MERTK: A NOVEL TARGET IN NON-SMALL CELL LUNG CANCER

by

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Thesis directed by Professor Douglas K. Graham

ABSTRACT

The American Cancer Society projects 221,200 new diagnoses and 158,040 deaths to occur in 2015 as a result of lung cancer. With a five-year relative survival rate that has only marginally improved from the 1970’s (12%) to today (18%), new treatments are desperately needed for these large numbers of patients. Molecularly targeted therapies have begun to answer this call, with therapeutics against EGFR, ALK, and ROS1 now improving outcomes for patients with these specific genetic aberrations. However, the molecular events underlying lung cancer are complex, and many more therapeutic targets remain unidentified or untargeted. MERTK, a receptor tyrosine kinase of the TAM (TYRO3, AXL, and MERTK) family, is over-expressed or ectopically expressed in a wide variety of cancers, resulting in activation of several canonical oncogenic signaling pathways. Our laboratory has therefore begun developing novel therapeutics against MERTK. Described in Chapter III is Mer590, a monoclonal antibody that causes internalization and degradation of MERTK from the cell surface, resulting in decreased colony formation and increased apoptosis. The subject of Chapter IV, UNC2025, is an ATP-competitive small molecule inhibitor that blocks the kinase activity of MERTK, resulting in inhibition of downstream signaling, increased cell death, decreased colony formation, and decreased tumor formation in murine models. Although both of these agents show promising pre-clinical efficacy as single agents, they will not reach their full potential clinically unless given as part of a rational drug combination. Therefore, the identification of AXL as a target that has synergistic activity when inhibited in conjunction with MERTK is discussed in Chapter V. AXL and MERTK
receptor levels are regulated in an inter-dependent manner, and a functional relationship is also present in which AXL inhibition allows for more potent inhibition by UNC2025 of MERTK kinase activity, downstream signaling pathways, and proliferative and colony forming phenotypes. Identifying rational combinations such as this will afford the novel MERTK compounds outlined in Chapters III and IV the greatest chance for success in clinical trials, and will help achieve the overarching goal of the laboratory – to add MERTK to the list of actionable oncogenic targets in clinical oncology.

The form and content of this abstract are approved. I recommend its publication.

Approved: Douglas K. Graham
ACKNOWLEDGEMENTS

This work would not have been possible without the contributions of many, to whom many acknowledgements are due.

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ABBREVIATIONS

ALK – anaplastic lymphoma kinase
ALL – acute lymphoblastic leukemia
ATCC – American Type Culture Collection
ATF1 – activating transcription factor 1
CCTSI – Colorado Clinical and Translational Sciences Institute
CSF1 – colony-stimulating factor 1
CREB – cAMP-responsive element binding protein
CXR – chest x-ray
DSMZ – German Collection of Microorganisms and Cell Cultures
ECD – extracellular domain
EGFR – epidermal growth factor receptor
ERK – extracellular signal-regulated kinase
FAK – focal adhesion kinase
FBS – fetal bovine serum
GIST – gastrointestinal stromal tumor
Grb2 – growth factor receptor binding protein 2
HRP – horseradish peroxidase
IAP – Inhibitor of apoptosis protein
IL-3 – interleukin 3
JAK – Janus-activated kinase
LDCT – low-dose helical computed tomography
MEK – mitogen-activated protein/extracellular signal-regulated kinase
MFI – median fluorescence index
mIgG1 – murine immunoglobulin
NLST – National Lung Screening Trial
NSCLC – non-small cell lung cancer
NTB – nitrotetrazoline blue chloride
PI – propidium iodide
PI3K – phosphatidylinositol 3-kinase
PPI – protein protein interaction
rhGas6 – recombinant human Gas6
RTK – receptor tyrosine kinase
SCLC – small cell lung cancer
Shc – Src homology and collagen
shRNA – short hairpin RNA
siRNA – small interfering RNA
STR – short tandem repeat
TAM – TYRO3, AXL, MERTK family
TCGA – The Cancer Genome Atlas
TKI – tyrosine kinase inhibitor
TP – therapeutic peptide
Tulp1 – tubby-like protein 1
CHAPTER I

INTRODUCTION

Lung Cancer Burden, Treatment, and Research

Current state of lung cancer:

Cancer is the second leading cause of death in America, second only currently to heart disease. However; cancer is expected to surpass heart disease in the next few years, and is already the leading cause of death in adults ages 40-79 [1]. The large mortality associated with cancer is due in no small part to the contribution from lung cancer. Lung cancer has been the leading cause of cancer-related death in males since the 1950’s and in females since the 1990’s. In the year 2011, the most recent year for which such data is available, more Americans died due to lung cancer than died from breast, prostate, and colorectal cancer combined. This large mortality of lung cancer is due to a combination of related factors, including typical late-stage presentation, low relative survival rates, and insufficient therapeutic options for these late-presenting patients. Optimization of conventional chemotherapy regimens has led to modest gains in survival over the past few decades, but studies suggest that new treatment strategies must be pursued in order to achieve more impressive clinical gains [2-4].

Role of early detection in lung cancer:

One strategy that has demonstrated preliminary benefit in clinical trials is the use of screening strategies to increase the early detection of lung cancer. The National Lung Screening Trial (NLST) randomly assigned current or recently-former heavy smokers to receive an annual screening examination by either low-dose helical computed

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1 A portion of this chapter has been previously published: Cummings CT et. al., Molecular pathways: MERTK signaling in cancer. Clin Cancer Res 2013;19:5275-80. (Reference 54).
tomography (LDCT) or chest x-ray (CXR) for three years [5, 6]. Participants in the LDCT arm had lower lung cancer mortality, lower overall mortality, and an earlier stage of disease detected by screening than those in the CXR arm. Additionally, LDCT was found to detect significantly more disease than CXR. Impressively, the number needed to treat for LDCT to prevent a death from lung cancer was in the same range as that of mammography for breast cancer. There are challenges to incorporating LDCT screening into standard practice, but this trial provided evidence of its utility in facilitating early detection of lung cancer when curative intent is still the goal.

Diagnosis and treatment of lung cancer:

Upon diagnosis, lung cancer is categorized as either small cell lung cancer (SCLC), representing approximately 15% of diagnoses, or non-small cell lung cancer (NSCLC), which consists of nearly 85% of all lung cancer diagnoses. NSCLC is then staged according to the TNM system, an acronym standing for its component parts of assessing the primary Tumor, regional lymph Nodes, and distant Metastases (Table 1.1) [7, 8]. Early stage lung cancer, stages I and II, involves those cases with no metastases, and either no or only very localized lymph node involvement. Although this accounts for only 30% of NSCLC diagnoses, surgery with the goal of total resection and disease cure is the treatment of choice, typically by removing a single lobe of the lung in a lobectomy procedure. Adjuvant chemotherapy after surgery is occasionally recommended, but targeted therapeutics are not indicated. In patients with stage III NSCLC, those with disease characterized by more advanced primary tumor invasion or lymph node involvement than stages I or II but still without distant metastasis, a combined approach is utilized [9]. Surgery is still an option for some patients, in conjunction with chemotherapy and/or radiation therapy, and a complete cure is still an achievable goal. However, patients with distant metastases, or stage IV disease, are normally treated
only with palliative intent [10]. Surgery is not typically an option, and patients predominantly receive systemic chemotherapy. Current standard of care involves platinum-based doublet chemotherapy, with the possible addition of the anti-VEGF antibody bevacizumab for tumors of non-squamous histology. The overall five-year relative survival of NSCLC is only 18%; however, for patients presenting with advanced, stage IV disease, the rate is 4% [1]. Although early detection strategies offer the promise of a smaller proportion of patients being diagnosed with such late stage disease, for those who do present with stage IV NSCLC, new therapeutic treatment options are urgently needed.

**Targeted therapeutics in NSCLC:**

Targeted therapies have recently been introduced into the treatment arsenal for advanced stage NSCLC to begin to address this problem [10]. As opposed to conventional cytotoxic chemotherapy directed against all rapidly dividing cells, these new targeted therapeutics are specifically directed against molecular aberrations altered within an individual patient’s tumor cells. EGFR small molecule tyrosine kinase inhibitors, including erlotinib, gefitinib, and afatinib, have been approved as first-line therapy in patients harboring an activating EGFR mutation, while crizotinib and ceritinib are first-line therapy for patients presenting with an activating ALK translocation. Additionally, crizotinib is also now being utilized for patients with the more recently identified ROS1 activating translocation [11, 12]. While these agents have transformed treatment outcomes for patients with genetic modifications amenable to these targeted therapies, only approximately 10% of patients have an activating EGFR mutation, and only approximately 4% and 1% have an ALK or ROS1 translocation, respectively [12-17]. For the remaining majority of patients, opportunities clearly exist to identify new therapeutic targets and compounds.
Table 1.1: TNM classification system for the diagnosis of NSCLC. Adapted from [8]

<table>
<thead>
<tr>
<th>Primary Tumor</th>
<th>Tumor ≤ 3 cm diameter, surrounded by lung or visceral pleura, without invasion more proximal than lobar bronchus</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Tumor ≤ 2 cm diameter</td>
</tr>
<tr>
<td>T1a</td>
<td>Tumor &gt; 2 cm but ≤ 3 cm in diameter</td>
</tr>
<tr>
<td>T1b</td>
<td>Tumor &gt; 3 cm but ≤ 7 cm, or tumor with any of the following features:</td>
</tr>
<tr>
<td>T2</td>
<td>Involves main bronchus, ≥ 2 cm distal to carina</td>
</tr>
<tr>
<td>T2a</td>
<td>Invades visceral pleura</td>
</tr>
<tr>
<td>T2b</td>
<td>Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung</td>
</tr>
<tr>
<td>T2b</td>
<td>Tumor &gt; 3 cm but ≤ 5 cm</td>
</tr>
<tr>
<td>T2b</td>
<td>Tumor &gt; 5 cm but ≤ 7 cm</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor &gt; 7 cm or any of the following:</td>
</tr>
<tr>
<td>T3</td>
<td>Directly invades any of the following: chest wall, diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium, main bronchus &lt; 2 cm from carina (without involvement of carina).</td>
</tr>
<tr>
<td>T3</td>
<td>Atelectasis or obstructive pneumonitis of the entire lung</td>
</tr>
<tr>
<td>T4</td>
<td>Separate tumor nodules in the same lobe</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor of any size that invades the mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina, or with separate tumor nodules in a different ipsilateral lobe</td>
</tr>
</tbody>
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<tr>
<th>Regional Lymph Nodes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No regional lymph node metastases</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension.</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)</td>
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<table>
<thead>
<tr>
<th>Distant Metastasis</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>M1a</td>
<td>Separate tumor nodule(s) in a contralateral lobe; tumor with pleural nodules or malignant pleural or pericardial effusion</td>
</tr>
<tr>
<td>M1b</td>
<td>Distant metastasis (in extrathoracic organs)</td>
</tr>
</tbody>
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<tr>
<th>Stage Groupings</th>
<th>IA</th>
<th>IB</th>
<th>II A</th>
<th>II B</th>
<th>III A</th>
<th>III B</th>
<th>IV</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>T1a-T1b</td>
<td>T2a</td>
<td>T1a, T1b, T2a</td>
<td>T2b</td>
<td>T1a, T1b, T2a, T2b</td>
<td>T4</td>
<td>Any T</td>
</tr>
<tr>
<td></td>
<td>N0</td>
<td>N0</td>
<td>N1</td>
<td>N0</td>
<td>N2</td>
<td>N0</td>
<td>Any N</td>
</tr>
<tr>
<td></td>
<td>M0</td>
<td>M0</td>
<td>M0</td>
<td>M0</td>
<td>M0</td>
<td>M0</td>
<td>M1a or M1b</td>
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Genetic heterogeneity of NSCLC:

Although only a small number of molecular aberrations are currently targetable clinically, many more have been identified, and a subset of these also has novel therapeutics in various stages of development. A recent comprehensive review reveals that over a dozen validated oncogenic drivers have been described in NSCLC (Figure 1.1, [18]). While the majority of these drivers have been discovered individually, attempts are also being made to broadly survey lung cancer tumors in the search for remaining unidentified drivers. The most complete investigation into characterizing the molecular profile of NSCLC has been performed by The Cancer Genome Atlas (TCGA) research group. This consortium has profiled large numbers of untreated cancers of multiple types by analyzing DNA copy number, somatic exon mutations, mRNA sequencing, mRNA expression, promoter methylation, whole genome sequencing, microRNA sequencing, and proteomics studies. They have analyzed the two main histological subsets of NSCLC, squamous cell lung cancer and adenocarcinoma [19, 20]. For the squamous cell lung cancer subtype, the TCGA analyzed 178 tumors. Of note, they found an average of 360 exonic mutations, 165 genomic rearrangements, and 323 copy number alterations per tumor. This translated to a mean somatic mutation rate of 8.1 mutations per megabase, which was the highest rate of any cancer TCGA had assayed to that point, when the analysis was published in 2012. Additionally, the group determined that mutations common in adenocarcinoma, namely in KRAS and EGFR, were rare or absent in squamous cell lung cancer, but that FGFR gene amplification was much more common in this histological subset. In 2014, the group published its results on 230 adenocarcinoma tumors, and again found extremely high rates of genetic aberration. The mean average mutation rate was calculated to be 8.9 per megabase, surpassing that previously found in squamous cell lung cancer and taking over the lead for highest average mutation rate of all cancers assayed by TCGA. Like in squamous
cell lung cancer, this analysis was able to identify genetically altered potential driver oncogenes, including previously identified mutations in *KRAS* and *EGFR*, as well as novel mutations or amplifications in *ERBB2, MET, NF1*, and *RIT1*. However, an especially interesting finding was revealed in the proteomics analysis. By assaying the activation status of the MAP kinase pathway as well as the mTOR/AKT pathway, they determined that many tumors had activation of one or both of these pathways in the absence of a detected genomic alteration in a driver oncogene. While mutations in such driver genes undoubtedly contribute a significant impact on cancer development and progression, and have led to successful therapeutics, much work remains to identify all contributing factors to NSCLC oncogenesis outside of this traditional ‘driver’ oncogene model.

**MERTK Receptor Tyrosine Kinase**

**Role of MERTK in normal and cancer cells:**

As suggested by the aforementioned TCGA proteomics analysis, coupled with the high mean somatic mutation rates of both histological subsets, the oncogenic mechanisms behind NSCLC are complex and identification of additional therapeutic targets has therefore become a major research focus. One potential target is MERTK, a receptor tyrosine kinase (RTK) in the TAM family with AXL and TYRO3. Normal functions of MERTK are in the facilitation of platelet aggregation, and in the immune system as an anti-inflammatory molecule with important functions in efferocytosis (Reviewed in [21]). In platelets, all three TAM family members are required for full stabilization of thrombi. An interesting mechanism was uncovered in which loss of any one TAM receptor prevented the surface accumulation and subsequent activation of the other two family members, resulting in decreased downstream signaling critical to maintenance of platelet aggregation [22]. In the immune system, MERTK plays
important roles in a variety of cell types, the most studied of which is in efferocytosis, or the ingestion of apoptotic material (Reviewed in [21, 23]). Through its ability to bind phosphatidylserine, MERTK bridges macrophages to apoptotic debris and allows for their engulfment, preventing apoptotic accumulation and subsequent autoimmunity [24]. Additionally, MERTK stimulates the production of anti-inflammatory M2 cytokines, as another mechanism of dampening immune responses [25]. Indeed, many of the effects of loss of MERTK in genetically engineered mice are autoimmune in nature (Reviewed in Discussion, “Identifying and Avoiding Potential Side Effects” section). While these physiologic functions of MERTK have only recently been defined in platelets and macrophages, its over-expression and potential activation in a wide variety of cancers, including NSCLC, indicate that MERTK signaling confers an advantage on the tumor cell [22, 24, 26-48]. Target validation studies, discussed below, suggest that MERTK inhibition is a viable strategy for decreasing tumor burden in pre-clinical models. Finally, clinically relevant agents are under development in an effort to add MERTK to the list of effectively targeted proteins in patients with cancer, and are the subject of Chapters III-V of this thesis.

