A Bio-Organic Approach to the Design and Synthesis of Tyrosine Kinase Inhibitors with Multi-Modal Imaging Capabilities

A thesis presented

by

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The Department of Chemistry and Chemical Biology

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Chemistry

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ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry in the Graduate School of Arts and Sciences of Northeastern University, September 2010
Abstract

Diabetes research has been one of the most active fields for years and will continue to be relevant due to the escalating prevalence of the disease in the US and worldwide.\textsuperscript{1} Previous research focused on managing and treating the complications of this chronic disease, much of which has been centralized around insulin control. The research described in this thesis concentrated on treating diabetes from a different viewpoint, developing a more integrated approach to treat factors of diabetes, metabolic syndrome and cardiovascular disease. Approaches to improving potent drug candidates involved developing structure activity relationships to determine areas in which improvements can be made. In addition, a prodrug strategy was employed improve upon the pharmacokinetic properties of a novel and potent drug candidate. At this time the research cannot be fully disclosed and will be presented at a later time in patents and journal articles through Novartis Pharmaceuticals.

The up-regulation of epidermal growth factor receptors have been implicated in tumors of numerous cancers including pancreatic, breast, and non-small cell lung. Despite the efficacy of the three FDA approved tyrosine kinase inhibitors (TKI), success has been hampered in part because it is currently difficult to quantitate the expression of epidermal growth factor receptor (EGFR) prior to treatment. Noninvasive imaging agents that provide such data could enable personalized medicine. As a result, a medicinal strategy was employed to develop and synthesize a non-invasive diagnostic TKI for the imaging of tissue function. This agent can provide evidence of drug efficacy, allow for disease monitoring, help predict optimal dosage, and lead to selection of patients who are likely to be responsive to the particular therapeutic approach. The proposed diagnostic tool could allow doctors and clinicians to perform a more rapid and
comprehensive evaluation of the cancer patient’s sensitivity toward the ErbB receptor therapy.
Acknowledgements

This thesis is a selected compilation of the research I have performed throughout my four years at Northeastern University. It is without saying that I will always be indebted to those who contributed in anyway to this thesis. Below are a few people who not only who played a significant role in this thesis but also in shaping who I am as a person.

The first three chapters of my thesis focus on a research project performed during my second co-op at Novartis Pharmaceuticals in Cambridge. I am forever thankful to all the people at Novartis who have allowed me to share the research, including Louise Kirman, Greg Bebermitch, James Waring, Brian Raymer, Robert Day, and Herb Schuster. In particular, I would like to thank my supervisor, mentor, and friend Louise Kirman who has been one of the most influential people in my life and without her constant support, this thesis would not be possible. Many thanks to all my colleagues at Novartis who have made my experience there so great.

The researched detailed in chapter 4 was performed during my first summer and during my last year at Northeastern, under the advising of Dr. Robert Hanson. Words cannot express how grateful I am to Dr. Hanson for taking a chance on a young inexperienced undergraduate, four years ago. His encouragement and guidance made me realized my passion in chemistry and for that I am extremely thankful. He is a great advisor who exudes such an enthusiasm about chemistry that is truly inspirational. I would also like to thank the Hanson lab, in particular Lenny Dao and Anton Kozhushnyan who have been extremely kind and helpful.

I would also like to thank a few people at Northeastern University especially Jordan Swift for being the most caring and nurturing academic advisor. Thanks to John Bottomy for providing me any and all equipment for my research and guidance throughout my college career.

I would also like to thank all my family and friends for their patience, understanding and
support through the process of writing this thesis. Thank you to Kelly Reiser and Rhiannon Thomas, we started our college careers together and four years later I am glad to say that we have built life long friendships that will only continue to become stronger. Special thanks to Steven Criscione for being an amazing person; having you in my life makes me a better person. Last, but not least, I would like to thank my mother for her unconditional love and unwavering support in all my endeavors.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcOH:</td>
<td>Acetic Acid</td>
</tr>
<tr>
<td>ACN:</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>aq:</td>
<td>Aqueous</td>
</tr>
<tr>
<td>Bn:</td>
<td>Benzyl</td>
</tr>
<tr>
<td>Boc:</td>
<td>Tert-butoxycarbonyl</td>
</tr>
<tr>
<td>Bz:</td>
<td>Benzoyl</td>
</tr>
<tr>
<td>DCM:</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEAD:</td>
<td>Diethylazodicarboxylate</td>
</tr>
<tr>
<td>DIAD:</td>
<td>Diisopropylazodicarboxylate</td>
</tr>
<tr>
<td>DMF:</td>
<td>N,N'-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO:</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EGFR:</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EtOAc:</td>
<td>Ethyl-acetate</td>
</tr>
<tr>
<td>FDG</td>
<td>2-[^{18}F]fluoro-2-deoxy-D-glucose</td>
</tr>
<tr>
<td>HB-EGF:</td>
<td>Heparin-binding Epidermal Growth Factor</td>
</tr>
<tr>
<td>^1H NMR:</td>
<td>Proton Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>HPLC:</td>
<td>High-performance Liquid Chromatography</td>
</tr>
<tr>
<td>LC-MS:</td>
<td>Liquid Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>mM:</td>
<td>millimolar</td>
</tr>
<tr>
<td>mmol:</td>
<td>millimole</td>
</tr>
<tr>
<td>NRG1:</td>
<td>Neuregulin-1</td>
</tr>
<tr>
<td>NIS:</td>
<td>N-Iodosuccinimide</td>
</tr>
</tbody>
</table>
o/n: Overnight
PET: Positron Emission Tomography
Rf: Retention Factor
Rt: Retention Time
RT: Room Temperature
SM: Starting Material
TFA: Trifluoroacetic acid
TEA: Triethylamine
TGF-α: Transforming growth factor- α
THF: Tetrahydrofuran
TKI: Tyrosine Kinase Inhibitor
TLC: Thin layer Chromatography
TFA: Trifluoroacetic Acid
Ts: Toluenesulfonyl
UV: Ultraviolet
µ-wave: microwave
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Addendum and Summary of Novartis Research
Summary and Significance of Research Performed at Novartis Institute

Diabetes research has been one of the most active fields for years and will continue to be relevant due to the escalating prevalence of the disease in the US and worldwide. Treatment and research of diabetes has mainly been focused on managing and treating the complications of this chronic disease. In particular, much of the research has been centralized around insulin control. The research described in this thesis was performed and supported by Novartis Pharmaceuticals, and concentrated on treating diabetes from a different viewpoint. Diabetes, though a singular disease, is often diagnosed and/or develops as one component of an array of disorders. A presumably underlying condition to diabetes is metabolic syndrome. Metabolic syndrome is also the precursor to cardiovascular disease, which manifests itself prior to diagnosis or during the lifetime of a diabetic patient. Thus, as diabetes is part of a cluster of disorders, our approach was to develop a more integrated treatment by pursuing a common target implicated in numerous facets of metabolic syndrome, cardiovascular disease, and diabetes.

Analogs synthesized prior to my research provided the potency benchmark required to inhibit the desired target. My aim was to improve upon the established scaffolds by optimizing the pharmacokinetic (PK) properties of the compounds to allow for clinical progression. Though the initial attempts did not yield a balanced compound with both optimal solubility and potency, we gained insight about the drug-target interactions, such as which groups are tolerated, locations that will increase solubility and locations that will increase potency. Most importantly, we were able to establish a method for reducing off-target effects, an extremely useful advance in any medicinal chemistry campaign. By iteratively changing one aspect at a time on the original scaffold, and following the SAR, we were also able to develop a potential prodrug strategy that could impact our goal to improved pharmacokinetic properties.
The introduction of assorted moieties as inert chemical derivatives for the prodrug strategy proved to be successful in increasing the plasma exposure of a lead compound. After establishing a standard method for the synthesis of such prodrugs, the PK and pharmacodynamic (PD) data revealed that one specific prodrug was the most effective in achieving the required exposure and functional activity. Based on these promising results, other analogs were synthesized to increase exposure in efforts to decrease the dose required to achieve optimum activity.

The research detailed in these three chapters of the fully disclosed thesis provided a stepping stone for this project to move forward as the previous potential candidates for clinical applications were stalled due to solubility limited exposure. Through meticulous SAR exploration and implementation of a prodrug strategy, we were able to dissociate the solubility-limited exposure from the lead compound, thus enhancing the pharmacokinetic and pharmacodynamic profile for progression into human testing.
Addendum:

Due to the significance of the research performed, it is at this time August 13, 2010 stated that the full thesis written by Helen Trinh Pham cannot be fully disclosed based on the confidentiality, legality, and security of the content. The research performed will be disseminated through other forms of communication such as patents, journal articles, and internal posters and papers by Novartis Pharmaceuticals at a later date.
Chapter 1: Introduction and Background
In 2007, one in eight deaths were attributed to cancer worldwide.\textsuperscript{1} By 2050, it is projected that the global spread of cancer will gradually increase to reach 27 million new cancer cases with an estimated death rate of 17.5 million.\textsuperscript{1} Currently, the most effective therapeutic agents to combat cancer are non-specific agents which target rapidly dividing cells, a trademark of cancer. These agents act through various approaches such as interfering with DNA synthesis, inducing DNA damage, inhibiting of RNA synthesis, and disrupting cytoskeleton formation.\textsuperscript{3} Though effective, these therapies are extremely toxic and present adverse side effects. The emergence of targeted cancer therapy in the late 1980’s provided alternative agents that were less toxic and better tolerated by the patient. These therapies focused on treating cancer by targeting enzymes, proteins, and/or molecules associated with cancer and/or tumor formation rather than reducing tumor growth through rapidly dividing cells. One such approach was the specific interference with a new class of proteins, protein kinases, responsible for regulating crucial signaling pathways.

