Targeting Vulnerabilities in Cell State and Calcium Signaling for the Treatment of Lung Cancer

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Targeting Vulnerabilities in Cell State and Calcium Signaling for the Treatment of Lung Cancer

Clark A. Jones

Dissertation submitted to the School of Pharmacy at West Virginia University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical and Pharmacological Sciences

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Keywords: Lung cancer, MTI-101, calcium homeostasis, EMT, GPCR

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Abstract
Targeting Vulnerabilities in Cell State and Calcium Signaling for the Treatment of Lung Cancer

Clark A. Jones

Lung cancer remains the deadliest of all cancers due to the high mutational burden associated with the disease. Combating mutational drivers and drug resistance proves to be essential in the development of novel therapies to improve patient outcomes. A first-in-class cyclic peptide known as MTI-101 has been shown to induce necrotic cell death in a caspase independent manner. MTI-101 was derived from the linear peptide known as HYD1 that was found in a high throughput screen to block cell adhesion with the extracellular matrix. The compound was further optimized and cyclized to the currently used MTI-101 that was found to be substantially more potent across multiple cancer types. In this dissertation, we sought to determine biomarkers of resistance to MTI-101 coupled with cell signaling pathways that become activated with the introduction of MTI-101 along with their contribution to cell death. The development of isogenic resistant lung cancer cell lines were utilized as tools to aid in the delineation of MTI-101’s effects on lung cancer. Chronic exposure to MTI-101 leading to the acquired resistance demonstrated changes of cell state to a clinically favorable mesenchymal-to-epithelial transition (MET) genotype and phenotype using in vitro and in vivo models. Selectivity of MTI-101 towards cancer cells was revealed with a lack of efficacy in healthy bronchial epithelial cells treated with a lethal cancer cell dose of MTI-101. Acquired resistance was found to have collateral sensitivity to standard of care agents while used as single agents and synergistic activity on wildtype cancer cells when used in combination with MTI-101. Simultaneous activation of second messengers calcium and cAMP alluded toward GPCR activation with MTI-101 treatment. Attenuation of calcium signaling protected from MTI-101 induced cell death while activation of calcium signaling exacerbated death. Decreased drug binding likely explained the lack of downstream signaling and protection from cell death in acquired resistant lung cancer cell lines. Incorporation of therapies that alter cell state and calcium signaling pathways in combination with current standard of care agents remain vital in positioning novel therapy options in the clinic for improved patient survival with the deadliest cancer.
Acknowledgements

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Chapter 1

Introduction and Literature Review

Lung Cancer

Lung cancer remains the leading cause of cancer related death that accounts for more deaths each year than colon, breast and prostate cancer combined[1]. The disease is characterized by a high mutational burden that includes some of the more common driver mutations such as ALK, EGFR, and KRAS[2]. The prominent cause of lung cancer originates from smoking tobacco products and its most common presentation occurs in people over the age of 65. Other risk factors can come from environmental factors such as exposure to radon, asbestos, air pollution and water supply contamination with arsenic[1]. Lung cancer effects are seen equally among genders, despite difference among races with African descents at disproportionately higher rates[3]. The disease is broken into two classes of diagnosis either being non-small cell lung cancer (NSCLC) which makes up about 85% of all cases, and small cell lung cancer (SCLC) which makes up about 15% of cases[1]. NSCLC is further divided into three subsections known as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. The survival rate remains dependent on the stage in which the cancer is diagnosed. Unfortunately, the majority of all new lung cancer cases are diagnosed at stage IV where the cancer has metastasized to multiple sites within the body with a five year survival at a dismal 1%[4]. Advancements in technology for earlier detection methods along with novel therapies aim to improve these statistics for treatment in the future.
The treatment for lung cancer is separated among the clinical presentation between NSCLC and SCLC along with the tumor stage. SCLC gets divided between a limited and an extensive stage. Limited stage SCLC can typically be treated with radiation coupled with chemotherapy while extensive stage SCLC are mostly treated with just chemotherapy to limit the progression but not cure the patient[5]. NSCLC has a more complex staging system as treatment options become more abundant for this subset of lung cancers. The TNM staging system is still used to identify the location(s) in which the tumor exists as either solely in the primary tumor site (T), spreading of the tumor to nearby lymph nodes (N) or metastasis to distal sites in the body (M). Additional staging within these groups is also used to further delineate the tumor characteristics and is broken into four substages. Stage I deals with a tumor only in the primary site under 5 cm, Stage II has the tumor spread to nearby lymph nodes, Stage III describes the tumor that has migrated to the center of the chest along with the lymph nodes, while Stage IV has metastasized to other parts of the body[6]. Stages I-III typically combine surgical removal of the primary tumor coupled with radiation and chemotherapy in attempt to prevent recurrence. Stage IV treatment options consist mostly of chemotherapy coupled with palliative care therapies unless the tumor contains markers that would suggest success in targeted therapies[7]. Recently, formidable advances have been made in targeted therapy for lung cancer. All lung cancers are sequenced at the time of diagnosis for markers that could correlate to responsiveness to current targeted therapy options. As stated before, one of the most common mutations in lung cancer is an epidermal growth factor receptor (EGFR) mutation that causes this receptor to be constitutively active. Tyrosine kinase inhibitors (TKIs) have been developed to selectively target this mutated EGFR present on cancer cells
while leaving healthy normal cells undisturbed. Frontline TKIs such as erlotinib have been developed to target these mutations and have improved patient survival on average 2 months and up to 8 years in the most extreme cases for late stage NSCLC[8]. In addition, third generation TKIs such as Osimertinib have been developed to target the most common TKI resistant mechanism T790M mutation to further improve survival outcomes from first and second generation TKIs by over 9 months progression free on average[9]. Furthermore, huge milestones have been breached within the last year as the first FDA approved drug targeting a KRAS mutation was developed known as sotorasib[10]. The incorporation of immunotherapies for the treatment of lung cancer has been on the rise for further therapy options. Currently, PD-L1 inhibitors remain at the forefront of immunotherapy options for NSCLC[11]. Despite the breadth of options for therapy, tumor heterogeneity proves to be a difficult hurdle to overcome in prevention of recurrence where therapy eventually fails. New combination strategies, advancements in detection methods, and novel drugs are vital for improved patient outcomes.

MTI-101

The peptide MTI-101 was originally derived from a parent compound known as HYD1 that was discovered in a high throughput screen to block cell adhesion to the extracellular matrix (ECM)[12], [13]. HYD1 is a 10 D-amino acid with the following sequence: KIKMVISWKG. As a single agent, it was also found to induce necrotic cell death via reduction in mitochondria membrane potential along with an increase in reactive oxygen species[14]. HYD1 was found to induce cell death independent of apoptosis explained by the absence of caspase activation[14]. Through further experimentation, the minimally active core of HYD1 was found to be MVISW.
To enhance potency, the peptide was cyclized using a β turn promoter scaffold backbone and the sequence was further modified to include three lysine residues on the non-recognition strand to improve water solubility to the optimized cyclic peptide known as MTI-101 with the following backbone sequence: NLeVVAW[15]. The full peptide is shown in Figure 1. MTI-101 was found to be substantially more potent than HYD1 and have anti-tumor effects across multiple cancers in vitro and in vivo including castrate-resistant prostate cancer and multiple myeloma[16], [17]. MTI-101 will be analyzed throughout this dissertation to investigate its potential role in the treatment of lung cancer.

Epithelial-to-Mesenchymal Transition

Non-cancerous cells undergo a transition that allow them to move and expand for normal physiological functions such as wound healing and development[18]. Cancer has found a way to hijack this transition in order to grow and metastasize to other parts of the body when resources become limited at the primary tumor site across multiple tumor types including but not limited to breast, prostate, and lung cancer[19]–[21]. This process is known as the epithelial-to-mesenchymal transition (EMT) illustrated in Figure 2. Epithelial like cells have high expression of tight junction proteins such as claudins, occludins, and E-cadherin that are utilized for cell-cell connections along with attachment to the basement membrane[22], [23]. This cell state has been shown to have enhanced sensitivity to anoikis by interaction with E-cadherin complexes which control the balance of survival or apoptosis based on connections from the basement membrane and adjacent cells[24], [25]. A mesenchymal phenotype consists of characteristics such as elongated motile cells that have greater resistance to anoikis[24].
Increased expression of proteins that allow for motility within the ECM such as Fibronectin, N-Cadherin, and Collagen give mesenchymal cells some of the necessary tools to invade and metastasize throughout the body[26], [27]. Cancer cells undergo EMT through stress stimuli that alter cell state by reducing epithelial like proteins and increasing mesenchymal proteins by means of survival. Common sources of induction of EMT include limited nutrients in the tumor microenvironment[28], [29], immune surveillance[30], along with drug treatment[31]. Unfortunately, lung cancer treatment with standard of care agents often induces a drug resistant phenotype that has undergone EMT[32], [33]. This EMT phenotype has become increasingly difficult to treat as it develops multidrug resistance in the clinic[34]. Targeting cell state may prove to be just as important as inducing cell death in cancer cells. Understanding commonalities between therapies that alter cell state will be vital in the treatment of these late-stage metastatic cancers.

**Calcium Homeostasis**

Calcium is tightly regulated within cells as it is essential for many physiological and pathological functions. Given its wide role in cellular functions, cells have intracellular calcium stores in order to have the resource readily available at a moment’s notice. The main location of intracellular calcium stores resides in the endoplasmic reticulum(ER)[35]. Sarco/Endoplasmic Reticulum Calcium ATPases (SERCA) are pumps that allow for cytoplasmic calcium to be shuttled against the concentration gradient into the ER to stock calcium up to a concentration of 1-2 mM despite resting cytoplasmic concentrations averaging around 100 nM[36]. Calcium is released from the ER by means of IP₃ receptors or ryanodine receptors controlled by direct
levels of IP$_3$ and cyclic ADP ribose respectively[37]. IP$_3$ is generated as a downstream signaling messenger of the Phospholipase C (PLC) pathway that is commonly activated by G-Protein Coupled Receptors (GPCRs) and Receptor Tyrosine Kinases (RTKs). Ryanodine receptors are directly modulated by calcium concentrations in the ER and cytoplasm. These ryanodine receptors can open in response to low cytoplasmic calcium levels as well as extremely high concentrations of ER calcium to prevent cell death[37].

While the ER serves as the main intracellular storage of calcium, the mitochondria has been shown to participate in cytoplasmic calcium buffering[38]. Calcium is transported into the mitochondria via the mitochondrial calcium uniporter (MCU). The mitochondria use calcium ions for the augmentation of ATP production[39], but have recently been shown to buffer cytoplasmic calcium levels[40]. The ER and mitochondria work in coordination with many enzymes and receptors to tightly regulate one of the most important second messengers in cells to organize cell signaling essential for survival.

Furthermore, intracellular calcium can regulate calcium levels by binding to enzymes such as calmodulin. Calmodulin is involved in a plethora of functions and has been shown to modulate enzymes, receptors and entire signaling pathways[41]. The activation of calmodulin occurs when intracellular calcium levels rise and bind to the four calcium binding pockets on the molecule transforming the protein into its active conformation[42]. The ER is able to communicate with the store operated calcium entry (SOCE) that exists as various receptors on the plasma membrane such as ORAI1 and TRPC that allow for the transport of extracellular calcium into the cell. When calcium stores are low in the ER, STIM1 dimerizes on the ER membrane and communicates with the SOCE channels on the plasma membrane to influx
extracellular calcium into the cytoplasm[43]. This signal can be terminated by calcium being restored in the ER and preventing STIM1 from dimerizing, causing the closing of the SOCE[44]. Calmodulin has also been shown to negatively regulate the IP\textsubscript{3} receptors on the ER membrane[45] as well as SOCE channels such as TRPC1[46] and ORAI1/STIM1 complex[47] when bound to calcium in its active conformation. Voltage gated calcium channels (VGCC) have their own variation of calcium regulation, but given lung cancer is a non-excitable cell, this dissertation will focus on calcium homeostasis around ligand gated calcium channels (LGCC).

Cancer cells modulate the calcium homeostasis pathway in order to survive and proliferate. Cell alterations consistent with malignancies include changes in receptors, enzymes, and tolerance/utilization of calcium within cells. Different types of cancer were shown to overexpress SOCE channels such as ORAI1, TRPC, and TRPV to accommodate an excessive need for calcium to allow malignant cells to divide rapidly and metastasize[48]. The knockdown expression of the MCU has been shown to decrease metastasis and invasion in breast cancer cells illustrating its potential role in tumorigenesis [49]. The calcium dependent enzyme calmodulin has also been shown to be involved with invasion while pharmacological inhibition of calmodulin has been shown to decrease cell motility and metastasis in cancer[50]. In addition, calmodulin overexpression in multiple tumor types including breast and [51] lung cancer [52] may explain this contribute to cancer progression and invasion. Further research is warranted to better incorporate calcium signaling in cancer treatment given its vast role in cellular functions. The role of calcium homeostasis in cancer progression and current strategies to target these pathways will be discussed further in Chapter 2 of this dissertation.
GPCR Signaling

G-Protein coupled receptors (GPCRs) are the most abundant family of membrane proteins responsible for a plethora of cell responses and regulations including but not limited to sensation, metabolism, immune response, motility and growth[53]–[56]. GPCRs exist as 7-transmembrane domain proteins that span the width of the plasma membrane[57]. There are over 1000 different GPCRs and each is specific to a particular ligand and cellular response[58]. Some examples of GPCR ligands include light energy, peptides, lipids, sugars, and proteins. There are approximately 100 GPCRs that currently do not have known ligands and are known as orphan GPCRs[59]. These cell surface receptors are linked to G-proteins on the intracellular domain that are responsible for signal transduction within cells. The G-proteins consist of an alpha (α), beta (β) and gamma (γ) subunit that form a heterotrimeric complex. Activation of the GPCR causes a conformation change that allows the G-protein complex to exchange GDP for GTP at the intracellular G-protein α subunit which causes the dissociation of the β and γ subunits from the complex and the α subunit[57]. The α subunit is the main signal transducer, but the βγ dimer has been shown to be involved in the negative regulation of α along with various ion channels[60], [61]. The full activity of signal transduction with the βγ dimer is still not fully understood. The better characterized signaling subunits of GPCRs is the α subunit that has been grouped into four different classes as follows: G\textsubscript{αq}, G\textsubscript{αs}, G\textsubscript{αi}, and G\textsubscript{α12/13}. The signaling pathway for the three most common subunits is shown in Figure 3. Each class of α subunits contain multiple isoforms but the high sequence homology within each respective class activates the same downstream effector molecules. The G\textsubscript{αq} is capable of activating the Phospholipase C (PLC) signaling pathway that utilizes calcium as a second messenger for its
cellular effect[62]. The G\(_{\alpha S}\) and G\(_{\alpha I}\) work in opposition of each other as G\(_{\alpha S}\) activates adenylyl cyclase (AC) that utilizes cyclic adenosine monophosphate (cAMP) as a second messenger while G\(_{\alpha I}\) negatively regulates AC to decrease cytosolic cAMP[63]. G\(_{\alpha 12/13}\) primarily target Rho guanine nucleotide exchange factor (Rho-GEF) which can activate rho-associated protein kinases (ROCKs) as secondary messengers to modulate cell functions[64]. The main role of G\(_{\alpha 12/13}\) has been described as rearrangement of cytoskeletal elements in excitable cells[65]. Also, all known G\(_{\alpha 12/13}\) subunits are coupled with additional \(\alpha\) subunits that primarily signal through the other subunit[65]. All GPCRs contain at least one \(\alpha\), \(\beta\), and \(\gamma\) subunit despite the numerous combinations of different isoforms coupled in each intracellular domain. The GPCR can be linked with any combination of the four \(\alpha\) family subunits as well (ranging from 1 to all 4) producing extremely diverse signals with each respective GPCR[66]. The most recent classification system has GPCRs divided among the following groups based on sequence and functional similarities: glutamate, rhodopsin, adhesion, frizzled/taste, and secretin[67]. Despite the differences of each class, all GPCRs are regulated by common factors.

The negative regulation of GPCR signaling remains vital for properly executing functions and tasks, given their wide role in cellular processes. Prolonged signaling is capable of inducing cellular stress, mutations, and even cell death[68]. GPCRs rely on multiple factors for proper signal transduction and termination in order to function correctly. GTPases mediate all GPCR signals by hydrolyzing GTP back to GDP on the \(\alpha\) subunit which favors the reassociation of the G-protein heterotrimeric complex with the GPCR[69]. Given this process is extremely slow, GPCR signals are often regulated by additional proteins. Regulators of G-protein signaling (RGS) allosterically modulate GTPases by accelerating the conversion of GTP to GDP to expedite the
termination of signal[69]. Sustained agonist binding to GPCRs induces receptor endocytosis via phosphorylation of the GPCR by GPCR kinases (GRKs) and coupling to β-arrestin scaffolding proteins[70]. Receptor endocytosis desensitizes the receptor from agonist stimulation and further signal transduction. The receptor can later be degraded internally by lysosomes or trafficked back to the plasma membrane for future ligand activation[71]. Activation of downstream effectors such as PKC and PKA have been shown to phosphorylate GPCRs regardless of ligand binding to stop further signaling of activated GPCRs along with the impedance of new receptor signaling[70]. GTPases, RGSs, GRKs, and downstream effector molecules all work in concert to relay stimuli messages properly with each GPCR. Given GPCRs are involved in many pathological cases, understanding how to modulate GPCR regulators may prove to play a pivotal role in drug development for disease states. Further information is provided on the role of GPCRs in cancer progression along with targeting strategies for novel therapies in Chapter 2 of this dissertation.
References


[45] L. Missiaen et al., “Calmodulin Increases the Sensitivity of Type 3 Inositol-1,4,5-trisphosphate Receptors to Ca2+ Inhibition in Human Bronchial Mucosal Cells,” p. 4.