**Discovery of MERTK:**

MERTK has been implicated in cancer pathogenesis since it was first cloned from a human B-lymphoblastoid expression library and from a human glioblastoma library [29, 37]. Although ectopically expressed in multiple lymphoid leukemia cell lines and patient samples, MERTK is absent from normal B and T lymphocytes – data which provided the first evidence that MERTK may be an attractive target for cancer therapeutic development [29, 31, 49].
Figure 1.1: The genetic landscape of non-small cell lung cancer. Ratio of relative frequencies of genetic alterations demonstrated to have a driver role in NSCLC. Adapted from [50].
MERTK sequencing from human and mouse revealed it to be the human ortholog of the chicken c-eyk gene, the cellular proto-oncogene of v-eyk responsible for the oncogenic properties of the RPL30 avian retrovirus, which induces lymphomas, sarcomas, and other tumor types in chickens [51, 52]. Unbiased gain-of-function retroviral insertion screens have also identified the oncogenic role of MERTK [53].

**MERTK chimeric receptor signaling:**

Following cloning of the MERTK cDNA, multiple groups investigated the signaling pathways downstream of MERTK activation (Figure 1.2, [54]). As a ligand had not yet been identified, early studies used receptor chimeras with the intracellular domain of MERTK fused to the extracellular domain of proteins with a known ligand or dimerization capability. An early strategy combined the transmembrane and cytoplasmic domains of MERTK with the extracellular domain of human colony-stimulating factor 1 (CSF-1) receptor (Fms; ref. [37]). In this model, CSF-1 treatment induced MERTK autophosphorylation, transformed NIH 3T3 cells, and activated phospholipase Cγ, phosphatidylinositol 3- kinase (PI3K), and p70 S6 kinase. Recruitment of growth factor receptor binding protein 2 (Grb2) and phosphorylation of Src homology and collagen (Shc) led to Raf-1, mitogen-activated protein/extracellular signal–regulated kinase (MEK), and extracellular signal-regulated kinase (ERK) activation.

Another MERTK chimeric protein was made by fusing the extracellular domain of CD8 to the intracellular domain of MERTK creating a constitutively active MERTK chimera [55]. This MERTK chimera conferred an interleukin 3 (IL-3)-independent phenotype to Ba/F3 pro-B lymphocytes.
Figure 1.2: The MERTK signaling network. Multiple pro-oncogenic signaling pathways have been implicated downstream of MERTK activation. These include pathways promoting survival, increasing migration, and inhibiting apoptosis. The ligands Gas6, Protein S, Tubby, Tulp1, and Galectin-3 all induce MERTK autophosphorylation to initiate the signaling cascades depicted below. More recently, MERTK has been shown to be regulated by two miRNAs, miR126 and miR335, and may also have direct effects on gene transcription. Inhibitors of MERTK, including ligand traps, a monoclonal antibody, and small molecule inhibitors, are currently in preclinical development, and are described more fully in Chapters III, IV, and V.
Downstream, the Grb2, MEK1, and ERK pathways as well as PI3K were found to be activated. NFkB-mediated transcription was increased as assessed by a luciferase-reporter assay with potential activation of anti-apoptotic signaling in tumor cells (reviewed in ref. [56]). The p38 pathway was also activated and p38 inhibition decreased proliferation in MERTK chimeric Ba/F3 cells. Similar to NFkB, p38 has complex effects on proliferation, migration, and survival in cancer cells (reviewed in ref. [57]).

Finally, a chimeric receptor composed of the extracellular and transmembrane domains of the EGF receptor (EGFR) fused to the intracellular domain of MERTK was constructed [58]. Ligand activation prevented apoptosis in the myeloblast-like 32D cells promoting IL-3-independence and stimulated ERK, AKT, and p38 activity. In this model, MERTK signaling had a primary role in cell survival and cytoskeletal alterations without a substantial effect on proliferation.

MERTK ligands:

Although studies using chimeric receptors provided important information on MERTK signaling, identification of authentic MERTK ligands permitted analysis in a more physiologic context. The first ligand described was Gas6, identified by purification of AXL-activating conditioned media. Gas6 binds MERTK, but with 3- to 10-fold lower affinity [59, 60]. Subsequently, Protein S was identified as a MERTK ligand that did not activate AXL [61, 62]. Both ligands are secreted by multiple cell types and are present in human blood, although total Protein S levels are 1,000-fold higher; however, modifications of Protein S may be necessary for it to activate MERTK [63-65]. More recently, a novel phagocytosis-based functional cloning screening strategy identified 3 new MERTK ligands: Tubby, tubby-like protein 1 (Tulp1), and Galectin-3 [66, 67]. All known MERTK ligands induce MERTK autophosphorylation, although to date, roles for Tubby, Tulp1, and Galectin-3 have not been studied in MERTK-driven cancers.
However, over-expression of Galectin-3 has been shown in many cancers and Galectin-3 is known to play roles in a wide variety of oncogenic processes, consistent with the possibility that these phenotypes may be mediated by MERTK signaling [68].

**MERTK signaling in leukemia:**

The ectopic expression of MERTK in pediatric acute lymphoblastic leukemia (ALL) led our laboratory to investigate full-length MERTK receptor oncogenic signaling in lymphocytes [31, 69]. A transgenic model was made expressing MERTK from the Vav promoter; lymphocytes at all stages expressed MERTK. Lymphoblastic leukemia and lymphomas resulted from this ectopic MERTK expression and stimulation of these cells with Gas6 induced MERTK autophosphorylation and downstream activation of ERK1/2 and AKT. In human T-cell lymphoblastic leukemia cell lines, Gas6 also activated MERTK, ERK1/2, AKT, STAT5, and STAT6 [32]. STAT activation within tumor cells contributes to pro-survival phenotypes [70-72]. MERTK knock-down by short hairpin RNA (shRNA) resulted in decreased levels of phospho-STAT5 and phospho-ERK. In addition, in acute myeloid leukemia cells that did not express AXL or TYRO3, Gas6 activated MERTK and resulted in the phosphorylation and activation of ERK1/2, p38, MSK1, cAMP- responsive element binding protein (CREB), activating transcription factor 1 (ATF1), AKT, and STAT6 [73]. shRNA knockdown of MERTK reduced the activation of p38, ERK, and CREB, further strengthening these signaling findings. Taken together, these studies broaden and define endogenous MERTK signaling pathways.

**MERTK signaling in NSCLC and other solid tumors:**

MERTK signaling observed in leukemia cells is also seen in solid tumor cells; additional novel downstream effectors have also been identified. Of particular interest, in NSCLC, p38, ERK, GSK3α/β, MEK1/2, AKT, mTOR, CREB, and ATF1 phosphorylation
were all induced following Gas6 addition [45]. shRNA-mediated MERTK inhibition resulted in decreased levels of CREB, Bcl-xL, Survivin, and phospho-AKT, and increased levels of Bcl-2, in response to serum starvation. These results suggest additional mechanisms in NSCLC by which MERTK may impact tumor cell survival.

MERTK also has important signaling functions in other solid tumor types, including glioblastoma, melanoma, and prostate cancer. In glioblastoma cells, shRNA mediated reduction of MERTK protein expression and also decreased levels of phosphorylated ERK and AKT [38]. In addition, MERTK expression correlated with Nestin and Sox2 expression in a glioblastoma spheroid culture model, indicating possible roles for MERTK maintaining cells in an undifferentiated state [40]. In melanoma, Gas6-induced MERTK activation resulted in p38, ERK, GSK3a/b, AKT, AMPK, STAT5, CHK-2, focal adhesion kinase (FAK), and STAT6 phosphorylation, whereas overexpression of MERTK increased the levels of phospho-AKT [43, 44]. Inhibition of MERTK by shRNA prevented the Gas6-induced increase of pAKT, pERK, and pSTAT6, decreased basal phosphorylation of AKT, mTOR, and p70S6 kinase, increased PARP cleavage, and decreased CDC42 activity. In prostate cancer cells, MERTK associates with and facilitates the activation of Ack1, a non–receptor tyrosine kinase. This process results in an Ack activity-dependent degradation of the Wwox tumor suppressor, suggesting yet another oncogenic mechanism, that is, control of a tumor suppressor [26]. An FMS-MERTK chimera expressed in a prostate cancer cell line induced Raf, MEK1/2, p90RSK, ERK, and AKT phosphorylation and increased c-Fos and c-Jun transcription factor expression [47].

**MERTK in migration and cellular trafficking:**

MERTK signaling has also been implicated in tumor cell migration and invasion. In NSCLC cells, FAK is phosphorylated in response to Gas6 [45]. Both total and
phosphorylated FAK and RhoA increase, whereas total and phosphorylated myosin light chain 2 are decreased in glioblastoma cells in response to shRNA-mediated MERTK inhibition, with impaired migration [39, 40]. Melanoma cell migration and invasion are also decreased by shRNA MERTK expression [43, 44]. With respect to cellular trafficking, a recent report links MERTK action to EGFR surface levels [74]. In the absence of MERTK, EGF treatment induced a higher rate of EGFR internalization and degradation, resulting in decreased levels of EGFR on the cell surface and reducing downstream signaling. Related to the concept of receptor trafficking, a recent study found MERTK to be located not only at the cell surface, but also in the nucleus. Consensus nuclear localization sequences have also been identified in the MERTK gene [75]. This raises the possibility that MERTK may have effects on gene transcription.

**MERTK target validation studies:**

Many studies have used shRNA to show critical oncogenic roles for MERTK in a variety of tumor types. Most germane to this discussion, in NSCLC MERTK inhibition increased apoptosis, decreased colony formation, increased chemosensitivity, and decreased tumor formation in a mouse model. Importantly, this paper also demonstrated that approximately two-thirds of NSCLC patient tumor samples were positive for MERTK expression, independent of histology [45].

In T-cell acute lymphoblastic leukemia, shRNA against MERTK resulted in increased apoptosis, decreased methylcellulose colony formation, increased sensitivity to cytotoxic chemotherapies, and delayed disease onset with increased survival in a murine leukemia model [31, 32]. Similarly, in acute myeloid leukemia MERTK inhibition resulted in increased apoptosis, decreased colony formation, and increased survival in a mouse model [73]. In glioblastoma, MERTK shRNA increased apoptosis and autophagy, decreased colony formation, increased chemosensitivity, altered
morphology, and decreased migration [38-40]. shRNA-mediated inhibition of MERTK in melanoma cell lines resulted in increased cell death, decreased proliferation, decreased colony formation in soft agar, decreased migration, and decreased tumor formation in xenografts [43, 44].

MERTK knockdown in breast cancer cells resulted in decreased formation of metastases and inhibited endothelial cell recruitment in mouse models [34]. In breast cancer, miR-126 and miR-335 negatively regulate MERTK expression; loss of these miRNAs contributes to breast cancer progression and metastasis in a complex mechanism. Loss of miR-126 increased MERTK expression. This increased MERTK resulted in increased levels of extracellular domain cleavage, and soluble MERTK was postulated to act as a ligand sink, sequestering Gas6 and preventing activation of TAM receptors on migrating endothelial cells. Decreased Gas6-induced signaling in endothelial cells increased their migration, resulting in tumor-associated angiogenesis.

Inappropriate or overexpression of MERTK, one of its ligands, or both, has been the most studied mechanism linking MERTK and cancer. However, recent studies in multiple myeloma, melanoma, renal cancer, and head and neck cancer have also identified mutation of MERTK as another possible mechanism [43, 76, 77]. Mutation has been recognized as a common method of activating multiple other receptor tyrosine kinases, and it will be interesting to determine the frequency and functional effects of these recently discovered MERTK mutations.

**Role of AXL and TYRO3 in NSCLC:**

AXL has been studied as a primary target in NSCLC, while the role of TYRO3 is as of yet unclear; even in the case of AXL research is limited thus far (Reviewed in [78]). AXL expression is detected in 48% - 93% of patient samples, correlates with an invasive phenotype in cell lines, and with lymph node status and clinical stage in patients [45, 79].
Our laboratory has previously demonstrated AXL inhibition to cause chemo-sensitization and inhibit in vivo tumor growth in murine models [45]. However; the role of AXL, and to a lesser extent TYRO3, has been much more fully characterized in the setting of primary therapeutic resistance, including in NSCLC. These studies are reviewed below, in the “Characterization of Resistance Pathways” section.

**Therapeutics in development:**

One strategy to inhibit MERTK is to use a ligand trap, consisting of the extracellular domain of MERTK or an antibody against Gas6, to prevent ligand-dependent MERTK activation [27]. While efficacious in vitro, the aforementioned high levels of endogenous MERTK ligands present in serum may present challenges for translation to in vivo utility, and targeting MERTK directly may be a more beneficial approach. A monoclonal antibody against MERTK, Mer590, has been used to decrease the levels of MERTK protein on the surface of glioblastoma cells, resulting in decreased tumor cell migration in vitro [39]. This antibody has subsequently been extensively characterized in NSCLC, and is the subject of Chapter III of this thesis. In addition, several multikinase small molecule inhibitors have been shown to have inhibitory effects against MERTK, including sunitinib, BMS-777607, and Compound-52 [80-82]. Recently, several MERTK-selective small molecule inhibitors have been developed by our group using structure-aided design algorithms. The first of these, UNC569, is a pyrazolopyrimidine derivative that functions as an ATP-competitive inhibitor [83]. It is highly selective for MERTK, has a favorable pharmacokinetic profile, and has shown promising pre-clinical activity against leukemia in vitro. A second-generation compound, UNC1062, is a pyrazolopyrimidine sulfonamide derivative of UNC569, which has reduced human ether-a-go-go-related gene potassium channel activity, and therefore a more favorable toxicity profile [84]. This compound has shown promising pre-clinical
activity in a melanoma model, blocking MERTK activation and downstream signaling via STAT6, ERK, and AKT, increasing apoptosis, decreasing colony formation in soft agar, and decreasing collagen matrix invasion [44]. Further pharmacokinetic optimization has ultimately led to the discovery of UNC2025, the first of our novel MERTK-selective inhibitors with 100% bioavailability, allowing for oral dosing and potential clinical translation. Characterization of this compound in NSCLC is the subject of Chapter IV of this thesis.