1.1 Protein Tyrosine Kinases

Constituting as one of the largest and most diverse gene families, protein kinases have emerged as one of the most intensely investigated class of therapeutic drug targets. Generally, protein kinases are a class of ATP-dependent cellular enzymes that catalyze the transfer of a phosphate group to amino acids in a specific peptide sequence of various substrates.\textsuperscript{4} It is estimated that there are approximately 518 protein kinases encoded in the human genome.\textsuperscript{5}

In previous years, targeting of protein kinases was largely overlooked as there was insufficient evidence linking kinase activity with cancer. In addition, there was much hesitation about drug specificity and toxicity.\textsuperscript{6} However, the field of protein kinase research was altered
dramatically with the success of the protein tyrosine kinase inhibitor imatinib (Gleevec; Novartis), the first proof-of-principle compound inhibiting Bcr-Abl kinase in chronic myelogenous leukemia.\(^5\) With the breakthrough of imatinib, increased efforts were directed towards the re-evaluation of kinases and their role in cancer. Research soon pinpointed numerous kinases thought to be implicated in cancer, among them was the epidermal growth factor receptor (EGFR).

Stanley Cohen, a biochemist, first discovered the epidermal growth factor (EGF) in 1962 while examining crude extract from mouse sub-maxillary glands.\(^7\) This discovery led to a Nobel Prize in Physiology and Medicine in 1986, and also to the identification of the EGF receptor, EGFR. In the early 1980s EGFR, along with three other members of the ErbB family (also known as the EGFR family or the HER family) were linked to cancer when the avian erythroblastosis tumor virus was found to encode the gene for EGFR.\(^8\) Since then, the ErbB family has been one of most well established and researched group of receptors, due to its significant role in signal transduction and dysregulation in cancer.

### 1.2. Normal EGFR activity

The ErbB family is comprised of four members, ErbB1 (known as EGFR or HER-1), ErbB2 (known as HER-2 or neu), ErbB3 (HER-3), and ErbB4 (HER-4). These four members are closely related, sharing common structural motifs consistent with transmembrane receptor tyrosine kinases such as an extracellular ligand binding domain, transmembrane region, and an intracellular tyrosine kinase domain. The extracellular domain functions to recognize and bind ligands that are capable of activating the receptor. This domain consists of four sub-domains, DI-IV, two of which (DI and DIII) are leucine-rich and participate in ligand binding, and two
others (DII and DIV) that are cysteine-rich and form intradomain disulfide bonds to facilitate the conformational change induced by ligand binding. The transmembrane region anchors the receptor to the lipid bilayer of the cell and functions to control EGFR kinase activity, ligand internalization, and receptor sorting. Lastly, the intracellular tyrosine kinase domain is essential for the activation of signal transduction. Through the hydrolysis of ATP, a phosphate group can be transferred to tyrosine residues and/or substrates in the active site. The homology in the tyrosine kinase domain is extremely high amongst the receptors, with the exception of ErbB3, which lacks intrinsic tyrosine kinase activity. Comparatively, the extracellular domains are less conserved, which is apparent in the different specificity in ligand binding. At present, there are at least 12 unique ligands known to bind to EGFR, ErbB3, ErbB4 including EGF, TGF-α, and NRGs 1-4. However there is no known ligand for ErbB2, described frequently as an “orphan” receptor.

Typically, EGFR activity is stimulated upon ligand binding which causes the receptors to undergo a transformation from an inactive conformation to an active conformation by movement of an activation loop. During activation, the loop, a highly conserved sequence of Asp-Phe-Gly (DFG), is rotated outward giving the “DFG out” conformation. This conformational change induces receptor dimerization that subsequently alters the transmembrane region, relieving some of the steric hindrance normally imposed on the intracellular tyrosine kinase domain. As a result, the tyrosine kinase activity is catalyzed by ATP, leading to autophosphorylation of the protein’s tyrosine residues, which then serve as docking sites for recruitment of downstream proteins to the membrane. This leads to the activation of several signal transduction cascades. Some of the pathways that are engaged by EGFR activation include the PI3K pathway, Ras/Raf/MEK/MAPK pathways, STAT3 pathway, and the FAK pathway. These pathways control a wide variety of
cellular processes including apoptosis, migration, growth, adhesion, and differentiation.\textsuperscript{8}

![Figure 1: The ErbB signaling network, used with permission from reference 8.\textsuperscript{8}](image)

Receptor dimerization determines which pathways will be activated based on the type of dimer complex formed. Such receptor association can occur as either homo- or heterodimerization. Given that ErbB3 is devoid of inherent tyrosine kinase activity, the homodimerization is inactive; therefore it can only function when bound to a different receptor. As the “orphan receptor”, the extracellular domain of ErbB2 is intrinsically in the active form, making it the preferred dimerization partner. One of the most potent complexes is the ErbB2-ErbB3 complex, which is interesting as they complement each other’s shortcomings. Other preferred combinations include ErbB2-ErbB4 and ErbB1-ErbB4 complexes.\textsuperscript{12} Based on receptor combinations, only certain tyrosine residues are phosphorylated, in each case subsequently
recruiting different proteins and activating various signal pathways, as depicted in Figure 1.1.

In normal, non-cancerous cells, this process of EGFR-moderated activation is highly regulated to prevent continuous signal cascade. One of the essential aspects of the regulation is receptor internalization. Following ligand binding, the ligand-receptor complexes form clusters on the cell surface. The membrane on either side of the cluster then slowly fuses together forming an endosome, a membrane bound compartment which sequesters internalized contents from the rest of the cell. The contents of the endosome may be recycled to the cell surface or may be labeled for degradation. The EGFR pathway is also modulated by tyrosine phosphatases which have the ability to terminate kinase activity by de-phosphorylating the tyrosine residues.

1.3. Mechanisms of EGFR Dysregulation in Cancer

Due to the many facets of regulation involved in EGFR cascade, there are numerous ways by which the pathway can be errantly interrupted. One of the most frequently observed causes of these signaling abnormalities includes fusion of a receptor with a partner protein, often as a result of chromosomal translocation. The partner protein contains a domain which can cause oligomerization of the ErbB receptors in the absence of a ligand, stimulating autophosphorylation and leading to the activation of downstream signal transduction pathways. A second mechanism of dysregulation observed in ErbB is caused by point mutations. These mutations, prevalent in non-small cell lung cancer (NSCLC), alter the binding sensitivity of the receptor to ligands, making it more susceptible to promiscuous binding. Random point mutations are typically observed with higher frequency in cancer patients, as the rapid cell proliferation associated with cancer increases the chances for mutation. Another means by which ErbB signaling can be disrupted is abnormal expression of the ErbB receptors and/or their
respective ligands, increasing the probability that signal transduction is activated continuously.\textsuperscript{14} Other dysregulation mechanisms can include impaired regulatory pathways such as the increased rate of receptor recycling within the receptor internalization mechanism, or impaired tyrosine phosphatases.\textsuperscript{6}

1.4. Cancers associated with dysregulated EGFR

The dysregulation of the ErbB family has been implicated in a wide range of cancers including breast, colorectal, ovarian, prostate, and non-small cell lung cancer.

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Range of Tumor Expressing EGFR (%)</th>
<th>Tumor Type</th>
<th>Range of Tumor Expressing EGFR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head and neck</td>
<td>80-100</td>
<td>Prostate</td>
<td>40-80</td>
</tr>
<tr>
<td>Colorectal</td>
<td>25-77</td>
<td>Bladder</td>
<td>53-72</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>30-50</td>
<td>Cervical</td>
<td>54-74</td>
</tr>
<tr>
<td>Lung</td>
<td>40-80</td>
<td>Ovarian</td>
<td>35-70</td>
</tr>
<tr>
<td>Esophageal</td>
<td>71-88</td>
<td>Breast</td>
<td>14-91</td>
</tr>
<tr>
<td>Renal cell</td>
<td>50-90</td>
<td>Glioblastoma</td>
<td>40-50</td>
</tr>
</tbody>
</table>

Table 1.1: Relative Rates of EGFR expression in various tumor types. Adapted from reference (3) \textsuperscript{3}

In such cancers, the mechanisms of dysregulation are activated causing undesirable continuous signal transduction, which leads to reduced apoptosis, increased cell proliferation, increased angiogenesis, increased motility, and the formation and maintenance of tumors.\textsuperscript{15} As a result, the overexpression of ErbB receptors in human solid epithelial tumor types correlates to poor disease prognoses and reduced survival.\textsuperscript{16} In addition, more aggressive cancer behavior has been associated with overexpression of ErbB receptors, particularly ErbB2 expression, as additional consequences of dysregulation include increased potential for invasion, metastasis,
and resistance to conventional therapies.\textsuperscript{7} As Table 1.1 reveals, the expression of EGFR in solid tumors is a common thread amongst many non-related cancers.\textsuperscript{3} However, Table 1.2 demonstrates that each receptor plays a distinguished role in each unique type of cancer. The combined knowledge of ErbB expression rates and receptor components, in different cancers can help to determine treatment options and assess the aggressiveness of the cancer.