Figures

Figure 1:

Figure 2:

EMT

MET

E-Cadherin

Claudins

Occludins

Loss of

Cell

Polarity

and Tight

Junctions

Cytoskeletal

Remodeling

Release of

MMPs

Invasion

Metastasis

Cell Motility

Elongation

TWIST

Fibronectin

Vimentin

Right
Figure 3:

**GPCR Signaling Pathways**

- **Ligand**
  - Gα
  - βγ

- **Receptor and G-protein**
  - Gα
  - βγ

- **Effector protein**
  - Adenylate cyclase
  - cAMP
  - Protein kinase A
  - Increase protein phosphorylation

- **Second messengers**
  - Phospholipase C
  - DAG
  - IP3
  - Ca²⁺ release
  - Protein kinase C

- **Later effectors**
  - Adenylate cyclase
  - cAMP
  - Protein kinase A
  - Increase protein phosphorylation
Figure Legends

Figure 1. Chemical structure of MTI-101

Figure 2. The transient epithelial-to-mesenchymal transition (EMT). Epithelial cells have high expression of tight junction proteins such as E-cadherin, claudin, and occluding. Loss of tight junction proteins coupled with increases in mesenchymal proteins such as TWIST, fibronectin, and vimentin cause loss of cell polarity along with extracellular matrix (ECM) remodeling leading to elongation of cells. Release of matrix metalloproteases (MMPs) allows for the final step in the EMT process in which cells gain the ability to invade in the ECM and metastasize in the body.

Figure 3. The three main signal transduction pathways in non-excitable cells of the G-protein coupled receptor (GPCR) Gα subunit. GPCR ligands bind to their respective GPCR to induce a conformation change creating an exchange of GDP to GTP at the Gα subunit. Gαs (green) activates adenylyl cyclase which uses cAMP as a second messenger along with Protein Kinase A (PKA) for cellular response while Gαi (orange) inhibits this process. Gαq (blue) activates Phospholipase C (PLC) that utilizes Inositol Triphosphate (IP3) and diacylglycerol (DAG) as second messengers that increase cytosolic calcium and Protein Kinase C (PKC) respectively for cellular response.
Chapter 2

Role of Calcium Homeostasis in Modulating EMT in Cancer

Clark A. Jones and Lori A. Hazlehurst *

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Keywords: calcium; EMT; cancer
Abstract

Calcium is essential for cells to perform numerous physiological processes. In cancer, the augmentation of calcium signaling supports the more proliferative and migratory cells, which is a characteristic of the epithelial-to-mesenchymal transition (EMT). By genetically and epigenetically modifying genes, channels, and entire signaling pathways, cancer cells have adapted to survive with an extreme imbalance of calcium that allows them to grow and metastasize in an abnormal manner. This cellular remodeling also allows for the evasion of immune surveillance and the development of drug resistance, which lead to poor prognosis in patients. Understanding the role calcium flux plays in driving the phenotypes associated with invasion, immune suppression, metastasis, and drug resistance remains critical for determining treatments to optimize clinical outcomes and future drug discovery.

Introduction

Calcium is one of the most important elemental molecules in the human body; therefore, its regulation is just as crucial. It plays a vital role in many physiological processes, including, but not limited to, muscle contractions, metabolism, phagocytosis, apoptosis, cell division, motility, and signaling [1,2,3,4]. Despite calcium’s expansive role within the body, unbound, cytosolic-free calcium is the only form that can be used for physiological and pathological functions. In order for cells to perform their everyday functions, adequate calcium levels must be met and maintained, leading to the tight regulation of calcium throughout the
body. Bones, therefore, act as reservoirs to store excess calcium for utilization when accessible extracellular levels are insufficient in circulation.

Central to calcium homeostasis are the parathyroid hormone (PTH) and calcitonin. When extracellular calcium is low in circulation, PTH is released by the parathyroid glands. This triggers the release of calcium from bone deposits into the bloodstream and the inhibition of calcitonin, a negative regulator of calcium [5]. On the other hand, calcitonin is activated when calcium exceeds its narrow threshold, thereby causing the redeposition of calcium to the bone, along with excretion by the kidneys [5]. Disease states, such as hypercalcemia and osteoporosis, occur when the regulatory factors of calcium homeostasis are not able to function properly [6]. Prolonged uncontrolled fluctuations can lead to severe consequences, including neurological problems, kidney failure, and even death.

In cancer cells, mutations and changes in the expression of calcium channels, pumps, and binding proteins have resulted in calcium levels that exceed the typical threshold of normal cells. These elevated calcium levels allow the cells to proliferate and become malignant [7]. Cells that gain the ability to break through the extracellular matrix (ECM) and metastasize to distal portions of the body are said to undergo a process known as the epithelial-to-mesenchymal transition (EMT). The many roles calcium plays in EMT are shown in Figure 1. EMT is a slow, transient process that involves the deterioration of cell–cell junctions and detachment from the basement membrane, where cells lose their polarity. In non-malignant cells, EMT is common for functions such as wound healing, growth, and development. However, in pancreatic, lung, and breast cancer cells, this malignancy process has been shown to lead to poor prognosis and increased tumor progression [8,9,10]. Common epithelial genes
that are downregulated for this transition include E-cadherin, claudins, and occludins, which are essential in forming junctions between cells and holding them in place. Mesenchymal genes that are upregulated include vimentin, N-cadherin, and matrix metalloproteases (MMPs), which provide cells with some of the necessary tools to metastasize away from the primary tumor. The reverse process of EMT is called mesenchymal-to-epithelial transition (MET). When nutrients become scarce, cancer cells hijack both the EMT and MET processes to survive. These cells use the EMT process to metastasize from the nutrient-deprived primary tumor and the MET process to recolonize in a distal, nutrient-rich environment. In this environment, they become epithelial, similar to cells attached to a basement membrane, and form cell–cell junctions once more. In this review, the central role calcium plays in EMT are discussed in the following areas: (1) Calcium Channels, (2) GPCR Signaling, (3) Interplay with Integrins, (4) Immune Evasion/Drug Resistance, and (5) Combination Therapy.

**Calcium Channels**

In a resting cell, intracellular calcium levels remain close to only 100 nM. This is substantially lower than extracellular calcium concentrations, which range from 1 to 2 mM [7]. Channels and pumps are responsible for maintaining such a tightly regulated concentration within the cell, allowing for a chemical gradient across the cell membrane. The two types of calcium channels that exist in cells are voltage-gated and ligand-gated. Voltage-gated calcium channels (VGCC) cause an influx of calcium into excitable cells, such as neurons [11]. Typically, a quick response is elicited by a VGCC, such as a neuronal signal relay or a muscle contraction. In
contrast, ligand-gated calcium channels (LGCCs) induce calcium influx in all other non-excitable cells, and usually generate a much slower and more prolonged effect, which can lead to either cell proliferation or apoptosis, depending on the levels of calcium flux and the cellular context \[12\]. The variety, localization, and abundance of these channels are tightly regulated, due to their wide range of functionality in different cell types.

The LGCCs are the first step in a cellular process known as store-operated calcium entry (SOCE). This is also the primary means by which malignant cells obtain calcium for cancer progression \[13\]. Recent studies have shown that proteins such as STIM1, on the endoplasmic reticulum (ER) membrane, and Orai1, on the plasma membrane, play an essential role in the function of these channels. STIM1 is responsible for signaling to the plasma membrane after cellular ER calcium stores are depleted \[11\]. Orai1 is utilized by the SOCE pathway via pore formation in the plasma membrane, which selectively allows the passage of calcium ions \[14\].

Another ion channel that allows calcium fluctuations to occur is called a transient receptor potential (TRP). The family of TRP channels is less selective than Orai1, and can allow the passage of other cations, such as sodium, potassium, and magnesium \[15\]. The literature supports the fact that both subtypes (TRPC and TRPV) are capable of forming complexes with STIM1 and Orai1 to create sustained calcium entry \[16,17\]. The overexpression of a variety of members from the TRP family has been correlated with increased EMT \[18\] and poor patient prognosis \[19,20\].

The dysregulation of these proteins is often linked to multiple cancers, which show increased production and localization to their respective membranes, to accommodate an increased need for calcium. For example, it has been shown that the overexpression of STIM1 in hepatocellular
carcinoma leads to increased cytoplasmic calcium and increased cellular proliferation [21]. In addition, there is evidence to support the overexpression of Orai1 in lung cancer correlates with increased cell proliferation, along with poor patient prognosis in the clinic [22]. Calcium channels play the central role in calcium fluctuations in cells, but are not the only means of initiating elevated cytosolic-free calcium. G-protein coupled receptors (GPCRs) are also capable of altering intracellular calcium by means of coordination with SOCE and calcium channels along with crosstalk among additional signal transduction pathways, such as receptor tyrosine kinases (RTK) [23].

**GPCR Signaling**

GPCRs are involved in a plethora of physiological functions, such as the regulation of behavior, the immune system, cell growth, motility, and sensory input [24,25,26,27]. Unfortunately, GPCRs are also involved in numerous disease states, including immune deficiencies, mental and metabolic disorders, lack of sensation, and cancer [28,29,30]. Due to these receptors’ wide-ranging role in so many diseases, it is not surprising that almost half of all FDA-approved drugs target GPCRs for their biological effects. The ligands in cells bind to the GPCR extracellularly to induce a conformation change that activates an intracellular G-protein. This activated G-protein is then capable of signaling through two mechanisms of action, as seen in Figure 2. The first mechanism involves adenyl cyclase, which produces cAMP as a secondary messenger. The second involves phospholipase C, which is capable of enzymatically hydrolyzing phosphatidylinositol 4,5 bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol 1,4,5
triphosphate (IP₃). IP₃ then binds to the IP₃ receptor on the ER membrane, causing calcium stores from the ER to empty into the cytoplasm and act as a second messenger to carry out the biological functions [31]. Activation of phospholipase C via G-protein signaling has also been shown to increase intracellular calcium by store depletion and the subsequent activation of TRPC channels [32,33].

Mutations in GPCRs, such as point mutations, overexpression, and silencing, have contributed to either cell death or tumor initiation and EMT. One of the more common alterations discovered after analyzing an mRNA database was GPCR overexpression, coupled with a more frequent mutation rate, which has been found across 20 different cancers, including 45 subtypes, as compared to non-cancerous tissues [34]. These overexpressed GPCRs encompassed all classes, including A, B, C, adhesion, and orphan GPCRs. Since GPCRs can signal to initiate biological processes, such as proliferation and migration, a significant increase in receptors would allow for greater signaling output in these pathways. One example of this phenomenon is found in the family of GPCRs known as chemokine receptors. One role chemokines play in tumorigenesis and EMT is the recruitment of tumor-associated macrophages (TAMs), which leads to the release of MMPs [35]. An illustration of this is shown in Figure 2. These MMPs enhance motility by breaking down proteins in the basement membrane and extracellular matrix, to allow cells to metastasize away from the primary tumor. Recently, several types of the GPCR, protease-activated receptor (PAR), have been found to be upregulated in cancer [36]. Interestingly, the overexpression of PAR was found to induce EMT via TGF-β signaling, leading to the loss of cell polarity [37]. Point mutations in the binding pocket of GPCRs can also be crucial in the viability of cancer cells. The mutations can alter
ligand–receptor affinity along with ligand selectivity, which can change the entire signaling pathway through its respective receptor [38]. After analyzing one specific GPCR known as adenosine A$_{2B}$ receptor, a previous study found altered agonist efficacy and potency, with 15 different point mutations [39]. While some of these mutations increased the agonist effect, others reduced or completely eliminated receptor activation, demonstrating how minute changes in the protein sequence can completely transform this GPCR’s capabilities. Recent work has also substantiated GPCRs’ role in the upregulation of EMT transcription factors, such as ZEB, Snail, and Twist, which are involved in numerous transitions, including cell polarity, cytoskeleton remodeling, migration, and invasion [36,40]. It has been challenging to delineate the actual function and various ligands of all GPCRs, due to their extensive crosstalk with the proteins and pathways that perform the vast majority of cellular functions.

**Integrins**

Integrins are transmembrane adhesion receptors that have a primary role in cell–cell and cell–ECM binding, with a secondary role in signaling as well. They are essential for physiological development and are crucial in each step of the development and progression of cancer [41]. The first step in EMT occurs when cell–cell connections are lost and the ECM gains motility. In order for these cells to avoid apoptosis via a process known as anoikis, they rewire their genetic makeup in order to survive on their own, despite losing these connections [42]. There is evidence to suggest that the crosstalk between GPCRs, calcium channels, and integrins, ultimately leading towards EMT, makes this possible (Figure 3). Research studies have shown

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that certain calcium-sensing G-proteins form signaling complexes with integrins to aid in cell
differentiation and movement in cancers. For example, a GPCR with augmented expression
from increased extracellular calcium, known as CXCR4, activates a GTPase, known as Rap1,
which modulates integrin inside-out signaling by binding talin to β-integrin. This enhances cell–
ECM connections, assisting in cellular migration and cytoskeleton rearrangement [43]. One
study found that the overexpression of Orai1 was linked to survival of breast cancer cells, a
finding that was associated with increased collagen–integrin interactions [44].

In addition, recent studies have found that calcium directly modulates integrin activity.
Integrin subunits are formed in the ER and then released into the cytoplasm, and the proper
folding of these proteins requires the presence of divalent calcium ions [45]. Additionally, the
alpha and beta subunits of integrins cannot localize or activate the conformation in the plasma
membrane without the calcium ions. Calcium bound to integrins during transport ensures
inactivation until the integrins are membrane bound and the calcium is displaced by additional
cations, such as magnesium and manganese, to form an active conformation. Calcium allows
the two subunits to dimerize in the ER and translocate to the plasma membrane via
endocytosed vesicles for proper functionality [45]. It was also found that manganese and
magnesium work in conjunction with calcium to help bridge the integrin–ECM connection,
allowing for integrin signaling and cell adhesion [46]. One study found that calcium located at
the filipodia of cells was able to modulate the signal transduction process in integrins that were
used in migration and invasion for the purpose of tumorigenesis [47]. Whether used in
complexes and signaling or directly in cells, calcium has been shown to interact with integrins
that regulate normal biological and pathological progressions.
Calcium in Immune Evasion and Drug Resistance

The most proliferative and aggressive cancer cells possess essential characteristics that enable them to evade immune surveillance [48, 49, 50, 51, 52] and become resistant to drug treatment [53, 54, 55, 56, 57, 58, 59, 60, 61, 62]. These characteristics also enable proliferative and aggressive cancer cells to undergo EMT. Cancer cells often contain mutated antigens on the cell surface that are typically marked as non-self by locally activated T cells. Tumor cells with similar antigen presentation are, therefore, disposed of via T-cell infiltration and elimination. The binding of the antigen receptor on the tumor cell to the antigen receptor on the lymphocyte activates phospholipase C, capable of generating IP$_3$ and inducing SOCE. This store-operated calcium is used as a second messenger in the lymphocyte to transcriptionally activate NFAT. The lymphocyte is then able to release the calcium-dependent perforin, which disrupts the cancer cells’ membranes, leading to cell death [63]. This process can be initiated in immune cells and blocked in cancer cells by the use of calcium signaling. Cancer cells have manipulated calcium signaling, so that the immune system is no longer able to recognize and dispose of these abnormal cells. The tumor microenvironment plays an essential role in this process, in conjunction with the cancer cells themselves. As previously mentioned in Figure 2, cancer cells are capable of recruiting TAMs through calcium-induced transcriptional regulations to aid in cancer progression. For example, one study found that TAMs colocalized from a wide array of breast carcinomas were involved in the enhancement of tumor progression by producing a chemokine known as CCL18. TAMs located in the breast tumor microenvironment that release
CCL18 were able to activate a GPCR known as NIR1. This triggered integrin clustering and ECM remodeling in tumor cells, which were associated with increased calcium signaling through IP₃ generation, metastasis in multiple sites outside the primary tumor in patients, and poor survival [64]. In addition, by analyzing calcium-dependent and perforin-dependent cytotoxicity in multiple tumor types [65], experiments have shown that the overexpression of Orai1 and excess calcium signaling in cancer cause a reduction in the cytotoxic effect of natural killer cells and the ability of cytotoxic T-lymphocytes to eliminate the cancer. In contrast, the inhibition of Orai1 via siRNA and reduced extracellular calcium decrease cancer cell proliferation and enhance the cytotoxicity of cytotoxic T-lymphocytes and natural killer cells to tumors [65].

While intrinsic factors of tumor cells interacting with the tumor microenvironment can help cells avoid immune detection, extrinsic factors outside these cells can affect cellular processes that lead to drug resistance as well.

Sources of extracellular stress, such as hypoxia, lack of nutrients, and drug pressure, contribute to the induction of EMT by forcing cells to a crossroad of either adapting or dying. Calcium signaling is an important contributor to this process. Small calcium deposits in the tumor microenvironment, known as microcalcifications, have recently been studied as potential links to EMT, and may be a source or product of this increased calcium. One study of prostate cancer found a correlation between microcalcifications and increased bone metastasis [66]. Another study found a similar correlation of microcalcifications and EMT markers, such as CD44 and vimentin, in breast cancer, which could predict a poor prognosis in patients [67]. Intracellular calcium fluctuations have been shown to play a role in epigenetic programs and transcriptional regulation to allow cells to survive in the most extreme cases. For example,
sustained calcium entry by means of SOCE has been shown to activate the NFAT/CREB pathway, which induces transcriptional modifications in cells for the progression of tumorigenesis [68]. In contrast, the inhibition of calcium flux causes cancer cells to have increased sensitivity to drug treatment [69]. Further research is still needed to assess whether inhibiting calcium flux results in better patient outcomes. On the cell surface, channels and pumps undergo mutations to improve survival in the face of drug treatments as well. For example, Stim1 was found to work in conjunction with Orai1 to mediate TGF-β, which induces a key transcriptional factor of EMT, called snai1, that corresponds with drug resistance [70]. In contrast, efflux pumps play a vital role in cell survival. One study illustrated the role of sorcin (SOLuble Resistance-related Calcium-binding ProteIN) overexpression in cancer, which modulates the co-overexpression of ABC (adenosine triphosphate-binding cassette) transporters serving as a survival mechanism by efficiently effluxing drugs out of the cells, in addition to sorcin’s ability to induce EMT [71]. Currently, sorcin is viewed as an oncogene that could be central to multidrug resistance in clinical settings and a viable target for new drug discovery [71]. Furthermore, persister cells in cancer are a common cause of treatment failure and patient relapse. Persister cells make up a very small proportion of malignant cells and have adapted to survive extremely lethal doses of drug treatment [72]. They can also remain in a state of dormancy for extended periods of time, which, in conjunction with a decelerated cell cycle, enables them to acquire mutations that allow for cellular progression [73]. Research has substantiated a consistent trait of these persister cells as having increased calcium signaling in parallel with cytoskeleton remodeling [74]. Understanding the vast array of mechanisms of resistance remains crucial for future drug discovery and optimizing combination therapy.
Combination Therapy

The heterogeneity of tumors within their microenvironment has proven to be one of the biggest obstacles in treatment. Tumors create diversity through cellular differentiation to obtain a more sustainable environment and prevent their eradication [75, 76, 77, 78, 79, 80, 81]. Evasion of immune surveillance, angiogenesis, ECM remodeling, blockage of apoptosis, and multidrug resistance are all ways in which tumors are able to proliferate and explain why they cannot be captured by a single cellular phenotype. Calcium remains at the center of all of these malignant processes (Figure 1). In order to optimize patient treatment, it is often beneficial to use combination therapy to combat the heterogeneity of the tumor microenvironment. Combination therapy is a practice used for almost all cancers and subtypes. Additional details of how more well-studied cancers, including breast, lung, and colon cancer, respond to combination treatment are presented below.