Characterization of Resistance Pathways

Molecularly targeted therapeutics have dramatically transformed the field of clinical oncology. However; despite impressive initial responses, the rapid development of drug resistance has limited long-term success with these therapies. Insight from the infectious disease field, one well versed in combating the development of drug resistance, has demonstrated that two factors are essential: an initial rapid decrease in disease burden, and subsequent prevention of resistant clonal outgrowth [85]. These goals are effectively achieved in a variety of infectious disease settings by utilizing multiple therapies in combination, and it is likely that this strategy will also be necessary with MERTK therapeutics in particular, and in clinical oncology in general.

Several resistance mechanisms have been discovered in cancer patients, most commonly involving secondary mutation of the drug target or activation of a bypass signaling pathway. With regards to the latter, two recent studies have demonstrated the ubiquity of these bypass mechanisms. Wilson et al demonstrated that ‘kinase-dependent’ cancer cell lines can be rescued from small molecule-mediated inhibition of their dependent kinase simply by administration of one or more growth factor ligands, including HGF, FGF, IGF, PDGF, NRG1, and EGF [86]. Additionally, in many of these cell lines, multiple ligands were capable of rescue. In a similar study, Harbinski et al
treated cancer cell lines dependent on MET, FGFR2, or FGFR3 activation with a library of 3,000 secreted proteins, and again found that many were capable of rescuing cells from inhibition of their driver kinase [87]. These studies suggest the existence of multiple redundant bypass signaling pathways poised to take over upon inhibition of the primary ‘dominant’ signaling pathway. Identifying and targeting these pathways may therefore be a successful strategy to prevent resistance to targeted therapeutics and induce long-term remissions in cancer patients.

A protein that has been repeatedly identified as activating a ‘bypass pathway’ in multiple cancer types, and against a wide variety of therapeutics, is the receptor tyrosine kinase AXL. In response to conventional chemotherapeutics, AXL up-regulation has been demonstrated in acute myeloid leukemia patient samples [88]. Similarly, shRNA against AXL induces chemosensitivity in NSCLC and astrocytoma cell lines [38, 45]. In addition to conventional chemotherapeutics, AXL also plays a well-established role in resistance to targeted therapeutics, including up-regulation in response to IGF-1R inhibition in a rhabdomyosarcoma cell line, to TRAIL inhibition in an esophageal cancer cell line, and to BCR-ABL/c-kit inhibition in chronic myeloid leukemia and gastrointestinal stromal tumor patient samples and cell lines [89-93]. However, up-regulation of AXL in drug-resistant cancers has been most well studied in the setting of ErbB family member inhibition. In response to long-term culture with the HER2/c-neu inhibitor lapatinib, AXL was found to be increased in a breast cancer cell line, while its inhibition re-sensitizes these cells to lapatinib [94]. Similarly, inhibition of AXL is capable of re-sensitizing previously resistant cell lines to lapatinib treatment in esophageal adenocarcinoma [95]. With respect to EGFR inhibition, an AXL monoclonal antibody in a NSCLC cell line, and an AXL small molecule inhibitor in a resistant head and neck cancer cell line, increased sensitivity to erlotinib [96, 97]. Two database-mining studies identified elevated AXL expression as a marker of resistance to EGFR-targeted therapeutics, in NSCLC and
triple negative breast cancer, respectively [98, 99]. Interestingly, AXL was found to co-immunoprecipitate with not only EGFR, but also with HER2/c-neu, HER3, MET, and PDGFR in the breast cancer study, suggesting that physical interaction may be a mechanism utilized by AXL to mediate drug resistance. Finally, using NSCLC and HNSCC models, including HNSCC patient samples, Brand et al demonstrated AXL as a marker of cetuximab resistance, where it was found to associate with and stabilize EGFR, while Zhang et al found AXL up-regulation in a large subset of matched pre- and post-treatment erlotinib-resistant patient samples, and utilized cell line and mouse models to demonstrate that inhibition of AXL re-sensitized to erlotinib [100, 101]. The broad range of these studies implicating AXL in drug resistance indicates that AXL inhibition in this setting may have widely applicable utility.

In Chapter V, we identify MERTK inhibition as a novel scenario in which AXL is up-regulated as a bypass signaling pathway, providing intrinsic therapeutic resistance. Further, data suggest that co-inhibition of AXL and MERTK is more efficacious than monotherapy, and may be a useful strategy in preventing intrinsic therapeutic resistance in subsets of NSCLC.

**Summary**

Successful treatment of NSCLC requires novel therapeutic options due to its typical late presentation, limited efficacy of cytotoxic chemotherapeutics, and the resulting extremely poor prognosis. Advances have been made through the introduction of targeted therapies; however, many more such treatment options are needed to account for the vast complexity of oncogenic mechanisms underlying NSCLC. The MERTK tyrosine kinase has been linked to the pathogenesis of cancer since it was first discovered by expression cloning from a neoplastic cell nearly two decades ago, and is now being advanced as a potential target for NSCLC, where it is expressed in
approximately two-thirds of patient tumor samples. It has been shown to activate a wide variety of pro-oncogenic signaling pathways, including those involving MAPK and p38, PI3K, Janus-activated kinase (JAK)/STAT, FAK/RhoA/MLC2, and Bcl-2 family members, contributing to increased proliferation and migration, and decreased apoptosis and chemosensitivity. Several types of MERTK inhibitors are currently in development, including ligand traps, a monoclonal antibody, and small-molecule tyrosine kinase inhibitors. Such agents are designed to exploit the selective requirement for MERTK in tumor cells. The hope is that these efforts will result in clinically available MERTK-targeted therapeutics in the near future, as well as a further understanding of how best to deploy them to avoid therapeutic resistance and achieve the best possible responses.
CHAPTER II
MATERIALS AND METHODS

Cell Culture and Treatment

All cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Recombinant human Gas6 (rhGas6, 885-GS) were purchased from R&D Systems. Carboplatin was purchased from Sigma (C2538). The A172, A549, G361, H226, H1299, H2009, HMCB, and U251 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA), the Colo699 and HCC15 cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), the SF188 cell line was obtained from the UCSF Brain Tumor Bank, and the Calu3, H157, H322, H358, H441, H460, H1650, H1975, H2126, H2228, H3122, H3255, HCC827, HCC4006, HCC4011, and LC-2/ad cell lines were obtained from Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). All parental cell lines and shRNA-transduced derivatives (polyclonal and clonal) used in these studies were subjected to short tandem repeat (STR) analysis and the profiles were compared to publically available databases to verify their authenticity. Validated STR profiles were not available for some of the cell lines (H157, H3122, HCC4011, SF188) but the profiles obtained did not match any other cell line. UNC2025 and UNC1653 were synthesized as previously described [102, 103].

Production of Monoclonal Antibody

The anti-MERTK monoclonal antibody (Mer590) was purified from a mouse hybridoma generated by fusion of FoxNY mouse myeloma cells to B-cells from Balb/C mice immunized with recombinant MERTK extracellular domain/Fc chimera as previously described [39].
Lentiviral Transduction and Isolation of Clonal Populations

Lentiviral vectors (pLKO.1) containing shRNA sequences targeting MER (shMer1, Oligo ID: TRCN0000000862), AXL (shAXL9, Oligo ID: TRCN0000000575), or non-silencing control GFP (shControl, catalog no. RHS4459) were obtained from Open Biosystems. Lentiviral particles were produced in 293FT cells and Colo699 target cells were transduced as previously described [45]. Polyclonal populations were maintained in selection medium containing puromycin (2 μg/ml). Stable clonal isolates were obtained by single-cell sorting using flow cytometry. Clonal populations were cultured in puromycin for 2-3 doubling times every 2-3 weeks.

siRNA Transfection

Non-targeting siRNA (Cat# D-001810-01-05), siRNA targeting MERTK (Cat# J-003155-09-0005 and Cat# J-003155-10-0005), and siRNA targeting AXL (Cat# J-003104-10-0005 and Cat# J-003104-12-0005) were purchased from Thermo Scientific. Cells were transfected with a final concentration of 25 nM siRNA and 1 μL/mL DharmaFECT 1 Transfection Reagent (Cat# T-2001-02).

Western Blotting

Adherent cells were either lifted with 0.02% EDTA in PBS and resuspended in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM Na3VO4) supplemented with protease inhibitors (Complete Mini, Roche Molecular Biochemicals), or washed with PBS and scraped into lysis buffer. Total protein concentrations were determined and western blotting was performed as previously described [45].
Antibodies for Western Blotting and Flow Cytometry

For western blotting, the following antibodies were obtained from Cell Signaling: pAKT (S473, Cat# 9271), AKT (Cat# 9272), EGFR (Cat# 2232), Enolase-1 (Cat# 3810), pERK1/2 (T202/Y204, Cat# 9106), ERK (Cat# 9102), MET (Cat# 3127), PARP (Cat# 9542), pSTAT6 (Y641, Cat# 9361), STAT6 (Cat# 9362), Survivin (Cat# 2808), TYRO3 (Cat# 5585), and α-tubulin (Cat# 2125). Additional antibodies used for western blotting include AXL (R&D Systems, AF154), pMERTK (Y749, Y753, Y754, PhosphoSolutions), FGFR1 (OriGene, TA301021), MERTK (Abcam 52968), and pTyrosine (4G10 Platinum, Millipore 05-1050). Horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit, Bio-Rad 170-6515; goat anti-mouse, Bio-Rad 170-6516; donkey anti-goat, Santa Cruz sc-2020) were used for enhanced chemiluminescence of western blots. The following antibodies were used for measurement of indirect immunofluorescence using flow cytometry: mouse monoclonal anti-MERTK (Caveo Therapeutics, CVO-590) and phycoerythrin-conjugated donkey anti-mouse (Jackson Immunoresearch, 715-116-150), or Mer590 and allophtocyanin-conjugated donkey anti-mouse (Jackson Immunoresearch, 715-136-150). All antibodies were used as recommended by the manufacturer unless otherwise specified.

Immunoprecipitation and Detection of Phosphorylated MERTK, AXL, and TYRO3

Cells were treated with pervanadate phosphatase inhibitor (0.12 mM Na₃VO₄ in 0.002% H₂O₂ in PBS) 1 minute (Colo699) or 5 minutes (all other cell lines), and then lysed. Lysates were incubated with antibodies against MERTK (R&D Systems, MAB8912), AXL (R&D Systems, AF154), or TYRO3 (Cell Signaling, 5585) and with rec-Protein G-sepharose 4B beads (Invitrogen 10-1242) overnight. Beads were then washed twice with lysis buffer, resuspended in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 5% beta-mercaptoethanol, 2% SDS, and 0.01% bromophenol blue), boiled, and
resolved on polyacrylamide gels. Blots were probed for phospho-MERTK or phospho-tyrosine, stripped, and re-probed for total MERTK (Abcam, 52968), AXL (R&D Systems, AF154), or TYRO3 (Cell Signaling, 5585).

**Co-immunoprecipitation**

Sub-confluent cultures were washed with PBS and scraped into a modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and 1X HALT protease inhibitor cocktail – Life Technologies, 87786). Lysates were quantified by Bradford assay, and 500 μg was incubated overnight with antibody against MERTK (Abcam, 52968), AXL (R&D Systems, AF154), or vehicle control. The next day, freshly BSA-blocked rec-Protein G-sepharose 4B beads (Invitrogen 10-1242) were added for three hours. Beads were then washed three times with lysis buffer, resuspended in 4X Protein Loading Buffer (Li-Cor, 928-40004), boiled, and run by western blot.

**Cycloheximide Protein Stability Assay**

Sub-confluent were treated with DMSO or UNC2025 (200 nM) in combination with the protein translation inhibitor cycloheximide (100 μg/mL) and lysed 0 - 24 hours later to monitor for protein degradation and stability.

**Assessment of Downstream Signaling**

For Figure 3.3, sub-confluent cultures (approximately 5 x 10^5 cells) were pre-treated with 2 μg/mL Mer590 or PBS vehicle control in serum-free RPMI culture medium for 24 hours. 200 nM rmGas6 was then added to each well for 10 minutes before cells were lysed. Lysates were quantified by Bradford assay and analyzed by western blot.
For Figure 4.2, sub-confluent cultures were starved in serum-free medium for two hours and then cultured in the presence of UNC2025 or vehicle control for an additional two hours prior to Gas6 stimulation for 10 minutes. Lysates were quantified by Bradford assay and analyzed by western blot (Figure 4.2A). Alternatively, cultures were treated with UNC2025 or vehicle control for 72 hours (Figure 4.2B), prior to lysis, quantification, and analysis on western blot. Additionally, cell cultures for Figure 5.2A were pre-treated with siRNA for 24 hours, prior to one hour with UNC2025 before lysis and analysis on western blot.

**Flow Cytometric Detection of Surface and Total Proteins**

Sub-confluent cultures (approximately 5 x 10^5 cells) were washed with PBS and lifted with 0.02% EDTA in PBS. Harvested cells were fixed in 4% paraformaldehyde (Figure 3.2C) or not (Figure 3.1), then washed in FACS wash buffer (2% FBS and 0.02% azide in PBS) prior to staining in 50 μl staining solution (1% FBS and 0.02% azide in PBS) containing primary antibody, murine immunoglobulin (mIgG1, R&D Systems, MAB002), or vehicle control, for 15-30 minutes at 4°C. Cells were washed again in FACS wash buffer, and then incubated in staining solution containing fluorophore-conjugated secondary antibody or vehicle control for 15-30 minutes at 4°C. Stained cells were washed in FACS wash buffer, resuspended in staining solution, and fluorescence of surface-bound antibodies was measured by flow cytometry. For assessment of total MER, cells were fixed in 4% paraformaldehyde, washed in FACS wash buffer, permeabilized in permeabilization/wash buffer (BD, 554723), resuspended in staining solution containing secondary antibody or vehicle control for 30 minutes at 4°C, washed in perm/wash buffer, and resuspended in staining solution before analysis by flow cytometry.
Quantitative PCR

RNA was extracted from cells using the Qiagen RNeasy Plus Mini Kit (Cat# 74134), and analyzed for quality on an Agilent 2100 bioanalyzer. RNA was then reverse transcribed to cDNA, and qPCR was performed on a Roche LightCycler 480 II. Primer/probe mixtures were ordered from Life Technologies (GAPDH: Hs02758991_g1, GAS6: Hs01090305_m1, AXL: Hs01064444_m1, MERTK: Hs01031973_m1). Results were calculated using the delta delta Cp method.

Apoptosis and Cell Death Assay

For Figures 3.4 and 3.5, sub-confluent cultures were treated with mIgG1 or Mer590 for 48 hours, then mIgG1 or Mer590 with or without carboplatin for an additional 72 hours. For Figure 4.3, treatment was with UNC2025, UNC1653, or DMSO vehicle control for 72 hours. Supernatants were collected and combined with cells after lifting with EDTA. Cells were then stained with 0.2 uM YO-PRO-1 and 1.5 uM propidium iodide (PI) (Invitrogen). Uptake of dyes was assessed by flow cytometry using an FC500 flow cytometer and CXP analysis software (Beckman Coulter).

MTS Assay of Cell Proliferation

Cellular proliferation was assessed using the Promega CellTiter 96 AQueous One Solution reagent (Cat# G3580). Cells were seeded at 2,000 cells/well on 96-well plates, pre-treated for 24 hours with siRNA (Figure 5.3B), and then treated for 72 hours with UNC2025 (200 nM) and/or R428 (200 nM, Figure 5.4A). Promega reagent was then added for one hour, and plates were read at an absorbance of 490 nm.
Re-plating Colony Formation Assay

For Figure 3.3C, sub-confluent cultures were treated with vehicle control or Mer590 for 72 hours. Cells were then lifted with EDTA and counted. One thousand viable cells, as determined by trypan blue exclusion, were cultured in 6-well plates. Colonies were stained with crystal violet and counted after 10 days.