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Expression</th>
<th>Expression Areas</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ErbB1</td>
<td>Overexpression</td>
<td>Head and neck, breast, bladder, prostate, kidney, non-small-cell lung cancer</td>
<td>Significant indicator for recurrence in operable breast tumours; associated with shorter disease-free and overall survival in advanced breast cancer; may serve as a prognostic marker for bladder, prostate, and non-small cell lung cancers</td>
</tr>
<tr>
<td></td>
<td>Overexpression</td>
<td>Glioma</td>
<td>Amplification occurs in 40% of gliomas; overexpression correlates with higher grade and reduced survival</td>
</tr>
<tr>
<td></td>
<td>Mutation</td>
<td>Glioma, lung, ovary, breast</td>
<td>Deletion of part of the extracellular domain yields a constitutively active receptor</td>
</tr>
<tr>
<td>ErbB2</td>
<td>Overexpression</td>
<td>Breast, lung pancreas, colon, oesophagus, endometrium, cervix</td>
<td>Overexpressed owing to gene amplification in 15-30% of invasive ductal breast cancers Overexpression correlates with tumour size, spread of tumour to lymph nodes, high grade, high percentage of S-phase cells, aneuploidy and lack of steroid hormone receptors</td>
</tr>
<tr>
<td>ErbB3</td>
<td>Expression</td>
<td>Breast, colon, gastric, prostate, other carcinomas</td>
<td>Co-expression of ErbB2 with ErbB1 or ErbB3 in breast cancer improves predicting power</td>
</tr>
<tr>
<td></td>
<td>Overexpression</td>
<td>Oral squamous cell cancer</td>
<td>Overexpression correlates with lymph node involvement and patient survival</td>
</tr>
<tr>
<td>ErbB4</td>
<td>Reduced expression</td>
<td>Breast, prostate</td>
<td>Correlates with a differentiated phenotype</td>
</tr>
<tr>
<td></td>
<td>Expression</td>
<td>Childhood medullo-blastoma</td>
<td>Co-expression with ErbB2 has a prognostic value</td>
</tr>
</tbody>
</table>

Table 1.2: Expression of ErbB and their role in cancer, adapted from reference (8).\textsuperscript{8}
1.5. Current Inhibitors of EGFR

The evidence linking ErbB tyrosine kinase receptors to cancer begins to validate the need for therapeutic intervention at this target. Strategies for the drug targeting of the ErbB receptor family, specifically EGFR and HER-2, as they are more prominently overexpressed, can be designed to perturb the extracellular ligand binding domain, intracellular tyrosine kinase domain, or downstream molecular signals. Thus far the most effective mechanisms of targeting of these receptors have been monoclonal antibodies and tyrosine kinase inhibitors. Over the past several decades many inhibitors within these two classes of molecules have been approved by the FDA. The most notable monoclonal antibodies include cetuximab (head and neck cancer; EGFR), panitumumab (colorectal cancer; EGFR), and herceptin (breast cancer; HER-2). These compounds act to bind the receptor thereby restricting ligand binding and preventing activation of signal transduction pathways. Despite the effectiveness of extracellular domain inhibitors, the intracellular domain may still remain active as previously described via a mechanism of dysregulation involving partner proteins.

To combat constitutive oligomerization through the fusion of partner proteins, several tyrosine kinase inhibitors have been approved including Erlotinib (NSCLC & pancreatic cancer; EGFR), Gefitinib (NSCLC; EGFR), and Lapatinib (breast cancer; dual EGFR/HER-2). These reversible inhibitors compete with ATP, in the ATP-binding site of the intracellular tyrosine kinase domain. Competitive binding prevents phosphorylation of the receptor’s tyrosine residues preventing activation of downstream signals. In addition to the approval of these compounds for treating specific cancers (noted in the parenthesis), these compounds may be efficacious in other cancers.
Currently, these EGFR-targeted compounds are utilized as a second line of defense against the cancer after traditional first line chemotherapy regiments have failed or the cancer has accumulated resistance. Typically cancer cells hijack the ErbB pathway as a way to circumvent the initial treatment, (mentioned briefly in Section 1.4). Through point mutations, a dysregulated ErbB pathway will allow the cancer cells to re-grow tumors and allow for potential metastasis. The objective for the use of ErbB inhibitors is to prevent the cancer cells from using this pathway to continue proliferation, with the ultimate goal of clinical remission or disease stabilization prolonging the life of patients suffering from cancer.
1.6. Limitations associated with tumor targeting using EGFR inhibitors

Several challenges associated with ErbB inhibitors have hampered the routine use of these therapeutic molecules for the treatment of cancer. One of the difficulties in using such inhibitors is validating their effectiveness in a clinical setting. Getfitinib, previously approved by the FDA after showing a 10% response rate, has since been withdrawn as it did not show statistical significance in efficacy for the treatment of NSCLC when tested in larger clinical trials. The inconsistency seen in the clinical trials of gefitinib and others therapies like erlotinib are attributed to several factors which may have worked together to provide irregular response rates. These factors include unsuitable clinical trial selection of patients in terms of protein expression levels; insufficient inhibition due to dose determining obstacles; resistance due to mutation acquired during treatment; and possible development of alternative pathways to allow for cancer cell survival.3

The use of EGFR therapy in any medical setting, particularly clinical trials, requires that patients have the prerequisite of EGFR overexpression in tumors.17 However, there is a lack of correlation between EGFR expression and response to treatment, another limitation to use the EGFR inhibitors to treat cancer. This is largely attributed to the absence of appropriate methods with which to effectively measure EGFR expression. Methods currently used to detect ErbB receptors, including immunohistochemistry and FISH (fluorescence in situ hybridization), provide conflicting data, indicating that EGFR expression is not a reliable biomarker and thus it alone cannot predict efficacy.18 Confounding this challenge is the fact that these inhibitors show higher response rates for a specific sub-population containing an activating mutation. This mutation may increase the sensitivity of the receptor by altering the intracellular kinase domain, yielding it more susceptible to inhibitor binding.3 These patients comprise a sub-population
who patients would greatly benefit from the EGFR directed therapy yet are difficult to identify as there is no non-invasive biomarker available for correlation to this mutation.

A third prevalent shortcoming of EGFR therapies is that, unlike chemotherapeutic agents, the selection of the most effective dose for EGFR inhibitors is not easily determined. The criterion for selecting a proper dose of chemotherapeutic agents is to first identify the maximum tolerated dose.\textsuperscript{19} However, this paradigm is not ideal for EGFR inhibitors as side effects are not uniformly consistent markers, and the most effective dose may not parallel the maximum tolerated dose. Though the optimal dose may be predicted from pre-clinical models, there are possible differences within each patient (such as EGFR turnover and expression, receptor ligand levels, ATP concentration etc.) that could affect dosing selection schedule, possibly leading to insufficient inhibition.\textsuperscript{20} Currently, to establish an optimal dose, a trial-and-error approach is utilized. A dose is given and the tumor size is measured. This process is very time consuming and could take from months to years to properly assess; time which is very valuable to the patient.

Drug resistance and the possible alteration in the disease state are all part of the aberrant nature of cancer, making it very difficult to monitor. These factors are also assessed via biopsy, therefore a more time and cost efficient method is needed to determine proper dose and to monitor the progression of the disease.

\textbf{1.7 Radio-labeled EGFR-imaging agent}

Considering the variability of the factors involved in the design of clinical trials for ErbB receptor inhibitors, it is apparent why there are inconsistencies in the measured response rates of test subjects. Although select patients may in fact benefit from the investigational therapies,
the overall inconsistencies in the population prevent the routine use of these therapeutics. This challenge and previously outlined challenges that persist in the arena of EGFR inhibitors highlight the need to develop a method by which those individuals who are most sensitive to the EGFR therapy can be recognized, optimal doses of the drug can be determined, and disease-monitoring may be completed in a time efficient manner. One possibility by which to achieve these goals is via the use of radio-imaging EGFR-TKI probes. These probes utilized in the context of “personalized medicine” (tailored approach to patient treatment) could resolve many of the obstacles previously described, thereby truly treating those who will benefit, but equally allowing those who will receive no benefit to try alternative methods in a time efficient manner.

By utilizing conventional imaging modalities such as positron emission tomography (PET) and single photon emission tomography (SPECT), radio-labeled EGFR-TKI probes would allow real time evaluation of cancer through the visualization of the tumor tissue morphology. Information about underlying cell biology, physiology, and biochemistry could reveal whether the tumor has an overexpression of EGFR, whether the therapeutic drug has reached its target, whether the function of the target has been modulated due to treatment, and whether the tumor has changed in size. The use of this therapy would benefit the drug discovery process by providing more consistent criteria for population and dose selection in clinical trials, thereby leading to more efficient and proper evaluation of drug candidates. In addition, this therapy would allow doctors an objective and rapid method for determining patients sensitive to the EGFR therapy, administering proper doses, and allowing for non-invasive disease monitoring. Also, the probes could provide evidence of reoccurrence and/or resistance providing for timely intervention.