Breast Cancer is one of the most common types of cancer and kills more women than any other cancer worldwide [82]. It has been characterized by its many mutations, including human epidermal growth factor 2 (HER2), breast cancer genes 1 and 2 (BRCA1/2), and phosphatase and tensin homolog (PTEN) [83]. Within these mutational subtypes, studies have found alterations of calcium channels and pumps that play a part in the proliferation of breast cancer cells, such as changes in expression and localization, and which allow for sustained calcium signaling [84]. Targeting unique cancer characteristics, such as driver mutations and calcium fluctuations, may be necessary for optimal treatment. Further evidence of this,
involving a calcium channel known as TRPV6, which is found to be overexpressed in breast cancer, along with many other cancers, has been found in clinical settings. Clinical trials are ongoing with a novel TRPV6 inhibitor, in conjunction with standard-of-care therapy, for multiple subtypes of breast cancer, since some current treatments, such as tamoxifen, have been shown to negatively affect the TRPV6 channel as well [85]. Many of the current standard-of-care chemotherapy agents also target calcium signaling for the treatment of breast cancer. Cisplatin and tamoxifen act by causing an overwhelming influx of calcium and ER store depletion that leads to cell death, while doxorubicin induces sustained calcium flux, which activates the proapoptotic BIM pathway and causes mitochondrial calcium overload [86].

Lung Cancer is heavily burdened by mutational drivers that make targeted therapy a promising avenue for drug development and impactful in terms of patient outcomes. Some of the more common mutations include epidermal growth factor receptor (EGFR), KRAS, and ALK. EGFR inhibitors remain the most well-developed targeted inhibitors, with strategies to target the most common resistance mutation, T790M, for this disease. Epidermal growth factor (EGF), the ligand for EGFR, was found to play a role in the calcium oscillations essential for EMT [87]. The evidence suggests that the blockage of cells from extracellular calcium via a calcium chelator known as EGTA increases the efficacy of EGFR inhibitors such as afatinib [74]. Immunotherapies are also on the rise in cancer treatment. One study found that blocking calcium channels can suppress the transcription of programmed death-ligand 1 (PD-L1) and enhance natural killers cells’ ability to eliminate the cancer [88]. Chemotherapies that target calcium signaling, similar to those used against breast cancer, such as cisplatin and doxorubicin,
were found to be synergistic in combination with EGFR inhibitors (gefitinib and erlotinib) and to prolong patient survival in the clinic [89].

Colon Cancer is the third most common cause of cancer-related death. Little is known about the causes, but the most prominent driver mutations include adenomatous polyposis coli (APC), BRAF, and KRAS [90]. SERCA channels are often targeted to deplete ER calcium stores. One SERCA channel blocker used in combination with standard-of-care drugs for colon cancer is called thapsigargin [91]. Stim1 and Orai1 inhibitors have also been used to increase patient survival rates [91]. In addition, one standard-of-care drug for colon cancer, known as 5 Fluorouracil (5-FU), acts by modulating calcium itself. 5-FU was found to signal through a calcium-dependent pathway in order to induce apoptosis [92].

By incorporating the role of calcium signaling in the development of new drugs, therapies can evolve to optimize cancer treatment. This has been shown in the treatment of other cancers, such as prostate, pancreatic, and glioblastoma. As with lung cancer treatment, cisplatin and paclitaxel alter calcium homeostasis and remain an effective first-line treatment for prostate cancer that has an overexpression of TRPV channels [93,94]. Pancreatic cancer is similar to colon cancer, with the infamous KRAS mutation and the overexpression of Orai1. An additional similarity between the two malignancies is the use of the calcium-induced apoptosis treatment of 5-FU [95]. Even glioblastoma, one of the most lethal cancers, has started to be treated with calcium-altering therapies. T-Type calcium channel blockers, combined with temozolomide (TMZ), are in the early stages of clinical trials for use in the treatment of high-grade gliomas [96,97]. A comprehensive table, containing calcium alterations across multiple cancers and calcium pathway-targeting therapies, is shown in Table 1. Combination therapy has
become especially important with the increasing knowledge of EMT and multidrug-resistant mechanisms. While some standard-of-care drugs directly target calcium pathways, targeting multiple broad mutations of the heterogenous population within the tumor microenvironment may effectively reduce drug resistance and improve long-term patient outcomes.

Conclusion

Cancer constantly evolves, with mutational burdens that are continually being explored. Therefore, understanding commonalities that lead to the development of a more malignant EMT phenotype is essential for drug discovery and the expansion of combination therapies. A plethora of evidence has substantiated the fact that calcium plays a critical role in the EMT process, leading to immune evasion and drug resistance. Calcium modulates cellular functions by inducing alterations in calcium channels; interacting with receptors; and remodeling the ECM signaling pathways, transcription, and epigenetics. This allows normal cells to transition into cancer cells. Insights into specific changes at a transcriptional and protein level will aid in the construction of new targeted therapies to optimize the eradication of the heterogenous tumor population. Since the EMT is a transient process, the reversal of a malignant phenotype or the blockage of its progression would also be a viable option for improving patient outcomes. Calcium signaling and its crosstalk with a multitude of additional signaling pathways can obscure correlative data as causative evidence. As a result, further research is needed to improve knowledge on delineating calcium’s many roles in the EMT process and cancer progression, to help those faced with this deadly disease.
Author Contributions

Writing—original draft preparation, C.A.J.; Writing—review and editing C.A.J. and L.A.H.;
Supervision and Funding acquisition, L.A.H. All authors have read and agreed to the published
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Conflict of Interest

The authors declare no conflict of interest.
References

1. Terrié, E.; Coronas, V.; Constantin, B. Role of the calcium toolkit in cancer stem cells. *Cell Calcium* 2019, 80, 141–151, doi:10.1016/j.ceca.2019.05.001.


70. Bhattacharya, A.; Kumar, J.; Hermanson, K.; Sun, Y.; Qureshi, H.; Perley, D.; Scheidegger, A.; Singh, B.; Dhasarathy, A. The calcium channel proteins ORAI3 and STIM1 mediate TGF-β


77. Carm, K.T.; Hoff, A.M.; Bakken, A.C.; Axcrona, U.; Axcrona, K.; Lothe, R.A.; Skotheim, R.I.; Løvf, M. Interfocal heterogeneity challenges the clinical usefulness of molecular


Figures and Tables

Figure 1:

Role of Calcium in EMT
Table 1: Calcium changes by cancer type as a result of therapies targeting calcium pathways. ↑ (increase in expression)

<table>
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<tr>
<th>Cancer Type</th>
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<th>Calcium Alterations</th>
<th>Calcium Altering Therapies</th>
<th>Reference</th>
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<tr>
<td>Lung</td>
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<td>Doxorubicin</td>
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<td>↑TRPV6</td>
<td>Cisplatin</td>
<td>[83,85,86]</td>
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<td>↑TRPV1</td>
<td>T-Type Calcium Channel Blocker Combined with TMZ</td>
<td>[96,97]</td>
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**Figure Legends**

**Figure 1.** Role of calcium in epithelial-to-mesenchymal transition (EMT). Tumor associated macrophages (TAMs); Extracellular matrix (ECM); G-protein coupled receptor (GPCR); T-cell receptor (TCR)

**Figure 2.** GPCR signaling induces EMT. This schematic illustrates the activation of a GPCR signaling pathway when bound to a ligand such as a chemokine. Both signal transduction pathways have the ability to induce EMT at a transcriptional level by activating genes such as Snail, Twist, and ZEB. GPCR signals that transcriptionally activate EMT genes are capable of recruiting TAMs to the tumor microenvironment. TAMs produce MMPs that allow these cells to invade and metastasize in the EMT process. Diacylglycerol (DAG); Inositol trisphosphate (IP$_3$); Cyclic adenosine monophosphate (cAMP)

**Figure 3.** Calcium alterations causative of EMT. This schematic illustrates the role of calcium signaling, which is required for progression from a normal epithelial cell to an EMT phenotype associated with metastatic cancer cells, by showing (1) The starting point of a healthy epithelial cell containing all tight junction proteins and physiological levels of GPCRs and calcium channels. (2) Gradual reduction in junction proteins and increased calcium channel production. (3) Gradual increase in mesenchymal proteins, such as Vimentin and N-Cadherin, and GPCRs acquiring mutations. (4) ECM dissociation through integrins. (5) Increased motility. (6) Invasion of a cancer cell into bloodstream, to metastasize away from primary tumor. Junctional adhesion molecule (JAM); Zinc finger E-box-binding-homeobox (ZEB)
Chapter 3

Emergence of Resistance to MTI-101 Selects for Favorable MET Genotype and Phenotype in EGFR Driven PC-9 and PTEN Deleted H446 Lung Cancer Cell Lines

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Simple Summary

MTI-101 is a first-in-class novel cyclic peptide shown to have anti-tumor activity in both multiple myeloma and castrate-resistant prostate in vivo cancer models. These data suggest the potential for a broad spectrum of anti-cancer activity for this class of compounds. To further delineate determinants of sensitivity and resistance that were not dependent on oncogenic drivers, two isogenic drug-resistant cell lines were generated with chronic exposure to MTI-101 in a non-small cell lung cancer (NSCLC) PC-9 (EGFR driven) and a small cell lung cancer (SCLC) H446 (PTEN deleted and c-MYC amplified). Our data indicate that the chronic exposure of MTI-101 selects for a stable mesenchymal-to-epithelial (MET) genotype and phenotype in both PC-9 and H446 lung cancer cell lines.

Abstract

MTI-101 is a first-in-class cyclic peptide that kills cells via calcium overload in a caspase-independent manner. Understanding biomarkers of response is critical for positioning a novel therapeutic toward clinical development. Isogenic MTI-101-acquired drug-resistant lung cancer cell line systems (PC-9 and H446) coupled with differential RNA-SEQ analysis indicated that downregulated genes were enriched in the hallmark gene set for epithelial-to-mesenchymal transition (EMT) in both MTI-101-acquired resistant cell lines. The RNA-SEQ results were consistent with changes in the phenotype, including a decreased invasion in Matrigel and expression changes in EMT markers (E-cadherin, vimentin and Twist) at the protein level. Furthermore, in the EGFR-driven PC-9 cell line, selection for resistance towards MTI-101 resulted in collateral sensitivity toward EGFR inhibitors. MTI-101 treatment showed synergistic
activity with the standard of care agents erlotinib, osimertinib and cisplatin when used in combination in PC-9 and H446 cells, respectively. Finally, in vivo data indicate that MTI-101 treatment selects for increased E-cadherin and decreased vimentin in H446, along with a decreased incident of bone metastasis in the PC-9 in vivo model. Together, these data indicate that chronic MTI-101 treatment can lead to a change in cell state that could potentially be leveraged therapeutically to reduce metastatic disease.

Introduction

Lung cancer is the deadliest of all cancers and accounts for more deaths each year than breast, prostate and colon cancer combined [1]. Upon diagnosis, lung cancer can be categorized into two subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Almost all SCLC patients are current or former tobacco smokers, and cases of SCLC typically have very aggressive tumors grouped with a poor five-year survival rate below seven percent [2]. There are few treatment options for SCLC patients because many of the identified molecular drivers are not currently druggable, along with the presence of considerable tumor heterogeneity, which likely contributes to the rapid emergence of drug resistant disease. The emergence of resistance and metastatic disease contributes to the 5-year survival rate of SCLC patients at a dismal 7 %. In contrast, NSCLC patients include smokers and non-smokers. In fact, 10-15 % of all NSCLC newly diagnosed patients have never smoked[3]. Many therapeutically actionable mutational drivers have been identified in this class of lung cancer such as EGFR and ALK mutations that allow for targeted therapies for these subsets of lung cancer patients. Despite advances in targeted therapeutics, metastatic NSCLC remains a deadly disease due to
the rapid emergence of drug resistance. Recent data indicate that one way to combat drug resistant profiles and alternating the schedule as single agents may lead to improved patient outcomes by delaying the emergence of an aggressive drug-resistant phenotype [4].

MTI-101 is a first-in-class novel therapeutic agent that was originally derived from a linear peptide known as HYD-1, found in a high throughput screen that utilized inhibition of cell adhesion as a phenotypic endpoint[5]. It was later found to induce cell death in cancer cells by altering calcium homeostasis in a caspase-independent manner[6]. Experimental evidence indicates that MTI-101 selectively targets cancer cells via activation of a TRPC complex and calcium entry, leading to cell death [7-8]. MTI-101 has shown strong activity in relapsed multiple myeloma primary patient specimens tested ex-vivo and castrate-resistant prostate in vivo models by reducing tumor volume and increasing survival with little observed toxicity at the efficacious doses used for in vivo studies [7,9]. Interestingly, resistance to HYD-1 in myeloma cell lines resulted in a compromised cell adhesion-mediated resistant phenotype, suggesting that resistance emerged at an overall cost to the cell, related to cell adhesion[10]. We hypothesized that this cyclized peptide known as MTI-101 may be an attractive therapeutic agent for metastatic lung cancer, as calcium signaling pathways are critical for metastasis[11]. Identification of phenotypic traits and changes in states due to genotypic changes that occur with acquisition of resistance to MTI-101 will enhance the understanding of how to clinically position this first-in-class molecule.
The primary site of a tumor will eventually be limited by resources which places an initial selection pressure to favor cells which are capable of invading the surrounding tissue or relocate to additional regions in the body to obtain the necessary nutrients for the survival of rapidly dividing cells. This cellular rearrangement called epithelial to mesenchymal transition (EMT) is found to take place in normal cells for tasks such as development and wound healing, but cancer cells can undergo these changes in response to stress stimuli[12-14]. This transition allows for epithelial like tumor cells to alter gene expression such as decreasing epithelial proteins, including E-cadherin, claudin, and occludin while increasing mesenchymal proteins such as Vimentin (VIM), N-cadherin, and fibronectin (FN1), allowing them to survive after losing contact from adjacent cells and the basement membrane[15]. EMT cells can undergo cytoskeleton rearrangement to become more motile and also produce proteins such as matrix metalloproteases (MMPs) that allow them to invade into new tissues and metastasize[16-17].

Clinically, malignant cells displaying an EMT phenotype after drug treatment correspond with a poor prognosis that leads to decreased relapse time and increased metastatic tumor sites[18-20]. This transition is transient, and cells can also change to a more epithelial like phenotype moving toward MET[21]. Patients that are treated with current standard of care therapies for lung cancer often experience relapse that give rise to drug resistant tumors that have undergone EMT[20]. Recent studies have shed light on EMT as a spectrum of cell states compared to a binary process[22]. Early models supported the claim of the existence of hybrid EMT models by developing mathematical equations to describe a unique model of the microRNA-based coupled chimeric modules that utilize ZEB and Snail mutual inhibition feedback circuits[23]. This phenomenon was further explained by Snail microRNA serving as a
reversible switch to initiate EMT, while ZEB microRNA acts as an irreversible switch to establish the mesenchymal state with the interplay of TGF-β signaling[24]. More recent studies in vivo have shown that due to the heterogeneity of tumor populations, there are six distinct EMT subpopulations that exist based on cell surface markers that differentiate cellular plasticity, invasiveness and metastatic potential[25]. Due to the unique mechanism of action of MTI-101, we sought to further understand the emerging drug resistant phenotype using in vitro and in vivo models. Emergence of resistant cell lines were analyzed in comparison to the wildtype (WT) to determine the genotype and corresponding phenotype the drug is selecting for in lung cancer. These data may allow for identification of new actionable vulnerabilities that emerge with chronic selection pressure of MTI-101 treatment that may inform rational therapeutic strategies to be further validated in pre-clinical lung cancer models prior to the development of clinical strategies.

**Materials and Methods**

**Cell Culture/Reagents**- PC-9 (Sigma #90071810) H446 (ATCC #HTB-171) and HCC4006 (ATCC #CRL-2871) lung cancer cell lines were used as parental cell lines. MTI-101 resistant cell lines PC-9R and H446-R were developed with chronic exposure to MTI-101 over a 6-month period. All cell lines were maintained in RPMI-1640 L-Glutamine with 10% Fetal Bovine Serum, and 1% penicillin/Streptomycin. Resistant cell lines were maintained with a constant level of MTI-101 in culture media (60 µM for PC-9R and 40 µM for H446-R). All cell lines were tested for mycoplasma every six months, and STTR analysis was performed on a yearly basis.
**MTT Assays** - PC-9 cells were seeded at 10,000 cells/180 µL media and H446 was seeded at 20,000 cells/180 µL media in each well of a 96 well plate. The following day, 20 µL of varying drug concentrations of MTI-101 dissolved in sterile water was added to each treatment group with 20 µL of sterile water added to the controls. 24 hours after the introduction of MTI-101, 50 µL of MTT dye was added to each well and incubated for one hour. Following incubation, the media/dye was aspirated off, and the cells were resuspended in 200 µL of DMSO and the absorbance of each well was read on a plate reader at 570 nm. A $\log_{10} (Dose)$ curve was generated using GraphPad to calculate the IC$_{50}$ values of each cell line.

