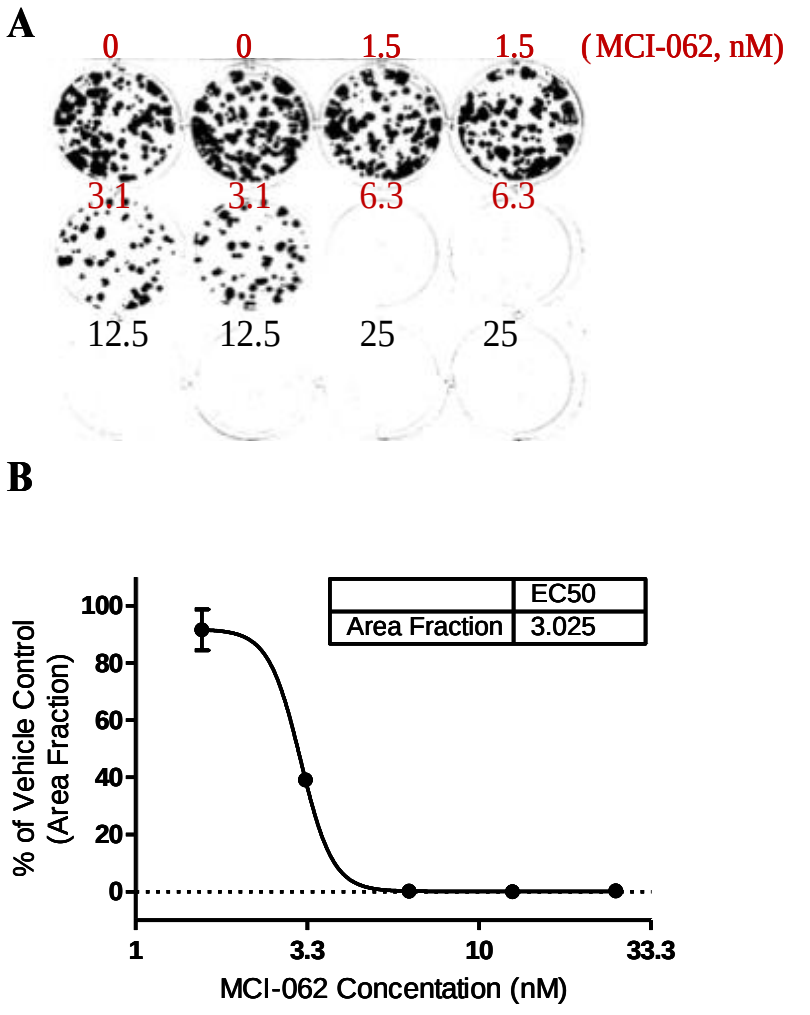
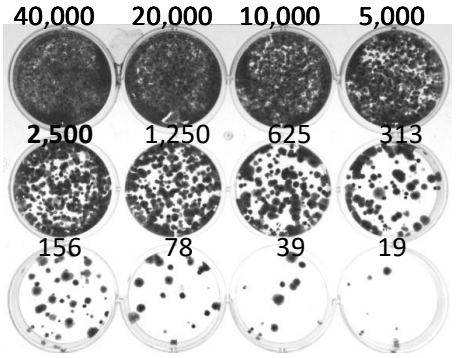


Figure 6. Pan-RAS inhibitor MCI-062 suppresses colony formation of A549 lung cancer cells in a dose-dependent manner. (A). Colony formation assay was performed in the presence of 80 cells of A549 in the treatment with 0, 1.5, 3.1, 6.3, 12.5, or 25 nM of MCI-062 for 10 days. **(B).** The EC50 was calculated in GraphPad Prism.