There have been several research groups which have attempted to develop radio-
labeled EGFR-TKI probes in efforts to provide a diagnostic tool. Previous efforts include the use of \(^{18}\text{F}\)FDG PET agents as surrogate markers, whereby the tracer metabolism was correlated to the success of inhibition by the therapeutic compound. However, this method fails to take into account possible inhibitor contribution to tracer metabolism, slow-growing tumors, metabolically less active tumors, and unselective uptake in adjacent tissues. Other efforts have focused on utilizing the FDA approved drugs containing an aminoquinazoline scaffold for derivatization into imaging probes. Atom replacement by introduction of a radionuclide (\(^{11}\text{C},^{18}\text{F},^{123}\text{I}\)) to this scaffold has been unsuccessful to date due the compounds’ limited accumulation in EGFR receptors, unselective uptake in nearby tissues, rapid metabolism, and clearance.

1.8 Rational drug design of a diagnostic anilinoquinazoline TKI

Our design for a versatile radio-imaging EGFR-TKI probe utilizes the 4-aminoquinazoline class of EGFR inhibitors as a template for derivatization in order to improve enzyme affinity and pharmacokinetic properties. This could potentially provide an effective tracer for various imaging technologies. Two general strategies were used for the preparation of the EGFR-TKI-linked imaging probe. These methods involve innovative features, purposefully designed into the imaging probe to overcome challenges seen with other derivatives.

In contrast to the agents previously reported, the imaging group is placed external to the aniline-quinazoline core, thereby more closely resembling the aforementioned FDA approved drugs and allowing the use of established synthesis protocols. In addition, the use of a polyethylene glycol (PEG) chain in the 6 or 7 position of the aniline-quinazoline core extends the substituent into a solvent accessible site of the target enzyme, allowing labeling groups to be present without reducing inherent biological activity of the inhibitor. The use of the PEG
group also enhances hydrophilicity, which prolongs systemic circulation, reduces non-specific binding. Lastly, the use of the extended PEG chain allows for facile addition of versatile imaging groups such as radionuclides and fluorescent dyes to be used. It also provides a moiety which can be tuned to further increase affinity and/or specificity without altering the core scaffold of the therapeutic agent.

Figure 1.3: General scaffold of EGFR-TKI linked radio-imaging probe.

Our initial investigation into the development tyrosine kinase inhibitors focused on the synthesis of 6/7 propargyloxy derivatives, as it provides a unique site of conjugation through a [3+2] cycloaddition allowing for the potential of radio-labeling. The propargyl linked anilinoquinazoline will then be conjugated to azido-substituted PEG-linked radio-imaging probes using Sharpless “click” chemistry. This method would introduce a 1,2,3-triazole group that does not alter the binding affinity as it is known that the 6 and 7 position are reach into a solvent pocket\textsuperscript{12}. This approach allowed for a divergent method to introduce a variety radio-labeling moieties by synthesizing various azido-PEGylated radioimaging reagents.

The second approach focuses on the development of linkers to allow for increased
binding affinity. The design of these compounds acted to closely mimic the heteroatom chain present in gefitinib and to further extend the linker into the solvent exposed area. In addition, the use of an alkyne at the end of the linker allows for divergent introduction of various radio-imaging groups. The comparisons of these compounds would allow for the evaluation of appropriate heteroatom and determination of the optimal length of linker.

**Approach One: Using [3+2] cycloaddition as a means to develop radio-labeled EGFR Inhibitors**

![Diagram 1.4: Proposed Approaches for the synthesis of TKI-linked radio-imaging probes](image)

**Approach Two: Incorporation of various N-heteroatom linkers to develop radio-labeled EGFR inhibitors**

![Diagram 1.4: Proposed Approaches for the synthesis of TKI-linked radio-imaging probes](image)

Figure 1.4: Proposed Approaches for the synthesis of TKI-linked radio-imaging probes

It is the expected that the different types of information made available by the various imaging modalities could complement each other, providing an in-depth image of the tumor tissue morphology. This detailed perspective could then allow for a more rapid and comprehensive evaluation of the cancer patient’s sensitivity toward the ErbB receptor therapy.
Chapter 2: Experimental Methods
2.1 Reagents and Instrumentation

All reactions were carried out in dry glassware unless otherwise noted. The glassware was dried by heating in a laboratory oven (113°C) for a minimum of 5 hours. All materials including the starting materials, were purchased from either Sigma Aldrich Company or Fisher Scientific and used as supplied. THF and toluene were distilled from sodium/benzophenone. All other solvents were purchased as anhydrous grade and used as received.

Analytical thin layer chromatography (TLC) was performed using silica gel 60Å precoated plates purchased from Sigma Aldrich, and visualized using a 254 nm / 366 nm UV lamp, phosphomolybdic acid or ninhydrin stains, or an iodine chamber. All column chromatography was performed on silica gel unless otherwise noted. Flash chromatography was performed on a Flash Master Solo (Argonaut Technologies) using ISOLUTE Flash Si II columns (Biotage). Melting points were determined using an Electrotherm capillary melting point apparatus and are uncorrected. $^1$HNMR spectra were obtained using Varian Mercury 300 (300MHz), Varian Mercury 400 (400MHz) or Inova 500 (500 MHz) spectrometer. Chemical shifts are reported relative to TMS at 0.00 ppm. NMR spectra chemical shifts are reported in parts per million downfield from TMS and referenced either to TMS, or to the residual solvent peak for chloroform-$d$, acetone-$d_6$, methanol-$d_4$, and THF-$d_8$. Coupling constants are reported in hertz.

2.2 Experimental Procedures

General procedures for the synthesis of 4-anilinoquinazolines containing 6/7 methoxy or hydroxy substituents have previously been reported in literature.$^{24}$ Modifications to the
syntheses were made due to the availability of reagents and ease of synthesis, and will be noted in the Results/Discussion section. The syntheses for compounds 7 to 11 and 12 were previously reported. The synthesis scheme is shown below for the synthesis of 4-(3-fluorophenylamino)-7-methoxyquinazolin-6-ol, 13.

**Methyl 3-methoxy-4-(prop-2-ynyloxy)benzoate (1):** Methyl 3-methoxy-4-hydroxybenzoate (18.22 g, 0.100 mol, 1 eq) was dissolved in acetone (200 mL) and potassium carbonate (48.8 g, 0.400 mol, 4 eq) was added. Propargyl bromide (80 wt% in toluene, 26 mL, 0.30 mol, 3 eq) was added, and the mixture was heated at reflux with stirring for 7 h. The mixture was filtered while hot, the filtrate was evaporated to dryness, and the residue was recrystallized from methanol, yielding 17.25 g, 78%, as white needles, mp 87-88°C. $^1$HNMR (400 MHz, chloroform-$d$) δ ppm: 7.67 (dd, 1 H, $J_1$ = 8.5, $J_2$ = 2.0 Hz); 7.57 (d, 1 H, $J$ = 2 Hz); 7.05 (d, 1 H, $J$ = 8.5); 4.83 (d, 2H, $J$ = 2.5 Hz); 3.927 (s, 3H); 3.90 (s, 3H); 2.54 (t, 1H, $J$ = 2.5 Hz).

**Methyl 5-methoxy-2-nitro-4-(prop-2-ynyloxy)benzoate (2):** Compound 1 (7.85 g, 0.0357 mol, 1 eq) was dissolved in dichloromethane (250 mL) and chilled with an ethylene glycol-dry ice slush. SnCl$_4$ (in dichloromethane, 1M, 53 mL, 0.053 mol, 1.5 eq) and fuming nitric acid (6 mL, approx 0.05 mol, 1.5 eq) were added slowly to the rapidly stirred cold solution. After ten minutes the solution was allowed to warm to room temperature and was stirred for 45 min. The mixture was poured into water, partitioned between water and ethyl acetate and the organic phase was washed twice with brine, dried over magnesium sulfate, filtered, the solvents removed by evaporation, and the residue was recrystallized from methanol. The product was isolated as pale yellow needles (8.38 g, 89%). Mp 111-112°C. $^1$HNMR (400 MHz, chloroform-$d$) δ ppm: 7.75 (s, 1H); 7.20 (s, 1H); 4.95 (d, 2H, $J$ = 2.4 Hz); 4.08 (s, 3H); 4.03 (s, 3H); 2.70 (t, 1H, $J$ = 2.4 Hz).
**Methyl 2-amino-5-methoxy-4-(prop-2ynyloxy)benzoate (3):** Compound 2 (1.00 g, 0.0038 mol, 1 eq) was dissolved in hot methanol (125 mL). Sodium dithionite (4.00 g, 0.023 mol, 6 eq) was added, followed by 10 mL of water. The slurry was maintained at 55-65°C for 1 h or until the reaction was complete. The reaction mixture was concentrated and partitioned between ethyl acetate and brine. The organic phase was dried over magnesium sulfate, filtered and the solvent evaporated. The residue was recrystallized from methanol, yielding 0.804 g, 90%. Mp 118-119°C. $^1$HNMR (400 MHz, chloroform-$d$) $\delta$ ppm: 7.31 (s, 1H); 6.30 (s, 1H); 4.76 (d, 2H); 3.85 (s, 3H); 3.65 (t, 1H).