**Western Blots** - All Cells were lysed in buffer composed of 50 mm Tris (pH 7.4), 5 mm EDTA, 150 mm NaCl, and 0.5% Triton X-100 containing 1 µg/mL leupeptin and aprotinin and 1 mm phenylmethyl sulfonyl fluoride. The protein content of cell lysates was quantified by using the Pierce BCA protein assay kit, and equal amounts of the total protein were dissolved in Laemmli SDS-PAGE sample buffer prior to separation by 15% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad), and Western blot analysis was performed by using standard techniques with enhanced chemiluminescence detection (Roche/Boehringer Mannheim, Indianapolis, IN). The following antibodies were used in this experiment: E-cadherin, Vimentin, B-catenin, TWIST1, B-actin (Cell Signaling, Cat #:3195T, 5741T, 8480T, 46702, 4967S) and TWIST2 (ThermoFisher 66544-1-IG).

**Invasion Assay** - PC-9 cells were seeded at 25,000 cells/mL media and H446 cells were seeded at 40,000 cells/mL. 100 µL was dispensed in each well of a 96 well round bottom plate. Spheroids were allowed to form for 48 hours. Then, 70 µL media was slowly aspirated off and the plate was placed on ice for 5 minutes. Next, 70 µL of Matrigel was slowly added on top of
each spheroid and placed in the incubator to allow gel solidification for 1 hour. Following
incubation, each well was topped with 100 µL media to prevent the gel from drying out and the
plate was placed on the Biotek Lionheart imager to track invasion over a 48-hour period. The
image montage was stitched and the most in focus z-parameter was selected for each time
point. Invasion distance was quantified by creating a primary mask on the original spheroid at
Time 0 and tracked over time divided by the Time 0 mask for the duration of the invasion assay.

**Anoikis Assay** - Apoptosis assays for anoikis were performed by assaying Annexin v/pl staining.
500,000 cells (50,000 cells/mL) were placed in 100 mm ultralow attachment dish (0.5% methyl
cellulose; Sigma-Aldrich) for 24 hours. The apoptotic cells were determined using propidium
iodide (pl, 2µg/ml; Thermo Fisher) and Annexinv V-APC (1 µg/ml; BD Biosystems) using a
FACScan flow cytometer (BD biosystems).

**RNA Extraction** - Quadruplicates of RNA were extracted from each WT and resistant lines of PC-
9 and H446 using RNeasy Plus Mini Kit (Qiagen, cat#74104). 2-3 million cells were isolated in a
dry pellet and resuspended and RNA was initially extracted using Trizol, and then further
purified using the Qiagen RNeasy Mini Protocol and RNA was stored at -80 degrees. The same
RNA isolation procedure was performed for the PC-9 4, 5, and 6 months removed from drug
(RFD).

**RNA-Seq Data Analysis** - RNA-Seq libraries were sequenced at Marshall University Genomics
Core with Illumina NextSeq 2000. Pair-end RNA-SEQ short reads were aligned to the whole
human reference genome (hg38) by subread v2.0.1[26]. The total number of reads aligned to
transcripts at gene level were summarized by featurecounts from Rsubread v2.2.0[27] with
built-in RefSeq gene annotation. Gene expression level was quantified by RPKM (reads per
kilobase of exon model per million mapped)\textsuperscript{[28]}. We used edgeR v3.30.3\textsuperscript{[29]} to define differentially expressed (DE) genes with the following criteria: p-value < 0.05, fold change >1.5, and CPM (count per million; log2)> 0. To determine the relationship between expression changes of PC-9 and H446 to their MTI-101 resistant counter parts, we employed Gene Set Enrichment Analysis through GSEA v4.0.2. We created three “rnk” files based on fold change of expression (treatment vs baseline) for the following comparisons: PC-9R vs PC-9, H446-R vs H446 and PC-9 RFD vs PC-9; a “rnk” file consist of one column for gene symbol and another for fold-change of expression. The rnk file was then supplied as input to GSEA to determine the significance of a preferential expression for selected gene sets from MSigDB in treatment condition over the baseline, or vice versa.

Transfections- PC-9 and H446 cells were transfected with a tomato red/LUC vector (pcDBA3.1 (+)/Luv2=tdT, Addgene #32904)\textsuperscript{[30]} by electroporation using an Amexa nucleofection kit and Amexa nucleofector instrument per manufacture’s recommendation.

In Vivo Animal Studies- (H446 tomato red/LUC) A single treatment MTI-101 study was conducted in SCID/BEIGE mice to understand the single agent effect of MTI-101 in lung cancer. The mice (6-8 weeks old Male/Female) were injected with 5 million H446 cells (transfected with tomato red/LUC) via tail vein, allowed to engraft for four weeks and imaged on IVIS for tumor location and progression. Fourteen (M/F) mice were separated into two groups dividing tumor burden, location of tumor, and gender evenly. One group was treated with 20mM histidine (vehicle control) while the other was treated with 10 mg/kg MTI-101 via IP injection three times a week for the duration of the study (200 days). Once mice met study endpoints per ACUC protocol, mice were euthanized, and kidneys were collected for further analysis. In the H446
model, cells engrafted consistently in the kidneys and thus this organ was processed for further analysis. Vimentin, E-cadherin, and β-catenin stains were completed on slices of these kidneys of both groups. (PC-9 tomato red/LUC) A similar study was also conducted using the EGFR Driven NSCLC PC-9. The mice (6-8 weeks old M/F) were injected with 2 million PC-9 cells (transfected with tomato red/LUC) via tail vein, allowed to engraft for three weeks and imaged on IVIS for tumor location and progression. Twenty-one mice were separated into two groups dividing tumor burden, location of tumor and gender evenly. One group was treated with 20mM histidine (vehicle control) while the other was treated with 10 mg/kg MTI-101 via IP injection beginning 3 weeks post tumor injection for 12 weeks. Once mice met study endpoints per IACUC guidelines, they were euthanized, and lungs were collected for further analysis of MET markers including Vimentin (Ventana #790-2917), E-Cadherin (Ventana #790-4497), and B-catenin (Cell Marque #224M-14). Lung tissue sections were also stained with H&E to confirm presence of tumor, and IHC was used to detect markers of MET. For IHC studies, tissues obtained from 6 mice from control and 6 mice from the MTI-101 treated cohort were analyzed. Fiji software was used to analyze mean intensity based on tumor area.

All in-vivo study designs were approved by the West Virginia University ACUC board.

Results

Development of Isogenic MTI-101 Resistant Lung Cancer Cell Lines

The development of isogenic-acquired drug-resistant cell lines is one strategy to identify determinants of sensitivity and resistance [31,32] to novel therapeutics. The isogenic cell lines allowed for paired analysis using RNA-SEQ to match the genotype to the emerging phenotype.
PC-9 and H446 WT cell lines were initially treated with a low dose (10 μM) of MTI-101. The surviving or resistant cells were treated each subsequent time with 10 μM MTI-101 until they were able to proliferate at approximately the same rate as the parental WT cell line. The dose was then increased by 10 μM increments until a stable drug-resistant variant emerged. H446 was able to reach a 40 μM resistant level whereas PC-9 was stable up to a 60 μM resistant level. Interestingly, increasing the drug concentration beyond these doses was not achieved, as cultures did not sustain growth with an increased selection pressure. The stable variants were then compared to the respective WT cell line using an MTT analysis to assess resistant levels. The IC50 value shown is an average (n = 3 independent experiments) using the log dose of MTI-101 and viability compared to the control with non-linear regression on GraphPad. H446 and H446-R had, respectively, an IC50 value of 16.71 ± 3.97 and 34.51 ± 10.86 μM (Figure 1A). PC-9 and PC-9R had an IC50 value of 37.47 ± 4.33 and 82.11 ± 12.37 μM (Figure 1B). It was surprising to us that we could not select for higher levels of resistance, suggesting that a robust drug resistance phenotype may emerge at a high cost to overall fitness. In order to determine whether MTI-101 demonstrates specificity toward lung cancer cells, a healthy bronchial epithelial cell line known as BEAS-2B was also treated with the same range of doses of MTI-101 to determine its sensitivity. The IC50 value for BEAS-2B with treatment of MTI-101 was found to be 116.07 ± 29.05 μM. Together, these data indicate that lung cancer cells demonstrate an increased sensitivity compared to normal epithelial lung cells toward treatment with MTI-101. During the development of the two isogenic MTI-101-resistant lung cancer cell lines, morphological images were taken using bright field microscopy (20X), comparing each to their respective WT cell lines (Figure 1C,D). The resistant cell lines appeared to be more clumped and
adherent to the culture dish than the WT that formed an even monolayer in vitro, suggesting increased cell–cell connections with resistance. This observation is suggestive of resistant cells converting to a more epithelial phenotype compared to the parental line. In parallel with morphological changes, resistant cells were also found to be significantly more sensitive to anoikis-induced apoptosis than their WT counterpart (Figure 1E,F).

**Bioinformatic Analysis Discovers Enrichment of MET in Resistant Cell Lines**

We applied RNA-seq analysis to determine differences in transcriptomes among PC-9 and H446 lung cancer cell lines and their MTI-101 resistant variants, PC-9R and H446-R, respectively. To determine the stability of changes in the transcriptome, the PC-9R line was removed from MTI-101 drug pressure and RNA was collected at four, five and six months post drug removal. PCA analysis based on gene expression revealed that replicates from the same cell lines were clustered together, confirming the high reproducibility of the data (Figure 2A). The x-axis from the PCA plot depicts the cell type difference, and the y-axis demonstrates the expression difference between parental and MTI-101-resistant lines. The PC-9 RFD samples were closer to PC-9R than PC-9, indicating that removing the drug pressure for six months failed to convert the transcriptome of the resistant line back to its parental line. Gene set enrichment analysis was performed to determine pathways differentially expressed between WT and resistant cells. The results revealed that EMT is the most downregulated hallmark gene set in the MTI-101 resistance lines when compared to their parental lines (Figure 2B,C). Consistent with the PCA analysis, PC-9 RFD compared to PC-9 was also downregulated for the EMT gene set (Figure 2D), illustrating its similarity with PC-9R and the stability of the transcriptome.
signature after relieving drug pressure. A Venn diagram analysis was conducted to compare all
the differentially expressed genes between resistant cell lines and the WT lines (Figure 2E). It
identified 329 genes commonly shared by all. Genes related to EMT in PC-9 were individually
analyzed, including SPARC, vimentin, fibronectin, TWIST2 and PLOD2 (Figure 2F). Similar
significant results were found in the negative expression of these EMT genes in the H446 cell
line as well (Supplemental Figure S1). In addition, these same genes were analyzed by relating
their expression to the patient overall survival through the lung cancer Kaplan–Meier KM
Plotter [33]. A low expression of TWIST1, fibronectin and PLOD2 predicted a significant
increased patient survival (Figure 2G). In contrast, no changes in survival were observed based
on the expression of SPARC or TWIST2 and the surprisingly low VIM expression as a single gene
correlated with poor outcomes. Interestingly, EMT was negatively enriched in both resistant
cell lines despite the vast differences in starting transcriptomes between the parental PC-9 and
H446 with the sole selection pressure of MTI-101 treatment.

Emergence of Resistance to MTI-101 Selects for a Stable Phenotype Associated with

Diminished Invasive Capacity and Increased Expression of Epithelial Markers

Based on differential gene expression profiling, one of the only transcription factors
shown to induce EMT that was differentially expressed in both cells lines was TWIST. Comparing
parental to resistant lines, TWIST1 was significantly decreased in the H446-resistant variant
(Figure 3A,B). In PC-9 cells, the basal level of TWIST1 is low and was further diminished in the
resistant line (albeit the quantification of three blots did not show significance, \( p > 0.05 \) T-Test).
In contrast, TWIST2 was significantly decreased in the PC-9 resistant variant (Figure 3C,D). The
original Western blots for individual runs are contained in the Supplemental Figure S2–S4 for TWIST1 and Supplemental Figure S5–S7 for TWIST2. The TWIST2 expression in H446-R did appear to trend towards an increased expression compared to H446, but was not found to be significant ($p > 0.05$, $T$-test $n = 3$). It is important to note that even though this difference is only two-fold, it could be compensating for a decreased TWIST1 expression in H446. For comparison, there was a ten-fold decrease in TWIST1 expression whereas only approximately a two-fold increased expression of TWIST2 comparing H446 to H446-R. In addition, the difference among resistant and sensitive cell lines was analyzed based on common EMT markers by Western blot analysis. PC-9 WT was found to have low E-cadherin levels, whereas PC-9R demonstrated a significantly increased expression (three-fold change) of E-cadherin at the protein level (Figure 4A,B). On the other hand, H446 WT was found to have a high expression of vimentin, whereas H446-R demonstrated a reduced expression by three-fold (Figure 4A,B). In addition, the invasion capacity was also measured for these cell lines. In both cases, the WT was found to have a significantly greater invasion capability in Matrigel over a 48-hour period than that of the resistant line (Figure 4C–F).

The development of each MTI-101-resistant cell line required approximately six months of constant drug pressure with gradual increased increments of dosing before a stable drug resistant line emerged. In order to determine the stability of this acquisition of a MET phenotype, the drug pressure of MTI-101 was removed from the resistant cells and analyzed over a six-month span. The epithelial marker E-cadherin was found to not fully revert back to the expression level of the WT after six months of being removed from the drug (RFD) by Western blot analysis (Figure 4G,H). The original Western blots for individual runs can be found
in Supplemental Figures S8-S10 for Figure 4A along with Supplemental Figure S11 for Figure 4G. Sensitivity to MTI-101 was analyzed by MTT comparing time from RFD compared to PC-9 WT and PC-9R. The IC₅₀ values of RFD obtained a slight increase in sensitivity from their original PC-9R, but never fully reverted to the PC-9 WT sensitivity at each time point for the six months tested (Figure 4I). The inability to invade into Matrigel was compared among PC-9 RFD 4, 5 and 6 months to the PC-9 WT and PC-9R. Similarly, the invasion capacity of PC-9 RFD demonstrated a stable MET phenotype when the drug pressure was removed for six months (Figure 4J). Together, these data indicate that MTI-101 selects in vitro for a genotype and phenotype that are predicted to be less invasive.

**MTI-101 Treatment In Vivo Recapitulates the In Vitro MET Phenotype**

In vivo animal models were conducted to determine whether MT-101 treatment in vivo induces an MET phenotype. The first study entailed the H446 WT tomato red/LUC cells being administered via the tail vein in SCID/BEIGE mice. Once mice met the study endpoints per IACUC protocol, they were euthanized, and the kidneys were extracted for further analysis. The kidneys were used for to stain for markers of MET in this model because it was the most common sight of engraftment for all mice in the study, while the lungs had little discernable tumor burden. The median survival of the treatment group was 89 days, compared to 68 days for the control, yet was not significant (p-value = 0.13 Figure 5A).

The H&E stains at 40X magnification confirmed that the cancerous portion of the kidney was being analyzed in these images for both the treatment and control. IHC was used to compare EMT markers. Vimentin was found to be greatly decreased whereas the membrane
localization of β-catenin was substantially increased in the treatment group (Supplemental Figure S12A,B), indicating that the treatment with MTI-101 was consistent with the transitioning of the cell state toward an epithelial phenotype (Figure 5C). E-cadherin was found to not be expressed in either group, which was consistent from the Western blots in vitro for this cell line. The 4X images of three mice from each group analyzed are shown in (Supplemental Figure S13).

PC-9 cells constitutively expressing tomato red and luciferase were injected via the tail vein into SCID/BEIGE mice. The two groups consisted of vehicle control and 10 mg/kg MTI-101 treatment three times a week for the duration of the study. A representative IVIS image for the engraftment of the study is shown in Supplemental Figure S14. PC-9 engraftment was detected in the lungs of all animals in the study. IVIS imaging indicated that the detectable distal site of tumor outside of the lung was found in the hind limbs. These multiple tumor sites were disproportionate between control and drug treatment cohorts during the treatment window, with three mice containing tumors in the bone within the control group and zero in the treatment group (Figure 5D), along with significant increases in E-cadherin expression despite no significant change in vimentin or survival with treatment of MTI-101 compared to the control (Figure 5B,E,F). Following the end of the study, only one mouse in the MTI-101 treatment group was found to have tumor in the bone. Together, these data indicate that, at a dose of 10 mg/kg administered three times a week, although there was no significant difference in survival between treatment and control groups, the drug treatment was shown to induce a similar change in cell state seen in vitro towards a MET phenotype.
MTI-101’s Effect in Context with Standard of Care Treatment for Lung Cancer

PC-9 cells are driven by an exon 19 deletion causing EGFR to be constitutively active [34]. The identification of activating mutations has led to the discovery of EGFR inhibitors, including the first generation tyrosine kinase inhibitor erlotinib and third generation tyrosine kinase inhibitor targeting an additional T790M mutation osimertinib [35]. PC-9R cells were found to have a significantly increased sensitivity to osimertinib and erlotinib single treatment than that of the parental PC-9 (Figure 6A,B). The IC₅₀ values of the PC-9 WT were 11.59 ± 0.09 and 25.91 ± 0.08 compared to the PC-9R of 4.57 ± 0.07 and 6.60 ± 0.06 nM, respectively, showing over a two-fold increase in sensitivity with both EGFR inhibitors. There was found to be no significant difference in sensitivity to cisplatin comparing PC-9 to PC-9R (Figure 6C). These data indicate that selection for resistance to MTI-101 drives collateral sensitivity to EGFR inhibitors. To determine whether MTI-101 is synergistic with EGFR inhibitors, PC-9 and HCC4006 cells were treated with varying concentration of both agents, and the combination index was determined using CompuSyn software (Version 1.0.1) A combination index significantly less than 1 is indicative of synergism between the two drugs. Osimertinib and erlotinib were found to have synergy with MTI-101 with the treatment of PC-9 and HCC4006 cells (Figure 6D,E). The PC-9R maintained a synergistic inhibition of growth when MTI-101 treatment was combined with osimertinib (Figure 6F). In contrast, selection for resistance in the H446 (SCLC) line did not result in collateral sensitivity to the standard of care agent cisplatin (Figure 6G). Cisplatin combined with MTI-101 was found to be synergistic in HCC4006, PC-9 and H446 cell lines (Figure 6H). However, cisplatin was only additive in H446-R, indicating that a switch in cell state did not maintain synergy with a DNA crosslinking agent (Figure 6I). Together,
these data indicate that, for EGFR-driven NSCLC, alternating treatment between MTI-101 and EGFR inhibitors may be a superior strategy compared to combination strategies. In contrast, a regimen consisting of a cisplatin and MTI-101 combination may yield the optimal outcome based on synergy studies and the emerging MTI-101-resistant phenotype.

