I. The Utility of Activation Domain Mimics as Targeted Inhibitors of Transcription

II. The Design and Implementation of a Peer-Led Module in Practical Research Ethics

by

Christopher Taylor

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Doctoral Committee:

Professor Anna K. Mapp, Chair
Professor E. Neil G. Marsh
Professor John Montgomery
Assistant Professor Matthew B. Soellner

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Chapter I

Introduction

A. Project Overview

Transcription, in which information stored in the coding regions of the cells DNA is converted into RNA is a fundamental cellular process.[1] As such, it is highly regulated in healthy cells. Aberrant transcription is a hallmark of cancer,[2,3,4] and is also associated with a host of other pathologies such as metabolic,[5,6] inflammatory[7,8] and neurological disorders.[9,10] The proteins responsible for recruiting the transcriptional machinery to the promoter, transcriptional activator proteins, contact their target proteins, coactivators, through transcriptional activation domains that are often relatively short contiguous groups of amino acids.[11,12] Because of the central role that transcription plays in regulating cell function and fate, the ability to cause a targeted increase or decrease in particular transcription programs has tremendous potential as both a therapeutic approach, and as a tool for biochemical investigations.

The design or discovery of ligands, particularly small molecule ligands, that target transcription has proven difficult and only a handful of such molecules are known.[12,13] Prominant obstacles include the promiscuous binding profile of many transcriptional activation domains, the low to moderate affinities of activator-coactivator interactions, and the lack of structural data, or even the identity of, the biologically relevent coactivators in most circumstances. High throughput screening

against the well characterized coactivators CBP and p300,[14,15] or against particular phenotypes, such as the death of cells which depend on overexpression of a particular gene,[16] have seen isolated successes. However, at the time this work was initiated, a general design rationale for small molecule transcriptional inhibitors had not emerged.

The project described in this dissertation grew out of the hypothesis that small molecules which mimic activation domains can be used as scaffolds for the development of transcriptional inhibitors. In this work, I will discuss the potential of small molecules that mimic the activation domain of the transcriptional activator ESX to inhibit the transcription of erbB2, an oncogene whose expression is mediated by ESX.[17,18,19] This will be followed by the description of a generalizable strategy for overcoming some of the limitations of small molecule transcriptional inhibitors.

The final portion of this thesis concerns the important issue of education in research practices. A growing body of work and suggests the widespread use of questionable practices by research scientists.[20,21,] This is supported by the accounts of outright fraud and sabotage involving graduate students and post-doctoral researchers have appeared in the scientific press.[22,23,24] Data from the National Research Council, and interviews with those involved indicates that these incidents are connected to percieved pressure on those involved. As part of a collaboration with another graduate student – Dr. Amy Danowitz – I designed, wrote, and lead a discussion module for incoming graduate students about research ethics, data interpretation, and conflict management.

B. Introduction to Transcription

Transcription is the process by which the information stored in the protein-coding regions of a cell's chromosomal DNA is converted to RNA, in case of proteing coding genes, messenger RNA (mRNA).[1] The information contained in the sequence of this mRNA is then used by the ribosome to synthesize protein with a particular sequence of amino acids. As protein synthesis is one of the principle ways in which cells regulate their internal processes, transcription plays a fundamental role in determining cell function and fate.[2,3]

The actual process of transcription involves the carefully orchestrated recruitment of a series of proteins and protein complexes to the target gene. Early in this process, transcriptional activator proteins localize to the DNA, usually in the promoter region just upstream of their target gene. These transcriptional activator proteins then recruit the other proteins or protein complexes required to initiate transcription.[1] The details of this process, however, are unclear. The direct targets of activators and the order and manner in which they are recruited remains an area of active investigation and debate.[12]

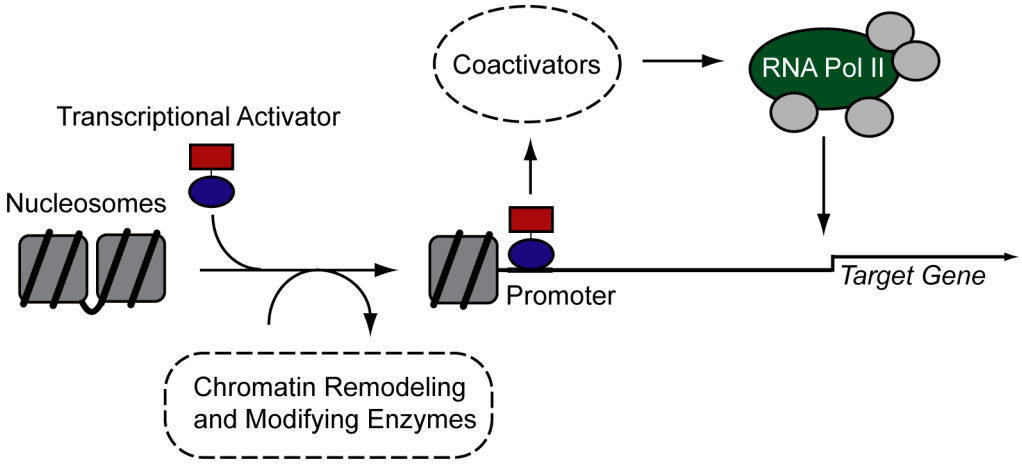


Figure I-1. Initiation of transcription by transcriptional activator proteins. The activator protein binds to a recognition site and recruits proteins or protein complexes that alter the chromatin structure to allow access to the target gene. The activator than recruits coactivators that in turn recruit RNA Pol II and the rest of the transcriptional machinery needed to for the Pre-Initation Complex (PIC) which can begin transcription.

The prevaling model suggests that activators must recruit multiple proteins or protein complexes in order to facilitate both promoter accessibility and formation of the pre-initation complex (PIC) that includes RNA Polymerase II (RNA Pol II), which carries out mRNA synthesis.[25,26] The first of these function is needed because most chromosomal DNA is stored as chromatin, DNA wound up around protein complexes called histones that render the DNA more compact and less accessible.[27] Protein complexes such as Swi/Snf and SAGA act to alter chromatin structure and location, making the target gene more accessible.[28,29] A second class of coactivators act as scaffolds that link transcription activators and the transcriptional machinery. This includes members of the mediator complex,[30,31] which is known in many cases to act as a bridge between DNA bound activator proteins and RNA Polymerase II. There appear to be some unifying features, such as the fact that recruitment of mediator is

generally required for the activation of transcription. However most of the details, such as the particular protein-protein contacts made or even the order of recruitment, are unknown for most transcriptional activators.

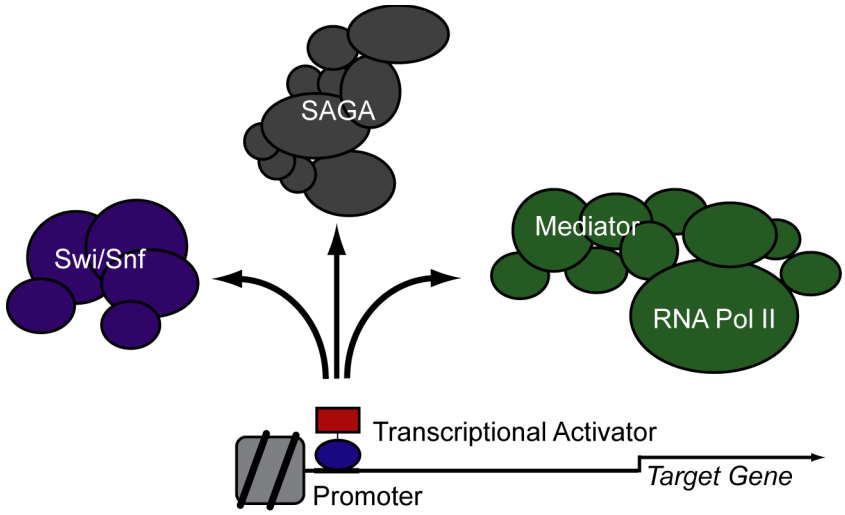


Figure I-2. Possible interacting partners of transcriptional activator proteins. In most cases, the biologically relevant binding partners of a particular activator are disputed or unknown. The current model suggests that activators must make contact with multiple proteins or protein complexes, likely incuding the three complexes shown here: Swi/Snf and SAGA are largely responsible for modifying histones or shifting their position in order to facilitate access to the promotor of the target gene, Mediator is responsible for recruiting and stabilizing RNA Pol II and the rest of the transcriptional machinery.

C. Structure and Function of Transcriptional Activators and Coactivators.

C.1. Transcriptional Activator Proteins

Transcriptional activators are modular proteins, minimally comprised of a DNA binding domain (DBD), which is responsible for localizing the activator to its cognate sequence in the promotor of its target gene(s), and one or more transcriptional activation domains (TADs) which are responsible for making contacts with other

members of the transcriptional machinery in order to initiate transcription.[1] The surprising degree of modularity found in transcriptional activators has been demonstrated in experiments where the activation domain of one transcriptional activator protein and the DNA binding domain of a different protein were shown to effectively initiate transcription.[1]

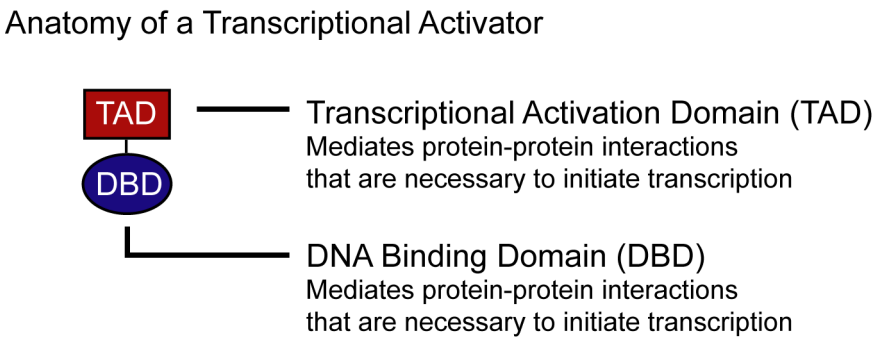


Figure I-3. Anatomy of a transcriptional activator. Activators are minimally composed of the two domains shown above.

In contrast to DNA binding domains, which often have well defined and crystalizable structures, transcriptional activation domains are generally poorly structured in solution and computational analyses indicate that they have a very high incidence of 'intrinsically disordered regions.[32] The largest class of activation domains – usually called 'acidic' or 'amphipathic' activation domains because of their interspersed arrangement of acidic and hydrophobic amino acids – are generally thought to form α-helices upon interaction with their coactivator targets.[12,33,34,35] In this model, the arrangement of amino acids in the TAD allows this domain to form a helix with a largely non-polar face for interacting with recognition surfaces on its target interaction partners, and a polar face that can remain solvent exposed. An example of this is the interaction between the activation domain of vp16 and the general

transcription factor TFIIH (figure I-4).[36]

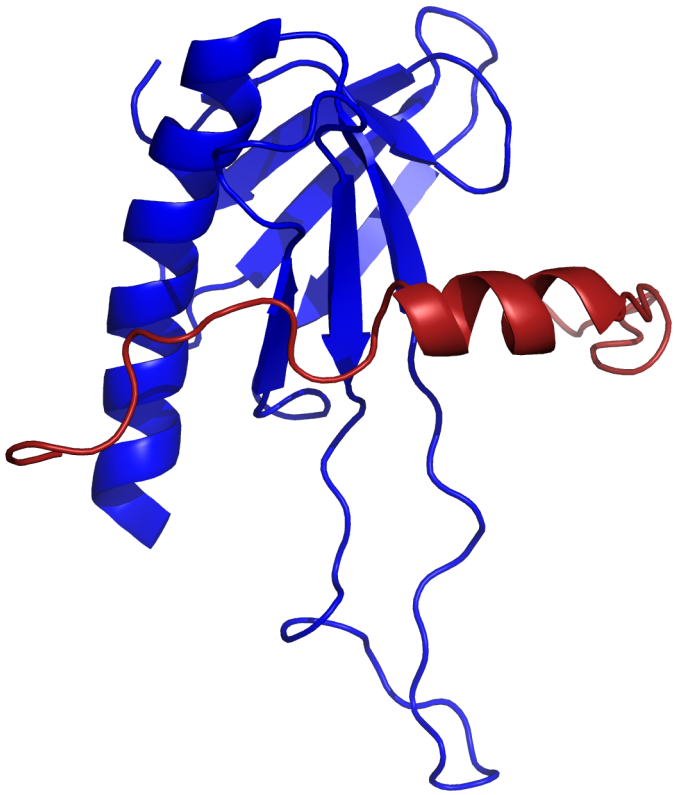


Figure I-4. Ribbon diagram of the VP16 activation domain. This activation domain (red, residues 412-490) is shown interacting with the tfb1 subunit of general transcription factor TFIIH (blue), resolved using NMR spectroscopy. PDB ID: 2k2u

Although the current model generally assumes a helix, and experimental results indicate at least some helical propensity for many activation domains, including vp16,[36,37] ESX,[38] p65,[39] and MLL and myb,[40] the degree to which most activators domains form perfectly canonical helices is unknown. It has been shown that individual activation domains are capable of interacting with multiple, non-homologous co-activators,[41,42] indicating that lock and key type rationales are an inadequate way to conceptualize activator-coactivator interactions. It has been proposed that the relatively unstructured nature of these domains in solution may play an important role in their function by allowing them to sample a wide range of

conformations and to facilitate interaction with the different proteins needed to initiate transcription.[32] For example, the potent activator VP16, which is often used in yeast as a model for activation in eukaryotes, has been seen to interact with or recruit a variety of proteins, including p300 and TFIID,[41] mediator subunit 15,[43] mediator subunit 25,[37] TFIIH,[36] and a combination fo TBP, TFIIB and the SAGA complex.[42]

In addition having promiscuous binding profiles, it has also been shown that many activator-coactivator interactions have affinities ranging from mid nanomolar to low micromolar. For example, a study of the affinities of the activation domains of the yeast activators Gal4 and Gcn4, as well as the human activator p53, and the viral activators HIV-TAT and VP16 for a variety of transcriptional coactivators found that none had binding affinities tighter than 100nM.[44] This is supported by a more recent analysis of the yeast activators Gcn4 and Gal4, and the viral activator VP16 found that even when bound to DNA, their dissociation constants for the coactivator mediator subunit 15 (Med 15) ranged from 100-320 nM.[43] The overall picture suggested by these facts is one in which activators form transient, moderate affinity interactions with a series of coactivators in order to recruit the necessary transcriptional machinery.

Although the DNA binding and transcriptional activation modules are the minimal requirements for a transcriptional activator, other structural features can be present. These often include regulatory elements reponsible for mediating activity and interaction with regulatory proteins or dimerization partners. For example, NFκβ subunit p65 contains a Rel-homology domain that, in addition to acting as a DNA binding domain, mediates contacts with Iκβα – an regulatory factor that inactives the DNA binding and nuclear localization functions of the RHD and holds p65 in the

cytosol in an inactive state until signals are received for its release.[45] The RHD also serves as a dimerization domain, allowing p65 to dimerize with p50 in order to effectively bind its consensus sequence in the promoter of its target genes.[46] Dimerization in conjunction with DNA binding is a common regulatory motif in transcriptional activation, which provides an additional level of control for the cell to selectively active particular genes controlled by a given activator.

C.2. Transcriptional Co-activator Proteins

Two well defined coactivators are the paralogs CBP and p300, which have largely overlapping functions within the cell.[47] Both CBP and p300 have multiple activator binding domains, each of which is known in turn to interact with as many as 20 different activators.[48] These two coactivators likely serve several roles in transcriptional activation. Because CBP/p300 are known to bind such a large number of transcriptional activators, and are also known to interact with general transcription factors such as TFIIB, it is thought the CBP/p300 may act as a bridge between targeted transcriptional activators and general elements of the transcriptional machinery.[49] Additionally, CBP/p300 are known to possess both intrinsic histone hcetyltransferase (HAT) activity.[50] This activity appears to be important for transcriptional activation, as ablation of HAT activity using small molecule inhibitors[51] or depletion of the cofactor CoA reduces the activation in systems which rely on CBP/p300 as a coactivator.[41] Finally, CBP/p300 also appear to be able to acetylate certain activators or even themselves, thereby regulating the timing and order of coactivator recruitment.[41,52]

An essential component of the transcriptional machinery that is contacted directly by transcriptional activators is the mediator complex. Mediator is unique to eukaryotes, and is largely conserved between yeast and mammals.[30] Although the structure and composition of the mediator complex appears to depend at least partially on promoter context, the complex is generally held to include 26 subunit proteins, with a total size of approximately 1.2 MDa.[30] Although a complete picture of the role of the mediator complex during transcription remains elusive, it is thought that the complex acts as a scaffold that, once recruited by transcriptional activator proteins, serves to mediate the recruitment and removal of the cofactors necessary for mRNA synthesis and termination of the finished mRNA transcript.[25,30,53] Mediator is instrumental in both the recruitment and the stabilization of RNA Pol II and its associated proteins at the promoter.[31,53]These functions are mediated by extensive contacts between RNA Pol II and the 'head' and 'middle' regions of the mediator complex.[30] The tail region of mediator is the least conserved between yeast and mammals. Although this region is dispensable for basal transcription, its loss renders genes unresponsive to transcriptional activator proteins indicating that the tail region is responsible for making the contacts that allow mediator to be recruited by activator proteins.[30]

D. Small Molecules and Peptidomimetics that Target Transcription

D.1. Potential Utility of Agents that Target Transcription

Given the importance of transcription as a cellular process, the association

between mis-regulated transcription and the development of diseased states in unsurprising. Cancer is commonly linked to the over-expression of one or more proteins that promote uncontrolled cell growth.[3,4] One such example is the trans-membrane tyrosine kinase erbB2, the over-expression of which is responsible for a particularly aggressive type of breast cancer.[54] In another, the transcription factor HIF-1α is responsible for mobilizing the cellular response to hypoxia, is over-expressed in many cancers, including those of the brain, mammary tissue and reproductive organs, where it is associated with increased patient mortality.[55] In addition to specific examples such as these, there is the general characterization of cancer as a disease of mis-regulated gene expression, in which healthy patterns of expression are disrupted, leading to core oncogenic attributes such as uncontrolled proliferation,[56,18] and repression of apoptosis.[57,7] Thus, interventions that target transcriptional processes could prove to be potent therapeutic tools for treating cancer. In principle, the ability to selectively up- or down-regulate particular genes or sets of genes could be used to activate tumor suppressive programs, or to reverse oncogenic phenotypes.

D.2 Challenges in the Development of Agents that Target Transcription

In spite of the potential usefulness of biomolecules or synthetic agents that target transcription, the obstacles to the development of a general and effective strategy for the exogenous control of transcription are formidable. On the one hand protein and (to a lesser extent peptide) based interventions offer the potential for a high degree of affinity and selectivity, but the obstacles to delivery include cell uptake and subcellular localization, and dosing issues such as oral availability.[58,59] On the

other hand, although the therapeutic use of small molecules to affect intracellular processes is well established, the design of small molecules that target transcription is a challenge in itself. The surfaces of protein-protein interactions are generally much larger than the surface area of most small molecules.[60,61] This may be especially problematic in cases such as transcription, where many of the interacting surfaces are relatively unstructured, rather than well-defined structures that fit into deep binding clefts. Furthermore, other than CBP/p300, little definitive information is available about the structures of most coactivators, which are largely composed of intrinsically disordered regions.[32] In many cases, the relevant coactivator targets of a given transcriptional activator are not known, and in cases like CBP/p300 where particular domains are targeted by many different activators, it raises serious questions about even the theoretical possibility for activator-specific inhibitors. For example, the CH1 domain of CBP is known to interact with at least 11 different activation domains, including NF-κβ subunit p65, the metabolic regulator HNF-4, the tumor suppressor p53, the epithelial specific growth inducing transcription factor ets-1, and HIF-1α, which mobilizes the cell's response to hypoxia and promotes the growth and vascularization of tumors.[48,62] It is therefore likely that an inhibitor that binds to the CH1 domain will inhibit the ability of any of these transcriptional activators to initiate transcription. A close example of this would be the small molecule chetomin, which perturbs the structure of the CH1 domain. Although it was identified as an inhibitor of HIF-1α mediated transcription, it has been shown to inhibit the activity of a Gal4-Stat2 construct.[63]

From a biological point of view, the potential for specificity for a single gene may be even more remote, but such exacting selectivity may be unnecessary. Because

any given activator or coactivator will probably be necessary for more than a single target gene, any intervention that targets the transcriptional machinery may directly affect many genes. This lack of perfect specificity for a single gene may not turn out to be a problem, as many activators regulate functionally related clusters of genes. Indeed, it may be beneficial. Recent developments with therapeutic combinations[64,65,66] support the idea that affecting several related systems is a more effective way to treat pathology that targeting a single cellular event – such as the activity of a single kinase, or the expression of a single gene. The redundancy of signaling networks (and the adaptability of pathologies such as cancer) mean that individual perturbations can often be overcome.

D.3. Initial Advances in the Design of Synthetic Activation Domain Mimics

Initial work in the design of synthetic agents that modulate transcription has focused largely on the design of synthetic activators. For these to be effective, they must possess the same minimal function as transcriptional activator proteins – namely, a DNA binding domain and a transcriptional activation domain. Non-biomolecule DNA binding domains have been develop using large polyamides made up of imidazole, pyrrole, and hydroxypyrrole subunit that are designed to bind to the minor groove of double-stranded DNA in a sequence specific manner.[67,68]

Most of the artificial activation domains known are peptides, or are based on peptide like polymers. Artificial activators are often identified by screening against known binding partners of transcriptional activators. For example, the Kodadek lab was able to identify potent artificial peptidic activation domains that function in yeast

by carrying out phage display against Gal80, a repressor protein that is known to bind to the yeast activator Gal4.[69] This same lab has also identified potent peptoid based transcriptional activators based on a screen against the KIX domain of CBP.[70] In another example, peptides were screened for the ability to bind to the yeast coactivator Med15.[71] In all of these example, peptides or peptoids that functioned as activation domains were identified, but success at identifying small molecules that act as activation domain mimics has been more rare.

D.4. Small Molecule Inhibitors of Transcription

Although a handful of small molecules that target activator-coactivator interactions have been identified in spite of the difficulties outlined in section D2, these molecules have mostly been developed using screens against the well characterized coactivators CBP and p300. For example, KG-501 was identified through an NMR based screen, in which the ability of a library of molecules to interact with the KIX domain of the coactivator CBP was assessed.[14] This molecule inhibits the CREB-CBP interaction with a Ki of approximately 90 μM, and interferes with transcription of a luciferase reporter under the control of CREB and the production of NR4A2 (a CREB target gene) mRNA, both at concentrations between 5 and 10 μM. The fungal metabolite Chetomin[63] was also identified as a potential transcripional inhibitor based on its ability to interfere with the interaction between a minimal HIF-1α binding region of the CH1 domain of CBP, and a 41 residue peptide derived from the activation domain of HIF-1α. Although chetomin is highly effective at disrupting this interaction and has prominant effects on the ability of Gal4- HIF-1α

to activate a reporter gene at concentrations as low as 5nM, these activity appears to be caused by the ability of chetomin to disupt the structure of CBP.

There are examples of inhibitors discovered using methods that do not depend on detailed knowledge of a coactivator. ICG-001, an inhibitor of β-catenin mediated transcription, was identified from a screen against β-catenin activity in a reporter system. ICG-001 reduces expression of a β-catenin controlled reporter gene, and the transcription of known β-catenin target gene *survivin* at concentrations as low as 10 μM.[15] The molecule dubbed 'wrenchnolol' was optimized from a lead molecule identified using a high throughput screen in which the ability of compounds to kill erbB2 overexpressing cells was assessed. Wrenchnolol appears to reduce erbB2 protein levels at 7 μM, and GST-pulldowns indicate that one of the cellular proteins that wrenchnolol interacts with is the putative ESX coactivator Med23.[16] Although these molecules validate the potential of small molecules to act as transcriptional inhibitors, they do not provide a generalizable design rationale.