**6-Methoxy-7-(prop-2ynyloxy)quinazolin-4(3H)-one (4):** Compound 3 (0.86 g, 0.0036 mol, 1 eq) was added to 10 mL of formamide and ammonium formate (0.35g, 0.0055 mol 1.5 eq), and the solution was heated at reflux for 5 h. The reaction mixture was poured into water and extracted 4x with ethyl acetate. The combined organic phases were washed twice with brine, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was recrystallized from methanol-ethyl acetate, yielding 0.331g, 43%. Mp 161-162°C, pale amber needles. $^1$HNMR (400 MHz, DMSO- $d_6$) $\delta$ ppm: 12.10 (br s, 1H); 7.99 (d, 1 H, $J= 3.5$ Hz); 7.47 (s, 1H); 7.23 (s, 1H); 4.98 (d, 2H, $J= 2.5$ Hz); 3.88 (s, 3H); 3.65 (t, 1 H, $J= 2.5$ Hz).

**4-Chloro-6-Methoxy-7-(prop-2ynyloxy)quinazoline (5):** To a suspension of 4 (0.21g, 0.9 mmol, 1 eq) in 1,2-dichloroethane (10 mL) were added carbon tetrachloride (0.8 mL) and triphenylphosphine (0.52g, 2.0 mmol, 2.2 eq). The reaction mixture was warmed to 80°C and the temperature maintained until all starting material had been consumed. The solution was cooled, evaporated to dryness and the residue was purified by column chromatography on silica gel (hexane-ethyl acetate, 7:3). Depropargylated 4-chloro-6-methoxy-7-hydroxy-quinazoline
(0.060g, 30 %) eluted first, followed by 5 (0.135g, 60%). The product was recrystallized from isopropanol to give colorless needles. Mp 207-209 °C, $^1$HNMR (400 MHz, chloroform-d) δ ppm: 8.89 ( s, 1H); 7.52 (s, 1H); 7.44 (s, 1H); 4.95 (d, 2H, $J= 2.5$ Hz); 4.086 (s, 3H), 2.595 (t, 1H, $J= 2.5$ Hz).

4-[(3-Fluorophenyl)amino]-6-methoxy-7-(prop-2ynyloxy)quazoline hydrochloride (6): To isopropanol (3mL) were added 5 (0.050 g, 0.20 mmol, 1 eq) and 3-fluoroaniline (0.22 g, 2 mmol, 10 eq) and the resultant solution was heated at reflux for 3 h. The reaction mixture was cooled and the resultant precipitate was collected by filtration, washed with cold isopropanol and dried, yielding 6 (0.062g, 0.17 mmol, 85%). Mp (HCl salt) 259-262 °C, $^1$HNMR free base (400 MHz, chloroform-d) δ ppm: 8.69 (s, 1H); 7.73 ( dt, 1H); 7.42 (s, 1H); 7.36-7.25 (m, 2H); 7.10 (s, 1H); 6.83 (td, 1H); 4.91 (d, 2H, $J= 2.5$ Hz); 4.04 (s, 3H,); 2.58 (t, 1H, $J= 2.5$ Hz).

7-((1-(2-(2-(2-(4-nitrobenzo[c][1,2,5]oxadiazol-7-ylamino)ethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)-N-(3-fluorophenyl)-6-methoxyquinazolin-4-amine (14): Compound 6 (10.0 mg, 0.027 mmol, 1 eq) was dissolved in a 1:1 solution of t-BuOH/H$_2$O (500 µL) and additional water (300 µL), the solution was then added to a 2 dram vial. N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (10.6 mg, 0.027 mmol, 1 eq) was dissolved in a 1:1 solution of t-BuOH/H$_2$O (500 µL) and acetone (200 µL) to afford a bright orange solution which was added dropwise to the solution in the vial. The reaction mixture was stirred for 30 min at 40°C. Copper(II) sulfate pentahydrate (6.9 µL, 0.27 µmol, 0.01 eq) and (+)-sodium L-ascorbate (26.7 µL, 1.35 µmol, 0.05 eq) were added and the reaction mixture was heated at 40°C o/n. TLC in 10% MeOH/EtOAc showed product formation, however with SM still present. Another one equivalent of the catalysts were added and the
reaction mixture was stirred over the weekend. After which the reaction mixture was poured into a biphasic mixture of water (15 mL) and ethyl acetate (15 mL). The organic layer was washed with water (2x, 15 mL). The combined organics were dried over MgSO$_4$, filtered and concentrated to afford a red solid. The material was purified using column chromatography (10 gram column. The column was primed with hexanes, followed by a 0-10% MeOH/EtOAC gradient, affording a slightly oily red solid (12 mg, 63%). R$_f$ (10% MeOH/EtOAc) = 0.27;

$^1$H NMR (500 MHz, acetone-$d_6$) δ ppm: 1.56 - 1.64 (m, 4 H); 3.50 - 3.68 (m, 8 H); 3.84 - 3.86 (m, 2 H); 3.94 (s, 3 H); 3.95 - 3.97 (m, 2 H); 4.59 - 4.69 (m, 2 H); 6.44 - 6.50 (m, 1 H); 6.80 - 6.89 (m, 1 H); 7.34 - 7.42 (m, 1 H); 7.50 - 7.55 (m, 1 H); 7.58 - 7.62 (m, 1 H); 7.73 - 7.76 (m, 1 H); 8.01 - 8.07 (m, 1 H); 8.22 - 8.25 (m, 1 H); 8.45 - 8.51 (m, 1 H); 8.57 - 8.64 (m, 1 H); LC-MS: Rt= 1.94 min [MH]$^+$ = 704.7

7-((1-(2-(2-(2-(5-dimethylaminonaphthalene1-sulfonamide)ethoxy)ethoxy)-ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)-N-(3-fluorophenyl)-6-methoxyquinazolin-4-amine (15): Compound 6 (10.0 mg, 0.027 mmol, 1 eq) was dissolved in a combination of 1:1 solution of t-BuOH/H$_2$O (500 µL) and water (300 µL), the solution was then added to a 2 dram vial. N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-(5-dimethylamino)napthalene-1-sulfonamide (12.5 mg, 0.027 mmol, 1 eq) was dissolved in a 1:1 solution of t-BuOH/H$_2$O (500 µL) to afford a yellow solution which was added dropwise to the solution in the vial. The RM was stirred for 30 min at 40ºC. Copper(II) sulfate pentahydrate (6.9 µL, 0.27 µmol 0.1 eq) and (+)-sodium L-ascorbate (26.7 µL, 1.35 µmol. 0.05 eq) were added and the reaction mixture was heated at 40ºC o/n. TLC in 10% MeOH/EtOAc showed product formation, however with SM still present. Another equivalent of the catalyst was added and the reaction mixture was stirred
for three days, after which the reaction mixture was poured into a biphasic mixture of water (15 mL) and ethyl acetate (15 mL). The organic layer was washed with water (2x, 15 mL). The combined organics were dried over MgSO₄, filtered and concentrated to afford a red solid. The material was purified using column chromatography (10 gram column; the column was primed with hexanes, then a 0-10% MeOH/EtOAc gradient) to afford a slightly oily yellow solid (10 mg, 51%). R_f (10% MeOH/EtOAc) = 0.25; ^1HNMR (500 MHz, methanol-d₄) δ ppm: 1.92 (s, 2 H); 2.86 (s, 7 H); 2.99 - 3.02 (m, 2 H); 3.30 (d, J=5.37 Hz, 4 H); 3.50 (d, J=4.39 Hz, 2 H); 3.58 (d, J=3.42 Hz, 2 H); 3.89 - 3.91 (m, 2 H); 4.00 (s, 3 H); 4.60 - 4.63 (m, 2 H); 5.35 (s, 2 H); 6.87 - 6.93 (m, 1 H); 7.23 (d, J= 7.32 Hz, 1 H); 7.34 - 7.42 (m, 2 H); 7.50 - 7.58 (m, 3 H); 7.77 - 7.80 (m, 2 H); 8.16 (d, J= 7.32 Hz, 1 H); 8.20 (s, 1 H); 8.32 (d, J= 8.30 Hz, 1 H); 8.48 - 8.54 (m, 2 H).