Clonogenic Assay of Colony Formation

Cells were plated at low density (500 cells/well) on 6-well plates in the presence of DMSO vehicle control, UNC2025, and/or R428. Cells were allowed to grow and form colonies for 10 days, with media replaced with new drug every 3-4 days. Colonies were then stained with crystal violet and counted on a GelCount colony counter.

Soft Agar Colony Formation Assay

For Figure 4.4, 15,000 cells per well were plated in 0.35% Noble agar in RPMI, and overlaid on 0.5% Noble agar in RPMI in 6-well plates. Agar layers were overlaid with 2 mL medium containing DMSO vehicle control, UNC2025, or UNC1653. This medium layer was aspirated and replaced three times per week. After two weeks of growth, medium was aspirated, and cells were stained with 200 µL nitrotetrazoline blue chloride (NTB) (Biosynth N-8100) overnight. Colonies were then counted using a GelCount colony counter and software (Oxford Optronix).

Subcutaneous Xenograft Model

All experiments involving animals were approved by the University of Colorado Institutional Animal Care and Use Committee. For Figure 4.5, two million H2228 cells or 5.5 million A549 cells were suspended in 100 µL PBS (with 50% Matrigel for the A549 cell line) and injected subcutaneously into the flank of athymic Nude-Foxn1nu mice.
Tumors were measured weekly with calipers, and volume was calculated using the formula \( \text{Volume} = \pi a^2 b / 6 \), where ‘a’ represents the shortest diameter measured perpendicular to the longest diameter, ‘b’. When tumors reached an average size of 100 mm\(^3\) (A549) or 150 mm\(^3\) (H2228), mice were randomized to groups and treated twice daily by oral gavage with saline or 50 mg/kg UNC2025 administered at a dose of 10 ml/kg.

**Statistical Analysis**

Statistical analyses were performed using Prism 5 software (GraphPad Software, Inc.). All data are representative of at least three independent experiments.
CHAPTER III
MER590, A NOVEL MONOCLONAL ANTIBODY TARGETING MERTK, DECREASES COLONY FORMATION AND INCREASES CHEMOSENSITIVITY IN NSCLC

Abstract

The successes of targeted therapeutics against EGFR, ALK, and ROS1 in non-small cell lung cancer (NSCLC) have demonstrated the substantial survival gains made possible by precision therapy. However, the majority of patients do not have tumors with genetic alterations responsive to these therapies, and therefore identification of new targets is needed. Our laboratory previously identified MERTK receptor tyrosine kinase as one such potential target. We now report our findings targeting MERTK with a clinically translatable agent – Mer590, a monoclonal antibody specific for MERTK. Mer590 rapidly and robustly reduced surface and total MERTK levels in multiple cell lines. Treatment reduced surface MERTK levels by 87%, and this effect was maximal within four hours. Total MERTK levels were also dramatically reduced, and this persisted for at least seven days. Mechanistically, MERTK down-regulation was mediated by receptor internalization and degradation, leading to inhibition of downstream signaling through STAT6, AKT, and ERK. Functionally, this resulted in increased apoptosis, increased chemosensitivity to carboplatin, and decreased colony formation. In addition to carboplatin, Mer590 interacted cooperatively with shRNA-mediated MERTK inhibition to augment apoptosis. These data demonstrate that MERTK inhibition can be achieved with a monoclonal antibody in NSCLC. Optimization toward a clinically available anti-MERTK antibody is warranted.

2 A portion of this chapter has been previously published: Cummings CT et. al., Mer590, a novel monoclonal antibody targeting MER receptor tyrosine kinase, decreases colony formation and increases chemosensitivity in non-small cell lung cancer. Oncotarget 2015;5:10434-45. (Reference 110)
Results

Mer590 decreases total cellular and surface MERTK expression:

We generated a novel monoclonal antibody, Mer590, against the extracellular domain of human MERTK in mouse hybridoma cells [39]. A 24-hour exposure to 0.5 μg/ml Mer590 significantly reduced MERTK total protein levels in four NSCLC cell lines, without affecting levels of the closely related receptor tyrosine kinase AXL (Figure 3.1A). Comparable results were obtained after 48 hours of Mer590 treatment (data not shown). Additional experiments with HCC15 cells demonstrated persistent knockdown of MERTK seven days after a single application of Mer590 (data not shown). As total MERTK decrease was consistent in all four NSCLC cell lines assayed, we selected two representative cell lines for further study: Colo699 because it does not express AXL and is MERTK-dependent, and H2009 as a representative cell line expressing both MERTK and AXL. Like total MERTK expression, surface MERTK expression as measured by flow cytometry was also decreased after Mer590 treatment, with a reduction of 87% after 48 hours of treatment in the Colo699 cell line (Figure 3.1B). Dose-response curves were generated, and indicate that a concentration of 6.25 ng/ml of Mer590 was sufficient to decrease MERTK surface levels by 50%, while a concentration of 50 ng/ml was sufficient to produce the maximal decrease in MERTK surface levels (Figure 3.1C). Finally, the kinetics of the Mer590-induced decrease in MERTK surface levels were determined, again by surface flow cytometry. At concentrations of 6.25 or 200 ng/ml of Mer590, maximal reduction of surface MERTK was achieved within four hours of Mer590 exposure, independent of the dose (Figure 3.1D).
Figure 3.1: A novel inhibitory anti-MERTK antibody, Mer590, reduces total cellular and surface expression of MERTK. (A) A549, H2009, HCC15, and Colo699 cells were cultured in the presence of 0.5 μg/ml Mer590, 0.5 μg/ml isotype control murine immunoglobulin (mlgG1), or PBS vehicle control for 24 hours. Western blot analysis demonstrated significant loss of MERTK protein expression after Mer590 treatment without affecting expression of the related receptor tyrosine kinase AXL. Tubulin was used as a loading control. (B) Colo699 cultures were treated with 0.5 μg/ml Mer590 or mlgG1 for 48 hours, and then stained for surface MERTK expression and analyzed by flow cytometry. Representative histograms (top panel) correspond to red and black rectangles overlaying the bar graph (bottom panel). (C) Colo699 cultures were treated for 48 hours with the indicated doses of Mer590 or with vehicle control and surface MERTK protein was detected by flow cytometry. (D) Colo699 cells were treated with 6.25 ng/ml Mer590, 200 ng/ml Mer590, or vehicle control for the indicated times and surface MERTK levels were determined by flow cytometry. Mean values and standard errors were derived from 3 independent experiments.
Mer590 induces receptor internalization of MERTK:

Possible explanations for the reduction of total MERTK protein levels in response to Mer590 treatment include promotion of MERTK extracellular domain (ECD) shedding, and induction of MERTK internalization and degradation. A number of antibodies have been developed to target the MET receptor tyrosine kinase, and both mechanisms of action have been demonstrated, depending on the specific antibody utilized [104, 105]. Additionally, the MERTK ECD can be cleaved from the cell surface under basal conditions, and ECD shedding is increased in response to stimulation with lipopolysaccharide or phorbol 12-myristate 13-acetate, posing the possibility that MERTK ECD shedding may also be induced by Mer590 administration [27]. To distinguish between these two mechanisms, levels of MERTK ECD in culture media with and without Mer590 were measured by western blot analysis (Figure 3.2A). Soluble MERTK protein was visualized at the expected molecular weight of 120-130 kDa [27]. Administration of Mer590 resulted in decreased MERTK ECD levels in the culture media compared to mIgG1 treated cells. Whole cell lysates were used to confirm efficacy of MERTK decrease by Mer590 (Figure 3.2B). The reduction of MERTK ECD in conditioned media would not be expected if the action of Mer590 was to increase receptor cleavage. The result is consistent with Mer590-induced receptor internalization, reducing surface MERTK available for cleavage.

To further test the hypothesis that Mer590 induces receptor internalization, cells were incubated with Mer590 at 4° C for 20 minutes. Although antibody binding occurs at this temperature, receptor internalization cannot [106]. Half of the samples were subsequently kept at 4° C and half were moved to 37° C for 20 minutes, a temperature permissible for receptor internalization. Cells were then fixed and either stained to detect surface MERTK, or permeabilized and stained to detect total MERTK. Median fluorescence intensity (MFI) levels were then determined by flow cytometry. After
moving to the permissive temperature, cell surface levels of MERTK decreased by 52.6% and 58.4%, in the Colo699 and H2009 cell lines, respectively, while total MERTK levels were decreased by only 24.9% and 20.0% (Figure 3.2C). If Mer590 induced receptor cleavage, an equal loss would be expected from the cell surface and total levels; however, the selective loss of MERTK from the cell surface is consistent with receptor internalization. The small loss of total MERTK levels may be due to lysosomal degradation, the end-point of receptor internalization, taking place within the 20 minutes at the permissive temperature prior to fixation. Together, the decreased MERTK ECD shedding into the media, and the decreased surface:total MERTK ratio, demonstrate that Mer590 promotes receptor internalization.

Mer590 prevents MERTK phosphorylation and downstream signaling:

MERTK is activated by several ligands, including Gas6, which induces receptor autophosphorylation and activation of a wide variety of downstream signaling pathways [54]. To determine if Mer590 interferes with these signaling processes, cells were pre-treated with Mer590 or vehicle control for 48 hours, then cultured with or without serum in the continued absence or presence of Mer590 for two hours. At this point, cells were stimulated with either Gas6 or vehicle control for ten minutes. Cells were lysed and total and phospho-MERTK levels assessed by immunoprecipitation and western blotting (Figure 3.3A). As expected, Gas6 induced MERTK phosphorylation; this was prevented by Mer590 treatment (lanes 5, 6, 11, and 12). Mer590 also reduced basal levels of phospho-MERTK in H2009 cells cultured in complete medium (lanes 7, 8), as well as residual levels of phospho-MERTK in serum starved H2009 cells (lanes 9, 10). In Colo699 cells, both phospho-MERTK and total MERTK were undetectable in samples treated with Mer590 (lanes 2, 4, and 6).
Figure 3.2: Mer590 induces internalization of surface MERTK. (A and B) Colo699 and H2009 cells were cultured overnight in RPMI containing 10% FBS. The next morning, the medium was replaced with serum-free RPMI containing vehicle control (PBS), isotype antibody control (1 μg/ml mlgG1), or Mer590 (0.5 or 1 μg/ml). After 24 hours, the culture supernatants were collected, filtered to remove any floating cells and cellular debris, and concentrated approximately 40-fold using Amicon Ultra-4 centrifugal filter units (Millipore UFC803096). Adherent cells were lysed separately. MERTK protein levels were analyzed by western blot. (A) MERTK protein levels in concentrated culture supernatants. Enolase-1 is excreted from NSCLC cells and was used as a loading control [107]. (B) MERTK protein levels in whole-cell lysates demonstrate significant loss of total cellular MERTK following Mer590 treatment without effect on expression of the related receptor tyrosine kinase TYRO3. Tubulin was used as a loading control. (C) Colo699 and H2009 cells were treated with Mer590 (2 μg/ml) for 20 minutes at 4°C to allow binding to MERTK without induction of internalization. Cells were then either kept at 4°C or shifted to 37°C for 20 minutes to allow for internalization before fixing in paraformaldehyde. Half of the samples were then stained for surface MERTK, while half were permeabilized and stained for total MERTK, and then analyzed by flow cytometry. Representative histograms are shown above, with quantification of median fluorescence index (MFI) values below. MFI values relative to samples kept at 4°C were determined, such that loss of MERTK in each compartment upon shifting to 37°C could be assessed.
To determine if inhibition of MERTK activation translated to reduced downstream signaling, cells were treated with Mer590 for 24 hours in the absence of serum, and then stimulated with Gas6 for ten minutes. Phosphorylated and total STAT6, AKT, and ERK levels were then assessed by western blot. Mer590 pre-treatment resulted in decreased levels of Gas6-activated STAT6, AKT, and ERK in the Colo699 cell line, and decreased levels of Gas6-activated AKT and ERK1/2 in the H2009 cell line (Figure 3.3B).

**NSCLC colony formation is reduced by Mer590 treatment:**

To determine the long-term effects of Mer590, we utilized a re-plating assay. In this experiment, cells were treated for 72 hours with Mer590 or vehicle control, and then counted. Equal numbers of live cells were then re-plated at low density in fresh media and allowed to grow and form colonies for 10 days in the absence of any treatment. This experiment determines the residual effects of Mer590 treatment on cells that have survived the initial treatment period, but may be compromised in their ability to re-populate when Mer590 is withdrawn. As demonstrated in Figure 3.3C, treatment with Mer590 significantly decreased Colo699 colony number by 27.8% (p=0.0353). These results were confirmed in a second cell line, H2009, in which administration of Mer590 reduced colony formation in the re-plating assay by 36.8% (p=0.0013).

**Mer590 enhances carboplatin-induced apoptosis:**

Carboplatin and cisplatin are commonly administered therapeutics as part of the standard of care regimen for patients with NSCLC [10]. We determined whether Mer590 increased induction of apoptosis in NSCLC cells in response to treatment with chemotherapy.
Figure 3.3: Mer590 inhibits ligand-dependent phosphorylation, activation of downstream signaling pathways, and colony formation in NSCLC cells. (A) Colo699 and H2009 cells were cultured in the absence (-) or presence (+) of 0.5 μg/ml Mer590 for 48 hours followed by 2 hours in growth media containing 10% (complete media, lanes 1,2,7, 8) or 0% fetal bovine serum (serum starve, lanes 3-6, 9-12) and with or without Mer590. Samples in lanes 5, 6, 11, and 12 were then stimulated with 200 nM rhGas6 for 10 minutes. All cultures were treated with 120 μM pervanadate prior to cell lysis in order to stabilize MERTK phosphorylation. MERTK was immunoprecipitated from lysates and samples were analyzed by western blot using phospho-specific and total MERTK antibodies. (B) Colo699 and H2009 cells were cultured for 24 hours in serum-free medium containing Mer590 (2 μg/ml) or PBS vehicle. 200 nM rmGas6 was added for 10 minutes and cell lysates were prepared. Phospho-STAT6, phospho-AKT, and phospho-ERK levels were determined by western blot. Blots were stripped and re-probed for total protein levels. (C) Colo699 and H2009 cells were treated with Mer590 (2 μg/ml) or vehicle control for 72 hours, then lifted, stained with trypan blue, and counted. One thousand live cells were re-plated in complete media without Mer590 and cultured. Colonies were stained with crystal violet and counted after 10 days. Mean and SEM from at least three independent experiments are shown in the histograms to the right (*P<0.05, **P<0.01).
Colo699 cells were treated with vehicle control, mlgG₁, or Mer590 in the presence or absence of 10 μM or 15 μM carboplatin. Live, early apoptotic, and dead cells were quantified by flow cytometry after staining with YoPro-1-iodide and propidium iodide (Figure 3.4A,B). Treatment with 1μg/ml Mer590 alone reduced the number of live cells from 71.5% to 60.0% compared to mlgG₁ treated cells (p=0.0214), while 10 μM or 15 μM carboplatin (plus mlgG₁) reduced the percentage of live cells to 51.6% and 49.6%, respectively (p=0.0015, p=0.0091). Treatment with a combination of Mer590 and 10 μM or 15 μM carboplatin resulted in the greatest induction of cell death, with only 40.3% and 31.5% live cells remaining, respectively (p=0.0009, p<0.0001). Induction of apoptotic cell death was confirmed biochemically by western blot analysis of PARP cleavage, which was highest in cells treated with both carboplatin and Mer590 (Figure 3.4C). Additionally, carboplatin alone induced a dose-dependent increase in pERK levels. However, Mer590 administration reduced carboplatin-induced pERK activation, potentially blocking a pathway used to escape carboplatin-induced apoptosis.