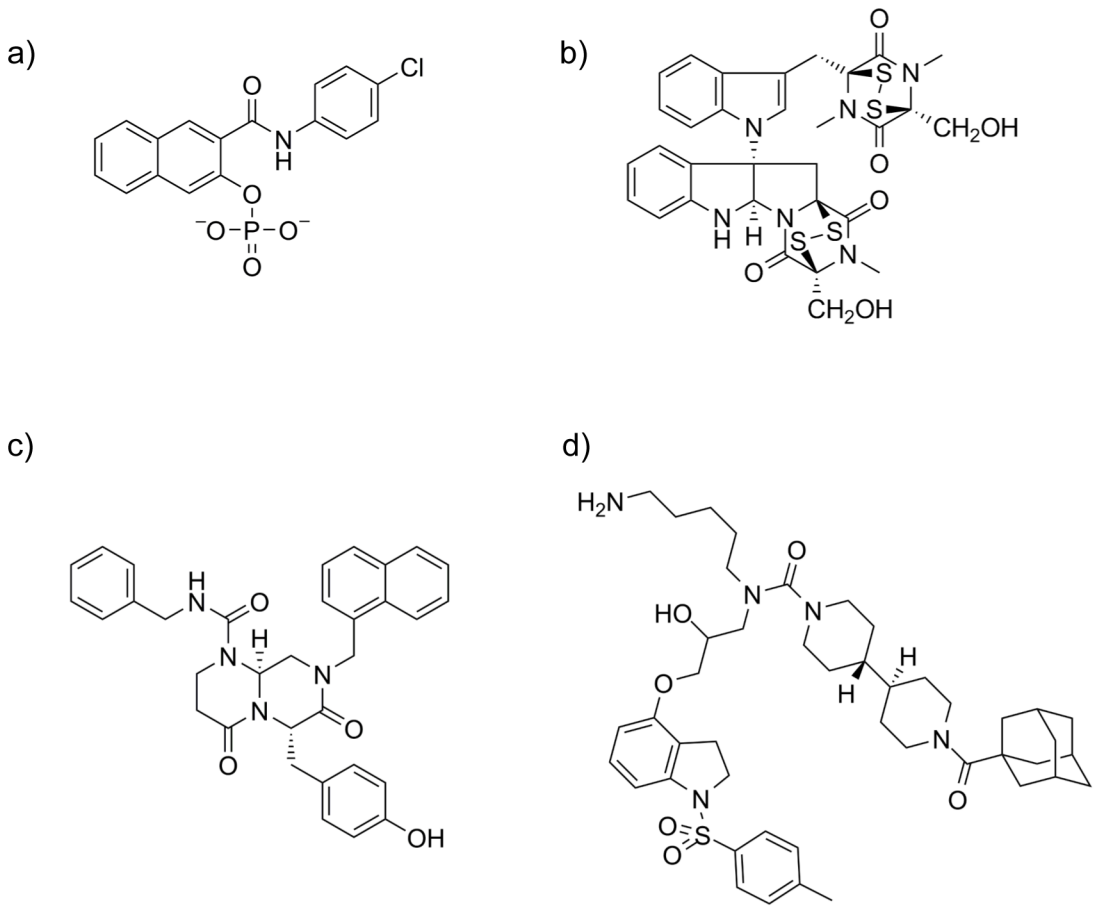


Figure I-5. Small molecule transcriptional inhibitors identified from high throughput screens. (a) KG-501 also interacts with CBP, but reduces CREB driven transcription. (b) Chetomin, which acts by destabilizing the CH1 domain of p300 (c) ICG-001 which interferes with β-catenin mediated transcription by blocking the activator binding site on CBP. (d) Wrenchnolol affects erbB2 expression by blocking a putative interaction between the transcription factor ESX and Med23.

D.5. The Development of Isoxazolidine-Based Activation Domain Mimics

Based on the model of activation domains as short, amphipathic sequences responsible for recruiting coactivators to the promoter, previous research in the Mapp lab identified isoxazolidines, such as **I-1** as potential activation domain mimics. These

molecules present amphipathic functionality in an approximately helical arrangement. In order to test the ability of these molecules to act as activation domain mimics, they were attached to a known ligand of the glucocorticoid receptor ligand binding domain (GR LBD). This allowed the isoxazolidine **I-1** to activate transcription of reporter genes controlled by a Gal4-GR LBD fusion protein in a dose dependent manner in mammalian cells.[72]

In support of their activity as activation domain mimics, these molecules appear to interact with the coactivator CBP in a manner similar to that of the natural activation domains MLL and Jun. Chemical shift perturbation of ^{1}H-^{15}N HSQC spectra of the KIX domain of CBP upon addition of **I-2** indicate that this molecule interacts with a large subset of the residues that interact with MLL and Jun, in effect, binding to the same site on CBP.[73] The ability of these molecules to mimic the interactions of natural TADs indicated that they would be effective as scaffolds for the development of transcriptional inhibitors.

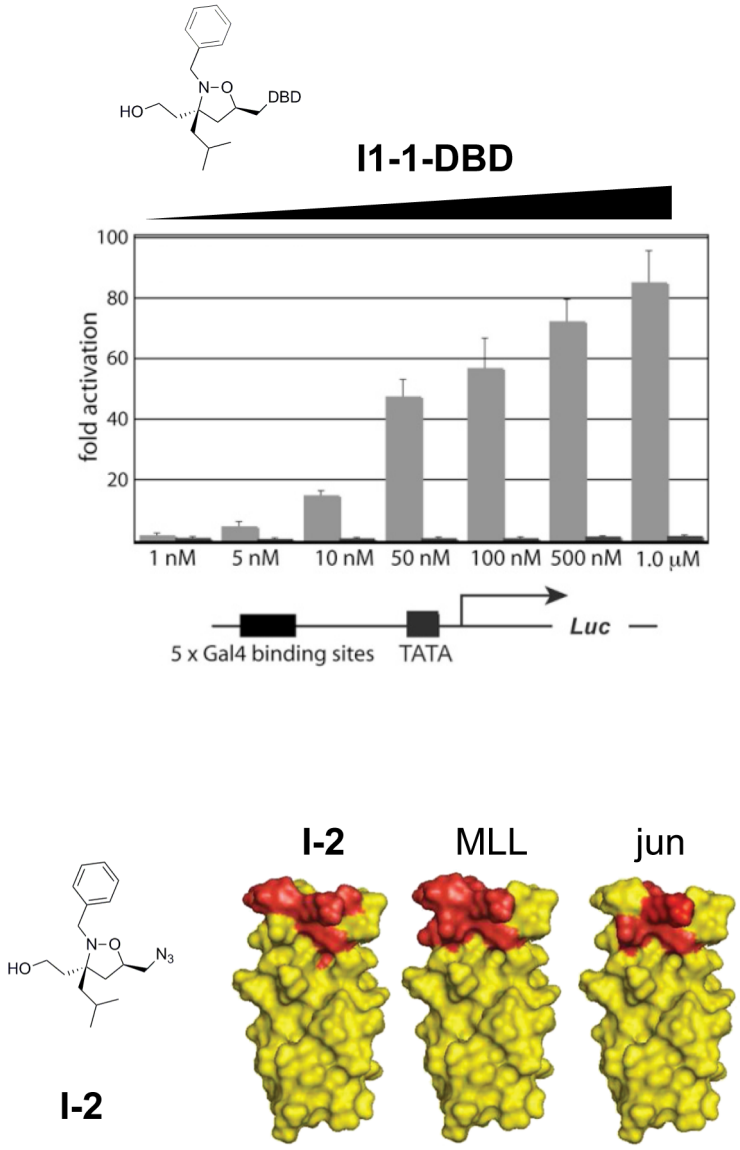


Figure I-6. Development of an isoxazolidine based TAD. Isoxazoldine-DBD conjugate **I-1** (DBD = oxidized dexamethasone) activates transcription in a dose dependent manner in mammalian cells. **I-2** interacts with the same region of the KIX domain of the coactivator CBP as the natural activation domains MLL and Jun. Figures from Buhrlage, S.J. et al. *ACS Chemical Biology* **2009**, *4*, 335-345 and Rowe, S. P. et al *J. Am. Chem. Soc.***2007**, *129*, 10654-10655. Used with permission.

E. The Regulation of the ErbB2 oncogene by ESX and the Role of the ErbB2 Protein

E.1. Biological Role and Clinical Relevance of the ErbB2 Protein

The model system chosen to test our rationale for inhibitor design was ESX driven overexpression of erbB2. ErbB2 (Her2) is a transmembrane tyrosine kinase growth receptor that is overexpressed in approximately 25% of breast cancers,[54] where it has been shown to drive an aggressive phenotype marked by more rapid metastasis and shorter life expectancy than breast cancers which do not over-express erbB2.[56,74,75] Furthermore, erbB2 over-expressing (erbB2+) cancer cells are known to undergo growth arrest and cell death if erbB2 expression is suppressed.[76,77,78]

ErbB2 drives oncogenesis through its interactions with other member of the erbB family of growth factor receptors. In addition to erbB2 this family also includes erbB1 (EGFR), erbB3 and erbB4. These receptors undergo ligand induced dimerization, forming hetero- or homo- dimers that undergo a series of autophosphorylation events, followed by transphosphorylation that allows the dimer to initiate cellular signaling programs.[79] ErbB1 and erbB3 both have known ligands that induce them to form dimers. In contrast, erbB2 is putatively ligandless.[75] This is supported by the observation that the conformation of the non-functional ligand binding domain of erbB2 is similar to the conformation of activated, ligand-bound domain of erbB1.[80] Perhaps because of this, erbB2 occupies a unique place in the erbB family. ErbB2 is known to form active heterodimers with both erbB1 and erbB3, which have been shown to strongly activate the Ras/Raf/MAPK (erbB2/erbB1)[81] and PI3K/Akt (erbB2/erbB3)[82] pathways. Although erbB1/erbB1 and erbB1/erbB3 dimers

are also possible, erbB2 appears to be the preferred dimerization partner for both of the proteins. ErbB2 containing heterodimers initiate stronger signaling responses, and are more resistant to endocytosis and proteolysis.[83,84,75]

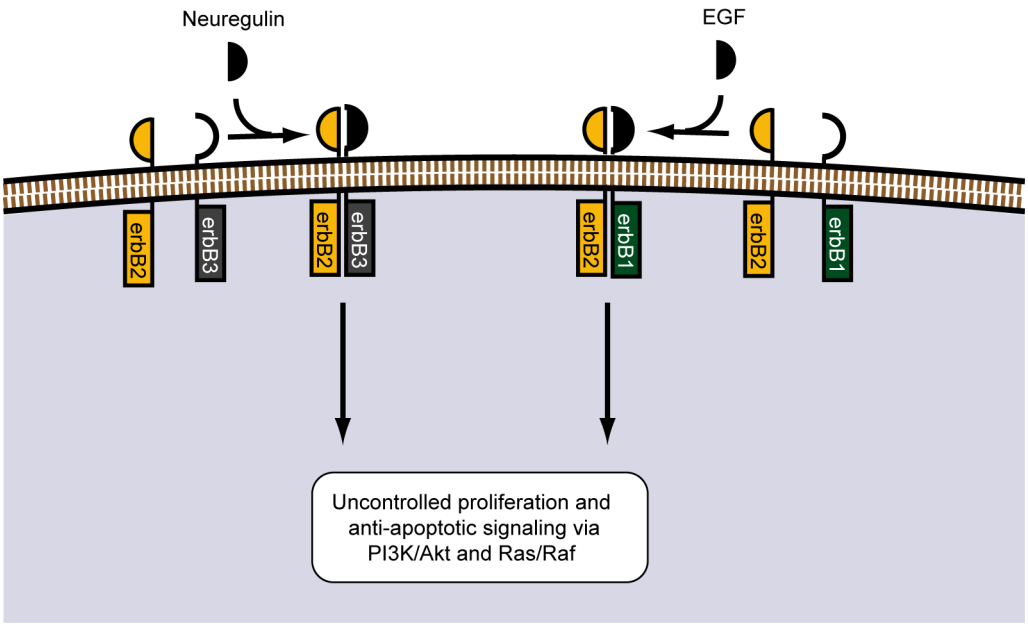


Figure I-7. ErbB2 is the preferred dimerization partner for erbB signaling. ErbB2 forms heterodimers with erbB1 and erbB3 that activate Ras and PI3K/Akt signaling pathways.

The role of erbB2 in the initiation and progression of cancer has been evaluated both phenotypically and biochemically. Clinically, patients with erbB2 overexpressing tumors have shorter life expectancy, are more likely to develop resistance to their chemotherapy and to experience relapse than patients with non erbB2+ breast cancers.[74,85] In both murine and cell culture models, induction of erbB2 overexpression is sufficient to transform previously non-tumorigenic cells.[86,75] Subsequently, cessation of erbB2 overexpression either leads cells to revert to an untransformed phenotype,[86] or to growth inhibition and death of erbB2+ tumors.[87,78]

E.2. Regulation of ErbB2 Transcription

The primary regulator of erbB2 overexpression appears to be the ets family transcriptional activator ESX.[18,17,19] The minimal activation domain of ESX that functions in a reporter assay when fused to a Gal4 DNA binding domain is $ESX_{129-159}$.[38] Within this region, mutagenesis indicates that residues 137-SWIIELLE-144 appear to be a hotspot which are essential to activity. Mutation of either Trp138, or either the leucines or isoleucines abrogated interaction with coactivator mediator subunit 23 (Med23) a putative target of ESX. Analysis of the NOE spectrum of this region indicates helicity,[38] and mapping the region 137-SWIIELLE-144 on to a helical wheel produces an amphipathic display, with a clearly defined hydrophobic surface for protein-protein interactions. As is discussed in later chapters, small molecules which mimic the hydrophobic surface of this activation domain are effective at curbing ESX driven erbB2 expression in erbB2+ cancer cells.

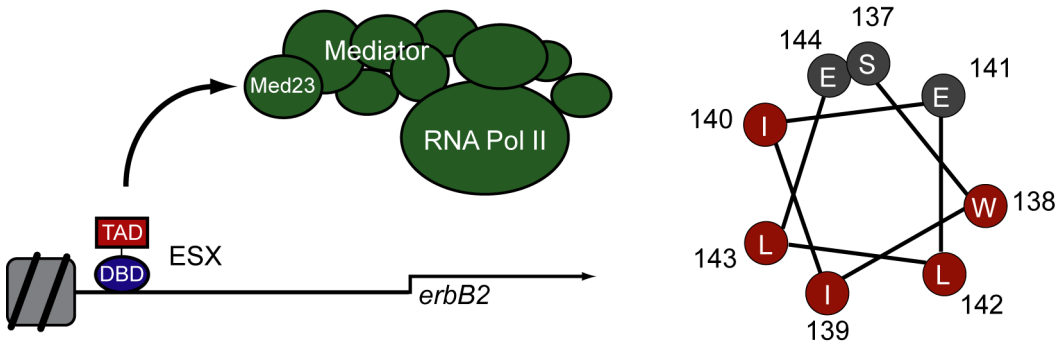


Figure I-8. The transcriptional activator ESX regulates transcription of erbB2. There is evidence that interaction between the TAD of ESX and the mediator subunit Med23 plays an important role in this process. The key region of the activation domain of ESX is thought to be the eight residue section 137-SWIIELLE-144. This region is putatively helical, and mapping it onto a helical wheel suggests the presence of a nonpolar surface for interacting with coactivator proteins such as Med23.

F. Research Misconduct in the Sciences

F.1. Misconduct Defined

Science, as a cumulative and often cooperative undertaking, relies on a reasonable amount of trust between different researchers. In the absence of such trust, the exchange of information and the dissemination of results that are essential to the present research paradigm become suspect and ultimately useless. Accordingly, funding agencies actively discourage research misconduct. The Office of Research Integrity, which is also responsible for investigating alleged misconduct and administering penalties to offenders, defines research misconduct in terms of falsification, fabrication, and plagiarism.[88]

> Research misconduct means fabrication, falsification, or plagiarism in proposing, performing, or reviewing research, or in reporting research results. (a) Fabrication is making up data or results and recording or reporting them. (b) Falsification is manipulating research materials, equipment, or processes, or changing or omitting data or results such that the research is not accurately represented in the research record. (c) Plagiarism is the appropriation of another person's ideas, processes, results, or words without giving appropriate credit. (d) Research misconduct does not include honest error or differences of opinion.

Although these definitions are generally used when misconduct is discussed, they are not comprehensive. They are not generally interpreted to include activities such as sabotage, the misrepresentation of experimental protocols, or the use of research designs that are known to be inadequate or inappropriate.

F.2. Occurrence of Misconduct

There are numerous well-document and highly publicized cases of research misconduct. These include extensive forgeries such as those of Hendick Schon at Bell labs,[89] in which Dr. Schon was found to have fabricated the data used in a series of papers published in several journals, including both Nature and Science. A more recent example of misconduct comes from a research lab at Columbia University,[90,23] which involved some 21 counts of, fabrication and falsification by a graduate student and lead to the retraction of 6 papers. Another recent example, albeit one that does not fit the technical definition of misconduct, took place at the University of Michigan.[22] In this case, it was determined that a post-doctoral researcher had been sabotaging a graduate students research for a period of several months, with total damages estimated at approximately $70,000. There is an emerging consensus in the literature that these cases represent only the 'tip of the iceberg' and that there are many instances that go unreported.[20,21,91]

The prevalence of research misconduct has been evaluated by administering anonymous surveys, in which researchers are asked about their own conduct and that of their colleagues. Although there is variation in the estimated frequency of misconduct depending on the definitions and methods used, the data from surveys consistently indicate a surprisingly high rate of questionable or inappropriate conduct. In an analysis of approximately 3,000 NIH funded researchers, Martinson et al.[91] determined that although only 0.3% of their respondents admitted to 'Falsifying or cooking research data,' 15% admitted to 'dropping observations or data points from analyses based on a gut feeling that they were incorrect,' and 13.5% admitted to using

inadequate or inappropriate research designs. A meta analysis of the literature on misconduct[20] concludes that the falsification rates reported by Martinson are probably artificially low, in part because the language used asks specifically about falsification and fabrication (which researchers are reluctant to admit to) rather than particular behaviors that fall under these categories. The overall results from the meta-analysis indicate that approximately 2% of respondents admit to having falsified or fabricated at least one piece of data themselves, while 15% had knowledge of a coworker who has done so. Frequency of other questionable practices (such as using inappropriate research designs, or reporting intentionally incomplete methodological data) were approximately 30% for self-admission, and 70% for observed behavior of coworkers. At this point, the causes of the discrepancy between the self-reported rates of misconduct and the rates of known or observed misconduct are largely speculative. In addition to the obvious explanation – that researchers are reluctant to admit to misconduct even under anonymous conditions – it is possible that the rates of known or observed misconduct include events that are suspected but unproven, or multiple reportings of single events or persons known to many researchers. Despite this caveat, data such at these indicate that researchers often act in ways that damage the implicit trust and openness that is part of the ethos of modern science.

G. Summary of Thesis

In Chapter II, I discuss advances that target the activity of transcriptional activator proteins by using small molecules designed to mimic a particular activation domain, thereby interfering with the ability of the native transcriptional activator

protein to initiate transcription of its target genes. The model system in which these advances are demonstrated is transcription of the oncogene erbB2, which is a well established driving force in breast cancers. Chapter III describes the development and application of a strategy that overcomes some of the inherant potency and selectivity limitations of using a small molecule to target the protein-protein interactions that regulate transcription. As a meta-research issue, Chapter IV is concerned with the development and implementation of a workshop designed to provoke discussion of good research practices among incoming graduate students. Appendix A details initial work on a project that will use peptidomimetics to inhibit the activity of NFκβ subunit p65, a second transcriptional activator protein which plays a key role in both inflammation and cancer progression. Finally, Appendix B describes the development of a novel methodology for the syntheses of alleneamides, which provide a versatile precursor to densely functionalized heterocycles such as those used for the design of small molecules that target components of the transcriptional machinery.

H. References

(1) Ptashne, M.; Gann, A. *Genes and Signals*; 1st edition (November 13, 2001) ed. Cold Spring Harbor Laboratory Press.
(2) Vogelstein, B.; Kinzler, K. W. Cancer genes and the pathways they control. *Nat Med* **2004**, *10*, 789-799.
(3) Albertson, D. G. Gene amplification in cancer. *Trends in Genetics* **2006**, *22*, 447-455.
(4) Hanahan, D.; Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **2011**, *144*, 646-674.
(5) Finck, B. N.; Kelly, D. P. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *Journal of Clinical Investigation* **2006**, *116*, 615.
(6) Feige, J. N.; Auwerx, J. Transcriptional coregulators in the control of energy homeostasis. *Trends in cell biology* **2007**, *17*, 292–301.
(7) Bassères, D. S.; Baldwin, A. S. Nuclear factor-κB and inhibitor of κB kinase

pathways in oncogenic initiation and progression. *Oncogene* **2006**, *25*, 6817-6830.
(8) Hoffmann, A. Natoli, G.; Ghosh, G. Transcriptional regulation via the NF-κB signaling module. *Oncogene* **2006**, *25*, 6706-6716.
(9) Thiel, G. Lietz, M.; Cramer, M. Biological activity and modular structure of RE-1-silencing transcription factor (REST), a repressor of neuronal genes. *Journal of Biological Chemistry* **1998**, *273*, 26891.
(10) Zuccato, C. Belyaev, N. Conforti, P. Ooi, L. Tartari, M. Papadimou, E. MacDonald, M. Fossale, E. Zeitlin, S. Buckley, N.; others Widespread disruption of repressor element-1 silencing transcription factor/neuron-restrictive silencer factor occupancy at its target genes in Huntington's disease. *The Journal of neuroscience* **2007**, *27*, 6972.
(11) Majmudar, C. Y.; Mapp, A. K. Chemical approaches to transcriptional regulation. *Current Opinion in Chemical Biology* **2005**, *9*, 467-474.
(12) Mapp, A. K.; Ansari, A. Z. A TAD Further: Exogenous Control of Gene Activation. *ACS Chem. Biol.* **2007**, *2*, 62-75.
(13) Lee, L. W.; Mapp, A. K. Transcriptional Switches: Chemical Approaches to Gene Regulation. *Journal of Biological Chemistry* **2010**, *285*, 11033-11038.
(14) Best, J. L. Amezcua, C. A. Mayr, B. Flechner, L. Murawsky, C. M. Emerson, B. Zor, T. Gardner, K. H.; Montminy, M. Identification of small-molecule antagonists thatinhibit an activator:coactivator interaction. *Proceedings of the National Academy of Sciences* **2004**, *101*, 17622–17627.
(15) Emami, K. H. Nguyen, C. Ma, H. Kim, D. H. Jeong, K. W. Eguchi, M. Moon, R. T. Teo, J.-T. Oh, S. W. Kim, H. Y. Moon, S. H. Ha, J. R.; Kahn, M. A small molecule inhibitor of B -catenin /CREB-binding protein transcription. *Proceedings of the National Academy of Sciences* **2004**, *101*, 12682–12687.
(16) Shimogawa, H. Kwon, Y. Mao, Q. Kawazoe, Y. Choi, Y. Asada, S. Kigoshi, H.; Uesugi, M. A Wrench-Shaped Synthetic Molecule that Modulates a Transcription Factor−Coactivator Interaction. *J. Am. Chem. Soc.* **2004**, *126*, 3461-3471.
(17) Eckel, K. L. Tentler, J. J. Cappetta, G. J. Diamond, S. E.; Gutierrez-Hartmann, A. The Epithelial-Specific ETS Transcription Factor ESX/ESE-1/Elf-3 Modulates Breast Cancer-AssociatedGene Expression. *DNA and Cell Biology* **2003**, *22*, 79-94.
(18) Chang, C.-H. Scott, G. K. Kuo, W.-L. Xiong, X. Suzdaltseva, Y. Park, J. W. Sayre, P. Erny, K. Collins, C. Gray, J. W.; Benz, C. C. ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. *Oncogene* **1997**, *14*, 1617 - 1622.
(19) Allander, S. V. Illei, P. B. Chen, Y. Antonescu, C. R. Bittner, M. Ladanyi, M.; Meltzer, P. S. Expression Profiling of Synovial Sarcoma by cDNA Microarrays. *American Journal of Pathology* **2002**, *161*, 1587-1595.
(20) Fanelli, D. How many scientists fabricate and falsify research? A systematic review and meta-analysis of survey data. *PLoS One* **2009**, *4*, e5738.
(21) Sovacool, B. K. Exploring Scientific Misconduct: Isolated Individuals, Impure Institutions, or an Inevitable Idiom of Modern Science? *Bioethical Inquiry* **2008**, *5*, 271-282.
(22) Maher, B. Sabotage! *Nature* **2010**, *467*, 516-519.
(23) Service, E. Columbia Lab Retracts Key Catalysis Papers. *Science* **2006**, *311*,