4.3 Colony Formation Assays with H358 Cells

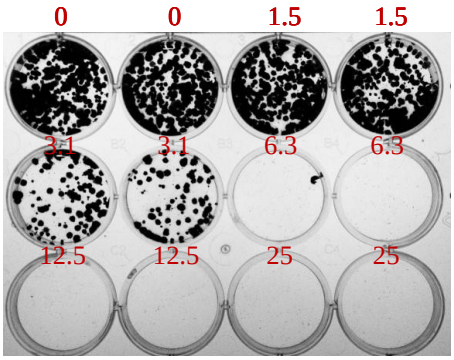
To determine the inhibitory effects of the novel RAS inhibitor MCI-062 in another type of KRAS mutant cells, the H358 ($KRAS^{G12C}$) lung cancer cell line was used in similar experiments as A549 cells. As shown in **Figure 7A**, the optimal condition in colony formation for H358 was 2,500 cells. The H358 cells responded similarly to the A549 cells in the colony formation assay (**Figures 7B and 7C**). EC50 of MCI-062 for H358 cells was determined using GraphPad Prism in the same manner as for the A549 cells and was found to be ~3.088 nM under their own optimal cell concentration.

The results from **Figure 6** and **Figure 7A** indicated that novel small molecule inhibitor MCI-062 could inhibit A549 ($KRAS^{G13D}$) cells and H358 ($KRAS^{G12C}$) cells with similar potency. Furthermore, these results demonstrate that MCI-062 inhibits the growth of lung cancer cells with both $KRAS^{G12C}$ and $KRAS^{G13D}$ mutations.



Serial dilution starting with H358 cells

B



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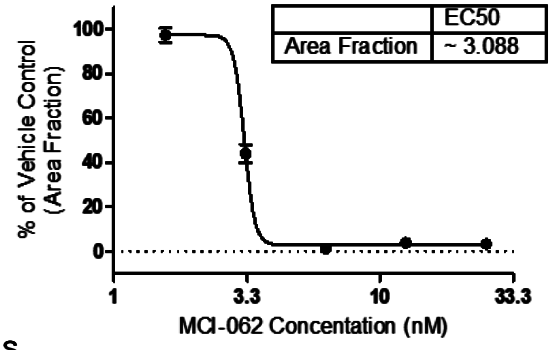

Dose-response of KRAS inhibitor, MCI-062, with H358 cells

Figure 7. Pan-RAS inhibitor MCI-062 suppresses colony formation of H358 lung cancer cells in a dose-dependent manner. (A). Colony formation in a serial dilution of H358 cells. **(B).** Colony formation assay was performed in presence of 2,500 cells of H358 in the treatment with 0, 1.5, 3.1, 6.3, 12.5, or 25 nM of MCI-062 for 10 days. **(C).** The EC50 was calculated using area fraction in GraphPad Prism.

CHAPTER V

DISCUSSION

The results of this project indicate that MCI-062 effectively inhibits the growth and proliferation of H358 and A549 lung cancer cells, which supports the original hypothesis. MCI-062's suppression of both cell lines suggests that MCI-062 extends KRAS inhibition from solely $KRAS^{G12C}$ to at least both $KRAS^{G12C}$ and $KRAS^{G13D}$, going beyond the capabilities of AMG 510 and other KRAS inhibitors being studied (20). As stated in the Introduction, the exact mechanisms of MCI-062 are still being studied and outside of the scope of this project. However, the research data suggest the potential for MCI-062 to have a novel but not-yet defined mechanism of inhibition distinct from other known inhibitors that are limited to $KRAS^{G12C}$ mutations, which encourages advanced preclinical investigation in animal models to support clinical evaluation.

Drugs developed for RAS or KRAS inhibition, such as MCI-062 and AMG 510, will potentially provide a cure to a specific population of lung cancer patients. As with most anti-cancer treatments, RAS inhibitors have the potential to lose efficacy due to drug resistance in patients. One potential solution to overcome such drug resistance is the development and use of combination therapies with inhibitors downstream of RAS to the RAF-MEK-ERK and PI3K/AKT/mTOR pathways and immunotherapies (6, 7, 17, 20, 25, 32). Many potential combination therapies have been employed in cancer patients,

especially in lung cancer and colon cancer (21, 32). Studies have shown that MEK inhibitors (MEKi) have a synergistic effect on suppressing tumor growth in combination with the KRAS inhibitor AMG 510 (20). The therapeutic efficacy of MCI-062 treatment in combination with immunotherapies, including anti-PD-1 and anti-PD-L1, is also promising, as previous results have indicated that the combination of KRAS inhibitor AMG 510 with anti-PD-1 has a significantly higher survival rate than both single treatment groups in a preclinical model (20). Other studies have demonstrated that KRAS inhibitor AMG 510 suppresses tumor growth in a dose-dependent manner, which is important for implementation in clinical use (20).