**Dual MERTK inhibition synergizes with carboplatin to induce cell death:**

As Mer590 increased induction of apoptosis in response to carboplatin, we hypothesized that greater MERTK inhibition would lead to an even more substantial increase in apoptosis induced by carboplatin. shRNA was used to stably knock down MERTK in the Colo699 cell line (Figure 3.5A). Both shRNA and Mer590 alone induced down-regulation of total MERTK. However, when administered together, shRNA and Mer590 reduced total MERTK further (Figure 3.5A). shRNA against MERTK combined additively with both 30 μM and 60 μM carboplatin (p=0.056 and p=0.055, vs. additivity), while Mer590 combined synergistically with 30 μM carboplatin (p=0.002) and additively with 60 μM carboplatin (p=0.079) (Figure 3.5B,C and Table 3.1).
Figure 3.4: Mer590 increases carboplatin-induced apoptosis and decreases compensatory downstream pro-survival signaling.
Figure 3.4: Mer590 increases carboplatin-induced apoptosis and decreases compensatory downstream pro-survival signaling. Colo699 cells were cultured in the presence of vehicle (PBS), isotype antibody control (mIgG1), or Mer590 at the indicated concentrations for 48 hours followed by an additional 72 hours of treatment with antibody ± carboplatin. Apoptotic and dead cells were identified by flow cytometric analysis of YO-PRO-1 and PI uptake. (A) Representative histograms are shown. Early apoptotic cells are stained with YO-PRO-1 but are impermeable to PI. Dead cells and cells in late apoptosis are permeable to both dyes. Viable cells are not stained by either dye. The percentages of live (lower left quadrant), apoptotic (triangular gate), and dead (both upper quadrants) are shown. (B) Mean values and standard errors from 7 independent experiments are shown. Results were evaluated for significance using 2-way repeated measures ANOVA and Bonferroni posttests (*P<0.05, **P<0.01, ***P<0.001). No significant differences between PBS and mIgG1 controls were observed. (C) Whole cell lysates were prepared and expression of the indicated proteins was determined by western blot analysis. Blots representative of 3 independent experiments are shown.
The combination of both shRNA and Mer590, in a dual-MERTK inhibition strategy, synergized with both 30 μM and 60 μM carboplatin (p=0.030 and p=0.009) (Figure 3.5B,C and Table 3.1). This finding was confirmed via western blot analysis of PARP cleavage, which was highest when dual MERTK inhibition was combined with carboplatin (Figure 3.5A). Interestingly, carboplatin treatment increased total levels of MERTK expression (Figure 3.5A), possibly as a compensatory survival response. The chemotherapy-induced MERTK up-regulation may reflect an increased reliance on MERTK signaling in the presence of carboplatin, and provide a rationale for carboplatin and MERTK-targeted agent combinations.

**Table 3.1: Dual MERTK inhibition (Mer590 plus shRNA) interacts synergistically with carboplatin to induce apoptosis and cell death in NSCLC cells.**

<table>
<thead>
<tr>
<th>Mer590</th>
<th>shMERTK</th>
<th>DualMERTK</th>
<th>30 μM Carboplatin</th>
<th>60 μM Carboplatin</th>
<th>Additive</th>
<th>Combination</th>
<th>P Value</th>
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<tr>
<td>0.18 ± 0.2</td>
<td>-</td>
<td>-</td>
<td>8.56 ± 1.4</td>
<td>-</td>
<td>8.72 ± 1.5</td>
<td>15.36 ± 1.4</td>
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<tr>
<td>-</td>
<td>1.76 ± 0.3</td>
<td>-</td>
<td>8.56 ± 1.4</td>
<td>-</td>
<td>10.16 ± 1.5</td>
<td>15.94 ± 2.2</td>
<td>0.056</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>2.45 ± 0.6</td>
<td>8.56 ± 1.4</td>
<td>-</td>
<td>10.79 ± 1.5</td>
<td>20.27 ± 2.9</td>
<td>0.03</td>
</tr>
<tr>
<td>0.18 ± 0.2</td>
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<td>-</td>
<td>-</td>
<td>13.65 ± 3.2</td>
<td>13.79 ± 3.3</td>
<td>19.71 ± 2.4</td>
<td>0.079</td>
</tr>
<tr>
<td>-</td>
<td>1.76 ± 0.3</td>
<td>-</td>
<td>-</td>
<td>13.65 ± 3.2</td>
<td>15.18 ± 3.1</td>
<td>22.61 ± 4.4</td>
<td>0.055</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>13.65 ± 3.2</td>
<td>15.79 ± 3.0</td>
<td>26.56 ± 4.3</td>
<td>0.009</td>
</tr>
</tbody>
</table>

3 The percentages of apoptotic and dead cells are derived from the polygonal gates labeled AC and D, respectively, in Figure 3.5. For the purpose of this comparison, the sum of the apoptotic and dead percentages were used (%ApD = red bars + hatched bars from Fig. 3.5). Baseline cell death observed in untreated samples was subtracted from raw %ApD. Synergism between carboplatin and a single MERTK inhibitor (Mer590 or shMERTK) was evaluated independently from synergism between carboplatin and dualMERTK inhibition (Mer590 + shMERTK). The expected %ApD for an additive interaction was determined using the Bliss additivity model and is shown (Additive) [108]. Statistically significant increases (student’s paired t test p value < 0.05) in the %ApD observed after MERTK inhibition in combination with carboplatin relative to the expected additive %ApD indicate synergy. Mean values and standard errors were derived from 4 independent experiments.
Figure 3.5: Mer590 has combinatorial effects with shRNA against MERTK to promote carboplatin-induced apoptotic cell death of NSCLC cells. Colo699 cells expressing shRNA against MERTK (shMERTK1) or non-targeting control vector (shNTV) were pre-treated with 0.5 μg/ml Mer590 or mIgG for 48 hours, followed by an additional 72 hours of treatment with antibody plus 30 μM carboplatin or vehicle control (PBS). (A) Whole cell lysates were prepared and expression of the indicated proteins (cPARP = cleaved PARP) was determined by western blot analysis. Blots representative of 3 independent experiments are shown. (B) Apoptotic and dead cells were identified by flow cytometric analysis of YO-PRO-1 and PI uptake. Representative histograms are shown. The percentages of apoptotic and dead cells are derived from the lower and upper polygonal gates, respectively. (C) Mean values and standard errors from 6 independent experiments are shown. Results were evaluated for significance using 2-way repeated measures ANOVA and Bonferroni posttests (*P<0.05, **P<0.01, ***P<0.001). No significant differences between PBS control and mIgG control were observed (data not shown).
Summary of Findings

Our group has previously validated MERTK as a potential therapeutic target in NSCLC by demonstrating that shRNA-mediated MERTK knockdown results in decreased pro-oncogenic signaling, synergy with standard chemotherapeutic agents, increased induction of apoptosis, and decreased colony and tumor formation in long-term assays [45]. In this study, we furthered this work by demonstrating that a clinically relevant therapeutic agent, a novel anti-MERTK monoclonal antibody, can phenocopy the effects of genetic inhibition of MERTK.

Mer590 treatment of NSCLC cells resulted in reduced surface and total levels of MERTK. This effect occurred rapidly (over one to four hours), lasted for up to seven days, and could be achieved with concentrations in the low ng/ml range. MERTK inhibition translated to a decrease in phosphorylated MERTK and a concurrent decrease in activation of downstream pro-oncogenic signaling molecules, including STAT6, AKT, and ERK. Functional effects of these biochemical changes included increased apoptosis and decreased colony formation, mediated by Mer590 as a single agent and in combination with carboplatin or shRNA against MERTK. These studies demonstrate the proof-of-principle that MERTK is targetable by a clinically relevant agent, and provide the foundation for further studies in carrying MERTK therapeutics into clinical practice.
CHAPTER IV
UNC2025, A NOVEL SMALL MOLECULE TYROSINE KINASE INHIBITOR
TARGETING MERTK, IS EFFICACIOUS IN PRE-CLINICAL MODELS OF NSCLC

Abstract

Treatment of non-small cell lung cancer (NSCLC) has been transformed by targeted therapies directed against molecular aberrations specifically activated within an individual patient’s tumor. However, such therapies are currently only available against a small number of such aberrations, and new targets and therapeutics are needed. Our laboratory has previously identified the MERTK receptor tyrosine kinase (RTK) as a potential drug target in multiple cancer types, including NSCLC. We have recently developed UNC2025 – the first-in-class small molecule inhibitor targeting MERTK with pharmacokinetic properties sufficient for clinical translation. Here we utilize this compound to further validate the important emerging biologic functions of MERTK in lung cancer pathogenesis, to establish that MERTK can be effectively targeted by a clinically translatable agent, and to demonstrate that inhibition of MERTK is a valid treatment strategy in a wide variety of NSCLC cell lines independent of their driver oncogene status, including in lines with an EGFR mutation, a KRAS/NRAS mutation, an RTK fusion, or another or unknown driver oncogene. Biochemically, we report the selectivity of UNC2025 for MERTK, and its inhibition of oncogenic downstream signaling. Functionally, we demonstrate that UNC2025 induces apoptosis of MERTK-dependent NSCLC cell lines, while decreasing colony formation in vitro and tumor xenograft growth in vivo in murine models. These findings provide further evidence for the importance of MERTK in NSCLC, and demonstrate that MERTK inhibition by UNC2025 is a feasible, clinically relevant treatment strategy in a wide variety of NSCLC sub-types, which warrants further investigation in clinical trials.
Results

UNC2025 selectively inhibits MERTK phosphorylation:

To test the selectivity of UNC2025 within the TAM family, levels of phosphorylated MERTK, AXL, and TYRO3 were assessed in H2228, A549, Colo699, and H1299 cells after one hour of treatment with UNC2025 or UNC1653, a negative control compound that lacks activity against MERTK [103] (Figure 4.1A). UNC2025 potently inhibited MERTK phosphorylation at doses in the low nanomolar range, with IC\textsubscript{50} values below 50 nM in all four cell lines studied (Figure 4.1B). In addition, UNC2025 was selective for MERTK over the related kinases AXL and TYRO3. Phospho-AXL was expressed in the H2228, A549, and H1299 cell lines, while Colo699 cells did not express AXL. Phospho-TYRO3 was expressed in all four cell lines, but was present at relatively higher levels in the H2228 and H1299 cell lines and was only weakly detected in the A549 and Colo699 cell lines. Regardless of expression levels, treatment with UNC2025 at doses up to 300 nM was far less potent against phospho-AXL or phospho-TYRO3 than against phospho-MERTK in any of these cell lines. Importantly, AXL and TYRO3 were two of the most potently inhibited kinases after MERTK in enzymatic assays characterizing the specificity of UNC2025 against a panel of over 300 kinases [102]. Additionally, UNC1653 did not decrease phosphorylation of MERTK at doses up to 300 nM (Figure 4.1C). Interestingly, total MERTK expression was increased in response to treatment with higher doses of UNC2025 in 3 of the 4 lines tested.

Oncogenic signaling downstream of MERTK is blocked by UNC2025:

MERTK is known to stimulate a large network of downstream signaling proteins, most commonly through the MAPK and PI3K/AKT pathways [54]. To determine whether treatment with UNC2025 abrogated these effects, cells were treated with UNC2025 or vehicle control prior to stimulation with the TAM-family ligand GAS6 (Figure 4.2A).
Figure 4.1: UNC2025 selectively inhibits MERTK activation. A) Structures of UNC2025 and UNC1653. B,C) H2228, A549, Colo699, and H1299 cells were treated with the indicated doses of UNC2025 (B) or negative control compound UNC1653 (C) for one hour prior to preparation of cell lysates. MERTK, AXL, or TYRO3 proteins were immunoprecipitated and phospho- and total proteins were detected by immunoblot.
Treatment with UNC2025 consistently decreased pAKT and/or pERK in all four cell lines tested; however, these effects were cell-line specific. In the A549 cell line, UNC2025 decreased basal phosphorylation of AKT; in the Colo699 cell line, UNC2025 decreased GAS6-induced phosphorylation of ERK; while in the H2228 and H1299 cell lines, UNC2025 treatment decreased phosphorylation of AKT and ERK in both un-stimulated and ligand-stimulated conditions.

In addition to its effects on short-term phosphorylation-mediated signaling, the long-term effects of UNC2025 on downstream signaling were assessed in NSCLC cell lines. Cultures were treated with 300 nM UNC2025 for 72 hours, lysed, and analyzed by western blot. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, was consistently decreased after UNC2025 treatment (Figure 4.2B). As Survivin has been shown to be a promising drug target, we sought to determine whether Survivin levels were decreased in response to UNC2025 treatment in additional solid tumor cell lines representing a variety of tumor types [109]. We treated four additional NSCLC cell lines, three glioblastoma cell lines, and two melanoma cell lines with UNC2025, and examined Survivin levels in whole cell lysates. In 10 of the 13 solid tumor cell lines assessed, Survivin levels were dramatically decreased. Survivin protein expression was decreased in six of the eight total NSCLC cell lines assessed, all three glioblastoma cell lines, and one of the two melanoma cell lines (Figure 4.2C).

**MERTK inhibition by UNC2025 induces apoptotic cell death:**

We next hypothesized that the potent effects mediated by UNC2025 on MERTK phosphorylation and downstream oncogenic signaling would translate to a functional decrease in survival of NSCLC cells. To test this hypothesis, NSCLC cells were treated with 300 nM UNC2025 or UNC1653 for 72 hours, stained with YO-PRO-1 and PI dyes, and analyzed by flow cytometry to assess apoptotic cell death.
Figure 4.2: UNC2025 Inhibits Signaling Pathways Downstream of MERTK.
A) Sub-confluent cell cultures were starved for two hours in serum-free medium, and then treated with UNC2025 (300 nM) or an equivalent volume of DMSO vehicle for an additional two hours prior to stimulation with Gas6 (200 nM) for 10 minutes. Lysates were prepared and phospho- and total AKT and ERK were detected by immunoblot.
B, C) Cells were treated with 300 nM UNC2025, UNC1653, or DMSO vehicle control for 72 hours. Lysates were prepared and Survivin was detected by immunoblot.
Treatment with UNC2025, but not UNC1653, significantly increased the fraction of apoptotic and dead cells in all four cell lines studied (Figure 4.3). UNC2025 increased cell death from basal levels of 23.9±4.2%, 13.8±2.2%, 9.9±1.6%, and 44.2±0.1%, in the H2228, A549, Colo699, and H1299 cell lines, to 40.5±3.8%, 50.3±7.1%, 32.9±6.2%, and 59.1±3.4%, respectively (p = 0.0428, p=0.0081, p=0.0224, p=0.0118). Levels of apoptosis were not significantly different from basal levels after UNC1653 treatment in any of the four NSCLC cell lines.