1533.
(24) Couzin, J. Truth and consequences. *Science* **2006**, *313*, 1222.
(25) Biddick, R. K. Law, G. L. Chin, K. K. B.; Young, E. T. The Transcriptional Coactivators SAGA, SWI/SNF, and Mediator Make Distinct Contributions to Activation of Glucose-repressed Genes. *Journal of Biological Chemistry* **2008**, *283*, 33101-33109.
(26) Soutoglou, E. Coordination of PIC Assembly and Chromatin Remodeling During Differentiation-Induced Gene Activation. *Science* **2002**, *295*, 1901-1904.
(27) Bryant, G. O. Prabhu, V. Floer, M. Wang, X. Spagna, D. Schreiber, D.; Ptashne, M. Activator Control of Nucleosome Occupancy in Activation and Repression of Transcription. *Plos Biol* **2008**, *6*, e317.
(28) Dechassa, M. L. Zhang, B. Horowitz-Scherer, R. Persinger, J. Woodcock, C. L. Peterson, C. L.; Bartholomew, B. Architecture of the SWI/SNF-Nucleosome Complex. *Molecular and Cellular Biology* **2008**, *28*, 6010-6021.
(29) Wu, P.-Y. J. Ruhlmann, C. Winston, F.; Schultz, P. Molecular Architecture of the S. cerevisiae SAGA Complex. *Molecular Cell* **2004**, *15*, 199-208.
(30) Chadick, J. Z.; Asturias, F. J. Structure of eukaryotic Mediator complexes. *Trends in Biochemical Sciences* **2005**, *30*, 264-271.
(31) Taatjes, D. J. The human Mediator complex: a versatile, genome-wide regulator of transcription. *Trends in Biochemical Sciences* **2010**, *35*, 315-322.
(32) Fuxreiter, M. Tompa, P. Simon, I. Uversky, V. N. Hansen, J. C.; Asturias, F. J. Malleable machines take shape in eukaryotic transcriptional regulation. *Nat Chem Biol* **2008**, *4*, 728-737.
(33) Volkman, H. M. Rutledge, S. E.; Schepartz, A. Binding Mode and Transcriptional Activation Potential of High Affinity Ligands for the CBP KIX Domain. *J. Am. Chem. Soc.* **2005**, *127*, 4649-4658.
(34) Hermann, S. How Transcriptional Activators Bind Target Proteins. *Journal of Biological Chemistry* **2001**, *276*, 40127-40132.
(35) Majmudar, C. Y.; Mapp, A. K. Chemical approaches to transcriptional regulation. *Current Opinion in Chemical Biology* **2005**, *9*, 467-474.
(36) Langlois, C. Mas, C. Di Lello, P. Jenkins, M. Legault, P.; Omichinski, J. G. NMR Structure of the Complex between the Tfb1 Subunit ofTFIIH and the Activation Domain of VP16: StructuralSimilarities between VP16 and p53. *Journal of the American Chemical Society* **2008**, *130*, 10596–10604.
(37) Vojnic, E. Mourão, A. Seizl, M. Simon, B. Wenzeck, L. Larivière, L. Baumli, S. Baumgart, K. Meisterernst, M. Sattler, M.; Cramer, P. Structure and VP16 binding of the Mediator Med25 activator interaction domain. *Nat Struct Mol Biol* **2011**, *18*, 404-409.
(38) Asada, S. Choi, Y. Yamada, M. Wang, S.-C. Hung, M.-C. Qin, J.; Uesugi, M. External control of Her2 expression and cancer cell growth by targeting a Ras-linked coactivator. *Proceedings of the National Academy of Sciences* **2004**, *99*, 12747–12752.
(39) Schmitz, M. L. dos Santos Silva, M. A. Altmannl, H. Czischl, M. Holakl, T. A.; Baeuerle, P. A. Structural and Functional Analysis of the NF-KB p65 C Terminus. *Journal of Biological Chemistry* **1994**, *269*, 25613-25620.
(40) De Guzman, R. N. Goto, N. K. Dyson, H. J.; Wright, P. E. Structural basis for cooperative transcription factor binding to the CBP coactivator. *Journal of*

molecular biology **2006**, *355*, 1005–1013.
(41) Black, J. C. Choi, J. E. Lombardo, S. R.; Carey, M. A Mechanism for Coordinating ChromatinModification and Preinitiation Complex Assembly. *Molecular Cell* **2006**, *23*, 809-818.
(42) Hall, D. B. The VP16 Activation Domain Interacts with Multiple Transcriptional Components as Determined by Protein-Protein Cross-linking in Vivo. *Journal of Biological Chemistry* **2002**, *277*, 46043-46050.
(43) Wands, A. M. Wang, N. Lum, J. K. Hsieh, J. Fierke, C. A.; Mapp, A. K. Transient-state Kinetic Analysis of Transcriptional Activator{middle dot}DNA Complexes Interacting with a Key Coactivator. *Journal of Biological Chemistry* **2011**, *286*, 16238-16245.
(44) Melcher, K. The strength of acidic activation domains correlates with their affinity for both transcriptional and non-transcriptional proteins. *Journal of Molecular Biology* **2000**, *301*, 1097-1112.
(45) Scheidereit, C. IκB kinase complexes: gateways to NF-κB activation and transcription. *Oncogene* **2006**, *25*, 6685-6705.
(46) Hoffmann, A. Natoli, G.; Ghosh, G. Transcriptional regulation via the NF-κB signaling module. *Oncogene* **2006**, *25*, 6706-6716.
(47) Goodman, R. H.; Smolik, S. CBP/p300 in cell growth, transformation, and development. *Genes & development* **2000**, *14*, 1553.
(48) Dames, S. A. Martinez-Yamout, M. De Guzman, R. N. Dyson, H. J.; Wright, P. E. Structural basis for Hif-1a/CBP recognition in the cellular hypoxic response. *Proceedings of the National Academy of Sciences* **2002**, *99*, 5271.
(49) Vo, N.; Goodman, R. H. CREB-binding protein and p300 in transcriptional regulation. *Journal of Biological Chemistry* **2001**, *276*, 13505.
(50) Harton, J. A. The Histone Acetyltransferase Domains of CREB-binding Protein (CBP) and p300/CBP-associated Factor Are Not Necessary for Cooperativity with the Class II Transactivator. *Journal of Biological Chemistry* **2001**, *276*, 38715-38720.
(51) Mantelingu, K. Reddy, B. A. A. Swaminathan, V. Kishore, A. H. Siddappa, N. B. Kumar, G. V. P. Nagashankar, G. Natesh, N. Roy, S. Sadhale, P. P. Ranga, U. Narayana, C.; Kundu, T. K. Specific Inhibition of p300-HAT Alters Global Gene Expression and Represses HIV Replication. *Chemistry & Biology* **2007**, *14*, 645-657.
(52) Karanam, B. Wang, L. Wang, D. Liu, X. Marmorstein, R. Cotter, R.; Cole, P. A. Multiple Roles for Acetylation in the Interaction of p300 HAT with ATF-2 †. *Biochemistry* **2007**, *46*, 8207-8216.
(53) Malik, S.; Roeder, R. G. Dynamic regulation of pol II transcription by the mammalian Mediator complex. *Trends in Biochemical Sciences* **2005**, *30*, 256-263.
(54) Slamon, D. J. Godolphin, W. Jones, L. A. Holt, J. A. Wong, S. G. Keith, D. E. Levin, W. J. Stuart, S. G. Udove, J. Ullrich, A.; Press, M. F. Studies of the HER-2/neu Proto-Oncogene in Human Breast and Ovarian Cancer. *Science* **1989**, *244*, 707-712.
(55) Semenza, G. L. Evaluation of HIF-1 inhibitors as anticancer agents. *Drug Discovery Today* **2007**, *12*, 853-859.
(56) Burstein, H. J. The distinctive nature of HER2-positive breast cancers. *New England Journal of Medicine* **2005**, *353*, 1652–1654.

(57) Burstein, E.; Duckett, C. S. Dying for NF-κB? Control of cell death by transcriptional regulation of the apoptotic machinery. *Current Opinion in Cell Biology* **2003**, *15*, 732-737.
(58) Crews, C. M. Targeting the Undruggable Proteome: The Small Molecules of My Dreams. *Chemistry & Biology* **2010**, *17*, 551-555.
(59) Berg, T. Modulation of Protein–Protein Interactions with Small Organic Molecules. *Angewandte Chemie International Edition* **2003**, *42*, 2462 –2481.
(60) Keskin, O. Gursoy, A. Ma, B.; Nussinov, R. Principles of Protein−Protein Interactions: What are the Preferred Ways For Proteins To Interact? *Chem. Rev.* **2008**, *108*, 1225-1244.
(61) Fletcher, S.; Hamilton, A. D. Targeting protein–protein interactions byrational design: mimicry of protein surfaces. *Interface* **2006**, *3*, 215-233.
(62) Zhong, H. Voll, R.; Ghosh, S. Phosphorylation of NF-kB p65 by PKA Stimulates Transcriptional Activity by Promoting a Novel Bivalent Interaction with the Coactivator CBP/p300. *Molecular Cell* **1998**, *1*, 661–671.
(63) Kung, A. L. Zabludoff, S. D. France, D. S. Freedman, S. J. Tanner, E. A. Vieira, A. Cornell-Kennon, S. Lee, J. Wang, B. Wang, J. Memmert, K. Naegeli, H.-U. Petersen, F. Eck, M. J. Bair, K. W. Wood, A. W.; Livingston, D. M. Small molecule blockade of ranscriptional coactivation of the hypoxia-inducible factor pathway. *Cancer Cell* **2004**, *6*, 33-43.
(64) Borisy, A. A. Elliott, P. J. Hurst, N. W. Lee, M. S. Lehar, J. Price, E. R. Serbedzija, G. Zimmermann, G. R. Foley, M. A. Stockwell, B. R.; Keith, C. T. Systematic discovery of multicomponent therapeutics. *Proceedings of the National Academy of Sciences* **2003**, *100*, 7977–7982.
(65) Lehár, J. Krueger, A. S. Avery, W. Heilbut, A. M. Johansen, L. M. Price, E. R. Rickles, R. J. Short III, G. F. Staunton, J. E. Jin, X. Lee, M. S. Zimmermann, G. R.; Borisy, A. A. Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nat Biotechnol* **2009**, *27*, 659-666.
(66) Hopkins, A. L. Network pharmacology: the next paradigm in drug discovery. *Nat Chem Biol* **2008**, *4*, 682-690.
(67) Nickols, N. G. Jacobs, C. S. Farkas, M. E.; Dervan, P. B. Modulating Hypoxia-Inducible Transcription by Disrupting the HIF-1–DNA Interface. *ACS Chem. Biol.* **2007**, *2*, 561-571.
(68) Dose, C. Farkas, M. E. Chenoweth, D. M.; Dervan, P. B. Next Generation Hairpin Polyamides with (*R*)-3,4-Diaminobutyric Acid Turn Unit. *J. Am. Chem. Soc.* **2008**, *130*, 6859-6866.
(69) Han, Y.; Kodadek, T. Peptides Selected to Bind the Gal80 Repressor Are Potent Transcriptional Activation Domains in Yeast. *Journal of Biological Chemistry* **2000**, *275*, 14979 –14984.
(70) Liu, B. Alluri, P. Yu, P.; Kodadek, T. A Potent Transactivation Domain Mimic with Activity in Living Cells. *Journal of the American Chemical Society* **2005**, *127*, 8254-8255.
(71) Wu, Z. Belanger, G. Brennan, B. B. Lum, J. K. Minter, A. R. Rowe, S. P. Plachetka,, A. Majmudar, C. Y.; Mapp, A. K. Targeting the Transcriptional Machinery with Unique Artificial TranscriptionalActivators. *Journal of the American Chemical Society 125*, 12390-12391.
(72) Rowe, S. P. Casey, R. J. Brennan, B. B. Buhrlage, S. J.; Mapp, A. K. Transcriptional Up-regulation in Cells Mediated by a Small Molecule. *J. Am.*

Chem. Soc. **2007**, *129*, 10654-10655.
(73) Buhrlage, S. J. Bates, C. A. Rowe, S. P. Minter, A. R. Brennan, B. B. Majmudar, C. Y. Wemmer, D. E. Al-Hashimi, H.; Mapp, A. K. Amphipathic Small Molecules Mimic theBinding Mode and Function of EndogenousTranscription Factors. *ACS Chemical Biology* **2009**, *4*, 335-345.
(74) Slamon, D. J. Clark, G. M. Wong, S. G. Levin, W. J. Ullrich, A.; McGuire, W. L. Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene. *Science* **1987**, *235*, 177-182.
(75) Yarden, Y. Biology of HER2 and its importance in breast cancer. *Oncology* **2000**, *61*, 1–13.
(76) Gordon, L. I. Burke, M. A. Singh, A. T. K. Prachand, S. Lieberman, E. D. Sun, L. Naik, T. J. Prasad, S. V. N.; Ardehali, H. Blockade of the erbB2 Receptor Induces Cardiomyocyte Death through Mitochondrial and Reactive Oxygen Species-dependent Pathways. *Journal of Biological Chemistry* **2008**, *284*, 2080-2087.
(77) Chen, J.-S. Lan, K.; Hung, M.-C. Strategies to target HER2/neu overexpression for cancer therapy. *Drug Resistance Updates* **2003**, *6*, 129-136.
(78) Menendez, J. A. Vellon, L. Mehmi, I. Oza, B. P. Ropero, S. Colomer, R.; Lupu, R. Inhibition of fatty acid synthase (FAS) suppresses HER2/ neu (erbB-2) oncogene overexpressionin cancer cells. *Proceedings of the National Academy of Sciences* **2004**, *101*, 10715–10720.
(79) Schulze, W. X. Deng, L.; Mann, M. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol* **2005**, *1*, E1-E13.
(80) Garrett, T. P. J. McKern, N. M. Lou, M. Elleman, T. C. Adams, T. E. Lovrecz, G. O. Kofler, M. Jorissen, R. N. Nice, E. C. Burgess, A. W.; others The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Molecular cell* **2003**, *11*, 495–505.
(81) Hendriks, B. S. Quantitative Analysis of HER2-mediated Effects on HER2 and Epidermal Growth Factor Receptor Endocytosis: DISTRIBUTION OF HOMO- AND HETERODIMERS DEPENDS ON RELATIVE HER2 LEVELS. *Journal of Biological Chemistry* **2003**, *278*, 23343-23351.
(82) Stern, D. F. ERBB3/HER3 and ERBB2/HER2 Duet in Mammary Development and Breast Cancer. *J Mammary Gland Biol Neoplasia* **2008**, *13*, 215-223.
(83) Amin, D. N. Sergina, N. Ahuja, D. McMahon, M. Blair, J. A. Wang, D. Hann, B. Koch, K. M. Shokat, K. M.; Moasser, M. M. Resiliency and Vulnerability in the HER2-HER3 Tumorigenic Driver. *Science Translational Medicine* **2010**, *2*, 16ra7-16ra7.
(84) Roepstorff, K. Grøvdal, L. Grandal, M. Lerdrup, M.; van Deurs, B. Endocytic downregulation of ErbB receptors: mechanisms and relevance in cancer. *Histochem Cell Biol* **2008**, *129*, 563-578.
(85) Hirsch, F. R. Varella-Garcia, M.; Cappuzzo, F. Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. *Oncogene* **2009**, *28*, S32-S37.
(86) Drebin, J. A. Link, V. C. Stern, D. F. Weinberg, R. A.; Greene, M. I. Down-Modulation of an Oncogene Protein Product and Reversion of the Transformed Phenotype by Monoclonal Antibodies. *Cell* **1985**, *41*, 695-706.
(87) Agus, D. B. Akita, R. W. Fox, W. D. Lewis, G. D. Higgins, B. Pisacane, P. I. Lofgren, J. A. Tindell, C. Evans, D. P. Maiese, K.; others Targeting ligand-

activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* **2002**, *2*, 127–137.
(88) Office of Research Integrity Definition of Research Misconduct.
(89) Beasley, M. R. Datta, S. Kogelnik, H.; Kroemer, H. REPORT OF THE INVESTIGATION COMMITTEE ON THE POSSIBILITY OF SCIENTIFIC MISCONDUCT IN THE WORK OF HENDRIK SCHÖN AND COAUTHORS.
(90) Office of Research Integrity Case Summary - Bengu Sezen.
(91) Martinson, B. C. Anderson, M. S.; de Vries, R. Scientists behaving badly. *Nature* **2005**, *435*, 737-738.

Chapter II

Inhibition of ErbB2(Her2) Expression with Small Molecule Transcription Factor Mimics[a]

A. Chapter Overview

Developing molecules that interfere with the protein-protein interactions that initiate transcription using methods such as high throughput screening, or structure guided design based on crystallographic data has proven difficult, owing to the poorly understood and complex nature of these interactions.[1,2] Furthermore, it has been difficult to draw generalizable lessons from the rare examples identified through those approaches, because most of these successes[3,4,5,6] have been had with activators for which at least one protein-protein interaction is well characterized, which is not the case for most transcriptional activators.[7]

Based on the prevailing model that transcriptional activation domains tend to form alpha helices when interacting with their coactivator targets,[2,8] we hypothesized that it would be possible to use a small molecule scaffold that is known to act as a general transcriptional activator, and turn it into a transcriptional inhibitor by tailoring its

a Parts of this chapter appear in Lee, L. W.; Taylor, C. E. C.; Desaulniers, J. P.; Zhang, M.; Hojfeldt, J. W.; Mapp, A. K. Inhibition of ErbB2(Her2) expression with small molecule transcription factor mimics. *Bioorganic & medicinal chemistry letters* **2009**, *19*, 6233-6236.

functionality to more closely resemble a specific activation domain as depicted in Figure II-1. An isoxazolidine scaffold previously shown to mimic the activity and binding profile of a transcriptional activation domain[9,10,11] was altered to mimic that of the transcription factor ESX, which regulates expression of the erbB2 oncogene.[12,13,14] When used against erbB2 overexpressing cancer cells, these transcriptional inhibitors lead to decreased expression of the cell surface growth receptor ErbB2 (Her2) and curb the proliferation of erbB2 overexpressing cells. These results demonstrate a versatile rationale for the design of small molecule transcriptional inhibitors.

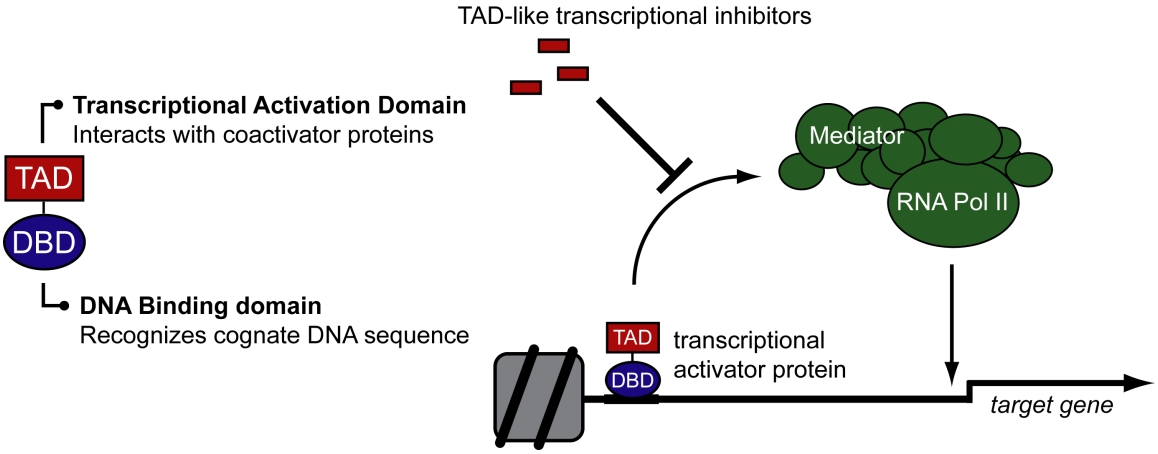


Figure II-1. The modular nature of activator proteins. The transcriptional activation domain (TAD) of an activator protein is responsible for establishing the protein-protein interactions that lead to recruitment of RNA Polymerase II, and transcription of the activator's target gene. Small molecules that mimic key regions of the TAD should interfere with its ability to recruit coactivators and initiate transcription.

B. Background

B.1. Transcriptional Activation Domains

Transcriptional activator proteins are minimally comprised of a DNA binding

domain (DBD)– which recognizes the activators cognate DNA sequence on the promoter – and a transcriptional activation domain (TAD) – which mediates the protein-protein interactions necessary to recruit the transcriptional machinery and initiate transcription[15] (Fig II-1a). The largest class of these activation domains, the 'acidic' or 'amphipathic' class, is characterized by interspersed acidic and hydrophilic residues.[1,2] Although these domains are often unstructured in solution, they are thought to form amphipathic alpha-helices in order to interact with their coactivator targets. The biologically relevent activator-coactivator interactions are a matter of continuing discussion in the literature,[2] but it appears that individual activators must interact with multiple proteins in a particular order to facilitate transcription.[16,17,18] In addition, coactivators generally interact with more than one transcriptional activator,[19,7,20] and some coactivators, such as the paralogs CBP and p300, are known to interact with more than a hundred different activators.[1] The multi-partner binding profiles of both activators and coactivators limit the utility of binding based assays for screening, and highlight the potentil utility of a design approach that is not based on a single putative activator-coactivator interaction.

B.2. Existing Small Molecule Transcriptional Inhibitors

Excluding ligand activated systems such as nuclear receptor mediated transcription, there are very few examples small molecule transcriptional inhibitors.[1,3] Most of the molecules identified are the results of high throughput screens, and are large, relatively planar, lipophilic molecules (Figure II-2). One example is ICG-001,[4] an

inhibitor of β-catenin mediated transcription that targets an activator binding site on CBP. ICG-001 reduces expression of a β-catenin controlled reporter gene, and the transcription of the known β-catenin target gene *survivin* at concentrations as low as 10 μM. KG-501[6] also targets CBP, and was identified through an NMR based screen, in which the ability of a library of molecules to interact with the KIX domain of the coactivator CBP was assessed. This molecule inhibits the CREB-CBP interaction with a Ki of approximately 90 μM, and interferes with transcription of a luciferase reporter under the control of CREB and the production of NR4A2 (a CREB target gene) mRNA, both at concentrations between 5 and 10 μM. A final example is the molecule dubbed 'Wrenchnolol' by Uesugi and coworkers,[21] which affects ESX mediated expression of erbB2. This molecule was optimized from the results of a high throughput screen for molecules that affected the viability of erbB2+ SkBr3 cells. Wrenchnolol appears to reduce erbB2 protein levels at 7 μM, and GST-pulldowns indicate that one of the cellular proteins that wrenchnolol interacts with is the putative ESX coactivator Med23. Although these molecules validate the potential of small molecules to act as transcriptional inhibitors, they do not provide a generalizable design rationale beyond that of screening small molecule libraries against known coactivator targets.