LC-MS: Rt= 2.23 min [MH]^+= 774.9

7-((1-(2-(2-(2-(2-(2-(azidoethoxy)ethoxy)ethoxy)-tributylstannyl)benzamide)ethoxy)ethoxy)ethoxy)-ethyl)-1H-1,2,3-triazol-4-yl)methoxy)-N-(3-fluorophenyl)-6-methoxyquinazolin-4-amine (16):

Compound 6 (20.0 mg, 0.056 mmol, 1 eq) was dissolved in a 1:1 solution of t-BuOH/H₂O (1 mL). The solution was then added to a 2 dram vial. N-(2-(2-(2-(2-(azidoethoxy)ethoxy)ethoxy)-ethyl)-4-(tributylstannyl)benzamide (34 mg, 0.056 mmol, 1 eq) was dissolved in a 1:1 solution of t-BuOH/H₂O (1 mL) to afford a clear solution which was added dropwise to the solution in the vial. The reaction mixture was stirred for 30 min at 40°C. Copper(II) sulfate pentahydrate (13 µL, 0.56 µmol, 0.01 eq) and (+)-sodium L-ascorbate (53 µL, 2.75 µmol, 0.05 eq) were added and the RM was heated at 40°C o/n. TLC in 10% MeOH/EtOAc showed product formation, however SM still present. Another equivalent of the catalyst was added and the RM was stirred for three days. The reaction mixture was poured into a biphasic mixture of water (15 mL) and ethyl
acetate (15 mL). The organic layer was washed with water (2x, 15 mL). The combined organics were dried over MgSO₄, filtered and concentrated to afford a red solid. The material was purified using column chromatography (10 gram column; prime column with hexanes; 0-10% MeOH) to afford a slightly oily yellow solid (17 mg, 34%). Rf (10% MeOH/EtOAc) = 0.35; ¹H NMR (500 MHz, methanol-d₄) δ ppm: 0.89 (t, J=7.32 Hz, 9 H); 1.09 (s, 3 H); 1.24 - 1.27 (m, 3 H); 1.34 (dq, 8 H), 1.46 - 1.63 (m, 6 H); 3.55 - 3.60 (m, 11 H); 3.87 (t, J= 4.88 Hz, 2 H); 4.00 (s, 3 H); 4.12 (d, J= 7.32 Hz, 2 H); 4.56 (t, J= 4.88 Hz, 2 H); 5.37 (s, 2 H); 6.89 (td, J₁= 8.30, J₂= 2.44 Hz, 1 H); 7.35 - 7.39 (m, 2 H); 7.49 - 7.57 (m, 3 H); 7.68 - 7.73 (m, 2 H); 7.78 (s, 1 H); 8.22 (s, 1 H); 8.48 (s, 1 H); LC-MS: Rt= 3.39 min [MH]+ = 936.5

7-((1-(2-(2-(2-(4-iodobenzamide)ethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)-N-(3-fluorophenyl)-6-methoxyquinazolin-4-amine (17): Compound 16 (10.0 mg, 0.011 mmol, 1 eq) was dissolved in THF (1 mL) to which solid N-iodosuccinimide (NIS) (2.87 mg, 0.012 mmol, 1.1 eq) was added. The reaction mixture was stirred at RT for two hours. With the addition of NIS the solution changed from a clear solution to a milky yellow solution. TLC in 10% MeOH/EtOAc showed full conversion to product. The material was concentrated to give a yellow sticky solid. The material was purified using column chromatography (10 gram column; prime column with hexanes; 0-10% MeOH) to afford a slightly oily yellow solid (5 mg, 62%). Rf (10% MeOH/EtOAc) = 0.13; ¹H NMR (400 MHz, methanol-d₄) δ ppm: 3.50 (d, J=5.13 Hz, 2 H); 3.54 - 3.59 (m, 11 H); 3.86 (t, J= 5.13 Hz, 2 H); 3.98 (s, 3 H); 4.57 (t, J= 4.76 Hz, 2 H); 5.35 (s, 2 H); 6.83 - 6.91 (m, 1 H); 7.34 (s, 2 H); 7.49 (d, J= 8.79 Hz, 3 H); 7.70 - 7.77 (m, 4 H); 8.20 (s, 1 H); 8.47 (s, 1 H).

Tert-butyl 4-(3-hydroxypropyl)piperazine-1-carboxylate (18): 1-Boc-piperazine (1.0
g, 5.36 mmol, 1 eq) was dissolved in ACN (25 mL), and potassium carbonate (0.963 g, 6.96 mmol, 1.3 eq) was added. The reaction mixture stirred for 30 min at RT, and 3-bromo-1-propanol (0.56 mL, 6.44 mmol, 1.2 eq) was added. The reaction mixture was heated to 60ºC o/n. The material was concentrated to afford a clear oil. The material was purified via a process called “salting out”. The material was dissolved in a minimal amount of acetone, to which a saturated solution of oxalic acid in acetone was added dropwise until a precipitate is formed. The white precipitate was cooled for 10 min, then filtered. The white needles like crystals were then de-salted via neutralization with saturated sodium bicarbonate. The material was washed with ethyl acetate (2x 25 mL). The combined organics were then dried over MgSO₄, filtered, and concentrated to afford white solid (1.003 g, 78%). ¹H NMR (400 MHz, chloroform-d) δ ppm: 1.44 (s, 9 H); 1.66 - 1.78 (m, 2 H); 2.45 (t, J= 4.40 Hz, 4 H); 2.60 (t, J= 5.50 Hz, 2 H); 3.35 - 3.48 (m, 4 H); 3.79 (t, J= 5.13 Hz, 2 H).

3-(4-(tert-butoxycarbonyl)piperazin-1-yl)propyl 4-methylbenzenesulfonate (19): Compound 18 (0.159, 0.650 mmol, 1 eq) was dissolved in DCM (3 mL) to which TEA (0.108 ml, 0.780 mmol 1.2 eq) was added. The reaction mixture stirred for 10 min at RT, and a solution of p-toluenesulfonyl chloride (0.136 g, 0.715 mmol, 1.1 eq) in DCM (1 mL) was added dropwise. The reaction mixture was stirred o/n at RT. The reaction mixture was quenched with 1N HCl until a neutral pH is reacted and diluted with DCM (10 mL) and washed with brine (2x, 15 mL). The organic layer was dried over MgSO₄, filtered, and concentrated to give a white sticky solid. The crude material was triturated with EtOAc and the solid obtained was used in further chemistry (60 mg) without further purification. Note that the product contains a sulfonamide by-product. ¹H NMR (400 MHz, chloroform-d) δ ppm: 1.45 (s, 9 H); 1.75 - 1.84 (m,
2H); 2.23 - 2.29 (m, 4H); 2.32 - 2.37 (m, 2H); 2.45 (s, 3H); 3.30 - 3.36 (m, 4H); 4.06 - 4.13 (m, 2H); 7.35 (d, J= 8.06 Hz, 2H); 7.79 (d, J= 8.06 Hz, 2H)

tert-Butyl-4-(3-(1-(3-fluorophenylamino)-6-methoxynaphthalen-7-yloxy)propyl)piperazine-1-carboxylate (20): Potassium carbonate (19 mg, 0.079 mmol, 1.1 eq) was added to a suspension of 4-(3-fluorophenylamino)-7-methoxyquinazolin-6-ol (20 mg, 0.0701 mmol, 1 eq) in DMF (1 mL). After the suspension was stirred for 10 min at RT, compound 19 (31.8 mg, 0.080 mmol, 1.15 eq) was added to the mixture. The material was heated to 95ºC o/n. The resulting material was concentrated and purified using flash chromatography (10 gram column; 0-15% MeOH/EtOAc) to afford a clear oil. This clear oil corresponds to the desired product, yet contains DMF (10%). The yield of the sample is 13.5 mg. 1H NMR (400 MHz, chloroform-d) δ ppm: 1.35 (s, 9H); 1.95 - 2.02 (m, 2H); 2.34 - 2.37 (m, 4H); 2.50 - 2.53 (m, 2H); 3.66 - 3.70 (m, 4H); 3.89 (s, 3H); 4.09 - 4.12 (m, 2H); 6.65 - 6.71 (m, 1H); 7.11 (s, 1H); 7.16 - 7.22 (m, 3H); 7.57 - 7.59 (m, 1H); 8.51 - 8.55 (m, 1H)

tert-Butyl-4-(2-(2-(2-(prop-2-ynyloxy)ethoxy)ethoxy)ethoxy)ethyl)piperazine-1-carboxylate (21): 1-BOC-piperazine (1.081 g, 2.79 mmol, 1 eq) was dissolved in ACN (40 mL) to which cesium carbonate (2.27 g, 6.9 mmol, 2.5 eq) was added. The reaction mixture stirred for 30 min at RT. After which, 2-(2-(2-(2-(prop-2-ynyloxy)ethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (0.520 g, 2.79 mmol, 1 eq) was added. The reaction mixture was heated to reflux o/n. The material was filtered and concentrated to afford a yellow oil. The material was purified via column chromatography (manual column; 25 g; 0-10% MeOH/DCM) to afford 21 as a yellow oil (0.525 g, 50%). 1H NMR (400 MHz, chloroform-d) δ ppm: 1.47 (s, 9H); 2.44 (t, J= 2.20 Hz, 4H); 2.60 (t, J= 5.50 Hz, 2H); 3.44 (t, J= 4.80 Hz, 4H); 3.61 - 3.73 (m, 14H); 4.21 (d, J= 2.20 Hz, 2H)
1-(2-(2-(2-(prop-2-ynyloxy)ethoxy)ethoxy)ethoxy)ethyl)piperazine (22): Compound 21 (0.402 g, 1 mmol, 1 eq) was dissolved in DCM (5 mL) to which TFA (2.5 mL) was added. The reaction mixture was stirred o/n at RT. The material was purified via column chromatography (15 g silica; 0-5% MeOH/DCM) to afford a yellow oil (0.297 g, 97%). $^1$HNMR (400 MHz, chloroform-$d$) δ ppm: 2.46 (s, 1 H); 2.86 (br. d, 1 Hz, 2 H); 3.05 (br. t, 4 H); 3.31 (br. t, 2 Hz, 4 H); 3.48 (s, 1 H); 3.60 - 3.71 (m, 14 H); 4.19 (d, $J=1.47$ Hz, 2 H)

3-(4-(2-(2-(2-(prop-2-ynyloxy)ethoxy)ethoxy)ethoxy)ethyl)piprazin-1-yl)propan-1-ol (23): Compound 22 (0.297 g, 1 mmol, 1 eq) was treated in the same manner as described for compound 18. After evaporation, 0.200 g of a sticky white solid was isolated, contaminated with unknown impurities that complicate the NMR spectra. The material was carried forward as crude material, since purification at this stage was problematic.