**UNC2025 decreases colony formation in a dose-dependent manner:**

A soft agar assay was utilized to determine the long-term functional effects of UNC2025 treatment on NSCLC colony formation. UNC2025, UNC1653, or vehicle control was administered three times per week. After two weeks of growth, colonies were stained and counted (Figure 4.4A). Treatment with UNC2025, but not UNC1653, significantly reduced colony formation of all four NSCLC cell lines assayed (Figure 4.4B). At a dose of 300 nM UNC2025, colony formation was reduced by 99.2%, 98.1%, 98.2%, and 80.1%, in the H2228, A549, Colo699, and H1299 cell lines, respectively (p<0.0001 for each).

Since UNC2025 robustly decreased colony formation in all four of the studied NSCLC cell lines, we sought to determine if this trend was conserved in a larger panel of NSCLC cell lines with different driver oncogene statuses. Cell lines harboring an EGFR mutation, an RTK fusion, a KRAS or NRAS mutation, and those with another or with no known driver were assessed in the soft agar assay. IC₅₀ values were calculated for each cell line (Figure 4.4C). Driver oncogene status did not correlate with sensitivity to UNC2025, as IC₅₀’s between 50 nM and 350 nM were observed in cell lines of each type studied.
Figure 4.3: UNC2025 induces apoptosis in NSCLC cells. H2228, A549, Colo699, and H1299 cells were treated with 300 nM UNC2025, UNC1653, or DMSO vehicle for 72 hours prior to staining with YO-PRO®-1 iodide (YO-PRO-1) and propidium iodide (PI) dyes and assessment of dye uptake by flow cytometry. Early apoptotic cells are stained selectively with YO-PRO-1, while late apoptotic and dead cells take up both dyes. A) Representative histograms for the H1299 cell line are shown. B) Mean values and standard errors derived from 3 independent experiments are shown (*P<0.05, **P<0.01).
Figure 4.4: UNC2025 inhibits colony formation in soft agar.
**Figure 4.4: UNC2025 inhibits colony formation in soft agar.** The indicated cell lines were cultured in 0.35% Noble Agar overlaying 0.5% Noble Agar in 6-well plates. Agar layers were overlaid with a medium layer containing UNC2025, UNC1653, or DMSO vehicle. The medium layer was aspirated and replaced three times a week for two weeks, after which time cells were stained with NTB and counted on a colony counter. A) Representative cultures of the H2228 cell line are shown. B) Mean colony numbers and standard errors derived from at least 3 independent experiments are shown (**P<0.01, ***P<0.001). C), IC$_{50}$ values were calculated from soft agar data generated using a large panel of NSCLC cell lines with different genetic driver mutations. IC$_{50}$ values (nM) are as follows: Driver Unknown or Other – Calu3: 90.7, Colo699: 69.6, H226: 57.3, H322: 190.4, H2126: 97.5. EGFR Mutant – H1650: 236.8, H1975: 70.0, H3255: 204.5, HCC827: 182.5, HCC4006: 88.8, HCC4011: 62.7. KRAS/NRAS Mutant – A549: 60.5, H157: 256.8, H358: 78.9, H441: 233.2, H460: 96.0, H1299: 157.6, H2009: 339.8. RTK Fusion – H2228: 91.6, H3122: 238.6, LC-2/ad: 37.4.
**UNC2025 inhibits tumor growth in murine models:**

As UNC2025 shows potent anti-tumor effects *in vitro*, and since it has the pharmacokinetic properties for *in vivo* administration, we determined the effects of UNC2025 *in vivo* in murine subcutaneous xenograft models. Mice were injected with H2228 (Figure 4.5A) or A549 (Figure 4.5B) NSCLC cells and tumors were established until an average size of approximately 100 cubic millimeters (A549) or 150 cubic millimeters (H2228) was reached. Mice were then treated twice daily by oral gavage with either saline vehicle or 50 mg/kg UNC2025 and tumor size was measured weekly. Tumor growth was significantly impaired in mice treated with UNC2025, as indicated by significantly smaller tumor volumes relative to mice treated with saline at every measurement point in the H2228 cell line (p=0.0193, p=0.0058, p=0.0011, and p=0.0034 after one, two, three, and four weeks of treatment, respectively). In the A549 cell line, tumor volume between the two groups were significantly different during the final two weeks of measurements (p=0.0021 and p=0.0015, respectively). At the end of the study period in the H2228 study, tumor volume was reduced by 70.2%, from 1183±258 mm$^3$ in saline-treated mice, to 352±51 mm$^3$ in mice that had received UNC2025. In the A549 cell line study, final tumor volume was reduced by 61.9%, from 373±57 mm$^3$ in saline-treated mice, to 143±28 mm$^3$ in UNC2025-treated mice.

**Summary of Findings**

MERTK was initially cloned from a B-lymphoblastoid expression library and its role in a wide variety of blood cancers has subsequently been extensively characterized [21, 29]. More recently, it has become increasingly appreciated that MERTK plays important roles in solid tumors as well. In NSCLC in particular, MERTK is aberrantly expressed in approximately two-thirds of patient tumors, and its activation results in increased downstream pro-oncogenic signaling.
Figure 4.5: UNC2025 inhibits NSCLC tumor growth *in vivo*.
Figure 4.5: UNC2025 inhibits NSCLC tumor growth in vivo. A) H2228 or B) A549 cells were injected subcutaneously into the flanks of Nude mice and tumors were established until an approximate average volume of 150 mm$^3$ (A) or 100 mm$^3$ (B). Mice were then treated twice daily by oral gavage with 50 mg/kg UNC2025 or 10 ml/kg saline vehicle. Tumors were measured weekly until study completion. In (A), data from two independent experiments were combined, for a total of 11 saline-treated mice and 12 UNC2025-treated mice, while in (B) there were 9 saline-treated mice and 10 UNC2025-treated mice. (*P<0.05, **P<0.01)
Genetic inhibition of MERTK by shRNA in NSCLC cells inhibits this signaling network, resulting in induction of apoptosis, decreased colony formation in vitro, enhanced chemosensitivity, and decreased tumor formation in vivo in a murine model [45]. As described in the previous Chapter, MERTK was further validated as an important target in NSCLC by the finding that Mer590, a monoclonal antibody targeting MERTK, decreases pro-oncogenic signaling, increases apoptosis, increases chemosensitivity, and decreases colony formation in NSCLC cell lines [110]. Importantly, two of the cell lines used in the current study, A549 and Colo699, were used extensively in the shRNA target validation and Mer590 monoclonal antibody studies, respectively. The consistency between the effects observed in these studies using three different mechanisms of MERTK inhibition provides strong support that the observed phenotypes are indeed MERTK-dependent.

MERTK has been extensively validated as a potential therapeutic target not only in NSCLC, but also in a multitude of other cancers. For this reason, our group recently began pursuing the synthesis of novel small molecule tyrosine kinase inhibitors targeting MERTK. We have generated a series of novel compounds representing the first-in-class MERTK-targeted small molecule inhibitors [44, 83, 84, 102, 103, 111, 112]. UNC2025 is the first compound of these inhibitors with pharmacokinetic properties suitable for in vivo study and potential clinical translation. Here, we have investigated the utility of UNC2025 in NSCLC and have demonstrated that UNC2025 is selective for MERTK over related TAM-family members AXL and TYRO3, inhibits downstream signaling through AKT, ERK, and Survivin, induces apoptotic cell death, decreases colony formation in vitro, and decreases tumor formation in vivo in murine xenograft models. Importantly, sensitivity to UNC2025 did not depend on driver oncogene status. Development of
MERTK-selective TKIs for clinical application may therefore provide a molecularly-targeted treatment option for patients without known oncogenic mutations or with a wide variety of alternative driver mutations.
CHAPTER V

AXL EXPRESSION CONFLICTS INTRINSIC RESISTANCE TO MERTK-TARGETED THERAPEUTICS

Abstract

Targeted therapeutics have made impressive inroads in the effort to improve survival of advanced stage NSCLC. However, cures are rarely achieved, due to either an up-front non-response to treatment, or an initial response eventually giving way to secondary acquired resistance. Resistance to targeted therapeutics in NSCLC has therefore developed into an active research field in its own right, with multiple mechanisms thus far identified. One of the most commonly identified has been up-regulation of a bypass signaling pathway; that is, a pathway capable of re-activating silenced oncogenic signaling pathways downstream of an inhibited therapeutic target. In this study, we identify AXL as one such mediator of resistance to MERTK-targeted therapeutics. AXL and MERTK receptor levels are shown to be coordinately regulated, and are also demonstrated to be in close physical proximity, resulting in co-immunoprecipitation. Additionally, a functional interaction between these receptors is identified by which inhibition of AXL sensitizes to UNC2025, a MERTK-targeted small molecule inhibitor. Finally, co-inhibition of MERTK and AXL with UNC2025 and the AXL small molecule inhibitor R428 demonstrated synergistic reduction of colony formation as compared to either single agent therapy. Overall, we identify AXL as mediating intrinsic resistance to MERTK inhibition, and propose MERTK:AXL co-inhibition as an efficacious combinatorial treatment strategy in NSCLC.
Results

Inhibition of MERTK or AXL increases expression of the alternate receptor:

The activation of receptor tyrosine kinases by ligand not only stimulates intracellular downstream signaling, but also receptor ubiquitination and degradation [113]. Predictably then, UNC2025, as MERTK small molecule tyrosine kinase inhibitor, not only inhibited MERTK activation but also caused increased expression of total MERTK, presumably by decreasing receptor ubiquitination and degradation (Figure 5.1A). Surprisingly, however, UNC2025 also caused increased levels of total AXL. TYRO3 expression, on the other hand, was decreased by UNC2025. Additional RTKs assayed, namely EGFR, MET, and FGFR1, remained unchanged. To demonstrate that the increase of total AXL expression was not due simply to an off-target effect of UNC2025 inhibiting phospho-AXL, cells were treated with UNC2025 for 24 hours and lysates immunoprecipitated for phospho-MERTK and phospho-AXL (Figure 5.1B). Although phospho-MERTK was substantially decreased, phospho-AXL levels were unchanged. As an additional control, siRNA against MERTK and against AXL was also utilized to confirm the specificity of the effects seen with UNC2025 (Figure 5.1C). siRNA against MERTK led to increased levels of total AXL, while siRNA against AXL led to increased levels of total MERTK. Interestingly, siRNA against MERTK in the H157 cell line strongly and reproducibly up-regulated total EGFR, in contrast to the effects of UNC2025 in this cell line. The implications of this finding are unclear, but may reveal complex interactions between MERTK and EGFR expression, as was suggested by a recent study demonstrating that loss of MERTK was associated with decreased stability of active EGFR at the cell surface in response to EGF treatment [74].
Figure 5.1: MERTK and AXL expression is inversely co-regulated. H1299 and H157 cells were treated with the MERTK-targeted small molecule inhibitor UNC2025, 200 nM (A,B), or siRNA (C), including non-targeting siRNA (siNTC), siRNA against MERTK (siMERTK9, siMERTK10), or siRNA against AXL (siAXL10, siAXL12) for 24 hours, and assessed by western blot for total levels of the receptor tyrosine kinases MERTK, AXL, TYRO3, EGFR, FGFR1, and MET (A,C) or immunoprecipitation and western blot for phospho- and total MERTK, AXL, and TYRO3 (B).

Increased MERTK and AXL expression is due to increased protein stability:

Two potential mechanisms for the up-regulation of MERTK and AXL following treatment with UNC2025 or siRNA are increased mRNA transcription and increased protein stability. To assay transcript levels, H1299 and H157 cells were treated with UNC2025 (Figure 5.2A) or siRNA against MERTK or AXL (Figure 5.2B), and mRNA was quantitatively analyzed by qPCR. UNC2025 did not significantly alter mRNA levels of MERTK, AXL, or GAS6 in either cell line analyzed. Likewise, siRNA against MERTK and against AXL strongly decreased levels of their target mRNA, but had no effect on mRNA levels of the alternate receptor or GAS6.

As increased transcription of MERTK and AXL was not responsible for their up-regulation, we considered the possibility that decreased degradation may be the relevant mechanism. Cells were treated with UNC2025 as well as cycloheximide, an inhibitor of
protein synthesis. As production of new protein is inhibited by cycloheximide, serial western blotting after its administration allows one to monitor the degradation of already-formed proteins, as an indicator of their stability. Both MERTK and AXL protein levels were gradually decreased over a period of 24 hours. However, in the presence of UNC2025, the degradation of both MERTK and AXL was significantly impaired, as at 24 hours post-cycloheximide treatment, levels were only marginally decreased (Figure 5.2C).

As previously mentioned, degradation of RTKs is closely tied to their activation. We therefore hypothesized that the presence of both MERTK and AXL was necessary for their full activation, and that loss of one receptor resulted in increased stability and therefore expression levels of the other receptor secondary to its decreased activation. One potential mediator of this mechanism is physical interaction, or heterodimerization, between MERTK and AXL. A co-immunoprecipitation experiment was therefore utilized to address this hypothesis (Figure 5.2D). Immunoprecipitation with an anti-MERTK antibody, followed by western blot, resulted in pull-down not only of MERTK, but also of AXL. Conversely, an AXL antibody pulled down not only AXL, but also MERTK. Notably, only a subset of total AXL was co-immunoprecipitated with the anti-MERTK antibody, and similarly, only a subset of MERTK was co-immunoprecipitated with AXL pull-down. This may indicate that at any moment, only a fraction of total MERTK and AXL are interacting with each other, or it may be simply an experimental artifact of dissociation between MERTK and AXL during the overnight immunoprecipitation step. Regardless, these findings indicate a potential physical interaction of MERTK and AXL, suggesting that these receptors may undergo co-activation via heterodimerization.
AXL inhibition sensitizes NSCLC cells to a MERTK inhibitor:

As MERTK and AXL expression and degradation appear inter-dependent, and a physical interaction of these receptors is suggested by co-immunoprecipitation assays, we sought to determine functional consequences of these findings. Effects of UNC2025 on phosphorylated MERTK, STAT6, AKT, and ERK were determined in the presence of control siRNA or siRNA against AXL (Figure 5.3A). In cells treated with non-targeting siRNA, UNC2025 induced a moderate decrease of phospho-MERTK and phospho-STAT6 in the H1299 cell line, and only phospho-MERTK in the H157 cell line, as phospho-STAT6 was not detected in this cell line. In both cell lines, phospho-AKT and phospho-ERK were unaffected by these relatively low doses of UNC2025. However, when AXL protein expression was blocked by pre-treatment with siRNA against AXL, UNC2025 induced a much more potent and dramatic decrease of phospho-MERTK, phospho-STAT6 (in the H1299 cell line), and phospho-AKT. In the absence of AXL, decreased phosphorylation of MERTK and downstream effectors were detected at lower doses of UNC2025, and maximal decreases of these phosphorylation levels were greater than when AXL was present.