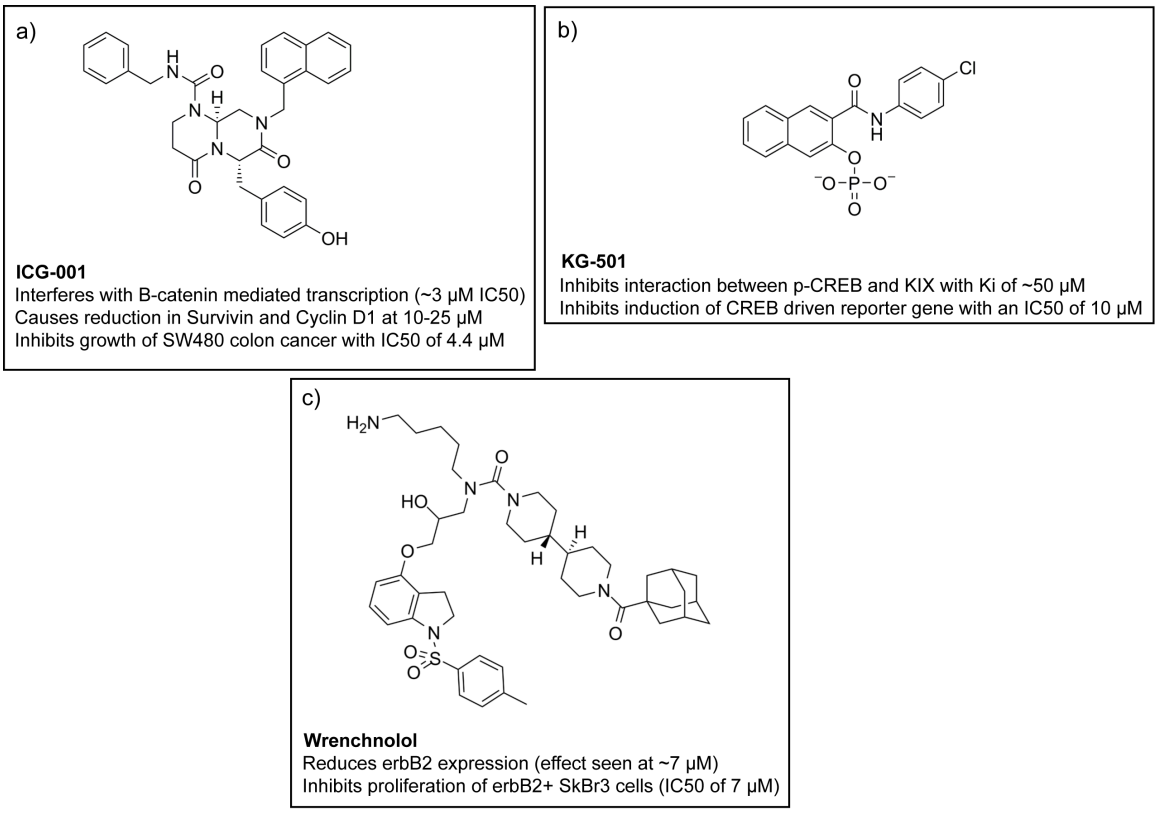


Figure II-2 Small molecule transcriptional inhibitors identified from high throughput screens. (a) ICG-001 which interferes with β-catenin mediated transcription by blocking the activator binding site on CBP. (b) KG-501 also interacts with CBP, but reduces CREB driven transcription. (c) Wrenchnolol affects erbB2 expression by blocking a putative interaction between the transcription factor ESX and Med23.

B.2. Using Small Molecule Transcriptional Activation Domain Mimics as Scaffolds for Transcriptional Inhibitors

In the course of developing small molecule-based transcriptional activators, this lab identified several amphipathic isoxazolidines that mimic the transcriptional activation domain (TAD) of endogenous amphipathic activators, the domain that interacts directly with the transcriptional machinery (Figure II-1). These isoxazolidines present a radial

display of functionality putatively similar to the active conformation of an activation domain. The molecule **II-1** not only interacts with the KIX domain of CBP, a known target of transcriptional activators, but does so in a manner similar to the endogenous activators MLL and Jun (Figure II-3a).[11] When localized to DNA, the isoxazolidine TADs up-regulate transcription in human cell culture up to 80-fold (Figure II-3b).[9] Thus, in the absence of a DNA-targeting moiety, we reasoned that this molecule could serve as a competitive inhibitor of activator-coactivator interactions. Furthermore, based on the shared structural characteristics of amphipathic activation domains, we hypothesized that this molecular scaffold could be used to develop inhibitors of specific activators responsible for pathological developments within the cell.

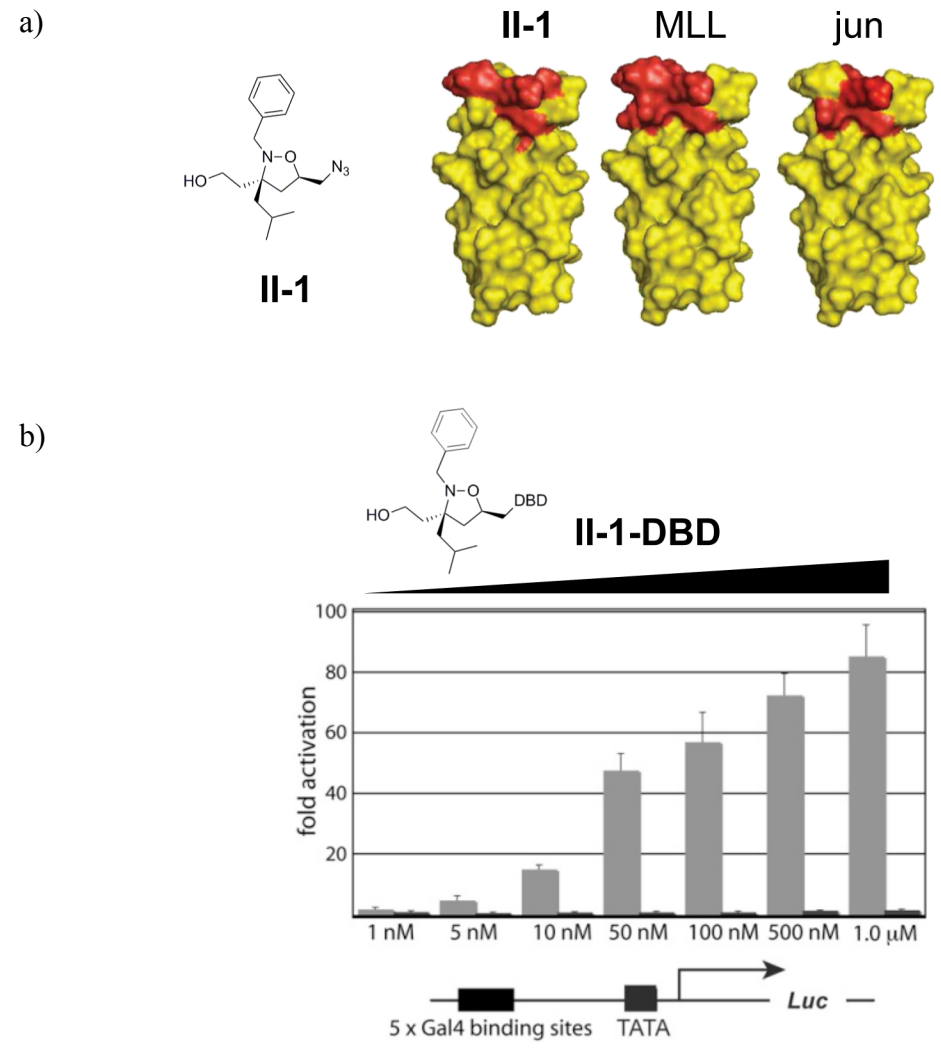


Figure II-3. Development of an isoxazolidine based TAD. (a) NMR chemical shift perturbation experiments with **II-1** and N15 labeled KIX domain indicate that **II-1** interacts with a region of KIX overlaps with those which interact with the endogenous activators MLL and Jun. (b) When **II-1** is attached to a DNA-localizing moeity, it causes dose dependent upregulation of transcription in mammalian cell culture. Figures from ' Buhrlage, S.J. et al. *ACS Chemical Biology* **2009**, *4*, 335-345 and Rowe, S. P. et al *J. Am. Chem. Soc.* **2007**, *129*, 10654-10655. Used with permission.

B.3. ESX Regulates Transcription of ErbB2

The activator chosen as a target for this study is ESX (ESE-1/ELF-3/ERT/Jen), an epithelial-specific transcriptional activator.[22] Several lines of evidence suggest that the ESX is a key regulator of ErbB2 expression. This include both correlative studies, in which ESX overexpression is seen to corellate with erbB2 levels in both primary tumors[13] and model systems.[12,23] They also include more direct evidence, such as ESX knockdowns (Figure II-4a) in which shRNA knockdown of ESX reduces erbB2 levels, and attenuates the growth (Figure II-4b) and migration of erbB2+ head and neck cancer cell lines. Analysis of ESX structure and activity via NMR and alanine scanning mutagenesis indicates a key region neccesary for activation (137-SWIIELLE-144).[21] The authors of this study propose that Tryptophan 138 is particularly important for ESX activity and that it is assisted by a combination of leucines and isoleucines which serve as the nonpolar face of the amphipathic binding surface. The polar side chains are thought to form a solvent exposed face that orients the TAD against its contact surface on coactivator targets.

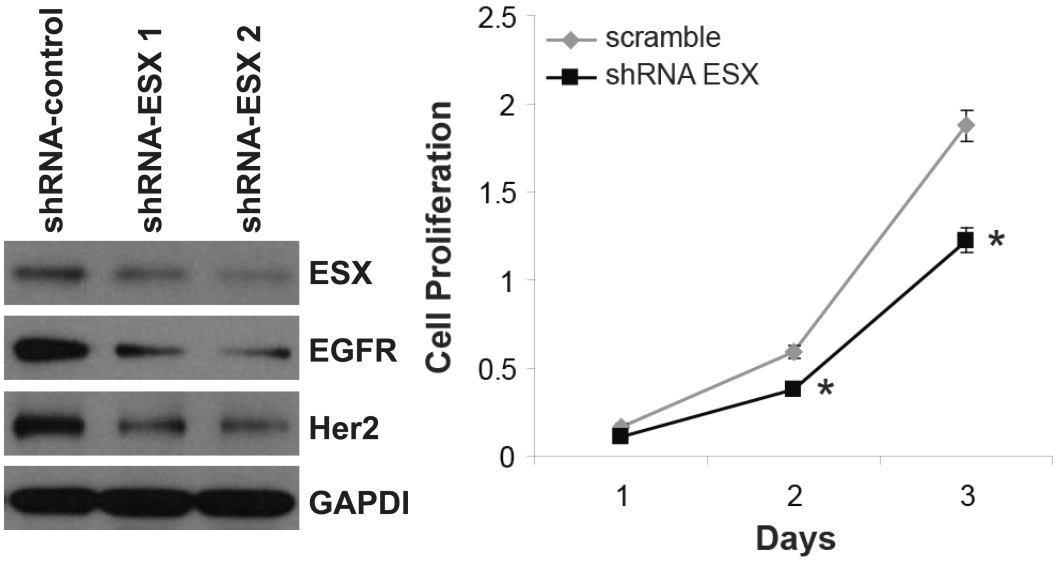


Figure II-4. Effects of ESX knockdown on erbB2+ cells. (a) Knockdown of ESX reduces expression of erbB2 and EGFR (erbB1) in Cal27 cells, an EGFR+ and erbB2+ head and neck cancer cell line. (b) Knockdown of ESX also reduces the proliferation of Cal27 cells compared to a scrambled shRNA.

B.4. ErbB2 Function and Relevance

ErbB2 is a transmembrane tyrosine kinase that is overexpressed in approximately 25% of breast cancers (erbB2+),[24] where it drives an aggressive phenotype marked by more rapid metastasis and shorter patient life expectancy than breast cancers that do not overexpress erbB2.[25,26,27,28] Unlike the other three members of the erbB family, a ligand for the extracellular domain of erbB2 has not been identified.[28] Instead, the extracellular domain of erbB2 appears to adopt a conformation that resembles the ligand- bound state of the other receptors.[29] Perhaps because of this, erbB2 is the preferred dimerization partner for erbB1 and erbB3.[30,31] The resulting dimers strongly activate signaling cascades

responsible for cell growth and survival, such as the oncogenic RAS/MAPK and PI3K/Akt pathways.[28] In particular, erbB2 and erbB3 appear to be extremely effective at inducing oncogenic signaling and tumor formation in a cooperative manner.[31,32,33,34] In carcinoma cells which overexpress erbB2, reduction of erbB2 levels has been shown to cause growth arrest,[35] reversal of transformation,[36] or cell death.[37]

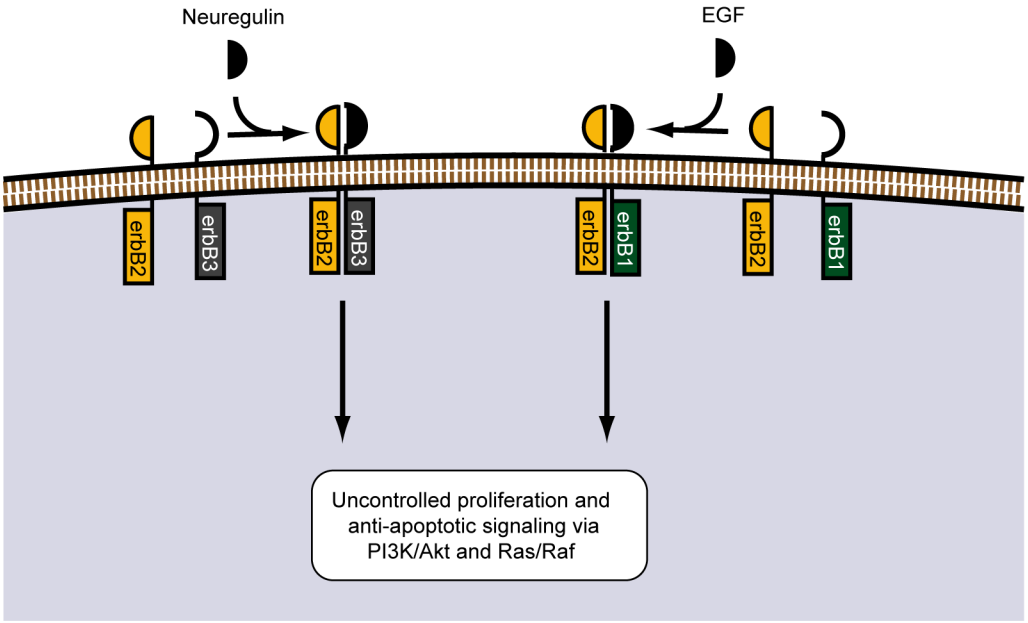


Figure II-5. ErbB dimerization. Membrane integrated erbB2 forms ligand induced heterodimers with erbB3 or erbB1. These dimers initiate oncogenic signaling cascades within the cell.

C. Design and Synthesis of Isoxazolidine Based ESX Mimics

C.1. Mimicking the ESX Activation Domain

As noted previously, the active conformation of the the ESX activation is thought

to be alpha-helical.[14] Mapping the putative hotspot of this activation domain onto a helical wheel (figure II-6b) suggests an amphipathic array of functionality similar to those found in other activation domains.[7,38] In order to mimic this, we used a combination of aliphatic leucine or isoleucine like functionality and a large aromatic group in place of the tryptophan side chain. For many of the molecules, some polar functionality was retained. This was done because previous studies have suggested that transcriptional activators, and perhaps inhibitors, function best with an amphipathic mixture of polar and non-polar functional groups.[11,39]

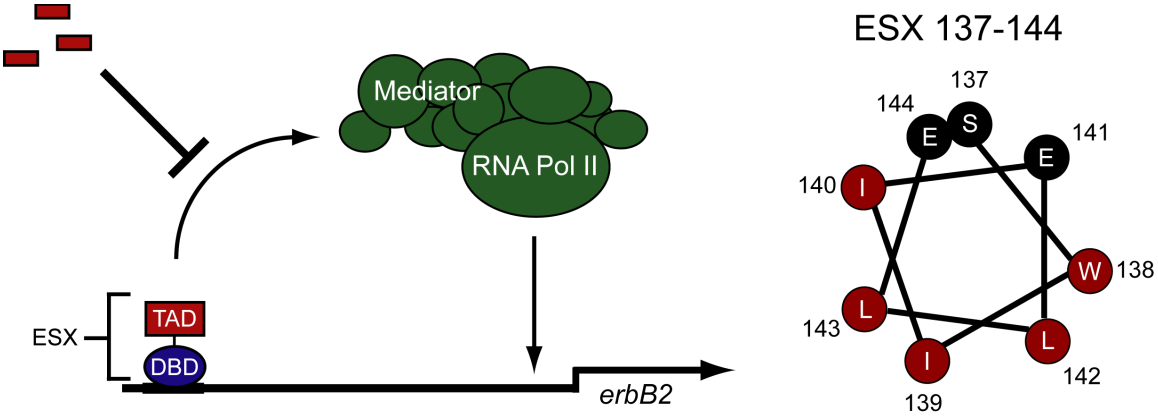


Figure II-6. The transcriptional activator ESX regulates transcription of erbB2.(a) Small molecules that mimic the activation domain of ESX should interfere with the activator's ability to recruit the transcriptional machinery and initiate transcription of erbB2. (b) The hotspot of the ESX activation domain is shown mapped on to a helical wheel. Polar residues are shown in black, while non-polar residues are shown in red to illustrate the amphipathic nature of the helix.

C2. Synthesis of ESX-Like Isoxazolidines.

The general route used to synthesize the isoxazolidines shown in figure I-10 is summarized below in figure I-7. In brief, an isoxazoline is generated via a 1,3-dipolar cycloaddition. This isoxazoline is then functionalized at the C3 position using an organometallic nucleophile. The N2 position was functionalized through either a reduction amination with the appropriate aldehyde, or a microwave assisted SN2 reaction. Although this route was iterative rather than convergent, it had the advantages of being reliable, and of using many common intermediates that could be easily prepared on on large (>10g) scales.

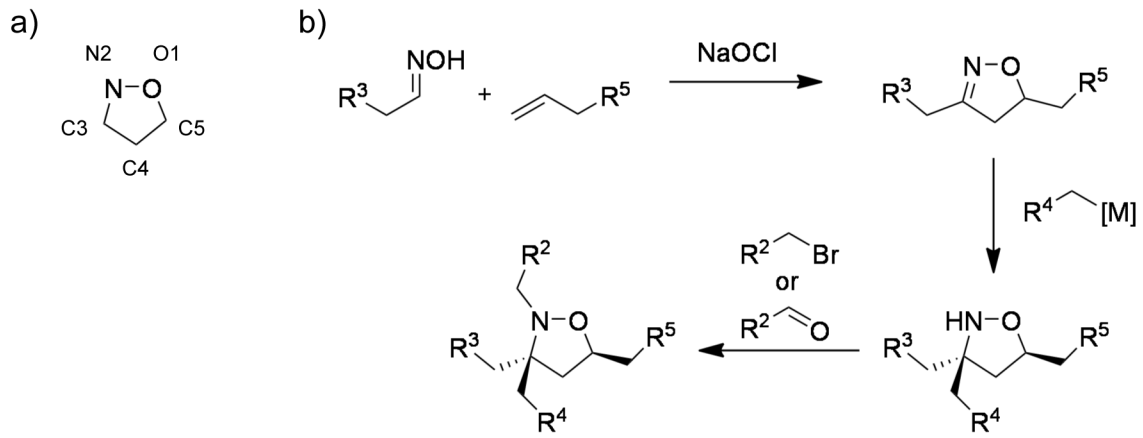


Figure II-7. General synthetic scheme for inhibitors. a) Notation used to describe positions on the isoxazolidine ring. b) The general synthetic scheme used to generate isoxazolidine inhibitors of erbB2 transcription. The initial 1,3-dipolar cycloaddition is followed by nucleophilic addition to C3, and then electrophilic functionalization of N2.

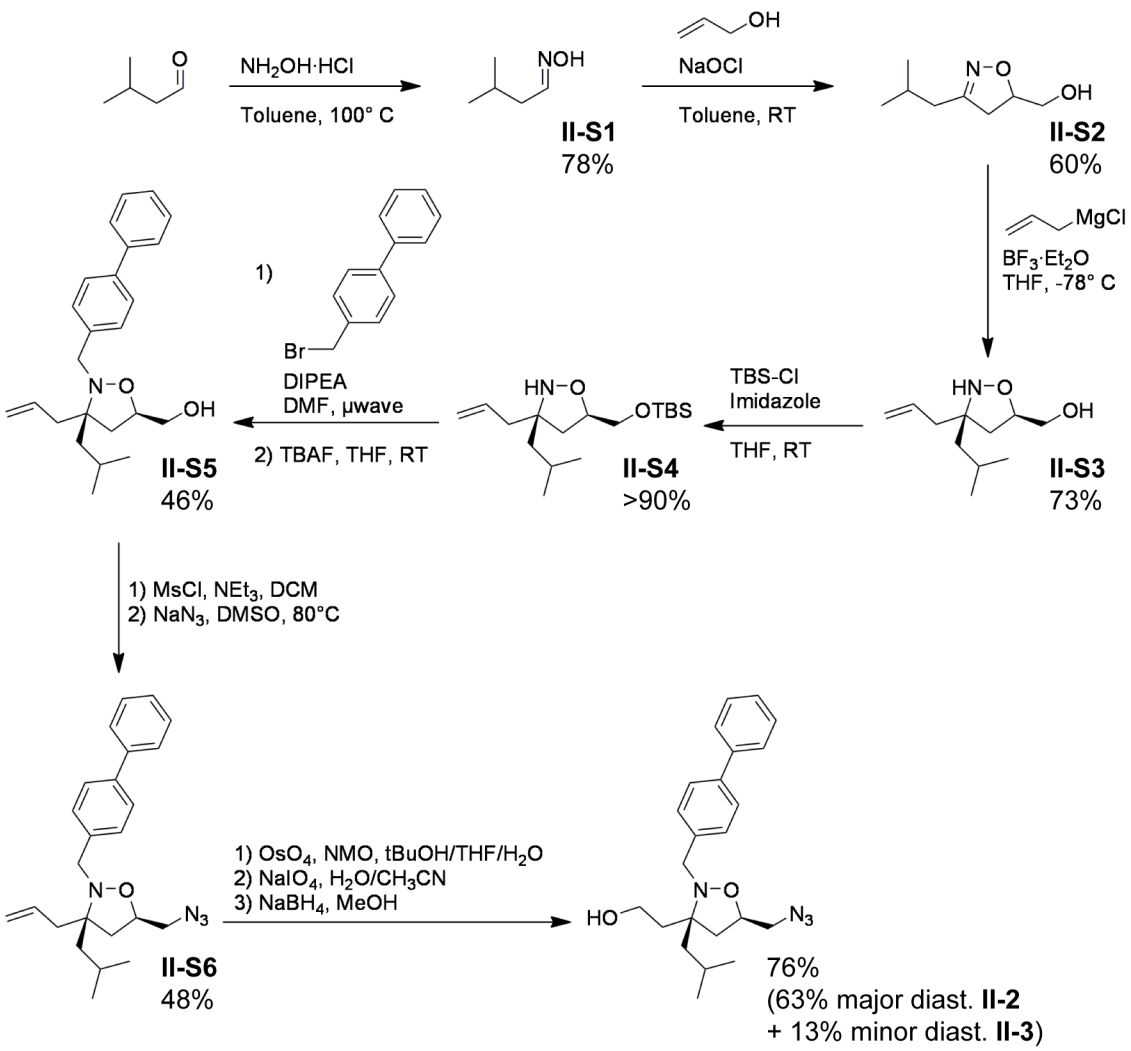


Figure II-8. Synthesis of **II-2** and **II-3**

A specific example of this route **II-2** is shown below. The route which followed the general synthesis depicted above was found to require the fewest protection/deprotection steps in this, and most of the other isoxazoldine syntheses. For those isoxazolidines with redox labile N2 substituents, a different general method was needed, owing to the tendency of the N2 functionalized intermediates to undergo side reactions during the final functional group changes. The synthesis of **II-4** provides an

example of this alternative route. It was found that the unprotected N2 position was surprisingly stable to electrophilic reagents such as methanesulfonyl chloride. This stability was also noted with reagents intended to functionalize the N2 position, thus the necessity for microwave activation, even when electrophiles such as allyl or benzyl bromide were employed. It is postulated that this is primarily due to the inductive effect of the neighboring oxygen. Replacement of the C5 alcohol with an azide was followed by functional group manipulation, and finally with N2 functionalization using a reductive amination. Although slightly longer than the original route, this alternative provided access to isoxazolidines **II-4** and **II-5.**