Discussion

MTI-101 is a cyclic peptide that was developed through the optimization and cyclization of the linear peptide referred to as HYD-1 [6]. The mechanism of action of this novel class of molecules includes the induction of cell death via calcium overload, a finding that was dependent on TRPC1/TRPC4/TRPC5 expression in multiple myeloma cell lines [8]. The cyclized peptide MTI-101 was shown to have activity using castrate-resistant prostate cancer in vivo models [9]. In addition, our laboratory recently demonstrated that selection for resistance to HYD-1, the linear derivative, in myeloma cell lines selected for a compromised cell-adhesion-mediated drug-resistant phenotype (CAM-DR) and that HYD-1 and MTI-101 are more potent in inducing cell death in specimens derived from myeloma patients that have relapsed in therapy compared to newly diagnosed samples [10]. In this paper, we sought to expand on the understanding of the activity of MTI-101 in the context of lung cancer. To better understand the mechanism of action and emergence of resistance, we developed two isogenic drug-resistant lung cancer cell lines. The first line, H446, is a SCLC cell line that is characterized as PTEN-deleted, and contains the overexpression of c-MYC [36,37]. The second cell line is characterized as NSCLC and harbors the exon 19 deletion of EGFR, leading to a constitutive activation of the tyrosine kinase EGFR [34]. In the development of MTI-101-resistant lines, morphological
changes were observed in response to the drug treatment. The parental cell lines were more spindle-shaped and consisted of an evenly dispersed monolayer in 2D culture. In contrast, the resistant cell lines display a more cobblestone morphology that clumped to form more cell–cell interactions, along with forming tighter junctions to the culture dishes, supported with the notable increased difficulty in trypsinizing these adherent cells. These characteristics of the resistant cells are consistent with the morphology of more epithelial-like cells that contain tight sheets of cobblestone morphology to form apicobasal polarity and minimize surface energy [38,39]. The change in morphology correlated with the functional inhibition of invasion into Matrigel. It has been shown that transcription factors such as Snail, Twist and nuclear β-catenin regulate the induction of EMT [40] and can lead to increases in invasive capabilities [41,42], as seen in the parental cell lines. Using an unbiased analysis of differential gene expression demonstrated that the hallmark of EMT was reduced in the resistant phenotype. The conversion to a more epithelial-like phenotype was stable as cells removed from the drug for six months retained markers of MET and failed to invade Matrigel. These data suggest a heritable change in cell state, rather than a transient drug-induced state. Importantly, conversion to a more epithelial-like phenotype was observed using two independent in vivo models. In the PC-9 cells, this finding correlated with a delay and reduction in detectable bone metastasis, likely attributed in part to the significantly increased E-cadherin expression. Current clinical trials are targeting EMT in combination with chemotherapies/targeted therapies in lung cancer to reduce drug resistance and metastasis [43]. One example is the repurposing of metabolic inhibitors such as simvastatin for the treatment of lung cancer via the attenuation of TGFβ1 [44,45]. In addition, a small molecule drug known as moscatilin has been shown to
reverse EMT in lung cancer cells without inducing cytotoxic effects by suppressing mesenchymal markers such as vimentin, Snail and Slug [46]. Despite the breadth of research targeting the EMT pathway in cancer, to the best of our knowledge, the literature has not presented a drug that selectively kills cancer cells in which a MET phenotype emerges as the drug-resistant population.

Bioinformatic analysis of the RNA SEQ data demonstrated a stable attenuation of expression of genes that are associated with the EMT hallmark in both MTI-101-acquired resistant cell lines. By comparing common differentially expressed genes leading to this negative EMT enrichment, insight can be gained on the cellular remodeling causative for transition toward an epithelial phenotype. One transcription factor that may be driving the MET phenotype with resistance is Twist. The genes Twist1 and Twist2 have both been shown to play a role in the promotion of EMT and invasion in cancer [47,48]. Twist binds to DNA to repress the expression of E-cadherin [49], thereby creating dissociation with β-catenin in the extracellular matrix, causing either the degradation or nuclear localization of β-catenin [40,50]. The activation of the WNT signaling pathway has been shown to activate nuclear β-catenin localization for the induction of EMT, and it is thus intriguing to speculate that WNT signaling may be a determinate of sensitivity to MTI-101-induced cell death [51]. A high expression of Twist1 has also been shown to correlate with the overexpression of fibronectin, vimentin [52] and SPARC [53], which is consistent with some of the most significantly decreased genes in the RNA SEQ data of resistant cells in both cell lines. Fibronectin and vimentin have been extensively studied as mesenchymal markers promoting invasion [20,54–57]. Fibronectin has also been shown to regulate anoikis resistance by stabilizing cells with the ECM once detached.
from adjacent cells [58]. A significantly decreased fibronectin expression in both resistant lines could be the cause of increased anoikis-induced apoptosis. There is controversy in the literature on the role of SPARC in cancer [59], but, specifically for lung cancer, it has been shown to be a poor prognostic indicator as it promotes tumor metastasis [60,61]. In contrast, it has also been shown that the loss of E-cadherin itself can induce transcriptional modifications that cause an induction of EMT in cells [62]. It is important to note that MTI-101 has been shown to shift a cell state to a more epithelial-like phenotype, but the EMT process has many intermediate stages of this transition [22]. Further research is needed to determine the heterogeneity of the resistant population and the existence of subpopulations through single cell sequencing to understand if an intermediate stage along this process is involved or if it is a full transformation into MET.

Given that Twist was the only EMT transcription factor identified as being downregulated in the MTI-101 drug-resistant cell lines by gene expression profiling, a partial transition to an epithelial state is likely. Importantly, when the drug pressure was removed, the transition state toward MET was stable with respect to the shift in the genotype and phenotype. Clear vulnerabilities were defined, including an increased susceptibility to anoikis and increased sensitivity to EGFR inhibitors. It is intriguing to postulate that Twist levels may be used as a biomarker for the sensitivity of the drug to push cells towards a vulnerable state. The goal of identifying key changes that could be leading to a less aggressive phenotype is to potentially target or modulate cancer cells to reduce the incidence of relapse and prolong survival.

Patient relapse is almost always inevitable with current standard of care options for metastatic lung cancer. The devastation that comes with drug-resistant tumors is (i) surgical excision of the tumor is not feasible at metastatic sites; and (ii) evasion of drug treatment. MTI-
MTI-101 may be the first of its kind to induce a more favorable and treatable relapsed tumor. Further studies are required to determine whether the acquisition of resistance to EGFR inhibitors that occurs via an EMT phenotype can be remodeled to a MET phenotype with treatment with MTI-101. Once the MTI-101-resistant cancer cells are given a drug holiday (removed from drug pressure), the shift to an MET phenotype has remained stable for at least six months. This window would be optimal for additional targeting or chemotherapy treatments to eliminate these surviving cancer cells. In lung cancer, resistance to standard of care agents typically yields a more aggressive and drug-resistant EMT phenotype [63,64]. Our findings are similar to what others have shown, such that the reversal of EMT to MET resulted in re-sensitizing cancer cells to tyrosine kinase inhibitors such as gefitinib [65]. The alterations created during MTI-101-acquired resistance enhanced the efficacy of erlotinib and osimertinib in PC-9. Further studies are required to determine whether alterations of drug treatment (i.e. EGFR inhibitor followed by MTI-101 and then a repeated cycle of alternating drugs) would increase survival outcomes in pre-clinical in vivo models of EGFR-driven tumors.

Overall, drug resistance remains a crucial hurdle in the development of new cancer treatments. Delineating changes that lead to drug evasion and understanding the acquired phenotype give insight into new possible avenues of targeted or combination therapy to optimize patient outcomes. MTI-101 has shown promising anti-cancer activity, along with alterations of a cell state to a more favorable MET phenotype. The acquisition of resistance to MTI-101 has shown unique qualities that have exposed vulnerabilities to the surviving cancer cells.
Conclusion

The emergence of resistance to MTI-101 led to genotypic and phenotypic changes that correlated with a MET phenotype using both in vitro and in vivo models in PC-9 and H446 cells. Phenotypic modifications included increased cell–cell connections through morphological changes and increased expression of epithelial-like proteins, while decreasing the expression of mesenchymal-like proteins with a reduction in invasion. The stability of the transformed state in vitro is intriguing and could allow for a double bind therapeutic strategy where the goal is to switch between cell states EMT-MET to keep the tumor from rapidly escaping targeted therapies, such as EGFR inhibitors. Interestingly, the phenotypic selection pressure was survival despite MTI-101 treatment, and this MET population arose concurrently. More studies are required to determine if MTI-101 depletes the EMT population, leading to the emergence of an MET genotype, or, rather, if selecting resistance coincides with the attenuation of calcium signaling and, conversely, if robust calcium signaling is required to support the EMT genotype and phenotype. Resistance to MTI-101 also conferred collateral sensitivity to EGFR inhibitors for PC-9 cells. In addition, MTI-101 combination therapy with standard of care agents erlotinib, osimertinib and cisplatin all displayed synergistic activity in eliminating the cancer cells. These alterations that arise in PC-9 and H446 cells with the sole pressure of MTI-101 suggest a shift to a clinically favorable and long-term mesenchymal-to-epithelial transition that could give insight into novel anti-cancer therapeutic targets and combination strategies to optimize patient outcomes. Future studies are required to determine if following treatment in vivo with a standard of care agent such as erlotinib with MTI-101 and the subsequent emergence of
resistance is sufficient to drive the tumor population back toward drug sensitivity and a more epithelial cell state.

**Supplementary Materials:**

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14133062/s1, Figure S1: Bioinformatic Analysis of RNA SEQ Predicts a Shift toward a MET Phenotype with Acquired Resistance to MTI-101 in H446 vs. H446- R. EMT genes’ fold change between H446 and H446-R; SPARC-FDR = 33.09 × 10−4, VIM-FDR = 6.36 × 10−4, FN1- FDR = 2.81 × 10−4, TWIST1- FDR = 1.60 × 10−4; Figures S2–S4: Original Western blots of TWIST1 (n = 3); Figures S5–S7: Original Western blots of TWIST2 (n = 3); Figures S8–10: Original Western blots for Figure 4A (n = 3); Figure S11: Original Western blot of Figure 4G; Figure S12: H446 In Vivo Single Treatment MTI-101. (A) B-catenin localized heavily inside the cell in the one kidney of the control group with B-catenin presence. (B) A representative image of the treatment group containing B-catenin almost exclusively extracellularly; Figure S13: All H446 In Vivo H&E and IHC Images at 4× Magnification. Kidneys were taken from the H446 in vivo study, fixed in formalin, paraffin blocked and then sliced for slides to analyze EMT markers. Three control and three treatment (10 mg/kg MTI-101) mice were picked at random to compare EMT markers. (A) H&E, (B) E-cadherin, (C) vimentin, (D) B-catenin; Figure S14: Representative IVIS Images of Tumor Location for each In Vivo Study.
**Author Contributions:** “Conceptualization, C.J. and L.H.; methodology, C.J. and L.H.; software, L.H., G.H.; formal analysis, C.J., S.D., G.H., L.H.; investigation, C.J., S.D., S.S., A.E., G.H., L.H.; resources, L.H., G.H.; data curation, C.J., S.D., W.C., G.H., L.H.; writing—original draft preparation, C.J.; writing—review and editing, C.J., L.H., G.H., S.D.; visualization, C.J., S.D.; supervision, L.H.; project administration, C.J., L.H..; funding acquisition, L.H. All authors have read and agreed to the published version of the manuscript.”

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**Informed Consent Statement:** Not applicable

**Institutional Review Board Statement:** No human studies were performed. All animal studies were approved by the West Virginia IACUC board (IACUC Protocol Number: 1907026586.3, approved 05-20-2021)
Data Availability Statement: RNA-Seq data were deposited to Gene Expression Omnibus: accession number GSE193455.

Conflicts of Interest: Lori Hazlehust is a co-founder of Modulation Therapeutics which has licensed MTI-101. “The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results”.

References


[58] H. Han et al., “Fibronectin regulates anoikis resistance via cell aggregate formation,” 


Figures

Figure 1:

A) Variable activity of drug

B) PC-9 vs PC-9R

C) H446

D) H446-R

E) Anokis Assay

F) Anokis Assay

Figure 2:

A) Hallmark Epithelial Mesenchmal Transition

B) PC-9

C) H446

D) PC-9 Remove from Drug

E) Resistant WT

F) Resistant WT

G) Resistant WT

H) Resistant WT

I) Resistant WT

J) Resistant WT
Supplemental Figure 1S:

H446

Supplemental Figure 2S-11S: Original Western blots can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14133062/s1

Supplemental Figure 12S:

A)
Supplemental Figure 13S:

A) H&E Stain

Control

Treatment #8

Treatment #14

Treatment #22

B) E-Cadherin Stain

Control

Treatment #8

Treatment #14

Treatment #22

C) Vimentin Stain

Control #15

Control #1

Control #3

D) B-Catenin

Control

Treatment #8

Treatment #14

Treatment #22

Supplemental Figure 14S:

H446

PC-9
Figure Legends

Figure 1: Development of Isogenic MTI-101 Resistant Lung Cancer Cell Lines. (A) 24 Hour MTTs were performed with varying drug doses of MTI-101 comparing H446 to its respective resistant cell line. IC50 values were reported as an average n = 3 independent experiments. (B) 24 Hour MTTs were performed with varying drug doses of MTI-101 comparing PC-9 to its respective resistant cell line. IC50 values were reported as an average n = 3 independent experiments. (C-D) Resistance to MTI-101 induces cell morphological changes to more adherent epithelial like cells compared to the respective WT on a microscope at 20x magnification. (E-F) Anoikis assay comparing WT to respective resistant cell line for apoptotic percentage in low attachment plate. Results reported as n = 3. Error bars represent SEM (significances denoted * for p<0.05, two-tailed T-test).

Figure 2: Transcriptome analysis indicated a downregulation in EMT in MTI-101 resistant lines. (A) PCA Plot of all Cell Lines based on gene expression. (B-D) GSEA analysis of genes sorted based on fold changes of PC-9R vs PC-9 (B), H446-R vs H446 (C), or PC-9R RFD vs PC-9 (D) from high (red) to low (blue) against MSigDB hallmark gene set “epithelial mesenchymal transition” (vertical bars). NES: normalized enriched score (E) Venn Diagram analysis of DE genes predicted for each resistance lines vs corresponding WT. (F) Bar plot for expression values of SPARC, VIM, FN1, TWIST2, and PLOD2 across PC-9, RFD, and PC-9R; FDR for differential expression from EdgeR indicated (G) Individual genes (SPARC, VIM, FN1, ACS3/TWIST1, TWIST2, and PLOD2) split by median on all lung cancer patient data and
predicted clinical prognosis in lung cancer. TWIST1 (ACS3), FN1 and PLOD2 are poor prognostic
indicators for clinical outcome in lung cancer.

Figure 3: MTI-101 acquired drug resistant lines demonstrate decreased expression of EMT
transcription factor TWIST at the protein level. (A) Western Blot probing for TWIST1 in WT and
Resistant Cells; Western Blot images were from the same blot but were spliced and rejoined
between PC-9R and H446 samples in TWIST1 and β-actin images to remove protein standard
marker for clarity (B) Quantification of Western Blot showing significant decreased protein
expression of TWIST1 in H446-R cells compared to H446 (C) Western Blot probing for TWIST2 in
WT and Resistant Cells (D) Quantification of western blot showing significant decrease in
protein expression of TWIST2 in PC-9 resistant cells compared to PC-9 WT. Completed as n = 3
independent experiments with reproducible representative images displayed for Western Blots.
Error bars represent SEM (significances denoted * for p<0.05, two-tailed T-test).

Figure 4: Emergence of Resistance to MTI-101 Selects for a Stable Phenotype Associated with
Diminished Invasive Capacity and Increased Expression of Epithelial Markers. (A) PC-9 and
H446 WT were compared to their respective MTI-101 resistant lines with respect to EMT
protein markers. E-cadherin is an epithelial marker and was found to be overexpressed in the
PC-9R cell line compared to WT. Vimentin is a mesenchymal marker and was found to be
substantially decreased in H446 resistant cells compared to WT. Both corroborate a transition
to a more epithelial like cell phenotype (MET). (B) Quantification of Western Blot (C) PC-9
Invasion assay comparing the WT to resistant cells’ ability to invade into the Matrigel over a 48-
hour time period (D) Quantification of PC-9 Invasion (E) H446 Invasion assay comparing the WT to resistant cells’ ability to invade into the Matrigel over a 48-hour time period. (F) Quantification of H446 invasion (G) Western blot comparing E-Cadherin expression when PC-9R is removed from drug pressure over a 6-month period (H) Quantification of Western Blot for E-Cadherin in PC-9 removed from drug (RFD) (I) IC50 values of MTTs run with MTI-101 treatment comparing PC-9 to its respective resistant and RFD cells (J) Invasion assay showing lack of invasion of PC-9 RFD even after six months compared to PC-9 wild type cell line. Completed as n = 3 independent experiments, reproducible representative images shown for invasion images and Western Blots. Error bars represent SEM (significances denoted * for p<0.05, two-tailed T-test).