This siAXL-mediated sensitization to UNC2025 in signaling assays translates to increased sensitivity to UNC2025 in a MTS cell proliferation assay (Figure 5.3B). In the presence of non-targeting siRNA, UNC2025 caused only a 28.9% or 12.4% reduction of cell proliferation after 72 hours, as compared to vehicle treated control cells, in the H1299 and H157 cell lines, respectively.
Figure 5.2: Receptor interaction and regulation of stability between MERTK and AXL.
Figure 5.2: Receptor interaction and regulation of stability between MERTK and AXL. Sub-confluent cultures were treated with siRNA for 8 or 24 hours (A) or UNC2025 for 24 hours (B) before RNA was harvested, converted to cDNA, and analyzed by quantitative PCR. (C) H1299 and H157 cells were treated with UNC2025 as well as the translation inhibitor cycloheximide. Cells were then lysed 0 - 24 hours later, and expression of MERTK and AXL analyzed by western blot. (D) MERTK or AXL was immunoprecipitated out of H1299 and H157 cell lysates overnight, followed by western blotting for both receptors.
However, pre-treatment with siRNA against AXL allowed UNC2025 to cause reductions of 42.9% and 43.5% in the H1299 cell line (pre-treatment with siAXL10 and siAXL12, respectively), and 44.4% and 27.8% in the H157 cell line (pre-treatment with siAXL10 and siAXL12, respectively). UNC2025, therefore, was capable of killing a larger proportion of NSCLC cells when they lacked AXL. These signaling and functional data indicate that AXL expression confers intrinsic resistance to the MERTK-targeted inhibitor UNC2025, and that inhibition of AXL is a mechanism to confer sensitivity to this therapeutic agent.

Figure 5.3: Inhibition of AXL sensitizes NSCLC cells to MERTK-targeted therapy. (A) Sub-confluent cultures were pre-treated with non-targeting siRNA or siRNA against AXL for 24 hours. UNC2025 (200 nM) was then added for one hour, and lysates were either immunoprecipitated for MERTK and western blotted for phospho- and total MERTK, or lysates were run directly on western blots and probed for phospho- and total-STAT6, AKT, and ERK. (B) Sub-confluent cultures were pre-treated with siRNA as in A, prior to a 72-hour treatment with UNC2025. Cell proliferation was measured by adding MTS dye for one hour, and absorbance was read at a wavelength of 490 nm.
Small molecule inhibitors against AXL and MERTK interact synergistically:

Given the functional interaction suggested in Figure 5.3B between siAXL and UNC2025, we sought to confirm these results and extend them to a more clinically relevant scenario. The AXL-targeted small molecule inhibitor R428 was therefore utilized in place of siRNA against AXL, to determine combinatorial activity with UNC2025. R428 is the most advanced AXL small molecule inhibitor in development, and is currently in stage Ib clinical trials for NSCLC (in combination with erlotinib or docetaxol) and acute myeloid leukemia [114, 115]. As in Figure 5.3B, co-inhibition of AXL and MERTK was more efficacious in the MTS assay than was monotherapy (Figure 5.4A). Treatment with R428 alone resulted in relative cell numbers of 92.6% and 102.8% in the H157 and H1299 cell lines as compared to DMSO vehicle treated cells after 72 hours of treatment. Likewise, UNC2025 alone resulted in relative cell numbers of 91.2% and 83.7% of DMSO control cells, in the H157 and H1299 cell lines, respectively. However; co-treatment with both agents resulted in only 67.8% and 63.5% of the number of cells in the DMSO control condition, again in the H157 and H1299 cell lines, respectively. This results phenocopies the interaction between siAXL and UNC2025 above, and suggests that the MERTK:AXL receptor combination is capable of inhibition by clinically relevant, translatable agents.

As a small molecule inhibitor, R428 has longer-lasting effects than the transient knockdown conferred by siRNA, allowing for study in a longer-term colony formation assay. H1299 and H157 cells were plated at low density (500 cells/well) on 6-well plates in the presence of DMSO vehicle control, or increasing concentrations of UNC2025 and/or R428. Cells were allowed to grow for 10 days, after which colonies were stained with crystal violet and counted. Similar to the MTS assay above, R428 alone or UNC2025 alone had sub-optimal effects on colony inhibition. However; the combination of UNC2025 and R428 synergistically reduced colony number as compared to treatment
with a single inhibitor, resulting in a maximal decrease in colony number of over 99% in both cell lines (Figure 5.4B, Table 5.1).

**Figure 5.4:** Small molecule inhibitors against MERTK and AXL synergistically reduce cell growth. (A) Sub-confluent cultures were treated with DMSO vehicle control, UNC2025 (200 nM), R428 (200 nM), or combination treatment with both inhibitors. 72 hours later, proliferation was measured by addition of MTS dye for one hour, followed by absorbance reading at 490 nm. (B) H1299 and H157 cells were plated at low density on 6-well plates in the presence of varying concentrations of UNC2025 and R428. Colonies were allowed to grow for 10 days, and then were stained with crystal violet and counted.
Table 5.1: Combination indices between UNC2025 and R428 in the clonogenic assay.

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<th>UNC2025 (nM)</th>
<th>R428 (nM)</th>
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<th>Expected Effect if Additive</th>
<th>Combination Index</th>
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<td></td>
<td></td>
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<td>H157</td>
<td>H1299</td>
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Summary of Findings

As discussed in the Introduction to this thesis, the use of combination therapy in clinical oncology is becoming more intensely studied as a means of avoiding the drug resistance issues that have plagued the otherwise successful clinical introduction of novel targeted therapeutics, including small molecule inhibitors. In this study, we identify AXL as conferring intrinsic resistance to the MERTK-targeted small molecule inhibitor UNC2025. Mechanistically, our data demonstrate a cooperative relationship between MERTK and AXL, and coordinated regulation of expression. Specifically, inhibition of

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4 Calculations made utilizing the Bliss additivity model based on the data presented in Figure 5.4B [108]. CI values below 1 are considered synergistic (highlighted gray in Table).
either receptor increases expression of the alternate receptor. These interactions were not due to changes in mRNA expression, but were rather secondary to effects on protein degradation. In conjunction with the finding that MERTK and AXL are capable of physical interaction, these data suggest that heterodimerization between MERTK and AXL is a relevant mechanism of dual receptor activation. siRNA and the small molecule inhibitor R428 were utilized to demonstrate improved efficacy of UNC2025 in the presence of concurrent AXL inhibition. Targeting AXL allowed for increased potency of UNC2025, both in its ability to decrease the activation of MERTK and downstream effectors, as well its ability to decrease proliferation and colony formation. Taken together, this paper identifies co-inhibition of MERTK and AXL as a novel treatment strategy in NSCLC, which may be efficacious in preventing intrinsic resistance to the MERTK-targeted small molecule inhibitor UNC2025.
CHAPTER VI

CLINICAL CORRELATES – THE CCTSI EXPERIENCE

In addition to the bench research described extensively in the preceding chapters, I have had the privilege of engaging in a wide variety of clinical experiences in order to supplement and enhance my graduate research education. I was fortunate to be accepted to the Colorado Clinical and Translational Sciences Institute (CCTSI), which is a program facilitating clinical involvement during the training period for basic science graduate students. My clinical preceptor for this program has been Dr. Christopher Baker, M.D., who also serves as a member of my thesis committee.

Biomedical research requires attention to detail and focus on intricate, molecular mechanisms, making it easy during the graduate school years to become hyper-focused on one’s particular topic and lose sight of the field in general. However, staying informed about the broader field of one’s research, including clinical applications and knowledge of related diseases, can inform a student’s own, focused research studies. It is in this light that I selected Dr. Baker as a clinical preceptor. As a pulmonologist at Children’s Hospital Colorado, Dr. Baker treats patients with a wide variety of lung diseases, both in the acute and long-term care settings. We have met on a monthly basis, and Dr. Baker has been a wonderful clinical instructor. He has allowed me to practice skills such as clinical history taking and physical examination, and is always ready to explain his reasoning behind a clinical decision or the mechanisms of a particular disease. Working with Dr. Baker has allowed me to view my project on lung cancer within its place in the wide spectrum of lung disease, and become more informed about the field of pulmonology than any one basic research experience could provide.

In addition to these direct benefits on my MD and PhD training, participation in the CCTSI program has had the additional benefit of assisting me in formulating my long-term career goals, through direct observation of physician scientists in action. My
long-term career goal is also to become a physician scientist, with approximately 80% of my time spent running a research laboratory, and 20% of my time spent in the clinic. I chose my research mentor, Dr. Doug Graham, and my clinical preceptor, Dr. Baker, in no small part due to the fact that they each model this career choice and know the skills I will need to be successful.

I believe that research and clinical experiences can inform each other, and I hope that my clinical observations will lead to pertinent research questions, while my laboratory research will give me a deeper understanding of the molecular underpinnings of disease I observe in the clinic. My experiences thus far regarding lung disease, including my thesis project on non-small cell lung cancer and my clinical experiences with Dr. Baker, have given me an appreciation of lung physiology and pathophysiology. In addition, these experiences have exposed me to a wide variety of clinical settings – useful information in selecting an ultimate career path. Our time in the various intensive care units of Children’s Hospital Colorado in particular have captured my interest and allowed me inside information on a potential career path I knew little of prior to participation in this program. Regardless of the specific area of study I choose in the end, the experiences and knowledge gained through the CCTSI program have augmented my growth and redoubled my motivations toward a career as a successful physician scientist.
CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

In the United States, males have a one in 13 chance, and females a one in 16 chance, of developing lung cancer in their lifetime [1]. This translates to hundreds of thousands of new lung cancer patients each year. While small subsets will benefit from targeted therapies against EGFR, ALK, or ROS1, the vast majority will have tumors driven by currently unidentified or untargetable oncogenic events. For these patients, new treatment options are desperately needed if relative five-year survival rates are ever to improve substantially from the 10-20% range they have been stuck in for decades. Personalized medicine holds promise to be able to provide this change, and indeed multiple new oncogenic mechanisms have been brought to light in the past several years, with many now undergoing pre-clinical or even clinical trial studies towards the development of novel therapeutics. MERTK was very recently identified as playing an important role in NSCLC, and has now begun down its own path towards clinical relevance. Exciting pre-clinical results have been obtained in the development of a monoclonal antibody, Mer590, and a series of ATP-competitive small molecule inhibitors, including UNC2025. Additionally, drug combination strategies, including the co-inhibition of MERTK and AXL, have begun to be identified, in an effort to more potently kill cancer cells and avoid the outgrowth of clones resistant to any one therapy. As detailed in the following sections, great strides have been made with each of these lines of investigation, yet much remains to be done before any patient will benefit from this work.
Pre-clinical Promise of Mer590

As described in Chapter III, Mer590 was the first clinically translatable therapeutic to show pre-clinical efficacy by targeting MERTK in NSCLC. In addition to its primary effects on colony formation, apoptosis, and downstream signaling, as detailed in the Results section of Chapter III, further insight can be gained from these studies. For example, the apoptosis and re-plating assays used in this study complement each other and highlight the ability of Mer590 to induce both short-term increases in apoptosis and the delayed effect of decreased colony forming potential even in cells that survive the initial treatment period. This residual defect in colony formation could theoretically be important in reducing the ability of cancer cells to re-populate leading to recurrence after a treatment cycle.

An additional feature of clinically relevant MERTK-targeted therapy is a potential increased efficacy of standard chemotherapeutics, which have previously been shown to interact synergistically with shRNA-mediated MERTK inhibition in NSCLC, and with small molecule-mediated MERTK inhibition in ALL [45, 112]. In this study, Mer590 interacted synergistically with carboplatin, a commonly used chemotherapy for NSCLC treatment. These data indicate the possibility that co-administration of MERTK-targeted agents with carboplatin may decrease tumor burden more effectively than carboplatin alone. Alternatively, utilization of MERTK-targeted agents may allow for dose-reduction of standard chemotherapy, with the goal of fewer or less severe side effects for NSCLC patients.

In addition to chemotherapy, Mer590 could also be administered in combination with alternative mechanisms of MERTK inhibition. Mer590 plus shRNA against MERTK synergized with carboplatin to cause increased apoptosis. A dual inhibition strategy against a single receptor has been successfully utilized against the ErbB family of receptors, where the antibodies cetuximab, panitumumab, or trastuzumab have been
combined with small molecule inhibitors, including gefitinib, erlotinib, afatinib, or lapatinib, in several clinical trials [116]. The combination of cetuximab and afatinib showed promising results in a phase Ib/II trial in NSCLC, and cetuximab paired with either gefitinib or erlotinib has generated promising data in patients with colon cancer. The most impressive clinical data thus far; however, have been generated in breast cancer, in which the combination of trastuzumab plus lapatinib has increased survival in two phase III clinical trials. In one trial, the effect was compared to lapatinib alone, while in the second, where the drug combination was given in combination with paclitaxel, survival was increased as compared to paclitaxel plus either targeted therapy alone.

In total, we have developed a novel monoclonal antibody, Mer590, which inhibits MERTK receptor tyrosine kinase, a recently validated target in NSCLC. We have completed critical pre-clinical experiments demonstrating the mechanism of action and the therapeutic potential of this antibody. Treatment with Mer590 is sufficient to induce apoptotic cell death and reduce colony formation and these effects can be augmented when given in conjunction with carboplatin or genetic inhibition of MERTK. Taken together, these data validate antibody-mediated targeting of MERTK as an attractive strategy for treatment of lung cancer that deserves further optimization and investigation.

**Translating Mer590 to Patients – the Next Steps**

Although data presented in Chapter III and summarized in the preceding section establish proof-of-principle that MERTK is targetable by a monoclonal antibody, much work remains to be done in order to translate these findings into clinical efficacy. Mer590 has two large hurdles to clear – the process of humanization, and the issue of receptor agonism. Fortunately, each of these potential pitfalls has been encountered and successfully overcome previously during the development process of other monoclonal antibodies, and can provide a guide for the path forward in the case of an
anti-MERTK monoclonal antibody.

Humanization of antibodies, while time-consuming and expensive, is now a well-established process [117]. What may appear as more difficult to overcome, on the surface, is the issue of receptor agonism. However, this too has been well studied, with antibodies against the MET receptor tyrosine kinase providing an illustrative example. First-generation MET antibodies, like Mer590, were bivalent, and as a result exhibited short-term agonism due to bringing into close proximity two RTKs, prior to their internalization and degradation ([118, 119] and data not shown). This resulted in brief periods of receptor activation followed by a longer period of receptor down-regulation. However, it was soon discovered that receptor activation and degradation were not inextricably tied together. In other words, an antibody did not have to behave as a ligand mimic and stimulate receptor dimerization and activation in order to promote the subsequent degradation that followed. Epitopes were identified on MET which when targeted promoted receptor internalization independent of activation [105, 120]. But translation of these findings to Mer590 would involve development of a completely new mAb targeting a new epitope – essentially starting back at square one. Fortunately, this was not the only solution found to overcome receptor agonism. Several independent research groups subsequently isolated the Fab portion of their respective MET antibodies, thereby creating a monovalent antibody-like fragment [121-123]. These proteins were incapable of inducing receptor dimerization, yet they retained the antagonistic function of reducing surface receptor levels. Mer590 may similarly benefit from such molecular biology techniques to induce monovalency, allowing it to act as a pure antagonist to MERTK.
UNC2025 Pre-clinical Efficacy

The development and pre-clinical efficacy of Mer590 suggested for the first time the possibility of targeting MERTK in NSCLC with a clinically-relevant class of therapeutics. However, these findings are at this point largely proof-of-principle in nature, and unlikely to translate to clinical utility in the very near future, as detailed in the preceding section. On the other hand, small molecule inhibitors, in particular UNC2025 and its derivatives, are much more promising clinical candidates. As described in Chapter IV, our group recently began pursuing the synthesis of novel small molecule tyrosine kinase inhibitors targeting MERTK. We have generated a series of novel compounds representing the first-in-class MERTK-targeted small molecule inhibitors [44, 83, 84, 102, 103, 111, 112]. UNC2025 is the first compound of these inhibitors with pharmacokinetic properties suitable for in vivo study and potential clinical translation.