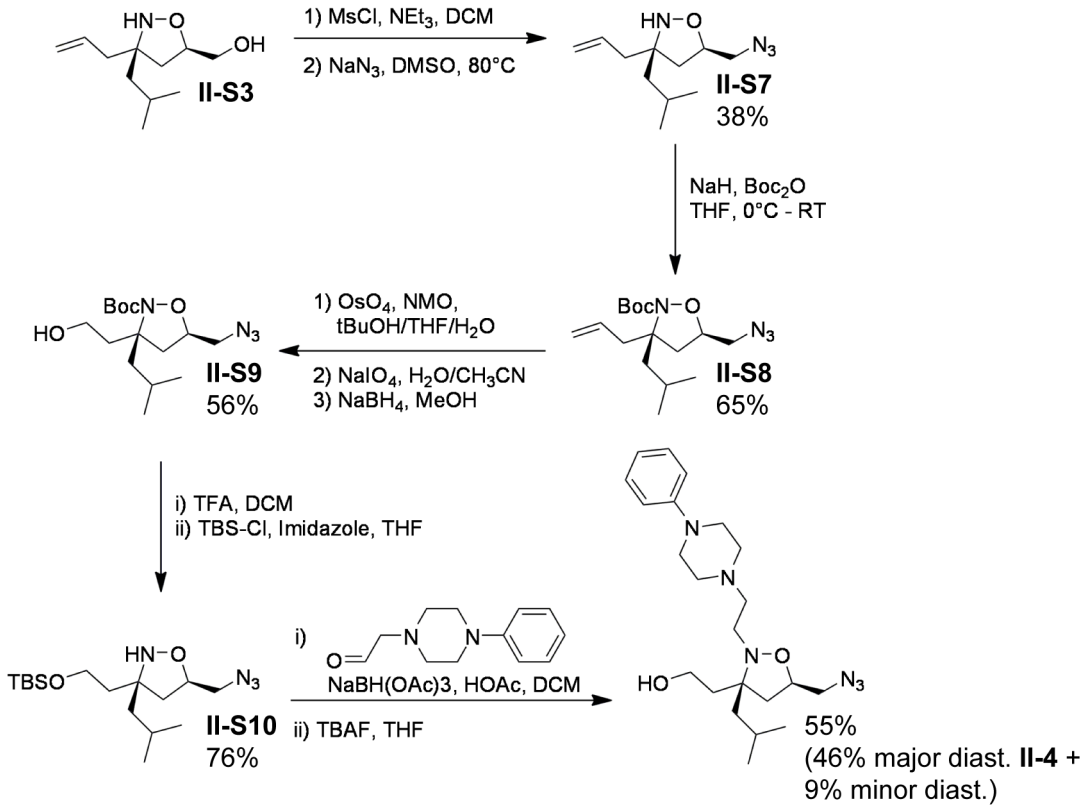


Figure II-9 Synthesis of **II-4**

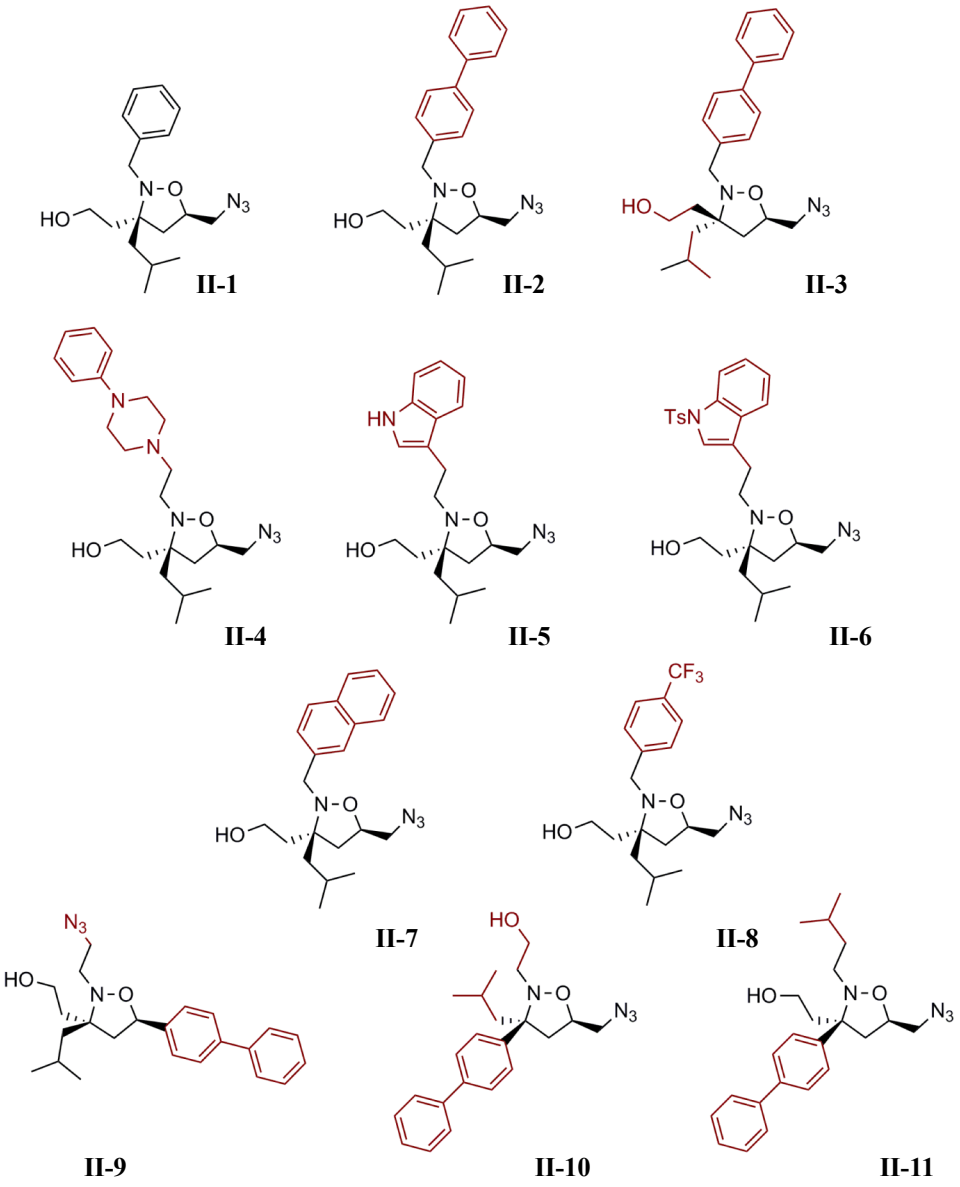


Figure II-10. Isoxazolidines **II-1** through **II-11.** The differences between each isoxazolidine and the generic TAD mimic **II-1** are highlighted in red.

Compound	vs. erbB2 mRNA	vs. erbB2 protein	vs. erbB2+ cells
II-1	-	-	
II-2	++	++	++
II-3	++	++	++
II-4	+	NA	+
II-5	+	+	
II-6	+	+	NA
II-7	-	+	NA
II-8	-	+	NA
II-9	-	+	+
II-10	-	+	+
II-11	+	+	++

Table II-1. Effectiveness of isoxazolidines **II-1** through **II-11.** Effects on curbing erbB2 expression and inhibiting the proliferation of erbB2+ cells. + indicates that some effect was seen, but that either the effect was minimal, or it required very high (≥50 μM) concentrations. ++ indicates substantial effect at concentrations of 20 μM or less. Effects on erbB2 mRNA were assessed using qPCR (Dr. Lori Lee). Effects on erbB2 protein were assessed using either Western Blot analysis (Dr. Lori Lee) or ELISA. Effects on erbB2+ cells were assessed using a WST-1 viability assay.

D. Evaluation of Isoxazolidine Based ESX Mimics

D.1. Effects on erbB2 Protein Levels

As shown in Table II-1, compounds were evaluated based on their ability to affect erbB2 mRNA production, protein levels, and their ability to attenuate the viability of erbB2+ SKBR3 cancer cells. These molecules include varying N2 substituents designed to better mimic the side chain functionality of tryptophan 138. They also include C5' variations and positional isomers of the early lead compound **II-2**. Because it has been shown that both peptidic and small molecule transcriptional activators are structurally

permissive,[40,41] we chose to investigate this for our inhibitors. These results were inconclusive. Although there may be some permissiveness, given the activity of **II-3** and **II-11**, both **II-9** and **II-10** had noticeably lower activity, indicating that the similarities in the activities of positional isomers are not as robust as those of activators.

Initial results indicated that **II-2** was the most effective of these molecules, and these results, in conjuction with the bench stability and ease of synthesis for this compound, lead to its being used for subsequent studies. Although some of the compounds which contained nitrogen in their N2 aromatic functional groups (such as **II-6** and **II-4**) were more intuitive tryptophan mimics, these compounds suffered from reduced activity and (in the case of indole containing compounds such as **II-5** and **II-6**) reduced benchtop stability owing to the redox sensitive nature of the indole functional group.

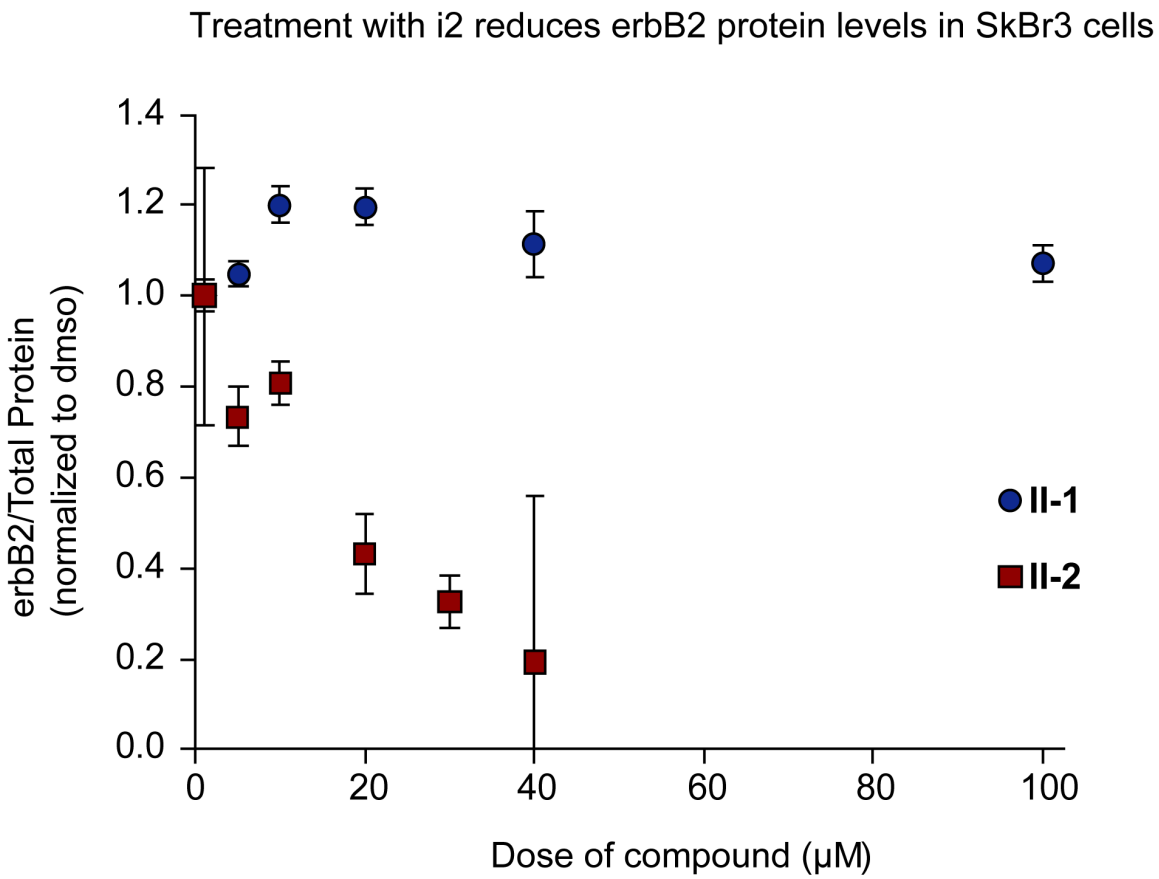


Figure II-12. Effects of **II-1** and **II-2** on erbB2 expression. Compound **II-1** has no effect on erbB2 expression in erbB2+ SkBr3 cells. In contrast, **II-2** has a pronounced effect. Results are from an ELISA, in which SkBr3 cells ($3x10^3$ cell per well) were dosed with indicated amounts of compound for 16 hours, at which point the cells were lysed, and erbB2 and total protein concentration were assayed as described in section F. Error bars indicate standard deviation.

The most reliable and most effective results were seen for **II-2**, and this compound was evaluated in more detail. Using an ELISA, **II-2** was shown to reduce erbB2 levels (normalized in each well to total protein amount) in a dose dependent manner (Figure II-12). Similar results were seen in HNSCC cell lines by collaborators Dr. Pan and coworkers (Figure II-13) using western blot analysis. Additional support for the effects of **II-2** comes from qPCR assays carried out by a colleague within the Mapp

lab (Dr. Lee, Figure II-13) which show erbB2mRNA levels reduced to approximately 60% of control levels at 10uM of **II-2**. Throughout these experiments **II-1** was used as a structurally related negative control. In contrast to **II-2**, it had no effect on erbB2 protein levels at concentrations as high as 100uM and minimal effects on erbB2 mRNA. Taken as a whole, these results indicate that **II-2** is effective at curbing overexpression of erbB2 in a variety of erbB2+ cancer cell lines.

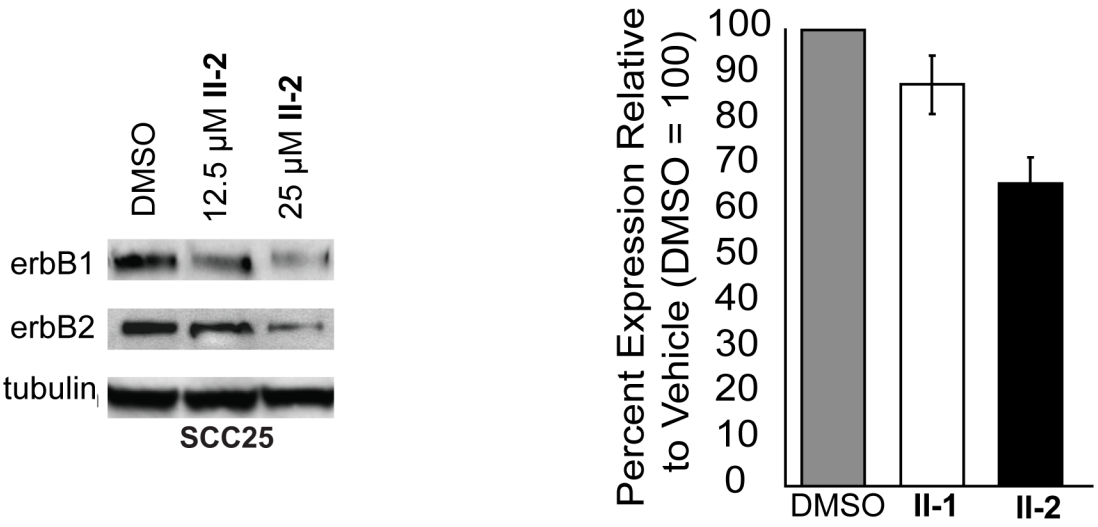


Figure II-13. Effects of **II-2** on erbB2 protein and mRNA. Effects of **II-2** as assessed by collaborating scientists. (a) **II-2** reduces expression of erbB2 and erbB1 (EGFR) in SCC25 HNSCC cells. (Dr. Quintin Pan and coworkers) (b) **II-2** reduces erbB2 mRNA levels when compared to either a DMSO control or **II-1**. (Dr. Lori Lee).

D.2. Effects on erbB2 Overexpressing Cells

As noted earlier, one of the reasons for choosing erbB2 as a model system was that reduction of erbB2 production or activity has been shown to cause growth arrest or death in erbB2+ cancer cells. We tested the ability of several potential ESX mimics,

including **II-2** to exert similar effects. In keeping with its activity against erbB2 levels, **II-2** was also effective against erbB2+ cancer cells. The SkBr3 cell line was chosen for our experiments, because this line is known to express both erbB2 and erbB3, and to respond to ligands that activate this dimer (such as neuregulin) with increased PI3K/Akt signaling, making it a useful approximation of the expected environment in primary erbB2+ cancer cells.[42] Isoxazolidine **II-2** inhibited the growth of SKBR3 cells with in a dose dependent manner, with an IC50 of 14 ± 1 μM, while isoxazolidine **II-1** was ineffective at concentrations up to 100 μM.

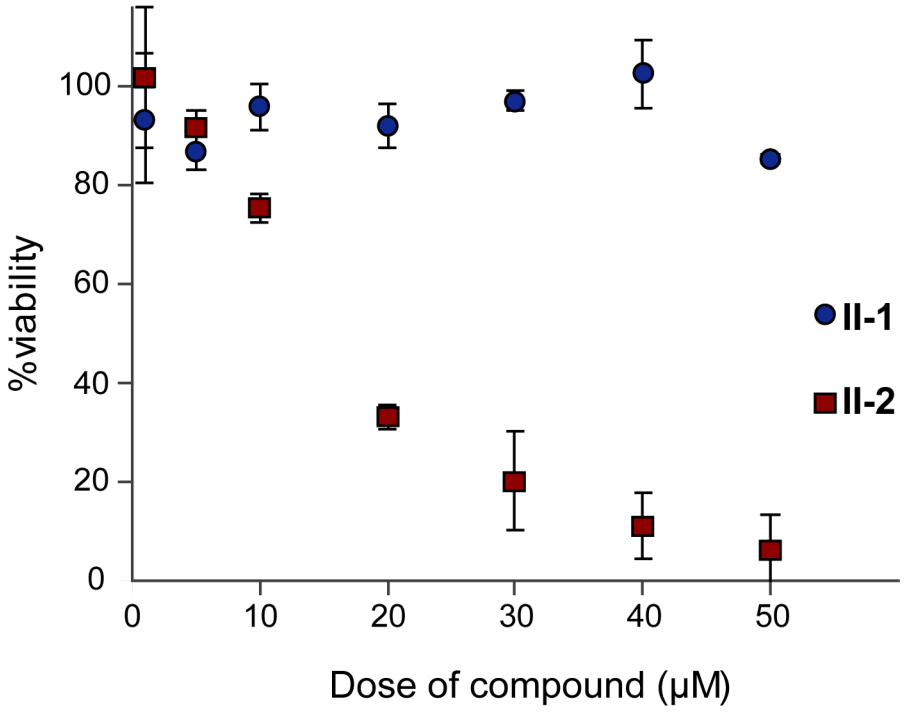


Figure II-14. II-2 reduces the viability of erbB2+ SkBr3 cells. **II-2** reduces the viability of erbB2+ SkBr3 cells in a dose dependent manner, while negative control **II-1** has minimal effect at similar concentrations. These data are from SkBr3 cells ($3x10^3$ per well) dosed for 24 hours with compound as indicated. Viability measurements were obtained using WST-1 reagent as described in section F. Error bars indicate 1 standard deviation.

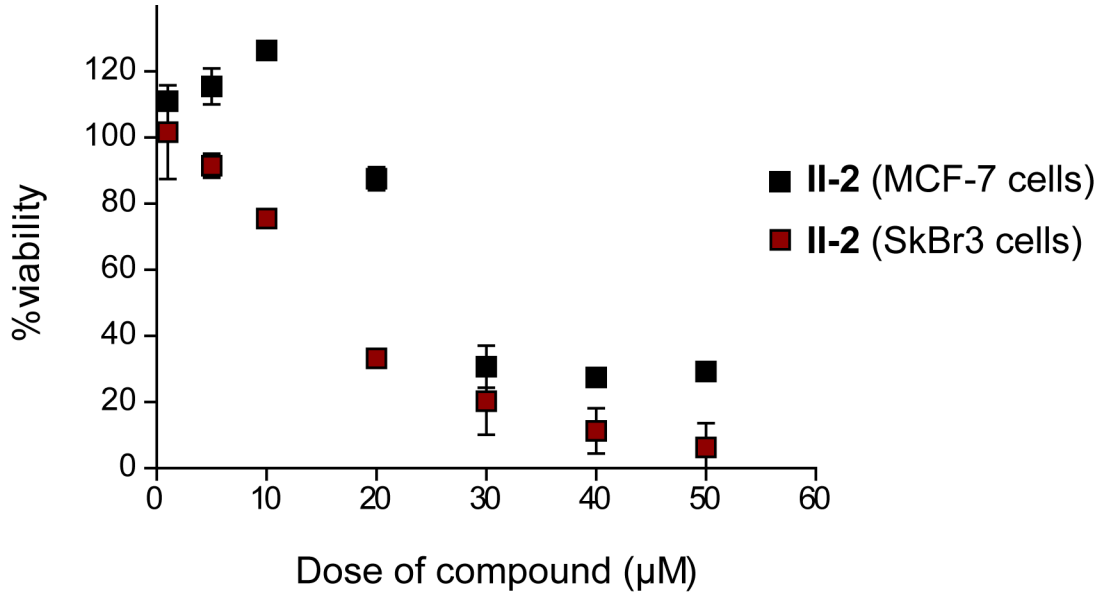


Figure II-15. Comparison of the effects of **II-2** (SkBr3 vs MCF-7).Comparison of the effects of **II-2** on erbB2+ SkBr3 cells vs. its effects on estrogen driven MCF-7 breast cancer cells. These data are from SkBr3 and MCF-7 cells ($3x10^3$ per well) dosed for 24 hours with compound as indicated. Viability measurements were obtained using WST-1 reagent as described in section F. Error bars indicate 1 standard deviation.

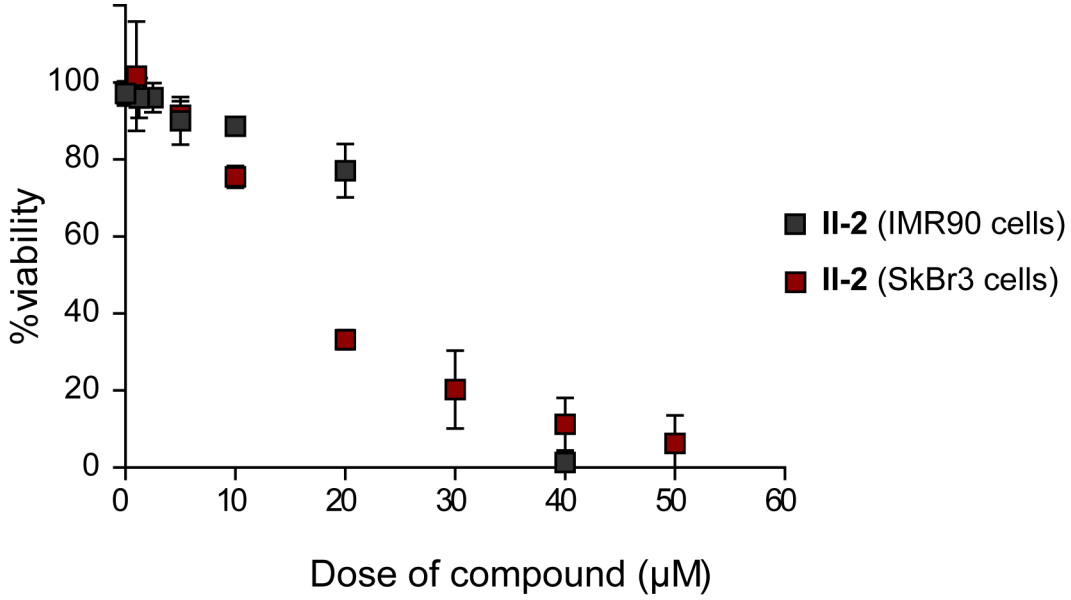


Figure II-16. Comparison of the effects of **II-2** (SkBr3 vs. IMR90). Comparison of the effects of **II-2** on erbB2+ SkBr3 cells vs. its effects on non-tumorigenic IMR90 cells. These data are from SkBr3 cells ($3x10^3$ per well) dosed for 24 hours with compound as indicated. Viability measurements were obtained using WST-1 reagent as described in section F. Error bars indicate 1 standard deviation.