Figure 5: MTI-101 Treatment In Vivo Recapitulates the In Vitro MET Phenotype. Kidneys were taken from the H446 in vivo study, fixed in formalin, paraffin blocked and then sliced for slides to analyze EMT markers. The lungs of the PC-9 study went through the same process. Three control and three treatment (10 mg/kg MTI-101 3 times per week IP injection) mice were picked at random from H446 and six of each in PC-9 were picked to compare EMT markers. (A) Survival curve shows no significant differences between MTI-101 treatment and control (n = 14, p>0.05 Log Rank Test) (B) Survival curve shows no significant differences between treatment and control in PC-9 study (n = 21, p>0.05 Log Rank Test) (C) H446 study tumor portions of the kidneys stained with H&E to confirm presence of tumor and EMT markers (vimentin, E-Cadherin and β-catenin) demonstrates in vivo tumor transformation to MET with chronic treatment with 10 mg/kg of MTI-101 (D) PC-9 study quantity of mice detected with tumor in the bone over
time course of study detected by IVIS and confirmed by H&E stains (E) Quantification of E-cadherin and Vimentin in IHC stains of PC-9 study (n=6 tissues stained from control and drug treated cohort) (F) PC-9 H&E and IHC stains of lungs show presence of tumor and a significant increase in E-cadherin expression but no significant change in vimentin or β-catenin between control and MTI-101 treatment. Average representative image shown for all IHC. Tumors engrafted for 4 weeks (H446) and 3 weeks (PC-9) prior to drug treatment. Error bars represent SEM (significances denoted * for p<0.05, two-tailed T-test).

Figure 6: Selection for resistance to MTI-101 demonstrates collateral sensitivity to EGFR inhibitors but not Cis-Platinum treatment. 72 Hour MTT Assays were performed with the single treatment of Osimertinib, Erlotinib, or Cisplatin comparing WT to their respective MTI-101 Resistant Cell Line. IC\textsubscript{50} values comparing PC-9 to PC-9R were significant with Osimertinib and Erlotinib (significance p<0.05,T-test) (A-C,G). 72 Hour MTTs using combination therapy with MTI-101 in PC-9, HCC4006 and PC-9R demonstrate synergism with EGFR inhibitors (D-F). 48 hour MTTs using combination therapy with MTI-101 and Cisplatin were synergistic in HCC4006, PC-9, and H446 cells (H) and additive in H446-R (I). Drug combination effects were determined by using CompuSyn software analysis. Shown is the mean CI of N=3 independent experiments. Error bars represent SEM (significances denoted * for p<0.05, two-tailed T-test).
Chapter 4

Targeted Anti-Cancer Therapeutic MTI-101 Induces Necrotic Cell Death Indicative of GPCR Activation and Calcium Dysregulation

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Abstract

A first-in-class cyclic peptide known as MTI-101 was found to selectively target and induce necrotic cell death in lung cancer cells correlated with concurrent calcium flux and cAMP activation. MTI-101 was shown to deplete ER calcium stores, followed by increased cytoplasmic calcium. Resistance to MTI-101 induced death was found to have decreased cationic channels, drug binding, and downstream effectors such as calcium and cAMP that could play an essential role in drug evasion. MTI-101 death was exacerbated with a CAM inhibitor (W7) and protected with an exogenous activation of CAM using CALP1 (CAM agonist). Meanwhile, inhibition of AC and cAMP activation was found to have no effect on MTI-101 cell death. Understanding the mechanism of MTI-101 could give insight into a novel GPCR target and its role in cancer. Additionally, the mechanism selecting for decreased calcium signaling could produce a less proliferative cell state leading to improved therapies and novel drug discoveries.

Introduction

As stated above, calcium seems to play a major role in MTI-101 induced cell death in multiple cancer types. Additionally, calcium remains essential in the progression of an epithelial-to-mesenchymal transition (EMT) in cancer by participating in roles including but not limited to remodeling of the extracellular matrix (ECM), invasion, G-protein coupled receptor (GPCR) signaling, immune evasion, and drug resistance[1]. The overexpression of calcium channels has been correlated with increased metastasis and proliferation. For example, ORAI3 and STIM1 have been found to mediate TGF-β induced Snai1 expression for the progression of
EMT[2]. Furthermore, TRPC1-6 overexpression was found to increase proliferation and invasion in several cancer types illustrating the importance these cationic channels play in EMT[3]. Given these findings, calcium channels are being viewed as potential targets for novel anti-cancer therapeutics. The role of calcium in current standard of cares is also being incorporated in the development of new combination therapy strategies along with giving insight into mechanisms of drug resistance. Targeted therapies exploit differences between healthy and malignant cells to selectively abrogate the unique and essential functions for cancer cells to survive. This excessive need for high levels of calcium can create vulnerabilities to changes in intracellular calcium within cancer cells that leaves them much more susceptible to calcium targeting therapies than the non-malignant cells. Recent studies have shown increased efficacy of standard of cares for advanced stage prostate cancer such as docetaxel and enzalutamide with the concurrent activation of a calcium influx channel known as TRPM8 using agonist such as menthol[4]. The role of calcium in MTI-101 induced cell death will be explored throughout this paper.

One way in which calcium signaling may be activated is through receptors located throughout the cellular membrane known as GPCRs. There are upwards of 1000 different GPCRs that each have a unique ligand and a particular signal[5]. GPCRs remain the most prominent druggable targets approved by the FDA with roughly 35% of drugs targeting these receptors[6]. This group of receptors has a variety of classes in humans including rhodopsin, secretin, glutamate, adhesion/taste, cAMP, frizzled/smoothened. Many of these receptors don’t currently have known ligands and are referred to as orphan GPCRs. The various signaling pathways of each GPCR is dependent on its bound G-proteins on the intracellular domain. The
different G protein subunits include Ga\textsubscript{s}, Ga\textsubscript{q}, and Ga\textsubscript{i}. The Ga\textsubscript{q} is capable of activating the Phospholipase C (PLC) signaling pathway that utilizes calcium as a second messenger for its cellular effect\cite{7}. The Ga\textsubscript{s} and Ga\textsubscript{i} work in opposition of each other as Ga\textsubscript{s} activates adenylyl cyclase (AC) that utilizes cyclic adenosine monophosphate (cAMP) as a second messenger while Ga\textsubscript{i} negatively regulates AC to decrease cytosolic cAMP\cite{8}. Further research is needed to determine the role these receptors play in physiological and pathological conditions.

**Materials and Methods**

**Cell Culture/Reagents**- PC-9 (Sigma #90071810) and H446 (ATCC #HTB-171) lung cancer cell lines were used as parental cell lines. MTI-101 resistant cell lines PC-9R and H446-R were developed with chronic exposure to MTI-101 over a 6-month period. All cell lines were maintained in RPMI-1640 L-Glutamine with 10% Fetal Bovine Serum, and 1% penicillin/Streptomycin. Resistant cell lines were maintained with a constant level of MTI-101 in culture media (60µM for PC-9R and 40 µM for H446-R). All cell lines were tested for mycoplasma every six months, and STTR analysis was performed on a yearly basis.

**Calcium Flux Assay**- (ER calcium indicator R-CEPIA1er) PC-9 WT and PC-9R cells both transfected with mCherry Calcium-measuring organelle-Entrained Protein IndicAtor 1 in the ER (R-CEPIA1er) were split and plated at a density of 10,000 cells per well in a 96 well plate with 100 uL complete media RPMI (+FBS, +P/S) and allowed to adhere and grow for 24 hours. The media was then aspirated off and the cells were washed with imaging media. 40 uL of imaging media containing 1x Dapi was added to each well and a baseline image was taken using Biotek Cytation 5 imaging system. Then, the control group received 40 additional uL of 1x Dapi imaging media and the treatment groups received imaging media containing 30 uM MTI-101.
The cells were imaged every 30 seconds for one hour and fluorescence intensity was analyzed for Dapi and CEPIA. (CEPIA excitation and emissions: 531 nM/593 nM wavelength)

(Cytoplasm Calcium Indicator X-Rhod-1 AM) PC-9 WT and PC-9R cells were split and plated at a density of 6000 cells/well in a clear, black bottom 384 well plate with 100 uL RPMI media (+FBS, +P/S) and allowed to adhere to plate and grow for 24 hours. The media was aspirated off and the cells were incubated at 37 °C with 4 uL/mL X-Rhod-1 AM (80 uL per well) for 45 minutes. After incubation, the dye was aspirated off and 80 uL RPMI media (+FBS, +P/S) was added to each well and incubated again at 37 °C for 30 minutes. Media was aspirated off and 40 uL imaging media containing 1x Dapi was added to each well and a baseline image was taken using Biotek Cytation 5 imaging system. Then, the control group received 40 additional uL of 1x Dapi imaging media and the treatment groups received imaging media containing 30 uM MTI-101. The cells were imaged every 30 seconds for one hour and fluorescence intensity was analyzed for Dapi and X-Rhod-1 AM. (X-Rhod-1 AM excitation and emissions: 580 nM/602 nM wavelength).

**cAMP Signaling Assay** - The fluorescent cAMP indicator (flamindo2) consists of a YFP (Citrine) fused with a cAMP binding domain mEPAC1 near the chemophore. When cAMP binds to cAMP binding domain, it induces a conformational change that reduces the YFP significantly. At saturation of cAMP (1mM), the YFP signal is reduced by 75% (4-fold). The flamindo2 plasmid was transfected in PC-9 WT and Resistant cells via electroporation and sorted for YFP+ by flow and gated with normal PC-9 cells. 8-bromo cAMP (SelleckChem, S7857) is a cell permeable cAMP analogue that still activates PKA (more cell permeable and phosphodiesterase resistance than cAMP) that was used as a positive control to show decreased YFP with increased cAMP
binding. PC-9 WT cells transfected with Flamindo2 were split and plated at a density of 10,000 cells per well in a clear, black bottom 384 well plate with 100 μL RPMI media (+FBS, +P/S). The cells were allowed to adhere and grow for 24 hours. Then, the media was aspirated off, and 40 μL 1X DAPI/Imaging Media was added to each well. A baseline image was taken using the Biotek Cytation 5 imaging system. The control group then received 40 additional μL of 1X DAPI/Imaging Media, and the treatment groups received 30 μM MTI-101/Imaging Media. The cells were imaged every 3 minutes for one hour, and fluorescence intensity was analyzed for YFP and DAPI (Flamindo2 excitation and emission spectra: 504/523nM).

cAMP Inhibition Assay

PC-9 WT cells were split and plated at a density of 10,000 cells per well in a clear, black bottom 384 well plate with 100 μL RPMI media (+FBS, +P/S). The cells were allowed to adhere and grow for 24 hours. The media was then aspirated off. Next, half the cells were incubated at 37 °C with 150 μM of a cell permeable non-competitive adenyl cyclase (AC) inhibitor SQ22536 (Tocris Cat #1435) in RPMI media (80 μL per well) for 45 minutes. The remaining cells were simultaneously incubated at 37 °C with RPMI media for 1 hour. After incubation, the media and SQ22536 were aspirated off, and 40 μL of 1X DAPI/Imaging Media (experimental control) or SQ22536 in 1X DAPI/Imaging Media were added. A baseline image was taken using the Biotek Cytation 5 imaging system. Then, the control group received 40 additional μL of 1X DAPI/imaging media, the SQ22536 single treatment group received 40 additional μL of SQ22536 in 1X DAPI, and the MTI-101 treatment groups received 1X DAPI containing 30 μM MTI-101. The cells were imaged every 3 minutes for one hour, and fluorescence intensity was analyzed for YFP and DAPI. There were four groups analyzed: 1X DAPI, SQ22536 in 1X DAPI,
MTI-101 in 1X DAPI, and MTI-101 and SQ22536 in 1X DAPI. Cell death was compared among the groups.

**Radioactive labeling of MTI-101**- MTI-101 was synthesized as previously described, and weighed (2 mg), dissolved in 1 mL freshly distilled tetrahydrofuran (THF), and 0.2 mL trichloroacetic acid added. After the peptide had dissolved, 50 uL of a 1 mCi/mL solution of [3H]H$_2$O (PerkinElmer) was added to the solution, and incubated while stirring for 2 hours. After two hours, a spatula of sodium bicarbonate (NaHCO3) was added and stirred until the evolution of neutralizing gas was evident. The THF was evaporated, and the peptide dissolved in 100% ethanol, cleaned using a C18 syringe column, and counted to validate labeling. [3H]MTI-101 was resuspended in ethanol to give a final concentration of 2 mg/0.5 mL.

**Binding Assay**- Two binding assays were performed to analyze changes in cells binding to MTI-101. The first binding assay used MTI-101 radiolabeled with tritium and standardized by protein content. Cells were seeded into a 6-well plate at 500,000 cells per mL with RPMI media. [3H]MTI-101 was added to the cells at 10 uL per well. After incubation period of 15 min, the media was aspirated, and the cells were washed with phosphate buffers saline (PBS) pH 7.4. RIPA buffer (500 uL) was added to each well, triturated, and placed into a 7 mL scintillation vial. Scintillation cocktail was added and counted using a scintillation counter (PerkinElmer).

The second binding assay used the linear peptide HYD1 conjugated with a GFP to make FAM-HYD1. Equal number of cells were plated and washed with a membrane dye (WGA, Texas Red), washed with PBS and then washed with 0.125 mg/mL FAM-HYD1 for 15 minutes. Cells were washed one last time with PBS and then fixed in 2 % PFA. Images were taken on EVOS
using GFP and Texas Red channels merged to show total amount and localization of FAM-HYD1 binding compared between WT and resistant cells.

**Calmodulin Inhibition Assay- W7 CAM Inhibitor**

PC-9 WT cells were split and plated at a density of 10,000 cells per well in a clear, black bottom 384 well plate with 100 uL RPMI media (+FBS, +P/S). The cells were allowed to adhere and grow for 24 hours. The media was then aspirated off. Next, half the cells were incubated at 37 °C with 25 μM W7 (Tocris Cat#0369) in RPMI media, a cell-permeable CAM antagonist (80 μL per well) for 1 hour. The remaining cells were simultaneously incubated at 37 °C with RPMI media for 1 hour. After incubation, the media and W7 were aspirated off, and 40 μL of 1X DAPI/Imaging Media (experimental control) or W7 in 1X DAPI/Imaging Media were added. A baseline image was taken using the Biotek Cytation 5 imaging system. Then, the control group received 40 additional μL of 1X DAPI/Imaging Media, the W7 single treatment group received 40 additional μL of W7 in 1X DAPI, and the MTI-101 treatment groups received 30 μM MTI-101/Imaging Media. The cells were imaged every 2 minutes for one hour, and fluorescence intensity was analyzed for DAPI. There were four groups analyzed: 1X DAPI, W7 in 1X DAPI, MTI-101 in 1X DAPI, and MTI-101 and W7 in 1X DAPI. Cell death was compared among the groups.

**Calmodulin Agonist Assay- CALP1 CAM Agonist**

PC-9 WT cells were split and plated at a density of 10,000 cells per well in a clear, black bottom 384 well plate with 100 uL RPMI media (+FBS, +P/S). The cells were allowed to adhere and grow for 24 hours. The media was then aspirated off. Next, half the cells were incubated at 37 °C with 100 μM CALP1 in RPMI media, a cell-permeable CaM agonist (80 μL per well) for 1 hour.
The remaining cells were simultaneously incubated at 37 °C with RPMI media for 1 hour. After incubation, the media and CALP1 were aspirated off, and 40 μL of 1X DAPI/Imaging Media (experimental control) or CALP1 in 1X DAPI/Imaging Media were added. A baseline image was taken using the Biotek Cytation 5 imaging system. Then, the control group received 40 additional μL of 1X DAPI/Imaging Media, the CALP1 single treatment group received 40 additional μL of CALP1 in 1X DAPI, and the MTI-101 treatment groups received 30 μM MTI-101/Imaging Media. The cells were imaged every 2 minutes for two hours, and fluorescence intensity was analyzed for DAPI. There were four groups analyzed: 1X DAPI, CALP1 in 1X DAPI, MTI-101 in 1X DAPI, and MTI-101 and CALP1 in 1X DAPI. Cell death was compared among the groups.

**RNA Extraction**- Quadruplicates of RNA were extracted from each WT and resistant lines of PC-9 and H446 using RNeasy Plus Mini Kit (Qiagen, cat#74104). 2-3 million cells were isolated in a dry pellet and resuspended and RNA was initially extracted using Trizol, and then further purified using the Qiagen RNeasy Mini Protocol and RNA was stored at -80 degrees. The same RNA isolation procedure was performed for the PC-9 4, 5, and 6 months removed from drug (RFD).

**RNA-Seq Data Analysis**- RNA-Seq libraries were sequenced at Marshall University Genomics Core with Illumina NextSeq 2000. Pair-end RNA-SEQ short reads were aligned to the whole human reference genome (hg38) by subread v2.0.1[9]. The total number of reads aligned to transcripts at gene level were summarized by featurecounts from Rsubread v2.2.0[10] with built-in RefSeq gene annotation. Gene expression level was quantified by RPKM (reads per kilobase of exon model per million mapped)[11]. We used edgeR v3.30.3[12] to define
differentially expressed (DE) genes with the following criteria: p-value < 0.05, fold change >1.5, and CPM (count per million; \log_2) > 0. To determine the relationship between expression changes of PC-9 and H446 to their MTI-101 resistant counter parts, we employed Gene Set Enrichment Analysis through GSEA v4.0.2. We created three “rnk” files based on fold change of expression (treatment vs baseline) for the following comparisons: PC-9R vs PC-9, H446-R vs H446 and PC-9 RFD vs PC-9; a “rnk” file consist of one column for gene symbol and another for fold-change of expression. The rnk file was then supplied as input to GSEA to determine the significance of a preferential expression for selected gene sets from MSigDB in treatment condition over the baseline, or vice versa.