3-(4-(2-(2-(2-(prop-2-ynyloxy)ethoxy)ethoxy)ethoxy)ethyl)piprazin-1-yl)propyl 4-methylbenzenesulfonate (24): Compound 23 (0.358 g, 1 mmol, 1 eq) was treated in the same manner as described for compound 19. After evaporation, 0.120 g of a sticky white solid was isolated, contaminated with unknown impurities that complicate the NMR spectra. The material was carried forward as crude material, since purification at this stage was problematic.

6-(3-chloropropoxy)-N-(3-fluorophenyl)-7-methoxyquinazolin-4-amine (25): In a sealed test tube, compound 13 (0.150 g, 0.526 mmol, 1 eq) was dissolved in DMF (3 mL) to which potassium carbonate (0.363 g, 2.63 mmol, 5 eq) and 1-bromo-3-chloropropane (0.260 mL, 2.63 mmol, 5 eq) were added. The RM was heated to 40ºC for 2 hr and then stirred o/n at RT. The material was washed with water and extracted with EtOAc (3x, 15 mL). The combined organics were dried over MgSO$_4$, filtered, and concentrated. The material was purified using
column chromatography (20 g of silica gel; 1:1 DCM/EtOAc as eluent) to afford compound 13, as a sticky solid (0.104 mg). $^1$HNMR (400 MHz, chloroform-$d$) $\delta$ ppm: 2.32 (quin, $J$=5.86 Hz, 2 H); 3.78 (t, $J$= 6.23 Hz, 2 H); 3.95 (s, 3 H); 4.23 (t, $J$= 5.86 Hz, 2 H); 6.78 - 6.86 (m, 1 H); 7.17 - 7.38 (m, 4 H); 7.62 (br. s., 1 H); 7.73 (d, $J$= 11.72 Hz, 1 H); 8.69 (s, 1 H)
Chapter 3: Results and Discussion
3.1. Preparation of the 6/7 proparglyoxy derivatives of EGFR-TKI

Despite the extensive syntheses of 4-anilinoquinazolines found in the literature, there is only one example of the introduction of a propargyloxy group at either the 6 or 7 position. The method utilized to prepare the 6/7-benzyloxy quinazoline was a debenzylation approach to provide the precursor for propargylation. An alternative approach could be to prepare the dimethoxy quinazoline and subsequently demethylate to give the desired precursor. However, both of these methods either introduce an additional step or were found to be non-reproducible on the scale required. We chose to rise to the challenge of introducing the propargyl group at the beginning of the 6 step synthesis understanding that most of the subsequent steps would require alteration from previous literature procedures due to the sensitive nature of the propargyl group towards reductive and acidic agents.

There is no apparent advantage between substitution at the 6 and 7 position, therefore the synthesis of both isomers were performed in parallel during the program; this thesis describes the synthesis of the 7 position derivatives, as seen in scheme 3.1. Alkylation of methyl-3-methoxy-4-hydroxybenzoate using propargyl bromide proceeded in quantitative yield, affording 1. Utilizing stannic chloride and fuming nitric acid in DCM, the propargyl ether was nitrated to give 2. These conditions were used as attempts using typical aryl nitration methodology (concentrated nitric acid in acetic acid at elevated temperatures), promoted hydration of the propargyl group. The nitro group was introduced in the 6-position as a result of steric hindrance and the para/ortho directing effects of the methylbenzoate and methoxy group. The subsequent reduction of the nitro-group to the amine was most successful with the most successful on a larger scale using sodium borohydride-nickel chloride and sodium dithionite, giving 3. Standard catalytic hydrogenation would not be applicable as it would result in the simultaneous reduction of the
propargyl group. Alternative approaches were explored including stannous chloride\textsuperscript{27}, sodium dithionite\textsuperscript{28}, raney nickel-hydrazine\textsuperscript{29}, zinc dust\textsuperscript{26}, and sodium borohydride-nickel chloride\textsuperscript{30}.

The resulting aniline was cyclized to give a quinzaolin-4-one scaffold using formamide-ammonium formate. The initial attempt, using thionyl chloride and DMF for the dehydrochlorination to yield 4, resulted in consumption of starting material to provide fluorescent byproducts which was confirmed by NMR. Since acid sensitivity may have lead to depropargylation, a more mild and non-acidic condition, (carbon tetrachloride-triphenylphosphine) was used. Similarly, we found depropargylated material, however, the generation of this byproduct was temperature dependent. Temperatures above 60ºC resulted in decomposition of the product. In addition, it was found that additional triphenylphosphine was needed to drive the reaction to completion as the SM were partial hydrates. The milder condition achieved the desired transformation to afford 5. The last step of the synthesis was a $S_nAr$ displacement of the chlorine with 3-fluoroaniline in isopropanol to yield propargloxy anilinoquinazoline, 6, as an HCl salt, in 85% yield.
Scheme 3.1: Synthesis of 6-Propargyloxy-4-(3-fluoroanilino)quinazoline.

Alongside the approach described above that carried the propargyloxy sidechain from the earliest part of the synthesis, we also prepared the materials through more conventional method, proceeding through the 7-O-benzyl intermediate. Using literature procedures we were able to synthesize 4-(3-Fluoroanilino)-6-methoxy-7-benzyloxy quinazoline with a moderate overall yield of 35% over 6 steps. Deprotection of the benzyl group using TFA and subsequent alkylation with propargyl bromide afforded the desired product, 6. It is important to note, deprotection prior to the placement of the aniline group proved to be unsuccessful, as hydrolysis of the chloroquinazoline functionality to give the quinazlinone also occurred. In the end, the published O-benzyl method proved to be higher yielding and more reproducible despite the additional step.
Scheme 3.2: Preparation of 6-linked O-Benzyl-4-(3-fluoroanilino)quinazoline.

3.2. Preparation of the [3+2] conjugated anilinoquinazoline and radio-imaging probes

Click chemistry is often perceived as a particular reaction (Huisgen [3+2] cycloaddition), but in actuality it is a philosophy which describes criteria for the generation of diverse molecules from simple and smaller units, similar to the biological process of synthesizing proteins from amino acids. The criteria advocates for simple reactions conditions, wide applicability, stereospecificity, etc.\textsuperscript{31} Proceeding under mild conditions with simple purification procedures, it is no surprise that Cu(I)-catalyzed alkyne-azole click reactions (aka Huisgen [3+2] cycloaddition) have become the staple “click reaction” for the formation of covalent bonds between two diverse building blocks.\textsuperscript{31} Both azide and alkynes are chemically benign in most
organic and biological environments and quite stable in terms of decomposition. The stability, specifically the kinetic stability, is one reason why these reactions often involve slight heating and long reaction times, as noted in the experimental section.\textsuperscript{31} The reaction itself is a 1,3 dipolar cycloaddition involving an azide and alkyne to give a substituted 1,2,3 triazole. The substitution pattern is dependent on the catalyst used; copper will exclusively give the 1,4 regioisomer, the preferred isomer due to steric factors.\textsuperscript{31} The triazole is also a unique heterocyclic compound, as it somewhat mimics an amide bond yet it has enhanced hydrogen bonding capabilities due to the increased dipole moment.\textsuperscript{31} With all of these factors combined, the Huisgen [3+2] cycloaddition was found to be the most elegant yet simple method to synthesize diverse EGFR-TKI based radio-imaging probes.

TKI compound 6 and a complementary azide were combined using a “click” reaction, seen in Scheme 3.3 using copper(II) sulfate pentahydrate and (+)-sodium L-ascorbate, to give three imaging probes, all in moderate yield. The (+)-sodium L-ascorbate acts as a reducing agent to reduce the copper (II) sulfate pentahydrate to produce Cu(I) \textit{in situ}. This approach is most commonly used, as the reaction using Cu(I) directly is often unpredictable. The azide components of these reactions were prepared by Professor Hanson using an azide polyethylene glycol amine and the respective imaging agents to provide RNH-XIV-51, RNH-XIV-55, RNH-XIV-57. The azide polyethylene glycol amine can be synthesized from commercial tetra-polyethylene glycol and through subsequent series of displacements to afford the precursors.
Scheme 3.3: Preparation of EGFR-TKI radio-imaging probes using Huisgen [3+2] cycloaddition.

The reaction was followed using TLC as fluorescent dyes allowed for convenient visualization on silica gel under the UV lamp, while the stannyl derivative was detectable using an iodine stain. Characterization of the final compounds was completed using various methods including MS, and $^1$HNMR. Mass spectral analysis showed the desired product was present, and $^1$HNMR revealed the triazole proton peak, appearing near 9 ppm. This was evidence the desired compound was synthesized, as both starting materials were pure. Below is Figure 3.4 depicting a
stacking of NMR plots of the starting materials and the desired product, with the triazole proton clearly present.