Cell Line	IC_{50}
SkBr3 (erbB2+)	14 ± 1 μM
MCF-7	27 ± 4 μM
IMR-90 (non-tumor)	24 ± 2 μM

Table II-2. Comparison of the IC50s of **II-2**. Comparison of the IC_{50}s of **II-2** in each cell line, data is from the same experiments as figures II-14 through II-16.

Isoxazolidine **II-2** was also tested on the cell line MCF-7, which is driven by estrogen dependent signaling, rather than erbB activity, and in the non-tumorigenic fibroblast cell line IMR-90. In both cases the IC50 against these cell lines were reduced when compared to SKBR3 cells. This reduction amount to approximately 2-fold, which, while significant, may limit the long term applications of this approach. Similar issues with selectivity in both cellular toxicity[43] and transcriptional selectivity[5] are present in the literature. A detailed examination of the rationale for selectivity issues with transcriptional inhibitors, and a solution to this problem is discussed at length in the next chapter.

E. Conclusions

Taken together, these data suggest that isoxazolidine **II-2** is a much improved mimic of the transcriptional activation domain of ESX relative to the generic TAD mimic **II-1,** validating our strategy for using an artificial TAD as a scaffold for the design of transcriptional inhibitors. Treatment of ErbB2+ cancer cells with isoxazolidine **II-2**

reduces erbB2 mRNA and protein levels, indicating that the molecule effectively interferes with the ability of ESX to activate transcription of erbB2. This molecule is also effective at attenuating the growth and viability of erbB2 overexpression cancer cells, which is consistent with the expected effects of an erbB2 transcriptional inhibitor.

However, **II-2**, like other small molecule transcriptional inhibitors, requires low micromolar concentrations for its cellular effects. This may be due to a combination of factors. Small molecules have much less surface area than is needed for most protein-protein interactions, and this impacts their ability to inhibit these interactions (ref). Transcriptional activation domains in particular have a complex, low affinity multi-partner interaction profile that may make it difficult design high affinity small molecule inhibitors.

F. Supporting Information

F.1. Materials

Unless otherwise noted, starting materials were obtained from commercial sources and were used without any additional purification. CH_2Cl_2 and THF were dried by passage through activated alumina columns and degassed by stirring under a dry N_2 atmosphere. Anhydrous DMF was purchased from Sigma-Aldrich (St. Louis, MO). All reactions were performed under anhydrous conditions (N_2 atmosphere) unless otherwise noted. Et_3N was distilled from CaH_2. Purification by flash chromatography was carried out with E. Merck Silica Gel 60 (230-400 mesh) according to the procedure of Still, Kahn, and Mitra.[1] ^1H spectra were recording in $CDCl_3$ at 400 MHz or 500 MHz and ^{13}C NMR were recorded at 100 MHz or 125 MHz, Reverse-phase HPLC purifications were performed on a Varian ProStar 210 equipped with Rainin Dynamax UV-D II detector (λ = 254 nm and λ = 214) using a C18 (8 x 100mm) Radial-Pak™ cartridge with a gradient of 0.1% TFA in H_2O and CH_3CN as the mobile phase. Normal-phase HPLC purification was performed on Varian ProStar 210 equipped with Varian ProStar UV-VIS 345 detector using a SiO_2 (25 x 100mm) Radial-Pak™ cartridge in hexanes/EtOAc. Molecules **II-1** through **II-11** were purified by reversed-phase HPLC prior to use in cell-based experiments.

F.2. Cell proliferation assays:

SkBr3 and MCF-7 cells were seeded at $3x10^3$ cells per well on 96 well plates in RPMI media supplemented with 2.5% fetal bovine serum (SkBr3) and 1:1 DMEM:F12 supplemented with 2.5% fetal bovine serum (MCF-7) respectively. After 24 hours, compound was added as a solution in DMSO (1% v/v final DMSO concentration). 24 hours after dosing with compound, cells were treated with WST-1 reagent (Roche, Basel, Switzerland) in accordance with manufacturer's instructions. Cell proliferation was calculated using DMSO-treated cells as 100%. IC_{50} values were calculated using equation **S2** in Origin 7.0 (OriginLab Corporation, Northampton, MA). Figures shown are from experiments carried out in triplicate, with error bars indicating the standard deviation.

y = a + (b-a)/(1+(x-c)^d)) **(equation S2)**

Where **a** is equal to the maximum effect observed, **b** is equal to the minimum effect observed, **c** is equal to the IC_{50}, and **d** is equal to the Hill slope. For determination of absolute IC_{50} minimum was set to 0% and maximum was set to 100%.

F.3. ErbB2 and p-erbB2 ELISA:

ELISA assays were performed based on those published elsewhere. In brief, SkBr3 cells were plated in 10% FBS at 3000 cells per well in 96 well plates. After

adhering overnight, media was changed to 2.5% FBS and compound was added. After 16 hours, media was removed carefully and cells were fixed and permeabilized with cold (-20º C) methanol. The cells were then washed twice with TBST, blocked for 2 hours at room temperature with superblock (TBST solution from Pierce), incubated with the primary antibody overnight at 4º C (as a 1:500 solution in superblock). Cells were then washed twice more with TBST and incubate with secondary antibody for 2 hours at room temperature (as a 1:1000 solution in superblock). After being washed 3 times with TBST, Slow TMB (Pierce) was added to measure antibody levels in accordance with the manufacturer's instructions and the absorbance at 370 nm was measured. The cells were washed 3 more times with TBST and total protein levels were measured at 560 nm using BCA reagent (Pierce). ErbB2 and p-erbB2 levels were normalized to total protein concentration by calculating (A370-A370blank)/(A560-A560 blank), where the A370 blank was from TMB treated cells not treated with a primary antibody, and the A560 blank was BCA reagent in wells that did not have cells plated in them. The resulting ratios were then normalized to cells treated with DMSO.

F.4. Synthesis and Characterization of Compounds II-1 through II-11 and Intermediates.

F.4a. Synthesis of Compounds II-2, II-3 and Intermediates Using the Scheme Shown in Figure II-8.

3-methylbutanal oxime (II-S1)

Hydroxylamine hydrochloride (27.8 g, 400 mmol) and sodium carbonate (42.4 g, 400 mmol) were combined in 400 mL toluene and heated to 70° C for 1 hour, with vigorous stirring. Reaction was cooled and isovaleraldehyde (27.7 mL, 250 mmol) was added slowly. The reaction was then heated to a light reflux (100° C) and stirred for 4 hours until complete by TLC. The crude mixtures was cooled , filtered through a scintered glass frit, and washed with toluene. The filtrate was concentrated under reduced pressure and distilled at 100° C under reduced pressure using an aspirator to yield **II-S1** a pale yellow oil (25.3 g, 78%).

(3-isobutyl-4,5-dihydroisoxazol-5-yl)methanol (II-S2)

Oxime **II-S1** (2.0 g, 20 mmol) and allyl alcohol (10.8 mL, 160 mmol) were combined in 400 mL toluene in a foil wrapped round bottom with vigorous stirring, cooled with an ice water bath. A 6% solution of sodium hypochlorite ('Chlorox bleach', 62 mL, 50 mmol hypochlorite) was added dropwise. The crude reaction was partitioned between toluene and water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S2** as a yellow oil (3.1 g, 60%)

3-allyl-3-isobutylisoxazolidin-5-yl)methanol (II-S3)

Boron Triflouride Etherate (1.85 mL, 14.7 mmol) was added to isoxazoline **II-S2** (0.771 g, 4.91 mmol) in 50 mL dry tetrahydrofuran under nitrogen at -78° C. After 15 minutes, allylmagnesium chloride (2M in hexanes, 7.35 mL, 14.7 mmol) was added dropwise. The

reaction was monitored via TLC and quenched at -78° C with 1:1 mixture of methanol and saturated acqueous ammonium chloride. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S3** (0.97 g, 73%).

3-allyl-5-(((tert-butyldimethylsilyl)oxy)methyl)-3-isobutylisoxazolidine (II-S4)

Tert-butyldimethylchlorosilane (0.772 g, 5.1 mmol) was added to a solution of isoxazolidine **II-S3** (0.72 g, 4.3 mmol) and imidazole (0.58 g, 8.5 mmol), in 40 mL dry tetrahydrofuran under nitrogen. Reaction was stirred at room temperature until completion. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S4** (5 g, >90%).

2-([1,1'-biphenyl]-4-ylmethyl)-3-allyl-3-isobutylisoxazolidin-5-yl)methanol (II-S5)

Isoxazolidine **II-S4** (0.95 g, 3 mmol), biphenylmethyl bromide (4.5 g, 18 mmol), and diisopropylethyl amine (3.2 mL, 18 mmol) where combined in 30 mL of dimethylformamide. This solution was heated using a conventional microwave in 10 second pulses at 20% power until the reaction was complete by TLC. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and passed through a short column of silica to remove as much DMF as possible. The resulting mixture was carried on crude.

Crude isoxazolidine was dissolved in 15 mL of 1M tetrabutylammonium fluoride in

tetrahydrofuran and stirred at room temperature until the reaction was complete via TLC. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S6** (0.51 g, 46%).

2-([1,1'-biphenyl]-4-ylmethyl)-3-allyl-5-(azidomethyl)-3-isobutylisoxazolidine (II-S6)

Methanesulfonyl chloride (0.17 mL, 2.5 mmol) was added to a solution of isoxazolidine **II-S5** (0.30 g, 0.8 mmol) and triethylamine (0.32 mL, 2.5 mmol) in 8 mL dichloromethane at 0° C. After 2 hours, the crude mixture was partitioned with water, and the organics were concentrated under reduced pressure. The crude product was dissolved in 8 mL dimethylsulfoxide, and sodium azide (0.78 g, 12 mmol) was added. The resulting suspension was stirred vigorously and heated to 80° C overnight. The crude mixture was diluted with water. The organics were extracted with ethyl acetate and concentrated under reduced pressure, then purified by column chromatography to yield isoxazolidine **II-S6** (0.16 g, 48% over two steps).

2-([1,1'-biphenyl]-4-ylmethyl)-5-(azidomethyl)-3-isobutylisoxazolidin-3-yl)ethanol (II-2 and II-3)

Osmium tetroxide (as a 2.5% solution in tert butanol, 0.4 mL, 0.04 mmol) was added to isoxazolidine **II-S6** (0.16 g, 0.4 mmol) and N-methylmorpholine-N-oxide (0.94 g, 0.8 mmol) in a mixture of tBuOH/THF/H2O (75%/20%/5%). Upon completion, reaction was quenched with sodium sulfite. The crude mixture was diluted with water. The organics

were extracted with ethyl acetate and concentrated under reduced pressure, then passed through a short column of silica to yield isoxazolidine which was carried on crude.

Crude isoxazolidine was dissolved in 5 mL of 1:1 water:acetonitrile, and cooled to 0° C. Sodium Periodate (0.17 g, 0.8 mmol) was added and the reaction was stirred for 30 minutes. The reaction was partitioned with water, and the organics were concentrated under reduced pressure. This crude mixture was dissolved in methanol (5 mL) and sodium borohydride (16 mg, 0.42 mmol) was added at 0° C. After 15 minutes the crude mixture was diluted with water. The organics were extracted with ethyl acetate and concentrated under reduced pressure, then purified by column chromatography to yield isoxazolidine (120 mg), 76% yield over 3 steps as a 5:1 (**II-2**:**II-3**) mixture of diasteromers which was seperated via HPLC.

F.4b. Synthesis of Compound II-1 and Intermediates Using the Scheme Shown in Figure II-8.

3-allyl-2-benzyl-3-isobutylisoxazolidin-5-yl)methanol (II-S11)

Run according to the protocol for **II-S5** with isoxazolidine **II-S4** (0.625 g, 2 mmol), benzyl bromide (1.37 g, 8 mmol), and diisopropylethyl amine (1.05 mL, 6 mmol), followed by 10 mL 1M tetrabutylammonium fluoride to yield isoxazolidine **II-S11** (0.45 g, 77% over two steps).

3-allyl-5-(azidomethyl)-2-benzyl-3-isobutylisoxazolidine (II-S12)

Run according to the protocol for **II-S6**, with isoxazolide **II-S11** (205 mg, 0.71 mmol),

methanesulfonyl chloride (0.16 mL, 2.1 mmol), and triethylamine (0.27 mL, 2.1 mmol). Followed by sodium azide (900 mg, 14 mmol) to yield isoxazolidine **II-S12** in (204 mg, >90% over two steps).

5-(azidomethyl)-2-benzyl-3-isobutylisoxazolidin-3-yl)ethanol (II-1)

Run according to the protocol for **II-2**, with isoxazoldine **II-S12** (204 mg, 0.65 mmol), Osmium tetroxide (as a 2.5% solution in tert butanol, 0.65 mL, 0.07 mmol) and N-methylmorpholine-N-oxide (152 mg, 1.3 mmol). This was followed by sodium periodate (214 mg, 1 mmol), and then followed by sodium borohydride (76 mg, 2 mmol) to yield isoxazolidine **II-1** (130 mg, 63% over 3 steps as a 5:1 ratio of diastereomers that was seperated via HPLC).

F.4c. Synthesis of II-8 and Intermediates Using the Scheme Outlined in Figure II-8.

3-allyl-3-isobutyl-2-(4-(trifluoromethyl)benzyl)isoxazolidin-5-yl)methanol (II-S13)

Run according to the protocol for **II-S5** with isoxazolidine **II-S4** (200 mg, 0.64 mmol), p-trifluorobenzyl bromide (0.51 mL, 3.2 mmol), and diisopropylethyl amine (0.34 mL, 1.9 mmol), followed by 6.4 mL 1M tetrabutylammonium fluoride to yield isoxazolidine **II-S13** (170 mg, 74% over two steps).

3-allyl-5-(azidomethyl)-3-isobutyl-2-(4-(trifluoromethyl)benzyl)isoxazolidine (II-S14)

Run according to the protocol for **II-S6,** with isoxazolidine **II-S13** (170 mg, 0.47 mmol), methanesulfonyl chloride (0.11 mL, 1.5 mmol), and triethylamine (0.19 mL, 1.5 mmol). Followed by sodium azide (305 mg, 4.7 mmol) to yield isoxazolidine **II-S14** in (169 mg, >90% over two steps).

5-(azidomethyl)-3-isobutyl-2-(4-(trifluoromethyl)benzyl)isoxazolidin-3-yl)ethanol (II-8)

Run according to the protocol for **II-2**, with isoxazolidine **II-S14**(160 mg, 0.42 mmol), Osmium tetroxide (as a 2.5% solution in tert butanol, 0.42 mL, 0.04 mmol) and N-methylmorpholine-N-oxide (100 mg, 0.85 mmol). This was followed by sodium periodate (135 mg, 0.63 mmol), and then followed by sodium borohydride (24 mg, 0.63 mmol) to yield isoxazolidine **II-8** (80 mg, 49% over 3 steps as a 5:1 ratio of diastereomers that was seperated via HPLC).

F.4d. Synthesis of II-7 and Intermediates Using the Scheme Outlined in Figure II-8

3-allyl-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-5-yl)methanol (II-S15)

Run according to the protocol for **II-S5**, with isoxazolidine **II-S4** (467 mg, 1.5 mmol), 2-(Bromomethyl)naphthalene (1.65 mL, 7.5 mmol), and diisopropylethyl amine (0.78 mL, 4.5 mmol), followed by 15 mL 1M tetrabutylammonium fluoride to yield isoxazolidine **II-S15** (285 mg, 56% over two steps).

3-allyl-5-(azidomethyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidine (II-S16)

Run according to the protocol for **II-S6**, with isoxazolide **II-S15** (270 mg, 0.80 mmol), methanesulfonyl chloride (0.18 mL, 2.4 mmol), and triethylamine (0.31 mL, 2.4 mmol). Followed by sodium azide (520 mg, 8 mmol) to yield isoxazolidine **II-S16** in (132 mg, 45% over two steps).

5-(azidomethyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-3-yl)ethanol (II-7)

Run according to the protocol for **II-2**, with isoxazoldine **II-S16** (120 mg, 0.33 mmol), Osmium tetroxide (as a 2.5% solution in tert butanol, 0.33 mL, 0.03 mmol) and N-methylmorpholine-N-oxide (77 mg, 0.66 mmol). This was followed by sodium periodate (107 mg, 0.5 mmol), and then followed by sodium borohydride (19 mg, 0.5 mmol) to yield isoxazolidine **II-7** (100 mg, 82% over 3 steps as a 5:1 ratio of diastereomers that was seperated via HPLC).

F.4e. Synthesis of Compound II-6 and Intermediates Using the Scheme Shown in Figure II-8.

2-(2-(1H-indol-3-yl)ethyl)-3-allyl-3-isobutylisoxazolidin-5-yl)methanol (II-S17)

Isoxazolidine **II-S4** (150 mg, 0.5 mmol), 3-(2-bromo-ethyl)-1H-indole (142 mg, 0.6 mmol), and pottasium carbonate (0.21 g, 1.5 mmol) were combined in 2.5 mL of acetonitrile. This solution was heated to 60° C under nitrogen until no further conversion was observed by TLC. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and chromatographed on silica to

yield isoxazolidine **II-S17** (45 mg, 21%).

3-allyl-3-isobutyl-2-(2-(1-tosyl-1H-indol-3-yl)ethyl)isoxazolidin-5-yl)methanol (II-S18)

Isoxazolidine **II-S17** (105 mg, 0.23 mmol) was dissolved in dry tetrahydrofuran and cooled to 0° C under nitrogen. Sodium hydride (12 mg of a 60% wt suspension in paraffin, 0.3 mmol) was added, followed by toluenesulfonyl chloride (50 mg, 0.27 mmol). The reaction was quenched by the dropwise addition of water and partitioned between water and ethyl acetate. The crude mixture was dried using sodium sulfate and concentrated under reduced pressure.

This mixture was then dissolved in 3 mL 1M tetrabutylammonium fluoride in tetrahydrofuran and stirred at room temperature until the reaction was complete via TLC. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S18** (75 mg, 66% over two steps).

3-allyl-5-(azidomethyl)-3-isobutyl-2-(2-(1-tosyl-1H-indol-3-yl)ethyl)isoxazolidine (II-S19)

Run according to the protocol for **II-S6**, with isoxazolidine **II-S18** (63 mg, 0.13 mmol), methanesulfonyl chloride (0.03 mL, 0.4 mmol), and triethylamine (0.05 mL, 0.04 mmol). Followed by sodium azide (85 mg, 1.3 mmol) to yield isoxazolidine **II-S19** in (50 mg, 74% over two steps).

5-(azidomethyl)-3-isobutyl-2-(2-(1-tosyl-1H-indol-3-yl)ethyl)isoxazolidin-3-yl)ethanol (II-6)

Run according to the protocol for **II-2**, with isoxazoldine **II-S19** (50 mg, 0.1 mmol), Osmium tetroxide (as a 2.5% solution in tert butanol, 0.1 mL, 0.01 mmol) and N-methylmorpholine-N-oxide (18 mg, 0.15 mmol). This was followed by sodium periodate (32 mg, 0.15 mmol), and then followed by sodium borohydride (6 mg, 0.15 mmol) to yield isoxazolidine **II-6** (20 mg, 38% over 3 steps as a 5:1 ratio of diastereomers that was seperated via HPLC).

F.4f. Synthesis of Compounds II-4, II-5, and Intermediates Using the General Scheme Shown in Figure II-9

3-allyl-5-(azidomethyl)-3-isobutylisoxazolidine (II-S7)

Carried out as **II-S6**, with isoxazolidine **II-S3** (880 mg, 4.4 mmol), methanesulfonyl chloride (0.91 mL, 13.2 mmol), and triethylamine (1.78 mL, 13.2 mmol). Followed by sodium azide (2.8 g, 44 mmol) to yield isoxazolidine **II-S7** (377 mg, 38% over two steps).

tert-butyl 3-allyl-5-(azidomethyl)-3-isobutylisoxazolidine-2-carboxylate (II-S8)

Sodium hydride (208 mg of 60% weight suspension in paraffin, 5.2 mmol) was added to isoxazolidine **II-S7** (377 mg, 1.9 mmol) in dry tetrahydrofuran at 0° C. The resulting mixture was stirred for 15 minutes, and then di-*tert*-butyl dicarbonate (1.74 g, 8 mmol)

and dimethylaminopyridine (324 mg, 2.65 mmol) were added. The reaction was stirred for 16 hours, after which it was partitioned between water and ethyl acetate, and the resulting organics were dried (sodium sulfate), concentrated under reduced pressure and chromatographed on silica gel to yield isoxazolidine **II-S8** (355 mg, 65%).

5-(azidomethyl)-3-(2-hydroxyethyl)-3-isobutylisoxazolidine-2-carboxylate (II-S9)

Run according to the protocol for **II-2**, with isoxazoldine **II-S8** (330 mg, 1 mmol), Osmium tetroxide (as a 2.5% solution in tert butanol, 1.0 mL, 0.1 mmol) and N-methylmorpholine-N-oxide (175 mg, 1.5 mmol). This was followed by sodium periodate (242 mg, 1.1 mmol), and then followed by sodium borohydride (34 mg, 0.9 mmol) to yield isoxazolidine **II-S9** (200 mg, 56% over 3 steps).

5-(azidomethyl)-3-(2-((tert-butyldimethylsilyl)oxy)ethyl)-3-isobutylisoxazolidine (II-S10)

Trifluoroacetic acid (1.5 mL) was added to a solution of isoxazolidine **II-S9** (190 mg, 0.6 mmol) in dichloromethane (25 mL). When complete (by TLC) the reaction was quenched with saturated aqueous potassium carbonate, partitioned between water and ethyl acetate, and concentrated under reduced pressure. The crude mixture was then dissolved in dry tetrahydrofuran (6 mL) and imidazole (81 mg, 1.2 mmol) and tert-butyldimethylchlorosilane (100 mg, 0.66 mmol) were added. Upon completion, the reaction was partitioned between water and ethyl acetate, and the resulting organics were dried (sodium sulfate), concentrated under reduced pressure and chromatographed on

silica gel to yield isoxazolidine **II-S10** (151 mg, 76%).

2-(2-(1H-indol-3-yl)ethyl)-5-(azidomethyl)-3-isobutylisoxazolidin-3-yl)ethanol (II-5)

Isoxazolidine **II-S10** (50 mg, 0.15 mmol), 3-(2-bromo-ethyl)-1H-indole (224 mg, 1 mmol), and triethylamine (0.064 mL, 0.5 mmol) where combined in 1 mL of N-Methyl-2-pyrrolidone. This solution was heated using a conventional microwave in 10 second pulses at 20% power until the reaction was complete by TLC. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and dissolved in 1 mL of 1M tetrabutylammonium fluoride in tetrahydrofuran and stirred until complete by TLC. The reaction was partitioned between water and ethyl acetate, and the resulting organics were dried (sodium sulfate), concentrated under reduced pressure and chromatographed on silica gel to yield isoxazolidine **II-5** (12 mg, 22% of a 5:1 diastereomeric mixture that was seperated using HPLC).

Preparation of 3-(4-phenylpiperazin-1-yl)propane-1,2-diol (II-S20)

1-phenylpiperazine (0.59 mL, 4 mmol), allyl bromide (0.34 mL, 4 mmol), and potassium carbonate (1.66 g, 12 mmol) were dissolved in DMF and stirred until the reaction was complete (TLC). The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and passed through a short column of silica to remove as much DMF as possible. The resulting mixture was dissolved in 20 mL of a mixture of tBuOH/THF/H2O (75%/20%/5%), Osmium tetroxide (as a 2.5% solution in tert butanol, 1.5 mL, 0.15 mmol) and N-methylmorpholine-N-oxide (0.70 g, 6 mmol)

were then added. The reaction was partitioned between water and ethyl acetate, and the resulting organics concentrated under reduced pressure and chromatographed on silica gel to yield 3-(4-phenylpiperazin-1-yl)propane-1,2-diol (249 mg, 18% yield over 2 steps).