Results

**MTI-101 Induces Intracellular Calcium Flux from ER Stores Correlated with Death**

It has previously been reported that MTI-101 induced cell death in U266 and H929 multiple myeloma cells through calcium flux leading to necrotic cell death[13]. To analyze this hypothesis in lung cancer, ER and cytoplasmic calcium levels were quantified during treatment of MTI-101. A genetically engineered probe called R-CEPIA1er was utilized to understand how ER calcium levels were altered by MTI-101. The R-CEPIA1er plasmid contains a calmodulin binding domain, ER localization sequence, and mCherry for red fluorescent protein (RFP) visualization and quantification. This plasmid was transfected via electroporation in the PC-9 WT and PC-9R cell lines and sorted by flow on RFP. When calcium binds to the calmodulin domain in the ER, the RFP fluoresces at five times the intensity to aid in the quantification of calcium level changes. The imaging cell media also contained 1X Dapi that lightly stains DNA
blue, but brightly fluoresces when the cell membrane becomes permeable after death.

Thapsigargin (SERCA blocker) was used as a positive control to validate the system since thapsigargin in the literature is known to deplete ER stores but also block the calcium reentry into the ER[14].

Images were taken at 5-minute intervals of the PC-9 WT CEPIA with a negative vehicle control of 1X Dapi, a positive control Thapsigargin (SERCA channel blocker) and a 30 µM MTI-101 treatment group (Figure 1A). PC-9 WT CEPIA was compared to PC-9R CEPIA. The WT cells showed a significant decrease of ER calcium after about five minutes of MTI-101 treatment illustrating the MTI-101 is capable of signaling for the depletion of ER calcium stores. Then, about fifteen minutes post treatment with MTI-101, calcium stores were replenished and correlated with an increase in Dapi intensity indicative of cell death in a representative single cell tracing (Figure 1C). The PC-9R CEPIA cell line showed no calcium flux with the same treatment of MTI-101 (Figure 1F). Thapsigargin caused a rapid decrease of calcium ER stores without replenishment or cell death, validating the experiment for both the WT and resistant cells (Figure 1D, Figure 1G). An average of 30 cells for each group was also quantified using RFP intensity comparing the three experimental groups of each cell line to show significance of calcium flux with MTI-101 treatment only in the PC-9 WT CEPIA (Figure 1E, Figure 1H). A high intensity Dapi threshold was established to quantify the comparison between WT and Resistant cell lines’ treatment and control groups. Significant death only occurred in the PC-9 WT CEPIA treatment group over the sixty-minute time analysis showing cell death occurred following calcium flux (Figure 1B).
MTI-101 Induces Cytoplasmic Calcium Flux Correlated with Cell Death

To increase the understanding of the mechanism of MTI-101 induced cell death, cytoplasmic calcium levels were also analyzed. X-Rhod-1 AM is a water soluble, cell permeable calcium indicator that combines the 8-coordinate tetra carboxylate chelating site with a xanthene chromophore to give a rhodamine like fluorophore that was used as a molecular tool to quantify calcium changes in the cytoplasmic with treatment of MTI-101. PC-9 WT cells incubated with X-Rhod-1 AM were found to have a significant spike of cytoplasmic calcium within a few minutes of treatment of MTI-101 followed by a gradual sustained increase in calcium correlated with cell death compared to that of the no treatment vehicle control in single cell analysis (Figure 2A, Figure 2B) and N=30 (Figure 2C). In contrast, the PC-9R cells were found to have no significant cytoplasmic calcium flux with treatment of MTI-101 nor cell death compared to its respective vehicle control in single cell analysis (Figure 2D, Figure 2E) and N=30 (Figure 2F) potentially playing a role in survival.

Transcriptome Analysis Substantiates Decreased Cationic Channel Activity in Resistance and Specifically TRP1,4, and ORAI1

Full genome RNA SEQ was conducted on PC-9 WT v Resistant cell lines. A heat map was constructed and analyzed for cation channel expression (Figure 3A). It was found that PC-9R cells had significantly reduced overall cation channel expression compared to PC-9. It was also found by analyzing individual cation channels that have been previously reported to be involved in the mechanism of MTI-101[15] such as TRPC1, TRPC4, and ORAI1 to be consistently down in
the PC-9R (Figure 3B-D). This data coupled with the calcium flux results support that resistant cell alterations are occurring upstream of calcium signaling that may be responsible for survival.

**Resistance to MTI-101 Correlated with Decreased Drug Binding**

Drug binding of MTI-101 was analyzed on PC-9, PC-9R, and BEAS-2B cells to potentially delineate changes on the cell surface that may lead to resistance. Both PC-9R and H446-R cells were found to have significantly decreased drug binding in an MTI-101 radiolabeled drug binding assay using tritium and standardized by protein concentration compared to their respective WT cells (Figure 4A, Figure 4B). The linear peptide of MTI-101 known as HYD1 was conjugated with FAM (GFP) and counterstained with a membrane dye WGA (Texas Red) to visualize localization and quantification of drug binding. PC-9R had substantially less drug binding than the PC-9 cells (Figure 5). The binding appeared to be extracellular and intracellular when the WGA was merged with the FAM-HYD1 stains. The intracellular binding could be due to significant overexpression of β-Arrestin in PC-9 and H446 compared to resistance that causes the receptor binding to agonist to be endocytosed (Supplemental Figure 1). BEAS-2B was also shown to have significantly less binding than the WT cancer cells lines (Figure 5), which would be consistent with lack of efficacy of MTI-101 in normal bronchial epithelial cells[16].

**MTI-101 Induces cAMP Activation**

PC-9 WT and Resistant cells were transfected with a cAMP detecting probe that decreases YFP signal when bound to cAMP. MTI-101 was found to induce cAMP activation in PC-9 WT similar to the positive control 8-bromo cAMP and significantly different than the negative vehicle control (Figure 6A-C). PC-9R cells illustrated no change in cAMP activity with
MTI-101 treatment (Figure 6D-F). This data indicates that MTI-101 induces cAMP activation in WT cells that is blocked in resistance. In this manner, MTI-101 has been shown to activate calcium signaling concurrently with cAMP which is a unique characteristic of GPCRs. Therefore, MTI-101 was proposed to have a mechanism in lung cancer of binding to a GPCR as an agonist that is capable of activating a calcium signaling pathway simultaneously with a cAMP signaling pathway shown in Figure 7. Inhibition of individual pathways will be analyzed to understand how MTI-101 induces cell death.

MTI-101 Death is not Correlated with Death Through cAMP Activation

An AC inhibitor (SQ22536) was used in combination to MTI-101 to show its effect on MTI-101 downstream signaling and its effect of death. The AC inhibitor is a cell permeable molecule that causes competitive inhibition of ATP binding to AC preventing the conversion of ATP to cAMP shown in Figure 8A. The 8-bromo cAMP (positive control) and MTI-101 single treatment were once again able to show that MTI-101 induced cAMP activity that was inhibited by the combination of MTI-101 treatment with the AC inhibitor similar to that of the negative vehicle control (Figure 8B). Interestingly, the blockage of MTI-101 cAMP induced activity did not alter cell death in PC-9 WT cells (Figure 8C).

The Effect of CAM on MTI-101 Induced Cell Death

PC-9 WT cells transfected with R-CEPIA1er, and PC-9 WT cells incubated with were both utilized in conjunction with a CAM inhibitor and agonist to understand how MTI-101’s mechanism would be altered in respect to an intracellular calcium regulator. The CAM agonist
CALP1 is capable of binding to CAM to induce its active conformation. CAM’s active conformation has been shown to prevent SOCE along with IP3R inhibition acting as a negative regulator of calcium flux. The proposed mechanism is the activation of CAM by an agonist will prevent MTI-101 induced calcium flux (Figure 9A). The CAM agonist combined with MTI-101 was found to completely protect cells from MTI-101 cell death compared to MTI-101 treatment alone (Figure 9C). The CAM agonist alone was also found to have no cytotoxic cellular effects as a single agent with no significant changes in death from the negative vehicle control (Figure 9C). At high dose MTI-101 treatment, CALP1 was found to significantly increase survival up to 24 hours (Supplemental Figure 2). In opposition, a CAM inhibition (W7) was utilized to see how antagonism of CAM would alter MTI-101 induced cell death. The CAM inhibitor is a cell permeable molecule that binds to CAM to prevent calcium binding and CAM activation essentially removing the negative regulator of calcium from involvement within the cell (Figure 9B). The CAM inhibitor alone was not capable of inducing cell death, but in combination with MTI-101 treatment the CAM inhibitor showed significantly increased cell death compared to MTI-101 treatment alone (Figure 9D). Therefore, the activation of CAM was shown to protect from MTI-101 induced cell death while antagonism of CAM exacerbates death.

**CAM Agonist Partially Protects from MTI-101 Induced Calcium Flux Correlated with Survival**

The PC-9 WT cells incubated with X-Rhod-1 AM and the PC-9 WT CEPIA cells were analyzed for calcium flux using the CAM agonist. MTI-101 combined treatment with the CAM agonist significantly delayed ER calcium depletion and reduced total ER calcium depletion compared to MTI-101 single treatment in single cell analysis (Figure 10A) and N=30 (Figure 10B).
In addition, combined CAM agonist and MTI-101 treatment delayed initial calcium flux and reduced overall cytoplasmic calcium increases when compared to MTI-101 single treatment in single cell analysis (Figure 10C) and N=30 (Figure 10D). The CAM Inhibitor combined with MTI-101 was found to have no significant changes in cytoplasmic calcium fluctuations compared to MTI-101 single treatment (Figure 11).

**Preliminary Evidence for MTI-101 Binding Target with GPCRs**

Full genome RNA SEQ was compared between PC-9, PC-9R, PC-9 RFD (removed from drug), H446, and H446-R with respect to significant differentially expressed GPCRs and was compiled in a heat map (Figure 12). The most interesting genes for GPCRs that were decreased in both resistant cell lines compared to their respective WTs and were retained down in the PC-9 RFD. The PC-9 RFD were characterized in Jones et al 2022 [16] and arose from the PC-9R cell line that was removed from MTI-101 drug pressure for 4, 5, and 6 months. A variety of Frizzled receptors (FZD2, FZD6, and FZD7) kept reappearing at disproportionate rates to other types of GPCR receptors that were decreased in expression in resistant cell lines and therefore were selected as top candidates for an MTI-101 binding target.

**Discussion**

Calcium remains tightly regulated in healthy cells, whereas cancer could come at a cost of decreasing calcium homeostasis pathways that lead to an abundance of calcium needed for proliferation and invasion of malignant cells. Targeting calcium pathways have been increasingly important with novel combination therapies that have proved to increase the
survival of patients[17]. RNA Sequencing data suggest that cationic channel activity was significantly decreased in resistance compared the WT PC-9 cancer cells. Previously, MTI-101 cell death was found to be blocked with inhibition of channels such as TRPC1, TRPC4, and ORAI1[15]. Interestingly in lung cancer, MTI-101 was found to deplete ER calcium stores, while increasing cytoplasmic calcium levels in lung cancer similar to the proposed mechanism in multiple myeloma[13]. Two spikes in calcium flux in the cytoplasm with MTI-101 treatment illustrate an initial source likely originating from ER stores and an additional source most likely originating from extracellular supply introduced via TRPC and ORAI1 channels. This process was found to be blocked in the MTI-101 resistant cells, likely suggesting upstream cell alterations that are leading to drug resistance.

Variation in drug binding was a forefront suspect that could lead to resistance. MTI-101 was found to be significantly hindered in binding to H446-R and PC-9R compared to their respective WTs. Binding was significantly less in the healthy BEAS-2B compared to both WT cancer cell lines as well which could be an explanation for the lack of efficacy seen in vitro of MTI-101. Resistance correlated with inhibited downstream effectors along with decreased binding suggestive that the binding target for MTI-101 could be downregulated, post-translationally modified to hinder localization to the membrane, or mutated to prevent drug binding.

In order to delineate the type of receptor that may be involved in MTI-101 induced cell death, changes in the second messenger cAMP were also analyzed to determine if a GPCR may be the binding target of MTI-101. Currently almost half of all FDA approved drugs target a GPCR[5] and a unique trait to these receptors is the capability of activating calcium and cAMP
simultaneously as downstream effectors for signal transduction[18]. MTI-101 was found to activate cAMP activity that was also blocked in resistant cells. Given GCPRs can signal through multiple downstream effectors, only one pathway may be responsible for cell death. Different inhibitors of these independent pathways were used to analyze effects of MTI-101 induced cell death. Inhibition of CAM in combination with MTI-101 was found to exacerbate cell death when compared to MTI-101 single treatment alone. A potential reason is that the negative regulator of increased calcium levels CAM cannot balance the increased cytoplasmic calcium and causes a further dysregulation of calcium that induces greater cell death. The CAM inhibitor alone is not capable of inducing cell death, demonstrating this effect remains dependent on the mechanism of MTI-101 signaling to induce greater cell death. In addition, a CAM agonist known as CALP1 was able to block MTI-101 induced cell death. Activation of CAM by binding calcium (or a calcium like protein, CALP1) has been found to bind to IP3 receptors and inhibit depletion of ER stores and SOCE[19]. GPCRs are also capable of activating a Gs subunit that uses cAMP as a second messenger, acting independently of the Gq pathway with calcium. An AC inhibitor was used that prevents AC from converting ATP to cAMP for signal transduction. The AC inhibitor combined with MTI-101 actively blocked cAMP formation, but did not alter observed cell death. This indicates that MTI-101 induced cAMP activation is not essential for cell death to occur.

The mechanism of GPCR signaling is still not fully understood as various combinations of G-proteins coexist with various ligands and regulators that make this signal transduction extremely complex. There are still orphan GPCRs that do not even have known ligands currently. Based on these findings, MTI-101 is proposed to bind to a GPCR that activates a Goq
and a G\textsubscript{as} subunit. The G\textsubscript{as} subunit is capable of activating AC to generate elevated levels of cAMP that appear to not be involved in MTI-101 induced cell death. The G\textsubscript{aq} subunit activates the PLC pathway responsible for increased cytosolic calcium. The negative regulator of calcium signaling known as calmodulin was likely able to inhibit IP\textsubscript{3}R mediated store depletion and SOCE entry but based on significance of calcium flux changes and when cell death occurred, calmodulin is thought to play the greatest role in protection by the inhibition of SOCE.

Precedence of this finding with sustained calcium signaling induced by MTI-101 has been shown with other drugs such as Fluoxetine. The selective serotonin reuptake inhibitor used to treat depression known as fluoxetine has been shown deplete ER stores and elevate cytoplasmic calcium through SOCE that lead to necrotic cell death via mitochondrial ATP depletion[20]. The sustained calcium signaling induced by MTI-101 may be attributed to receptor endocytosis via β-Arrestin that was found to be significantly higher in the drug sensitive WT cells. This may also be a means of resistance found in PC-9R and H446-R as they have significantly decreased β-Arrestin expression.

Several frizzled receptors are significantly decreased in the resistant cells and are retained down in the PC-9 RFD. Frizzled receptors have been shown to activate both calcium and cAMP signaling when bound to agonists[21], [22]. In addition, frizzled receptors have been shown to induce β-catenin nuclear localization used for the induction of EMT through increased expression of TWIST and further repression of E-cadherin[23]. Treatment/resistance to MTI-101 has previously been shown to reduce TWIST and E-cadherin expression in vitro and in vivo along with prevention of nuclear localized β-catenin in vivo[16]. This combined data supports that MTI-101 may be binding and activating a frizzled receptor that is down regulated in drug
resistance. Further research is warranted to delineate the binding target of MTI-101. Genetic manipulation of receptors with a GPCR Lenti-array CRISPR CAS9 library is an approach to unbiasedly target individual GPCRs and determine if they remain vital in MTI-101 induced cell death. The initial screen will analyze the effect of frizzled receptors expressed in these cell lines and expand from there. Pharmacological inhibition of individual G-protein subunits combined with MTI-101 could further support what G-protein subunits may be contributing to death as well. Understanding the mechanism of MTI-101 could lead to optimized combination treatment that target resistance along with the discovery of new drugs that target cell state and cancer specific characteristics.

Conclusion

Overall, MTI-101 was found to induce necrotic cell death in lung cancer cells correlated with concurrent calcium flux and cAMP activation. Resistance to MTI-101 was found to decrease cationic channels, drug binding, and downstream signaling effectors such as calcium and cAMP. A negative regulator of calcium, CAM, was found to exacerbate cell death when inhibited, and block cell death when activated, illustrating its involvement in the mechanism of MTI-101 induced cell death. In contrast, inhibition of AC and cAMP activity was found to have no effect on MTI-101 induced cell death. A drug such as MTI-101 that has shown to selectively target cancer cells and select for a population that decreases calcium signaling may alter cancer cells to a less aggressive and more treatable cell state in the clinic to improve patient outcomes.
Supplemental Materials

Supplemental Figure 1: β-Arrestin Significantly Decreased in MTI-101 Resistance (A) Fold change of β-Arrestin expression between PC-9 and PC-9R using full genome RNA SEQ (B) Fold change of β-Arrestin expression between H446 and H446-R using full genome RNA SEQ.