The continuation of this project might contribute to the development of a novel targeted therapy for *KRAS*-driven cancer patients, a population in desperate need of effective treatment options, and reach the goal of personalized medicine in these patients. Potential future directions include comparing the therapeutic efficacies of MCI-062 and AMG 510 in more lung cancer cell lines, studying the duration of the drug response and its interaction with approved EGFR, RAF, and MEK kinase inhibitors, and testing MCI-062's therapeutic efficacy in combination with immunotherapies. MCI-062 may also serve as a useful probe in studying KRAS inhibition in cancer treatment by providing powerful information for developing optimal targeted therapies for cancer patients.

CHAPTER VI

CONCLUSIONS

The results of this project demonstrate that MCI-062 inhibits the growth of lung cancer A549 cells with $KRAS^{G13D}$ mutation and H358 cells with $KRAS^{G12C}$ mutation. This study suggests that MCI-062 might exhibit therapeutic action against *KRAS*-mutant lung cancers even when they express different mutant isoforms, which supports the notion that MCI-062 acts as a pan-RAS inhibitor. This unique property may help advance the field of targeted treatment of patients with lung cancer involving *KRAS* mutations regardless of whether their specific mutation is $KRAS^{G13D}$, $KRAS^{G12C}$, or one of the other many isoforms that have been described in lung cancer and other cancer types..

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APPENDICES

Appendix Table KRAS inhibitors and other inhibitors for RAS downstream signaling in clinical trials

Compound	Mode of action	Phase	Cancer(s)	NCT No.[a]
Tipifarnib	Inhibits FT	II	HRAS mutant urothelial carcinomas	02535650
Tipifarnib	Inhibits FT	II	HRAS mutant squamous NSCLC	03496766
Rigosertib	Prevents RAS from interacting with downstream effectors	III	MDS	02562443
AMG 510	Locks KRAS G12C in an inactive state	I/II	Solid tumors with KRAS G12C mutations	03600883
MRTX849	Locks KRAS G12C in an inactive state	I/II	Solid tumors with KRAS G12C mutations	03785249
JNJ-74699157 (Phl)	Inhibits KRAS G12C	I	Solid tumors with KRAS G12C mutations	04006301
LY3499446	Inhibits KRAS G12C	I/II	Solid tumors with KRAS G12C mutations	04165031
siG12D LODER	RNA interference (RNAi)	I	Locally advanced/inoperable pancreatic	01188785
siG12D LODER[b]	RNAi	II	Locally advanced pancreatic	01676259
AZD4785	Antisense oligonucleotide targeting KRAS mRNA	I	Advanced KRAS-dependent tumors	03101839
BI 1701963[c]	Inhibits SOS1	I	Advanced cancers with KRAS mutations	04111458
TNO155	Inhibits SHP2	I	Advanced solid tumors	03114319
RMC-4630	Inhibits SHP2	I	Relapsed/refractory solid tumors	03634982
RMC-4630[d]	Inhibits SHP2	Ib/II	Solid tumors	03989115
V941/mRNA-5671[e]	Functions as mRNA vaccine against KRAS G12D, G12V, G13D and NRAS G12C	I	Advanced NSCLC, CRC and pancreatic with KRAS mutations	03948763

Abbreviations: CRC, colorectal cancer; FT, farnesyltransferase; MDS, myelodysplastic syndrome; NSCLC, nonsmall cell lung cancer.
[a]NCT No., ClinicalTrials.gov identifier.
[b]In combination with chemotherapy.
[c]With or without trametinib.
[d]In combination with cobimetinib.
[e]With or without pembrolizumab.