5-(azidomethyl)-3-isobutyl-2-(2-(4-phenylpiperazin-1-yl)ethyl)isoxazolidin-3-yl)ethanol (II-4)

2-(4-phenylpiperazin-1-yl)acetaldehyde was prepared by adding sodium periodate (140 mg, 0.65 mmol) to 3-(4-phenylpiperazin-1-yl)propane-1,2-diol (103 mg, 0.44 mmol) in 4.4 mL of 1:1 water:acetonitrile. After 1 hour, the crude was partitioned between water and ethyl acetate, and the resulting organics concentrated under reduced pressure. This crude mixture was then dissolved in dichloromethane and added to isoxazolidine **II-S10** (30 mg, 0.09 mmol), sodium triacetoxyborohydride (74 mg, 0.35 mmol) and acetic acid (1 μL). After 6 hours, this reaction was partitioned between water and ethyl acetate, and the resulting organics concentrated under reduced pressure and dissolved in 1mL 1M tetrabutylammonium fluoride in tetrahydrofuran and stirred overnight. The reaction was partitioned between water and ethyl acetate, and the resulting organics were dried (sodium sulfate), concentrated under reduced pressure and chromatographed on silica gel to yield isoxazolidine **II-4** (20 mg, 55% of a 5:1 diastereomeric mixture that was seperated using HPLC).

F.4g. Synthesis of Compound II-9 and Intermediates Using the Scheme Shown in Figure II-8.

5-([1,1'-biphenyl]-4-yl)-3-allyl-3-isobutylisoxazolidine (II-S21)

Run according to the protocol for **II-S2**, with (1.53 mL, 11.7 mmol) oxime **II-S1**, (1.18g, 11.7 mmol) p-bromostryene, and NaOCl (30 mL, 24 mmol, 6%). Carried on crude. According to the protocol for **II-S3,** crude isoxazoline fro the step above (3 g, 10.6 mmol), allylmagnesium chloride (16 mL of a 2M solution, 32 mmol), BF3 (4 mL, 32 mmol) were reacted together to yield isoxazoldine **II-S21** (1.575 g, 41% over 2 steps).

methyl 5-([1,1'-biphenyl]-4-yl)-3-allyl-3-isobutylisoxazolidin-2-yl)acetate (II-S22)

Run according to the protocol for **II-S5** with isoxazolidine **II-S21** (0.204 g, 0.63 mmol), methyl bromoacetate (0.82 mL, 8.6 mmol), and diisopropylethyl amine (0.56 mL, 3.2 mmol) to yield isoxazolidine **II-S22** (0.15 g, 60%).

5-([1,1'-biphenyl]-4-yl)-3-allyl-3-isobutylisoxazolidin-2-yl)ethanol (II-S23)

Diisobutylaluminium hydride (0.51 mL of a 1.5 M solution in toluene, 0.76 mmol) was added to a solution of isoxazolidine **II-S22** (0.15 g, 0.38 mmol) in toluene at 0° C. After 1 hr the reaction was partitioned between water and ethyl acetate, and the resulting organics were dried (sodium sulfate), concentrated under reduced pressure and chromatographed on silica gel to yield isoxazolidine **II-S23** (0.10 g, 72%).

5-([1,1'-biphenyl]-4-yl)-3-allyl-2-(2-azidoethyl)-3-isobutylisoxazolidine (II-S24)

Run according to the protocol for **II-S6**, with isoxazolidine **II-S23** (100 mg, 0.27 mmol), methanesulfonyl chloride (0.063 mL, 0.82 mmol), and triethylamine (0.11 mL, 0.82 mmol). Followed by sodium azide (260 mg, 4 mmol) to yield isoxazolidine **II-S24** (35 mg, 33% over two steps).

5-([1,1'-biphenyl]-4-yl)-2-(2-azidoethyl)-3-isobutylisoxazolidin-3-yl)ethanol (II-9)

Run according to the protocol for **II-2**, with isoxazoldine **II-S24** (31 mg, 0.08 mmol), Osmium tetroxide (as a 2.5% solution in tert butanol, 0.08 mL, 0.008 mmol) and N-methylmorpholine-N-oxide (14 mg, 0.12 mmol). This was followed by sodium periodate (26 mg, 0.12 mmol), and then followed by sodium borohydride (6 mg, 0.16 mmol). Upon completion, the reaction was partitioned between water and ethyl acetate, and the resulting organics were dried (sodium sulfate), concentrated under reduced pressure and chromatographed on silica gel to yield isoxazolidine **II-9** (15 mg, 47% over 3 steps).

Synthesis of Compound II-10 and Intermediates Using the Scheme Shown in Figure II-8.

4-bromobenzaldehyde oxime (II-S26)

Run according to the protocol for **II-S1** with 4-bromobenzaldehyde (37.1 g, 200 mmol) hydroxylamine hydrochloride (20.9 g, 300 mmol) and sodium carbonate (33.8 g, 319 mmol) to yield **II-S26** (28.7 g, 72%)

(3-(4-bromophenyl)-4,5-dihydroisoxazol-5-yl)methanol (II-S27)

Run according to the protocol for **II-S2** with oxime **II-S26** (14.9 g, 75 mmol), allyl alcohol (25.6 mL, 375 mmol), NaOCl (250 mL, 200 mmol, 6% solution) to yield **II-S27** (5.55 g, 29%).

3-(4-bromophenyl)-5-(((tert-butyldimethylsilyl)oxy)methyl)-4,5-dihydroisoxazole (II-S28)

Run according to the protocol for **II-S3** with isoxazolidine **II-S27** (5.55 g, 22 mmol), tert-butyldimethylchlorosilane (3.62 g, 24 mmol) and imidazole (3.75 g, 55 mmol) to yield isoxazolidine **II-S28** (7.14 g, 89%).

3-([1,1'-biphenyl]-4-yl)-5-(((tert-butyldimethylsilyl)oxy)methyl)-4,5-dihydroisoxazole (II-29)

Tetrakis(triphenylphosphine)palladium (80 mg, 0.07 mmol) was added to a solution of isoxazolidine **II-S28** (604 mg, 1.42 mmol), phenylboronic acid (207 mg, 1.7 mmol) and potassium carbonate (784 mg, 5.7 mmol) in 14 mL 1:1 water:dimethoxyethane. The resulting mixture was heated to 80° C. Upon completion, the crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S29** (492 mg, 82%).

3-([1,1'-biphenyl]-4-yl)-5-(((tert-butyldimethylsilyl)oxy)methyl)-3-(2-methylallyl)isoxazolidine (II-S30)

Run according to the protocol for **II-S3** with isoxazoline **II-S29** (2.5 g, 6.8 mmol), 2-

methylallylmagnesium chloride (42 mL of an 0.5M solution, 21 mmol), BF3 (2.64 mL, 21 mmol) to yield isoxazolidine **II-S30** (656 mg, 23%).

3-([1,1'-biphenyl]-4-yl)-5-(((tert-butyldimethylsilyl)oxy)methyl)-3-isobutylisoxazolidine (II-S31)

Palladium (II) acetate (27 mg, 0.04 mmol) was added to isoxazolidine **II-S30** (365 mg, 0.86 mmol) dissolved in triethoxysilane (0.4 mL, 2.15 mmol) under nitrogen in a sealed tube. After 16 hours, the crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S31** (180 mg, 49%).

3-([1,1'-biphenyl]-4-yl)-2-allyl-3-isobutylisoxazolidin-5-yl)methanol (II-S32)

Isoxazolidine **II-S31** (0.18 g, 0.43 mmol), allyl bromide (0.3 mL, 3.4 mmol), and diisopropylethyl amine (0.56 mL, 3.2 mmol) were combined in 4 mL of dimethylformamide in a sealed microwave safe reaction vessel. This solution was heated using a conventional microwave in 10 second pulses at 20% power, with four additional aliquots of allyl bromide added to replace any evaporation. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and passed through a short column of silica to remove as much DMF as possible. The resulting mixture was carried on crude.

Crude isoxazolidine was dissolved in 1.2 mL of 1M tetrabutylammonium fluoride in tetrahydrofuran and stirred at room temperature until the reaction was complete via

TLC. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S32** (75 mg, 50% over two steps).

3-([1,1'-biphenyl]-4-yl)-2-allyl-5-(azidomethyl)-3-isobutylisoxazolidine (II-S33)

Run according to the protocol for **II-S6**, with isoxazolidine **II-S32** (73 mg, 0.21 mmol), methanesulfonyl chloride (0.05 mL, 0.63 mmol), and triethylamine (0.08 mL, 0.6 mmol). Followed by sodium azide (195 mg, 3 mmol) to yield isoxazolidine **II-S33** in (60 mg, 76% over two steps).

3-([1,1'-biphenyl]-4-yl)-5-(azidomethyl)-3-isobutylisoxazolidin-2-yl)ethanol (II-10)

Run according to the protocol for **II-2**, with isoxazoldine **II-S33** (46 mg, 0.12 mmol), Osmium tetroxide (as a 2.5% solution in tert butanol, 0.12 mL, 0.012 mmol) and N-methylmorpholine-N-oxide (21 mg, 0.18 mmol). This was followed by sodium periodate (40 mg, 0.18 mmol), and then followed by sodium borohydride (9 mg, 0.24 mmol). Upon completion, the reaction was partitioned between water and ethyl acetate, and the resulting organics were dried (sodium sulfate), concentrated under reduced pressure and chromatographed on silica gel to yield isoxazolidine **II-10** (21 mg, 46% over 3 steps).

3-([1,1'-biphenyl]-4-yl)-3-allyl-5-(((tert-butyldimethylsilyl)oxy)methyl)isoxazolidine (II-S34)

Run according to the protocol for **II-S3** with isoxazoline **II-S29** (0.96 g, 2.63 mmol),

allylmagnesium chloride (3.9 mL of a 2M solution, 7.9 mmol), BF3 (0.99 mL, 7.9 mmol) to yield isoxazolidine **II-S34** (932 mg, 86%).

3-([1,1'-biphenyl]-4-yl)-3-allyl-2-isopentylisoxazolidin-5-yl)methanol (II-S35)

Sodium cyanoborohydride (0.5 g, 8 mmol) was added to isoxazolidine **II-S34** (0.67 g, 1.6 mmol) and isovaleraldehyde (2.44 mL, 22.7 mmol) in 8 mL methanol and 0.15 mL water at 0° C, which was then brought to a pH of 2 using 1M hydrochloric acid. Upon completion, the crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and passed through a short column of silica. The resulting mixture was carried on crude.

Crude isoxazolidine was dissolved in 16 mL of 1M tetrabutylammonium fluoride in tetrahydrofuran and stirred at room temperature until the reaction was complete via TLC. The crude mixture was partitioned with water, and the organics were concentrated under reduced pressure and purified by column chromatography to yield isoxazolidine **II-S35** (527 mg, 90% over two steps).

3-([1,1'-biphenyl]-4-yl)-3-allyl-5-(azidomethyl)-2-isopentylisoxazolidine (II-S36)

Run according to the protocol for **II-S6**, with isoxazolidine **II-S35** (495 mg, 1.35 mmol), methanesulfonyl chloride (0.32 mL, 4.1 mmol), and triethylamine (0.53 mL, 4.1 mmol). Followed by sodium azide (876 mg, 13.5 mmol) to yield isoxazolidine **II-S36** (224 mg, 43% over two steps).

3-([1,1'-biphenyl]-4-yl)-5-(azidomethyl)-2-isopentylisoxazolidin-3-yl)ethanol (II-11)

Run according to the protocol for **II-2**, with isoxazoldine **II-S36** (191 mg, 0.49 mmol), Osmium tetroxide (as a 2.5% solution in tert butanol, 0.49 mL, 0.05 mmol) and N-methylmorpholine-N-oxide (90 mg, 0.75 mmol). This was followed by sodium periodate (160 mg, 0.75 mmol), and then followed by sodium borohydride (29 mg, 0.75 mmol). Upon completion, the reaction was partitioned between water and ethyl acetate, and the resulting organics were dried (sodium sulfate), concentrated under reduced pressure and chromatographed on silica gel to yield isoxazolidine **II-11** (120 mg, 62% over 3 steps).

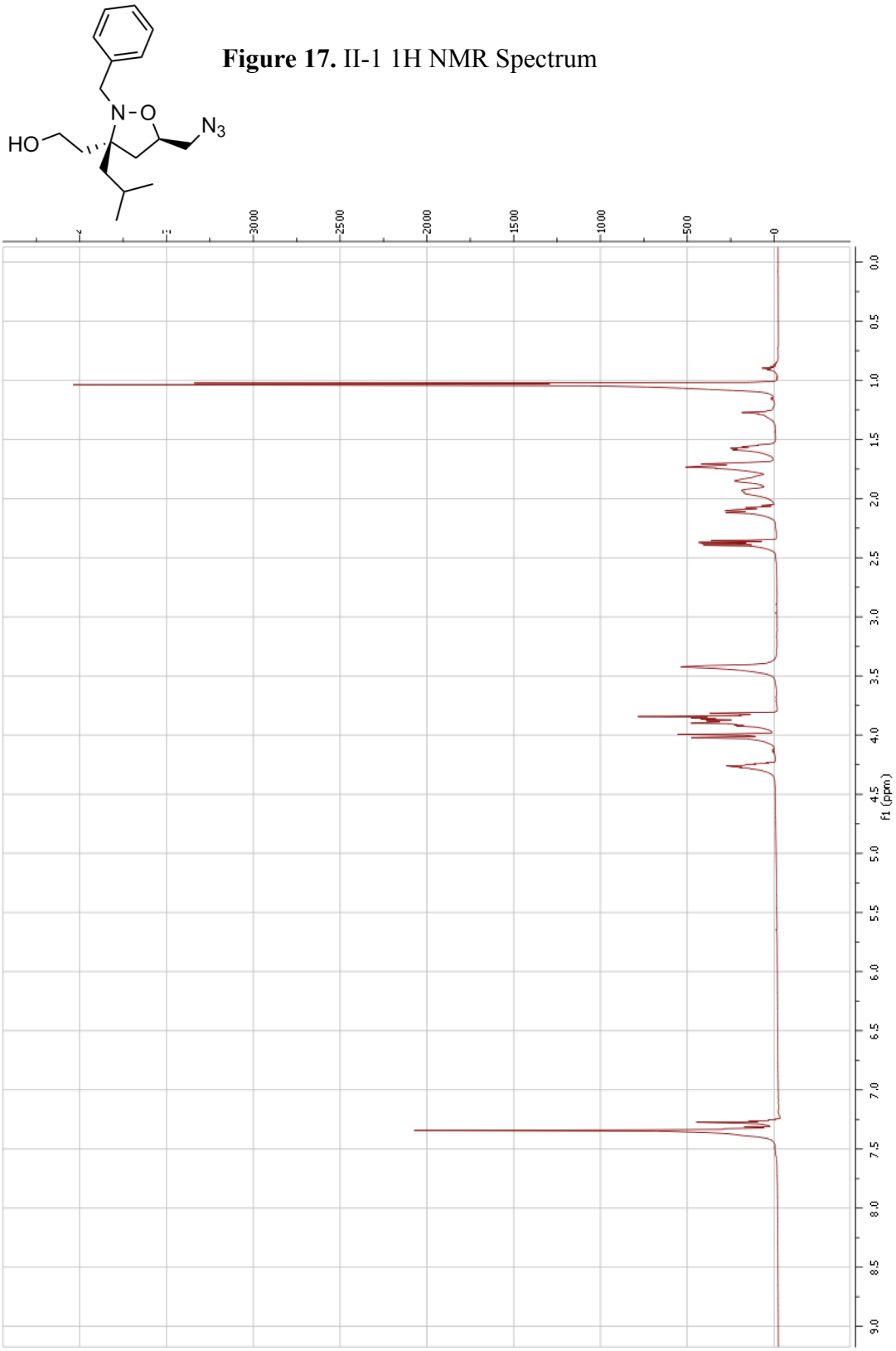


Figure 17. II-1 1H NMR Spectrum

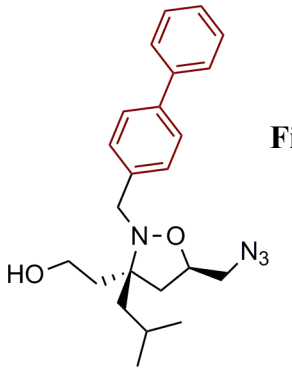
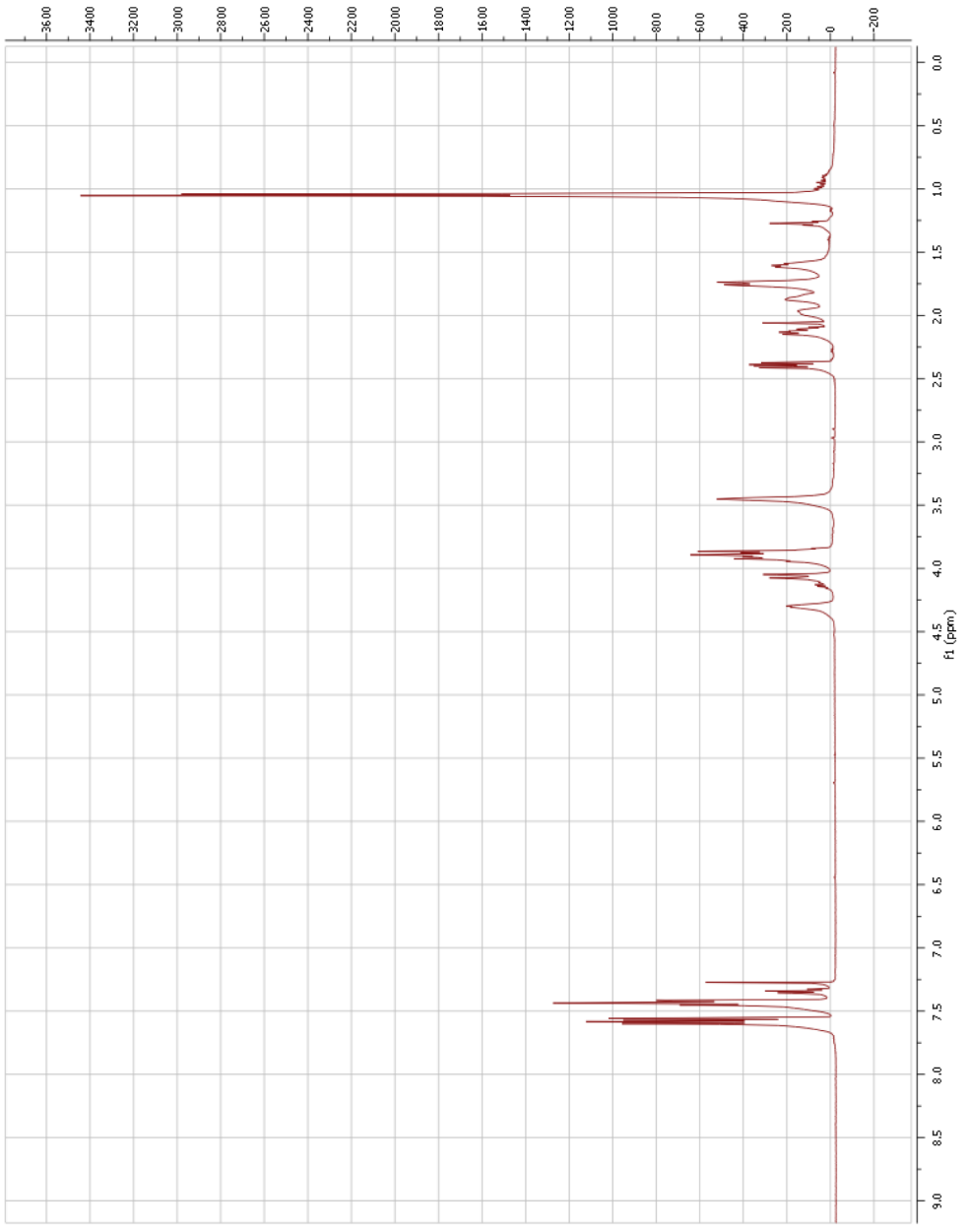


Figure 18. II-2 1H NMR Spectrum



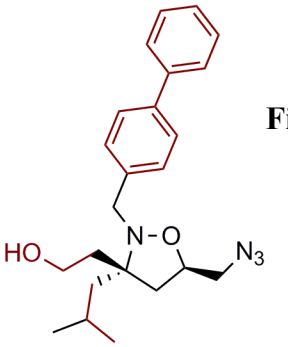
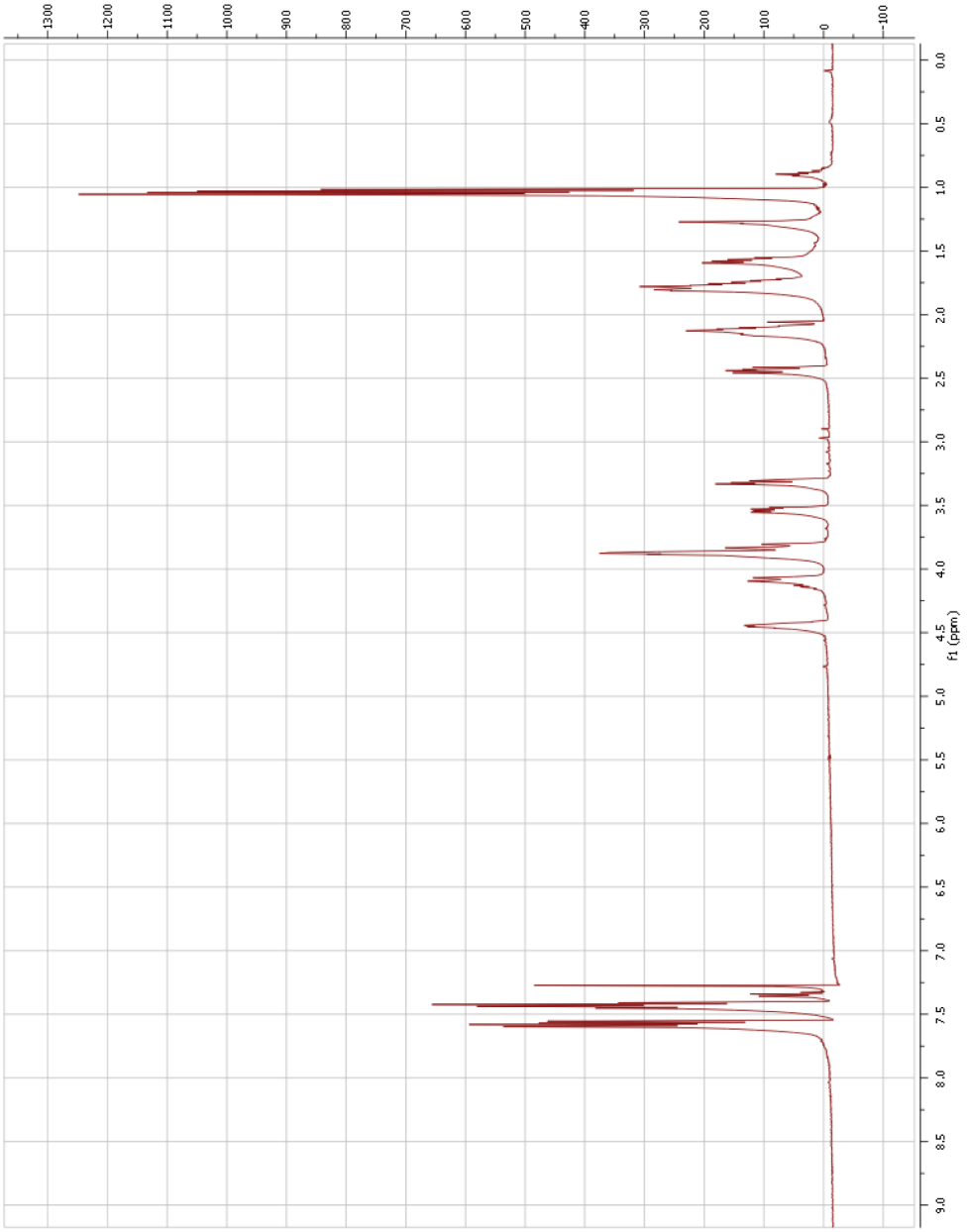