Figure 3.4: Stacking of the $^1$HNMR of the showing a distinct triazole peak at 9 ppm.

The stannyl derivative 16 was converted to the corresponding iodo derivative, as shown in Scheme 3.4 via halodestannylation using NIS. The material was instantly converted to the iodo derivative evident by the a new lower $R_f$ spot corresponding to the desired product. The resulting NMR of the isolated product was devoid of the alkyl peaks associated with the tributyl-stannane group. In addition, the observed aromatic pattern of the benzyl group, that of a 1,4 disubstituted benzene, indicates the desired product was made, rather than a destannylation product.
Scheme 3.4: Dehalostannylation of compound 16.

3.3. Alternative linkers for EGFR-TKI radio-imaging probes

The rationale behind the synthesis of the functionalized alternative linkers is to develop a more divergent approach compared to the [3+2] cycloaddition method, allowing for alterations in chain length, heteroatom, and radio-imaging modality applicable to a library synthesis for SAR development. There has yet to be an established optimal chain length. It is believed that a tetraethylene glycol is long enough to reach the solvent accessible area, however, modifications will be performed to find the best length. Studies have shown that anilinoquinazolines with basic side chains show greater binding affinity due to increased solubility, bioavailability, and reduced protein binding. As a result, piperazine was the basic heterocycle utilized when making this series of linkers. It was also placed using the same spacing seen with the morpholine of
gefitinib away from the anilinoquinazoline core. In addition, in a study in which they compared several N-heteroatom substituted anilinoquinazolines, the pKa of piperazine was shown to be 7.9-5.4, similar to morpholine with a pKa of 7.2-5.2. Furthermore, solubility was also evaluated, showing that piperazine increased the aqueous solubility at pH 7.4 by 3-fold compared to morpholine. Interestingly, triazole was also evaluated, showing a pKa of 5.3 and a solubility of about 100-fold less than piperazine. This validates that the possible use of other linkers which may provide better physicochemical properties, perhaps increasing binding and allowing for a better visualization of tissue morphology.

For the initial synthesis of the linker, it was envisioned that piperazine should be introduced to the anilinoquinazoline core first, allowing for the subsequent introduction of various polyethylene glycol chain lengths. Alkylation of 1-Boc-piperazine with 3-bromo-1-propanol gave 18. The product was purified as the oxalic acid salt, conveniently converted back to the free base. The 7-hydroxy-4-anilinoquinazoline was then reacted with 18 under Mitsunobu conditions unsuccessfully. Despite several attempts to optimize the procedure, e.g. changing the order of addition, and the use of polymer based DIAD, numerous inseparable products were detected including side products assumed to be triphenylphosphine oxide and several DIAD decomposition compounds. However, the major compound was believed to be piperazine homocoupling based on MS data. The MS did not detect any product, although that is not to say there was not any, as it could have been fragmented or was extremely small. Nonetheless, it was apparent that any successfully made product would be extremely hard to separate.
Scheme 3.5: Attempted Mitsunobu coupling to link the aniline-quinazoline core to the N-heteroatom linker

Literature precedent revealed that the Mitsunobu is not the most efficient pathway to achieve the desired transformation. Though a more elegant method, the yield was moderate compared to the high-yielding alkylation. To perform the alkylation, 18 was converted to the corresponding tosylate giving 19. The tosylate was then used to alkylate 13 using conditions involving potassium carbonate and DMF. The alkylation proved to be successful by NMR to afford 20. The drawback to this procedure is that DMF was still present even after purification and that the yield was low, possibly due to product loss during the aqueous workup. Despite these drawbacks this reaction showed that alkylation was a successful pathway in coupling the heteroatom to the anilinoquinazoline.
Throughout this series of reactions, it was noticed that solubility of the core anilinoquinazoline was quite poor in most organic solvents. Some of the reactions required co-solvents to completely dissolve the starting material. Knowing this, it was obvious that the reaction strategy needed to be altered in order to use the anilinoquinazoline in the last steps to prevent any solubility issues leading to poor yields. Therefore the alkyne linked tetraethylene glycol chain was coupled to the Boc-piperazine using typical alkylation conditions. Tetraethylene glycol was used, as it is twice the length of the chain of erlotinib, which is suspected not be long enough to reach the solvent accessible site. The resulting compound 21 was then deprotected, alkylated and subsequently tosylated to give 24. This compound was used to alkylate 13 using the same alkylation conditions (potassium carbonate and DMF). However this reaction was not successful in this case. Attempts were also performed using the same starting materials, dioxane, and NaH. NaH was added to create the alkoxide ion allowing for a stronger nucleophile. However, this reaction also proved to be unsuccessful. Possible reasons may be the purity of compound 24. It was quite difficult to purify this linker via column chromatography due to its polarity. Unseparated impurities were carried through at every step. In addition, it was observed that the linker did not dissolve in DMF and required DCM for
solubility.

Pathway C: Alkylation Approach using alkyne linked PEG chain analog

![Scheme 3.7: Attempted alkylation of the aniline-quinazoline core to alkyne linker](image)

A different strategy was ultimately employed to obtain the desired product. This pathway involved alkylation of the hydroxy group of the anilinoquinazoline, converting it to a chloropropoxy derivative, compound 25. Subsequent nucleophilic displacement of the aliphatic chlorine using compound 22 and KI in dioxane also proved to be unsuccessful in obtaining the desired product. The linker utilized was from the same batch as the previous failed attempts. This suggests the linker may be reason why these attempts have yielded no product.

Pathway D: Nucleophilic Substitution

Scheme 3.8: Nucleophilic substitution used as a method to couple the anilino-quinazoline core to alkyne linker.
Chapter 4: Conclusions and Future Directions
4.1 Conclusions

This chapter presents the various approaches towards the synthesis of diagnostic EGFR-TKI linked radio-imaging probes. The [3+2] conjugation approach was shown to be successful for the divergent synthesis of three diagnostic probes (14, 15, 17). On the other hand, the synthesis of the alternatively linked EGFR-TKI probes proved to be more challenging than anticipated. To this point, the synthesis did not achieve the same level of success as the previous approach, mainly in part to impure linkers. However, despite this obstacle, the alkylation step provided evidence that this approach has the potential to ultimately achieve the desired coupling between the anilinoquinazoline and alternate linkers. It is envisioned that the alkylation approach would then allow for a more versatile library synthesis of EGFR-TKI radio-imaging probes with higher binding affinity.

4.2 Significance of Research

Diagnostic EGFR-TKI linked radio-imaging probes are an important contribution to the field of cancer treatment. Currently, the FDA approved TKI inhibitors provide about a 40-50% response rates in patients with challenges in establishing dosing and monitoring of disease progression. These imaging probes were designed to allow for rapid and objective identification of patients who would benefit from ErbB-targeted therapy while allowing others to try alternative therapies. In addition, optimal dosing could be evaluated using these probes allowing for maximum inhibition of EGFR. Furthermore, disease progression can be monitored with these agents providing timely intervention if resistance occurs. By providing a tool by which doctors and clinicians can rapidly evaluate patient sensitivity towards ErbB-targeted therapy, we can improve the patient’s chance of survival.
4.3 Future Directions

Several EGFR-EKI imaging probes were successfully synthesized and will be evaluated in proof-of-concept studies. First, the biological activity of these compounds will be evaluated with the focus on binding affinity. It is envisioned that the fluorescent dyes (compound 14 and 15) could then be viewed under fluorescent microscopy to obtain the initial information about the biodistribution and metabolism following in vivo experiments. If the information provided reveals that these agents contain the characteristic properties of an ideal imaging probe, the “cold” iodo compound would be used to establish the parameters for SPECT imaging followed by the synthesis of the “hot” imaging agent.

It is anticipated that these compounds may not have the binding affinity needed for imaging which is why the alternative approaches to linker synthesis were performed in parallel. An alternative pathway to increase binding affinity elsewhere in the scaffold includes derivatizations of the anilino group. Literature precedent suggests that compounds with a functionalized anilino group (such as lapatinib) bind more tightly to the receptor as the extended sidechain binds to a hydrophobic pocket present in a DFG out configuration of EGFR. If all these alterations do not provide a binding profile consistent with an ideal imaging probe, other scaffolds of EGFR-TKI need to be evaluated as possible new templates for imaging probes.
References:


Supplemental Data
Figure A-I: Compound 6

Pulse Sequence: 42ps
Solvent: CDCl3
Ambient temperature: 25°C
Magnet: 400 MHz "proton"

Relax delay: 1.000 sec
Pulse: 3.4 degrees
Acq. Time: 1.325 sec
Width: 6.92 ± 0.2 Hz

DOB: 3 repetitions

Residual: 0.149 ppm
Data Processing:
Line broadening: 6.2 Hz
RT: 1000
Total time: 49 min, 59 sec

H22 H26

H35, H37

H29, H30

H31, H32

H23, H24

H38

MeOH

H25, H26

H37
Figure A-2: Compound 14
Figure A-4: Compound 16