One mechanism by which MERTK inhibition may mediate anti-tumor activity is inhibition of Survivin expression. We have previously demonstrated decreased total levels of Survivin in response to shRNA-mediated MERTK inhibition, and here confirm this finding with UNC2025-mediated MERTK inhibition [45]. Survivin plays important roles in several critical aspects of oncogenesis, where it functions to promote tumor cell survival, proliferation, metastasis, and chemoresistance, and can also contribute to tumor angiogenesis [109]. Additionally, Survivin is an extremely cancer-specific target, as it is expressed widely in a multitude of cancer types, but not in the vast majority of normal adult tissues. These qualities have made Survivin a top target for new therapy development, and several novel therapies are currently in clinical development. However, early results have not been as impressive as hoped, and additional strategies to target Survivin, including potentially via UNC2025-mediated MERTK inhibition, are needed. Additionally, as MERTK-targeted therapeutics transition to the clinic, pharmacodynamic biomarkers indicating response of the tumor to treatment will become
a necessity. Survivin has previously been used as a tumor biomarker due to its tumor-specific expression. We have demonstrated decreased Survivin levels following treatment with UNC2025 in a wide variety of solid tumor cell lines, including the majority of NSCLC lines assayed. Decreased Survivin expression is a more consistent indicator of UNC2025-mediated MERTK inhibition than changes in phospho-AKT and phospho-ERK levels, and is more easily measured than phospho-MERTK. It is conceivable that Survivin levels could be determined pre- and post-UNC2025 administration as an early marker of tumor response to facilitate clinical decisions regarding therapeutic strategy.

In addition to MERTK, important roles for AXL have also been characterized in NSCLC [45, 78]. AXL expression has been correlated with lymph node status and clinical stage in patient tumor samples and can mediate resistance to EGFR-targeted therapy [79, 100, 101]). Several TKIs against AXL are in various stages clinical development [21]. The data presented here suggest that continued development of MERTK-selective TKIs may allow a second TAM-family member to be targeted clinically.

UNC2025 is a novel TKI that is selective for MERTK within the TAM family, inhibits MERTK’s downstream signaling network, induces apoptotic cell death and inhibits colony formation independent of driver oncogene status, and decreases tumor growth in vivo in murine models. These promising effects mediated by UNC2025 in pre-clinical studies demonstrate that MERTK-targeted TKIs deserve further investigation and optimization toward clinical trials.

**Biomarker Development and Rational Combinations**

UNC2025 has shown promising pre-clinical properties toward becoming a clinical candidate, specifically its excellent pharmacokinetic and pharmacodynamics profile, and its efficacy in multiple murine models. However, questions remain regarding how to most effectively deploy UNC2025 in NSCLC patients. Particularly, which patients are
most likely to benefit from UNC2025, and which therapeutics should be co-administered with UNC2025, are pressing issues. As detailed in Chapter V, progress has been made on the co-administration front, with the identification of AXL inhibition as synergizing with MERTK inhibition. Additionally, MERTK inhibition by Mer590 and/or shRNA synergized with carboplatin, as detailed in Chapter III. Apoptosis and induction of PARP cleavage were both markedly increased when MERTK inhibition was combined with this conventional chemotherapeutic. Finally, in the initial target validation paper identifying MERTK expression in NSCLC, MERTK inhibition by shRNA synergized with cisplatin, resulting in less cell proliferation as measured by the MTT assay [45].

Identification of synergistic co-targets for MERTK inhibition has resulted in several promising leads. However, progress in identification of a rational biomarker for sensitivity to MERTK therapeutics has not as of yet been so fruitful. As discussed in the Introduction, MERTK was initially identified in NSCLC patient samples by immunohistochemistry. Positive MERTK staining did not correlate with histology, with approximately two-thirds of patient samples having some level of MERTK expression in both adenocarcinoma and squamous cell carcinoma samples [45]. Additionally, as detailed in Chapter IV, sensitivity to MERTK therapies does not correlate with driver oncogene status. In a large panel of cell lines, sensitivity to UNC2025 did not correlate with the presence or absence of activating EGFR mutations, KRAS/NRAS mutations, RTK fusion, or even no known driver mutation. While this is potentially an advantage, in that patients with a wide variety of genetic mutations may benefit from MERTK-targeted therapy, it presents a problem for the design of clinical trials. Early clinical trials for TKIs did indeed utilize simply receptor expression as their biomarker for enrollment. In the case of imatinib, this was expression of the BCR-ABL fusion gene. Fortunately, BCR-ABL is present in approximately 95% of chronic myelogenous leukemia, and did in fact correlate with drug sensitivity, allowing imatinib to become the poster-child for modern
cancer drug development success [124]. However, the story became more nuanced when imatinib was introduced into clinical trials for gastrointestinal stromal tumors (GIST). In this scenario, it was determined that mutation rather than simply expression of the target (c-kit in the case of GIST) was important in determining TKI sensitivity. Similarly, initial trials with the EGFR inhibitor gefitinib were conducted in NSCLC with patients over-expressing EGFR [125-128]. This included a large proportion of all NSCLC patients, from 40-80% depending on the estimate. However, responses were sub-optimal and unpredictable, and subsequent analysis again identified receptor mutation as critical in predicting drug sensitivity. Since this time, the identification of biomarkers prior to the initiation of a clinical trial has become prioritized. Therefore, identification of markers of MERTK sensitivity is of critical importance.

Identifying and Avoiding Potential Side Effects

For MERTK-targeted therapy to be useful clinically, side effects must be minimized. Evidence providing support that this is possible comes from mouse knockout studies. Mice lacking all three TAM family members display normal embryonic development, but eventually suffer from blindness, sterility, hyper-proliferation of B and T cells, and multiple additional autoimmune manifestations [129, 130]. However; single knockout mice lacking only MERTK have a much milder phenotype, retaining their fertility and having much less severe autoimmune effects. The role of MERTK on macrophages is thought to be central to each of these side effects, as macrophages lacking MERTK cannot appropriately clear apoptotic cell debris, leading to increased self-antigen accumulation and the resulting autoimmune phenotypic effects (Reviewed in [23]). Because the effects of MERTK loss are late-onset, it is likely that brief bursts of MERTK-targeted therapy will not result in significant autoimmunity manifestations. Additionally, it is possible that drug holiday windows could be used in treatment
regimens, to allow the immune system to clear any apoptotic debris that had built up during administration of the MERTK-targeted therapeutic. On a related note, there is even evidence that inhibition of MERTK on macrophages could prove useful in tumor inhibition. Studies utilizing mice transplanted with MERTK knockout bone marrow have indicated that loss of MERTK from immune cells favors a switch from a pro-tumor M2 phenotype to an anti-tumor M1 phenotype [131]. Ultimately, however, detailed pre-clinical studies will determine the exact conditions under which anti-MERTK therapy is tolerable in patients.

Cooperative Activity between MERTK and AXL

As detailed in the preceding section, one issue under investigation surrounding the entrance of MERTK-targeted therapeutics into the clinic is determining the optimal co-therapeutics to be given in combination with UNC2025 or other MERTK-targeted agents. Data in Chapter V suggests that co-inhibition of MERTK and AXL may be a viable treatment strategy in subsets of NSCLC, due to their potential heterodimerization and demonstrated functional cooperation. Co-immunoprecipitation between MERTK and AXL is a novel finding of this work, and functional interactions between TAM family members are poorly understood currently. Multiple studies on knockout mice provide the best evidence for functional relationships between TAM family members. For instance, disrupted spermatogenesis, vaginal atresia, and autoimmunity are all progressively more severe and/or common in triple TAM-knockout mice as compared to mice with one or more TAM family members intact [129, 130, 132]. Studies on platelets have shown that all three TAM family members must be present for thrombus stabilization [22]. In this study, it was shown that loss of any one TAM receptor prevented surface expression of the remaining two TAM receptors, thereby preventing their ability to be activated by Gas6. Similarly, phosphorylation of MERTK on
macrophages by apoptotic cells is compromised when AXL and TYRO3 are genetically knocked out [133]. Finally, two studies have shown co-immunoprecipitation of AXL and TYRO3. Brown et al demonstrated in Rat2 cells that the presence of AXL or TYRO3 caused increased phosphorylation of the alternate receptor in response to Gas6, and that these two receptors co-immunoprecipitate [134]. MERTK was not investigated as it was not expressed in these cells. Additionally, Pierce et al showed in GnRH neurons that survival and migration phenotypes are more severe when both AXL and TYRO3 are knocked out, and that these receptors co-immunoprecipitate [135]. Again, the role of MERTK was not investigated. Therefore, to the best of our knowledge, our current report is the first to demonstrate co-immunoprecipitation of MERTK and AXL.

**Potential Efficacy of Inhibition of Heterodimerization**

While targeting AXL and MERTK concurrently is already possible with available ATP-competitive small molecule inhibitors, other mechanisms of inhibiting this interaction and preventing AXL-mediated intrinsic resistance to MERTK therapeutics are also possible, including disruption of the physical interaction between AXL and MERTK. Therefore, identification and subsequent disruption of the binding interface between these receptors presents a future direction for this project. Protein-protein interactions (PPIs) play critical roles in nearly every aspect of cell signaling, including oncogenic signaling, and have therefore become important targets for development of novel therapeutics. In contrast to enzymes, including the heavily-targeted receptor tyrosine kinase family, PPIs are generally more difficult to target with small molecule inhibitors, and present unique challenges [136]. The interaction surface does not typically contain obvious binding pockets, such as the ligand or substrate binding pockets on RTKs and enzymes. Research has determined however that targetable pockets can be found even on surfaces which appear ‘flat’, due to the flexibility of the surfaces and alterations in
their structure between unbound and bound states with the compound or with the protein interaction partner. A second challenge in targeting PPIs is that the interaction surface is typically much larger than a small molecule compound. However, this concern has been alleviated by the discovery of ‘hot spots’, or sub-regions within an interaction surface that account for the majority of the binding affinity, as well as by the finding that interaction surfaces can be conformationally altered by binding of an inhibitor at a spatially distinct, allosteric site. Indeed, in spite of these and other initial concerns surrounding the druggability of PPIs, a large number of small molecule PPI inhibitors have shown promising efficacy in various cancer models [136, 137]. Examples include the PLX inhibitors which can allosterically block BRaf-CRaf heterodimerization, the AKT inhibitor MK-2206 which stabilizes the auto-inhibitory PH domain interaction with the catalytic domain, and the RAS inhibitor DCAI which inhibits the ability of SOS1 to interact with and activate RAS by exchanging GTP for GDP [138-141].

In addition to small molecules, peptides have also been successfully utilized to target PPIs. Peptides are much easier to rationally design and produce than small molecules, however they present their own drug-design challenges due to poor pharmacokinetic properties, including poor stability in vivo and an inability to be administered orally, as well as poor cell permeability. Nonetheless, these obstacles have also been overcome, through the use of chemical modifications to increase stability and carriers to improve delivery, resulting in a wide variety of efficacious therapeutic peptides (TPs). Specifically, RAS, EGFR, AKT, ERK, NF-kB, c-Myc, p53, Smac/Diablo, CDK inhibitors, and the Bcl-2 family have all been successfully targeted by TPs (Reviewed in [142, 143]). Examples exist for TPs acting both as mimics or ‘sinks’ of endogenous protein epitopes, thereby functioning as either an activator of a downstream protein’s normal function, or as an inhibitor by sequestering an upstream protein away from its downstream effectors. Targeting the MERTK:AXL interaction through the use of
small molecule inhibitors or TPs is a novel treatment paradigm that could be pursued in order to sensitize NSCLC cells to traditional kinase inhibition.

In addition to identifying and targeting the PPI between MERTK and AXL, a second, albeit riskier, potential future direction for this project involves examining various MERTK and AXL isoforms for their ability or inability to participate in heterodimerization and to mediate cooperative effects on the other receptor. Alternative splicing, the process by which multiple protein isoforms are generated from a single mRNA transcript, is estimated to occur in up to 94% of human genes [144, 145]. Many of the alternative proteins generated from alternative splicing have been demonstrated to have pro-oncogenic functions. Oltean and Bates have reviewed several examples of such variants, citing examples that affect each of the six original hallmarks of cancer (sustaining proliferative signaling, evading growth suppressors, inducing angiogenesis, activating invasion and metastasis, enabling replicative immortality, and resisting cell death), as well as the two ‘emerging’ hallmarks of deregulating cellular hyperenergetics and avoiding immune destruction [146, 147]. MERTK and AXL both have several identified isoforms to be investigated. It is conceivable that one or more of these variants may lack a region required for the cooperative interactions identified between MERTK and AXL in Chapter V. This would allow for classification of these various splice variants as promoting either sensitivity or resistance to MERTK therapeutics, which may have important effects on prognosis or treatment plans. AXL in particular could benefit from this sort of analysis, as it has been identified repeatedly as playing important roles in drug resistance to a variety of therapeutics, and has additionally often been found to physically interact with the receptors targeted by these agents, as summarized in the introduction to Chapter V. Identifying splice variants of AXL with a predilection towards RTK heterodimerization and intrinsic resistance may have broad implications in the care
of NSCLC patients. Therapies targeting alternative splicing are becoming more widely studied, and would have utility in this scenario if such variants of AXL could be identified.

**Final Impressions**

Investigation of the role of MERTK in NSCLC has covered much ground over the past few years, from identifying its expression in a series of patient samples, to the development and classification of multiple novel therapeutics ongoing today. Indeed, there is optimism that a small molecule inhibitor against MERTK may enter clinical trials in the near future. However, as discussed, much work remains to be done before clinical success targeting MERTK is achieved. The current model for developing targeted therapeutics involves identifying a mutated or translocated oncogene. If cancer biology is to move beyond targeting these classical ‘driver’ oncogenes, and begin to address the much deeper and more subtle underlying complexity within NSCLC circuitry, obstacles must be overcome. Specifically, the issue of developing a clinical biomarker for a non-mutated receptor is arguably the greatest obstacle to overcome before introducing MERTK-targeted therapeutics into NSCLC clinical trials. Ligand:receptor autocrine loops have been demonstrated to have predictive value for sensitivity to some therapeutics, as have gene expression profile arrays. Whether either of these or another approach will prove fruitful in elucidating a biomarker for sensitivity to MERTK receptor therapeutics remains to be seen. In addition to biomarker identification, ongoing work remains to be done in the areas of rational drug combination development, and investigation of alternative methods of targeting MERTK in cells, such as blocking its interaction with other network components. These and other open research questions ensure that studying the role of MERTK in NSCLC will have benefits for years to come, and the hope remains that this body of work will soon culminate in improved survival outcomes for patients with advanced stage NSCLC.
REFERENCES