Supplemental Figure 2: CALP1 Protects from MTI-101 induced cell death up to 24 hours. PC-9 cells treated with MTI-101 or MTI-101+CALP1 for 24 hours and calculated cell viability of percent viability compared to vehicle control via MTT assay. Error bars represent SEM (significances denoted * for p<0.05, two-tailed T-test).
References


Figures

Figure 1:

A) Control

B) Death Over Time

C) PC-9WT

D) PC-9R

E) Calcium Flux Correlated with Cell Death

F) Flux Correlated with Cell Death

Figure 2:

A) PC-9 WT Control

B) PC-9 WT Treatment

C) NQD

D) PC-9R Control

E) PC-9R Treatment

F) NQD
Figure 3:

**A)**

![Image of a heatmap with PC-9R and PC-9 labels.](image1)

**B) TRPC1, C) TRPC4, D) ORAI1**

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**Figure 4:**

**A) Binding Assay**

![Graph showing binding assay results for PC-9 and PC-9R.](image2)

**B) Binding Assay**

![Graph showing binding assay results for H446 and H446-R.](image3)
Figure 5:

WGA

FAM-HYD1

Merge

Figure 6:

A) PC-9 WT Control

B) PC-9 WT Treated

C) N138

D) PC-9R Control

E) PC-9R Treated

F) N138
**Figure 7:**

![Diagram of Lung Cancer with Death Through cAMP Activation](image)

**Figure 8:**

**A)**

![Diagram of Lung Cancer with Death Through cAMP Activation](image)

**B)**

![Graph of PC-9 WT-Flamindo2 CAMP Activity NF-36](image)

**C)**

![Graph of % Death](image)
Figure 9:

A) CAM Activation Promotes Survival

B) CAM Inhibition Increases Cell Death

C) MTI-101 Induced Calcium Flux

D) Single Cell N=30

ER Calcium

Cytosplasmic Calcium

Figure 10:

A) PC-8 Single Cell CAM Agonist (ER Calcium)

B) ER Calcium

C) PC-8 Single Cell CAM Agonist (Cytosplasmic Calcium)

D) Cytosplasmic Calcium
Supplemental Figure 1:

A) B)

Supplemental Figure 2:

CAM Agonist/MTI-101 MTT

Figure 11:

Figure 12:
Figure Legends

**Figure 1: MTI-101 Resistance Blocks ER Calcium Flux Correlated with Cell Death (A)** PC-9 WT ER calcium flux compared to PC-9R over time in images with vehicle control, positive control Thapsigargin, and treatment of MTI-101 (B) Percent death after MTI-101 treatment in Calcium flux assay shows only PC-9 WT MTI-101 treatment cell death following calcium flux (C-E) PC-9 WT ER calcium flux cells over time comparing no treatment (negative vehicle control), treatment with Thapsigargin (positive control) and MTI-101 (experimental group) in graphical form (N=30) (F-H) PC-9R ER calcium flux cells over time comparing no treatment (negative vehicle control), treatment with Thapsigargin (positive control) and MTI-101 (experimental group) in graphical form (N=30).

**Figure 2: MTI-101 Resistance Blocks Cytoplasmic Calcium Flux Correlated with Cell Death (A-C)** PC-9 WT cytoplasmic calcium flux cells over time comparing no treatment (negative vehicle control), and MTI-101 (experimental group) in graphical form (N=30) (D-F) PC-9R cytoplasmic calcium flux cells over time comparing no treatment (negative vehicle control) and MTI-101 (experimental group) in graphical form (N=30).

**Figure 3: Transcriptome Analysis Indicates Decreased Cationic Channel Activity in Resistance (A)** Heatmap from RNA SEQ of differentially expressed cationic channels in PC-9 compared to PC-9R (B-D) Individual cationic channel expression shows PC-9R expression of TRPC1, TRPC4, and ORAI1 are significantly decreased compared to PC-9.
Figure 4: MTI-101 Resistance is correlated with Decreased Drug Binding (A) A radiolabeled MTI-101 binding assay found significantly decreased drug binding in PC-9R compared to PC-9 (N=3) (B) Significantly decreased drug binding was also found in H446-R compared to H446 (N=3). Reads in counts per minute (CPM) standardized by protein concentration (ug/uL). Error bars represent SEM (significances denoted * for p<0.05, two-tailed T-test).

Figure 5: FAM-HYD1 binding Assay shows less drug binding in PC-9R and BEAS-2B cells compared to PC-9. PC-9, PC-9R, and BEAS-2B cells were stained with WGA membrane dye (RFP), FAM-HYD1 (GFP), and merged images (RFP+GFP) to visualize quantity and location of drug binding.

Figure 6: MTI-101 Induces cAMP Activation that Remains Blocked in Resistance (A-C) PC-9 WT cAMP activation using Flamindo2 cAMP indicator (YFP) in cells over time comparing no treatment (negative vehicle control), 8-bromo cAMP (positive control), and MTI-101 (experimental group) in graphical form (N=30) (D-F) PC-9R cAMP activation in cells over time comparing no treatment (negative vehicle control), 8-bromo cAMP (positive control), and MTI-101 (experimental group) in graphical form (N=30).

Figure 7: Proposed Mechanism of MTI-101 in Lung Cancer. MTI-101 binds to and activates a GPCR that is coupled intracellularly to a G_q and a G_s subunit. G_q generates the production of IP_3 that binds to its receptor on the ER causing a depletion of ER stores. ER store depletion causes STIM1 to dimerize and form a complex with SOCE channels that influx extracellular calcium into
the cytoplasm. This overload of intracellular calcium from the ER and SOCE cause mitochondrial calcium overload, ATP depletion, and loss of the mitochondrial membrane potential leading to rapid necrotic cell death in lung cancer cells. The Gs signaling pathway is also proposed to be activated by MTI-101 causing the generation of cAMP that is not involved in the rapid drug induced cell death.

**Figure 8: MTI-101 Death is not Correlated with Death through cAMP Activation**

(A) Model of cell permeable AC inhibitor’s role in MTI-101 signaling. The AC inhibitor (SQ22536) binds downstream of MTI-101 induced GPCR activation to AC to prevent ATP conversion to cAMP (B) PC-9 WT cAMP activation using Flamindo2 cAMP indicator (YFP) in cells over time comparing no treatment (MTI-101 negative control), AC Inhibitor (combination treatment negative control), 8-bromo cAMP (MTI-101 positive control), and MTI-101 (experimental group 1) and AC Inhibitor/MTI-101 combination treatment (experimental group 2) in graphical form (N=30) (C) Percent death of cAMP assay comparing no treatment (MTI-101 negative control), AC inhibitor (combination treatment negative control), MTI-101 treatment (experimental group 1), and AC Inhibitor/MTI-101 combination treatment (experimental group 2).

**Figure 9: MTI-101 Death is Correlated with Death through Calcium Flux Modulated by Calmodulin**

(A) Model of cell permeable CAM agonist (CALP1) and its role in MTI-101 signaling. The CAM agonist binds to CAM to induce its active conformation and negatively regulate calcium flux by binding to IP3R to prevent IP3 binding and ER store depletion along with inhibition of SOCE channel opening (B) Model of cell permeable CAM Inhibitor (W7) binds to
CAM to stabilize its inactive conformation and prevents calcium binding that can no longer participate in the negative regulation of calcium flux within cells (C) Percent death of calcium flux assay comparing no treatment (MTI-101 negative control), CAM agonist (combination treatment negative control), MTI-101 treatment (experimental group 1), and CAM agonist/MTI-101 combination treatment (experimental group 2) (D) Percent death of calcium flux assay comparing no treatment (MTI-101 negative control), CAM inhibitor (combination treatment negative control), MTI-101 treatment (experimental group 1), and CAM inhibitor/MTI-101 combination treatment (experimental group 2).

Figure 10: CAM Agonist Partially Protects from MTI-101 Induced Calcium Flux (A) Single cell analysis and (B) N=30 of ER calcium fluctuations using R-CEPIA1er in PC-9 cells comparing negative vehicle control, MTI-101 treatment, and CAM agonist/MTI-101 combination treatment (C) Single cell analysis and (D) N=30 of cytoplasmic calcium fluctuations using X-Rhod-1 AM in PC-9 cells comparing negative vehicle control, MTI-101 treatment, and CAM agonist/MTI-101 combination treatment.

Figure 11: CAM Inhibitor Does Not Significantly Alter MTI-101 Induced Calcium Flux. Analysis of cytoplasmic calcium fluctuations using X-Rhod-1 AM in PC-9 cells comparing negative vehicle control, MTI-101 treatment, and CAM agonist/MTI-101 combination treatment (N=30).
Figure 12: Evidence for Frizzled Receptor as MTI-101 Binding Target. Heat map using full genome RNA SEQ comparing differentially expressed GPCR genes between PC-9, PC-9 RFD, PC-9R, H446, and H446-R.
Chapter 5

Conclusion and Future Directions

Conclusion

Lung cancer remains the leading cause of death from all cancers, but recent discoveries and earlier detection methods have improved therapy options along with patient survival[1]. Education and prevention of smoking has also contributed to the recent decline in cases over the years[2]. The heavy mutational burden coupled with tumor heterogeneity enables lung cancer to maintain drug resistance despite advances in novel therapy options. Furthermore, late-stage detection in lung cancer enhances the tumor’s capability of evading treatment. Current therapy options target common mutational drivers such as EGFR or KRAS (i.e., osimertinib and sotorasib) as well as systemic chemotherapy options such as etoposide and cisplatin. Recent advances have also been made in the incorporation of immunotherapies to target lung cancer such as pembrolizumab. Selectively targeting markers or attributes for lung cancer may prove to be only half the battle as drug resistance and metastasis still remain the prominent causes of relapse. Targeting or reversing cell state may be the solution to treatment of late-stage and/or drug resistant tumors.

A cyclic peptide, derived from a less potent linear parental compound HYD1, known as MTI-101, was found to selectively target cancer cells and induce genotypic and phenotypic changes consistent with a more epithelial like cell. Given HYD1 was originally found in a high throughput screen that blocked cell adhesion, MTI-101 resistance could explain this attribute with decreased fibronectin expression along with increased anoikis sensitivity. MTI-101 may
select for a population that reduces cell-ECM connections and relies heavily on cell-cell connections to maintain survival (more sensitive to anoikis) and therefore creates a less stiff tumor population that become less invasive and more treatable. Cell-ECM connections are an integral part of tumorigenesis and malignancies that cause increased stiffness in cancer[3]. Decreased cell-ECM connections (fibronectin, laminin, collagen) in conjunction with increased cell-cell connections (claudins, occludins, E-cadherin) have been connected with a less stiff cancer type that displays decreased invasiveness and tumor aggressiveness[3]. MTI-101 could block cell adhesion with the ECM (decrease in fibronectin) and increase cell-cell connections with E-cadherin allowing for a less stiff phenotype. Tumor stiffness relies on excessive calcium signaling[4] which MTI-101 was also found to select for attenuated calcium, consistent with decreased tumor stiffness. Prolonged exposure to MTI-101 induced phenotypic cell alterations including decreased mesenchymal protein expression, increased epithelial protein expression, inability to invade in Matrigel, increased epithelial morphology, increased sensitivity to anoikis, and inability to form distal tumor sites in vivo. These in vitro and in vivo models provide substantial evidence to confer a change in cell state within cancer cells to a more favorable MET phenotype, independent of mutational driver, with the sole pressure of MTI-101.

MTI-101 is suspected to selectively target cancer cells exposing a vulnerability of mutations acquired by malignant cells that downregulated calcium homeostasis pathway genes to accommodate proliferation and migration. A healthy bronchial epithelial cell line known as BEAS-2B was found to have minimal cytotoxic effects when treated with the same lethal dose of MTI-101 as the lung cancer cell lines. Resistance to MTI-101 in lung cancer cells was found to decrease cation channel expression such as TRPC1, TRPC4, and ORAI1 which has been
previously reported to play a role in MTI-101 induced cell death[5]. Treatment with MTI-101 was shown to decrease ER calcium stores while increasing cytoplasmic calcium levels.

Concurrently, MTI-101 was reported to generate cAMP in a second signaling pathway. The simultaneous activation of calcium flux and cAMP alludes to the activation of a GPCR on the plasma membrane. Interestingly, resistance to MTI-101 blocked this calcium flux and cAMP generation concluding that cellular modifications are taking place upstream of these signaling pathways. Decreased MTI-101 drug binding in resistance conferred that MTI-101’s target may be mutated, knocked down, or not trafficked to the membrane.

Given GPCRs’ have the capability of signaling through multiple G-protein subunits, the rapid death induced by MTI-101 may be attributed to only one G-protein signal transduction. Inhibition of AC was found to block cAMP generation with MTI-101 treatment but was not able to protect from death. In contrast, a calmodulin agonist was able to reduce MTI-101 induced calcium flux and protect from cell death. In addition, inhibition of calmodulin was reported to exacerbate cell death. While activated calmodulin was able to delay/decrease overall ER store depletion as reported in the literature by binding to the IP$_3$R and competitively inhibiting IP$_3$[6], the effect of calmodulin activation with cytoplasmic calcium levels was suspected to be more significant in respect to cell death and likely the main source of protection. Activated calmodulin by calcium has been reported in the literature to impede SOCE channels on the plasma membrane[7]. Resistance to MTI-101 has been shown to downregulate SOCE proteins such as TRPC1, TRPC4, and ORAI1 which may have the same negative regulation of calcium flux seen with a calmodulin agonist that is capable of protecting from cell death. It may also be that
the receptors responsible for this signal transduction pathway may be downregulated in resistance and the need for these proteins is no longer as essential.

The ultimate goal for any drug discovery process is to analyze and develop a drug that can safely be administered in the clinic with a positive therapeutic outcome. Understanding mechanisms or resistance along with combined efficacy among standard of care agents remains crucial in mobilizing a drug past pre-clinical models. Standard of care agents such as osimertinib and cisplatin have been reported to have synergism when combined with MTI-101 treatment. In addition, MTI-101 resistant lung cancer cells have shown collateral sensitivity to single treatment of standard of care agents. Given treatment with standard of care agents for lung cancer have been reported to induce EMT, and MTI-101 has been shown to induce MET, these combined treatments may be vital in double bind studies to target varying attributes in cell state[8]. This combination strategy may alleviate drug resistance commonly presented in single agent treatment and improve patient survival rates. Further research into the mechanism of MTI-101 induced cell death in lung cancer is needed to determine its role in MET along with its clinical applications.
Future Directions

The MTI-101 induced change in cell state to a more epithelial like phenotype provides characteristics and transcription factors that could lead to the development of future drug targets in lung cancer. The RNA SEQ database comparing WT to resistance in MTI-101 could be mined for further delineation of factors modulating the cell state transition. Drugs that inhibit or induce these factors could result in a sustained transition as seen in the RFD cell line. TWIST1 and TWIST2 were found to be promising targets of inducing EMT in the WT cancer cells. There are currently small molecules in preclinical stages such as berberine hydrochloride and thymoquinone that have been shown to target and inhibit TWIST[9]. Using these molecules in combination with standard of care agents such as osimertinib and erlotinib could prove to have synergistic effects with lung cancer treatment as seen with MTI-101 combination therapy. The gene signature may also inform decisions on targeting calcium pathways in lung cancer given MTI-101 resistance selects for attenuation of calcium signaling that could be playing a role MET. Small peptides that inhibit calcium signaling such as SOCE channel inhibitors in lung cancer[10] may be investigated in combination with standard of care agents to illustrate a potential combination strategy for cancer treatment.

Evidence suggests that MTI-101 activates a GPCR on the plasma membrane that induces calcium overload leading to cell death. An unbiased approach in attempt to delineate the binding target of MTI-101 would be a GPCR library screen. A Lenti-Array GPCR Crispr library containing gRNAs to knock out 435 different GPCRs will be analyzed with effects on MTI-101 treatment. Endpoints of death will be observed, and positive hits will be GPCR knockouts that confer resistance to lethal WT cancer cell doses of MTI-101. Positive hits will further be
screened by drug binding assays, calcium flux assays, and MTTs with treatment of standard of care agents. While a library screen is one method to determine the MTI-101 binding target, it implies that there is only one target of MTI-101 and no compensatory mechanisms are involved. Another approach would be to further delineate signaling pathways involved in MTI-101 induced cell death.

Natural ligands of GPCRs typically do not lead to cell death, so how is MTI-101 different? The literature suggests that sustained activation of GPCRs can lead to necrosis, autophagy, and apoptosis[11]. There are several explanations to GPCR sustained signaling. First, synthesized ligands have been created to bind in a different manner to a GPCR than their natural ligand. Ligands that irreversibly bind to a GPCR via covalent linkage or bind with a higher affinity have been shown to have sustained activity on the receptor. For example, fingolimod is a synthetic compound that has been shown to bind to sphingosine-1-phosphate (S1P) receptor (GPCR) at a higher affinity than its natural ligand S1P to produce a sustained signal through G_{ai} after internalization[12]. Activated GPCRs are subjected to phosphorylation and receptor mediated endocytosis with the recruitment of β-arrestins[13]. β-arrestins were found to be overexpressed in both WT cells compared to their respective MTI-101 resistant cell lines. The overexpression of β-arrestin has been shown to induce EMT by increasing cell signaling responsible for proliferation and motility[13]. β-arrestin inhibitors have been developed and shown to decrease receptor mediated endocytosis signaling such as sustained cAMP generation[14],[15]. Combination treatment with β-arrestin inhibitors with MTI-101 may delineate if sustained calcium signaling is enabled by receptor mediated endocytosis. Pharmacological inhibition or genetic manipulation of G-protein subunits may also be
implemented to examine the role of the different G-proteins. Given MTI-101 is proposed to induce cell death via calcium overload, a $G_{aq}$ inhibitor or knockout will provide a valuable starting point. Since GPCRs are involved in most phycological communications on the cell membrane, ablation of entire G-protein subunits could produce unintended consequences for the cells. A healthy control that is not sensitive to MTI-101 such as BEAS-2B should also be subjected to the same G-protein knockdown or knockout to remedy these concerns.

MTI-101 has been described here to show promising effects in combination with lung cancer standard of care agents. Osimertinib, Erlotinib and Cisplatin all induce EMT driven resistance in the clinic. Meanwhile, MTI-101 has been shown to induce MET characteristics *in vitro* and *in vivo*. A double bind strategy alternating between MTI-101 and standard of care agents may be implemented to understand if evolving resistance to one drug enhances sensitivity to the other and prolong survival. A pilot study should be conducted to understand at what point MTI-101 treatment changes EMT markers within tumors. This time point will establish the intervals between the two treatments. Alternation of treatment with MTI-101 with standard of care agents may provide an extended window for subsequent treatments and ultimately prolong survival.
References