Figure 19. II-3 1H NMR Spectrum



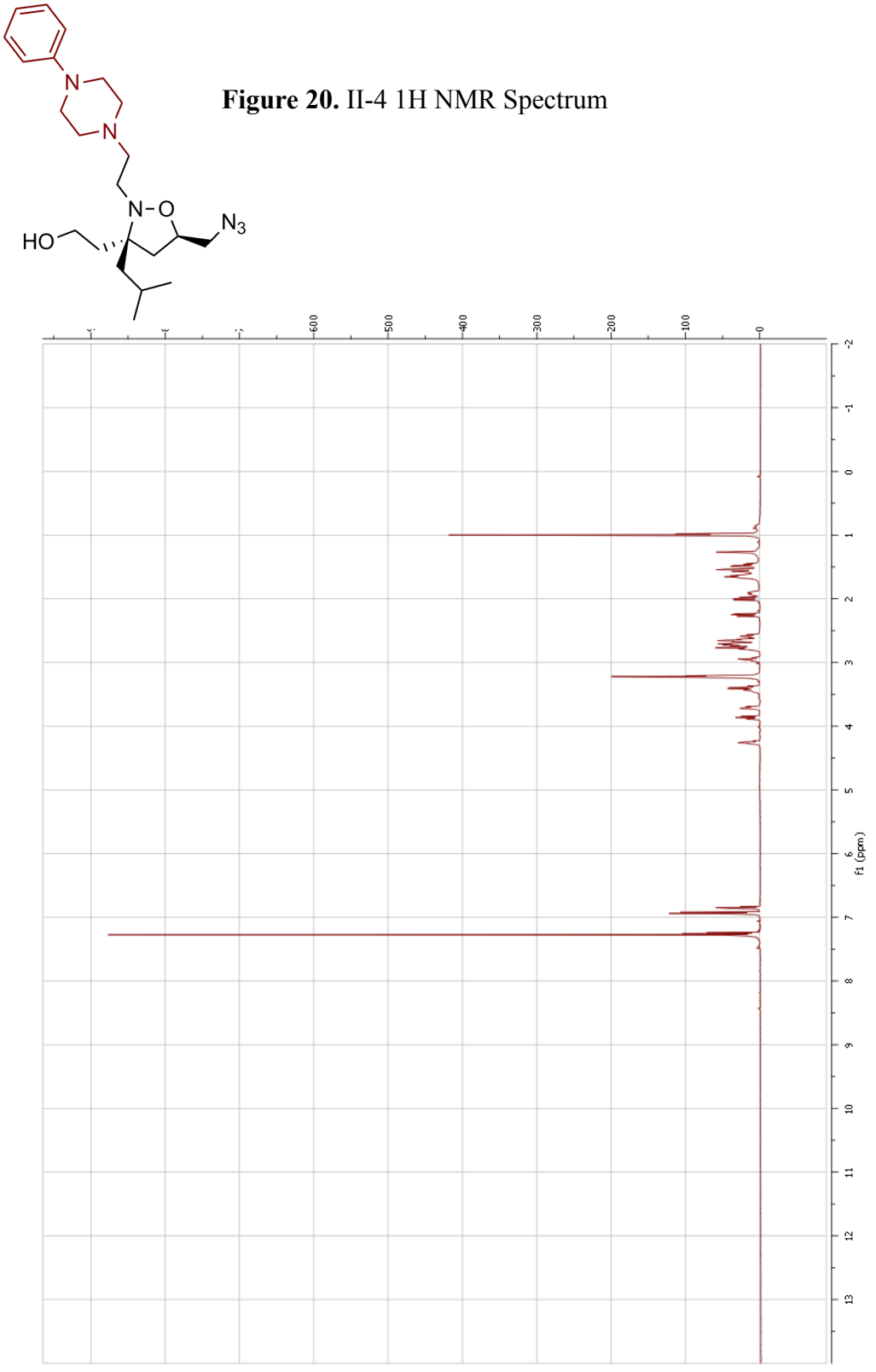


Figure 20. II-4 1H NMR Spectrum

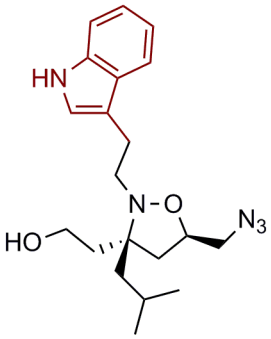
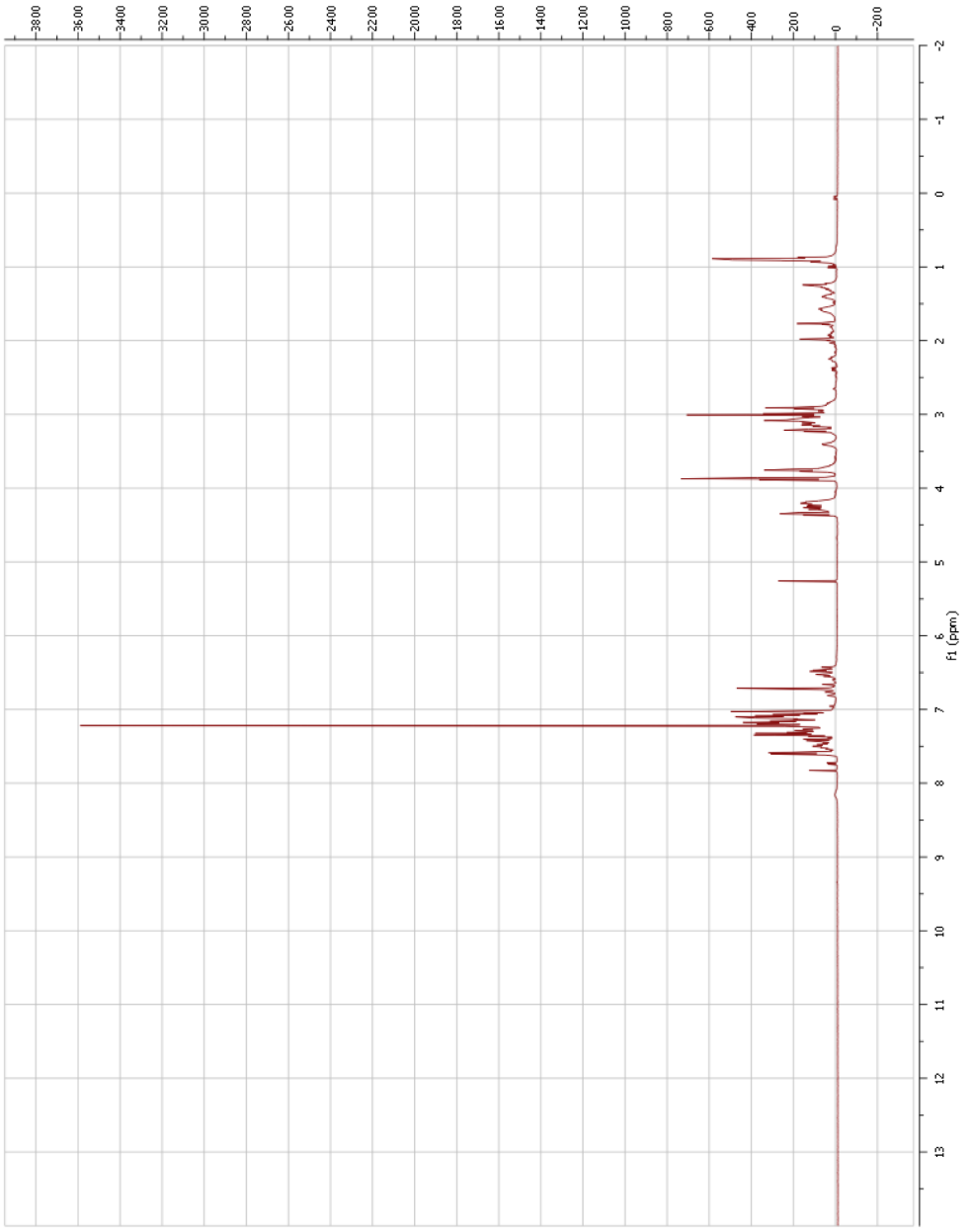


Figure 21. II-5 1H NMR Spectrum



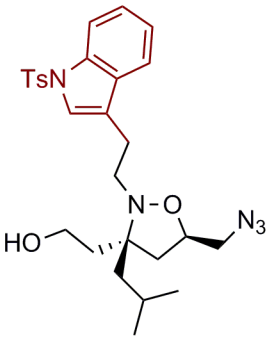
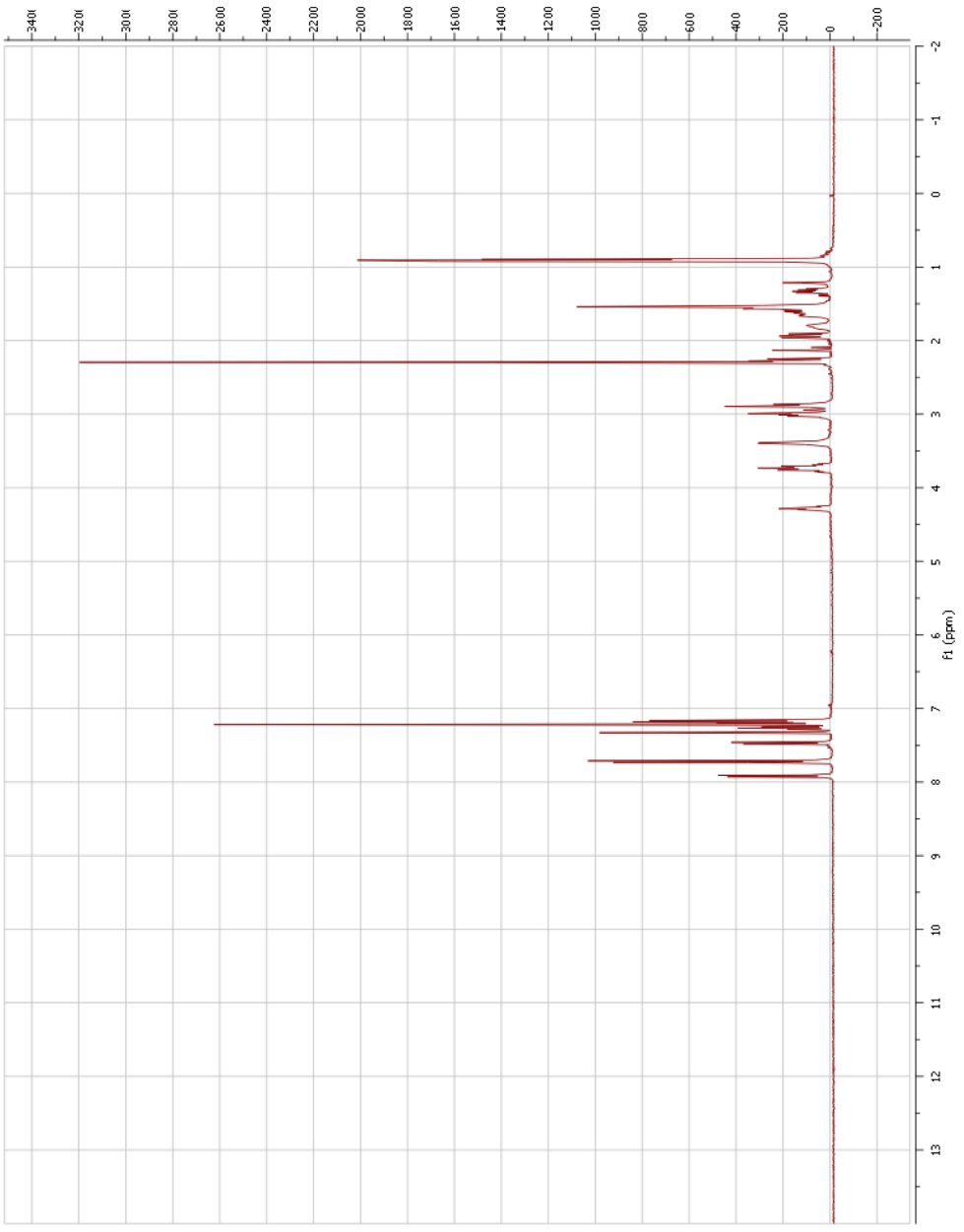


Figure 22. II-6 1H NMR Spectrum



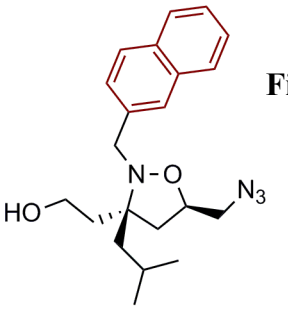
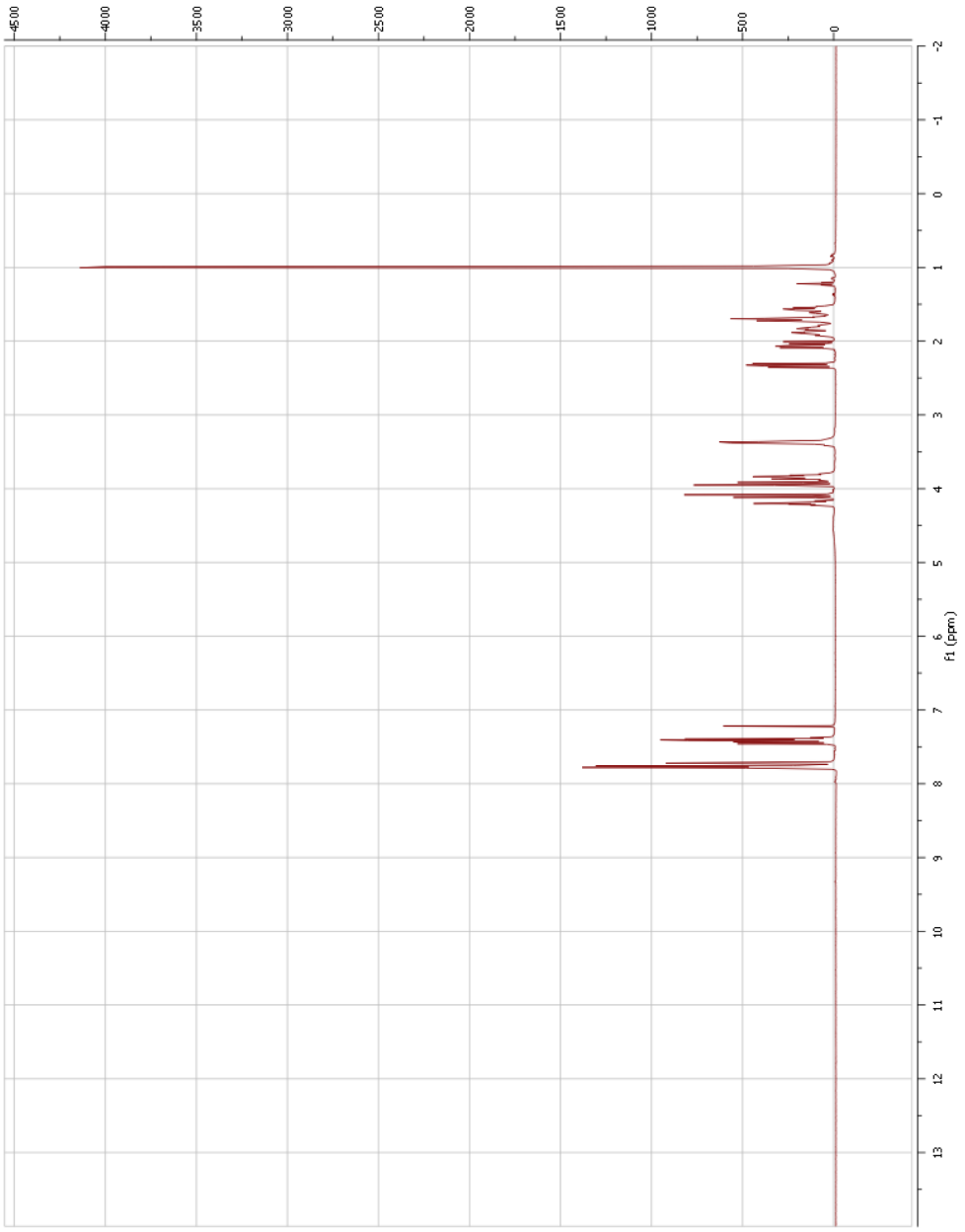


Figure 23. II-7 1H NMR Spectrum



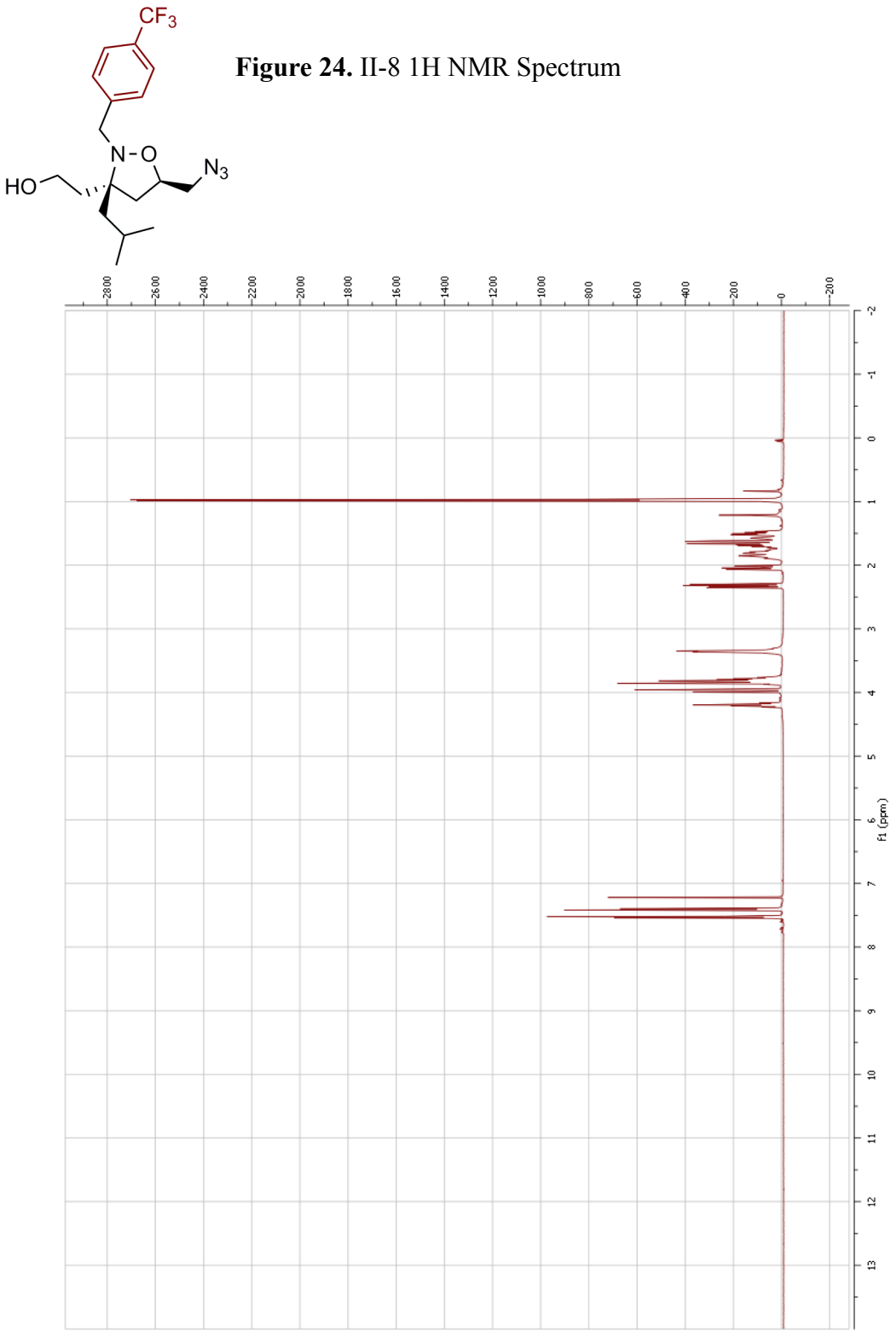


Figure 24. II-8 1H NMR Spectrum

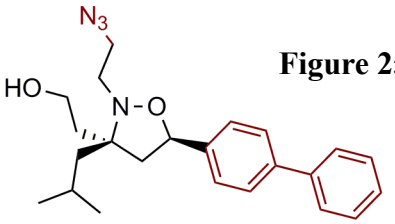
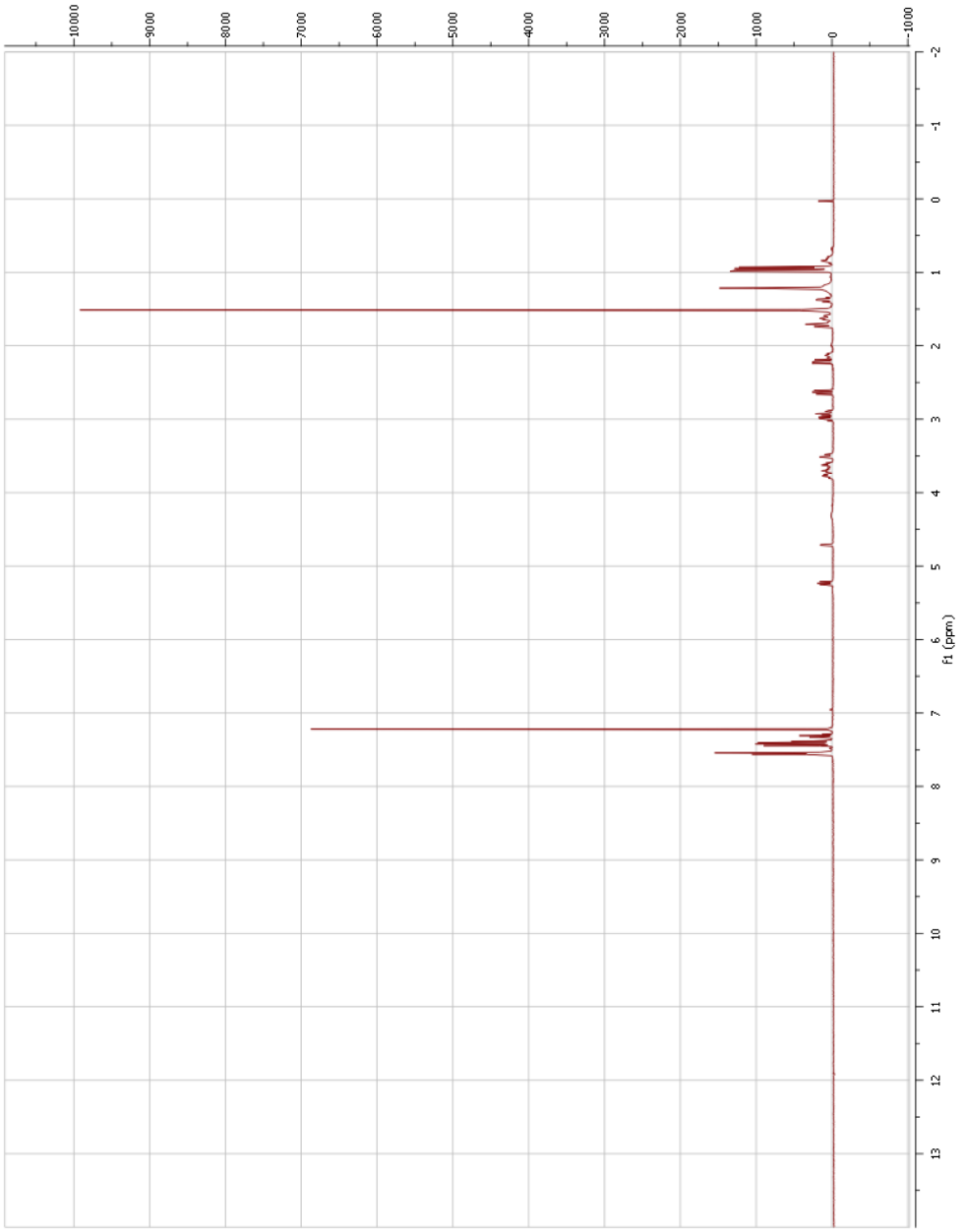


Figure 25. II-9 1H NMR Spectrum



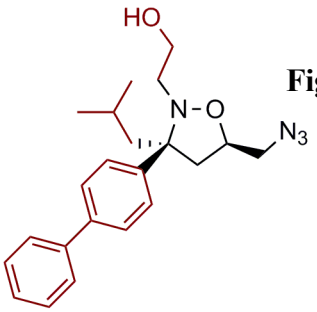


Figure 26. II-10 1H NMR Spectrum

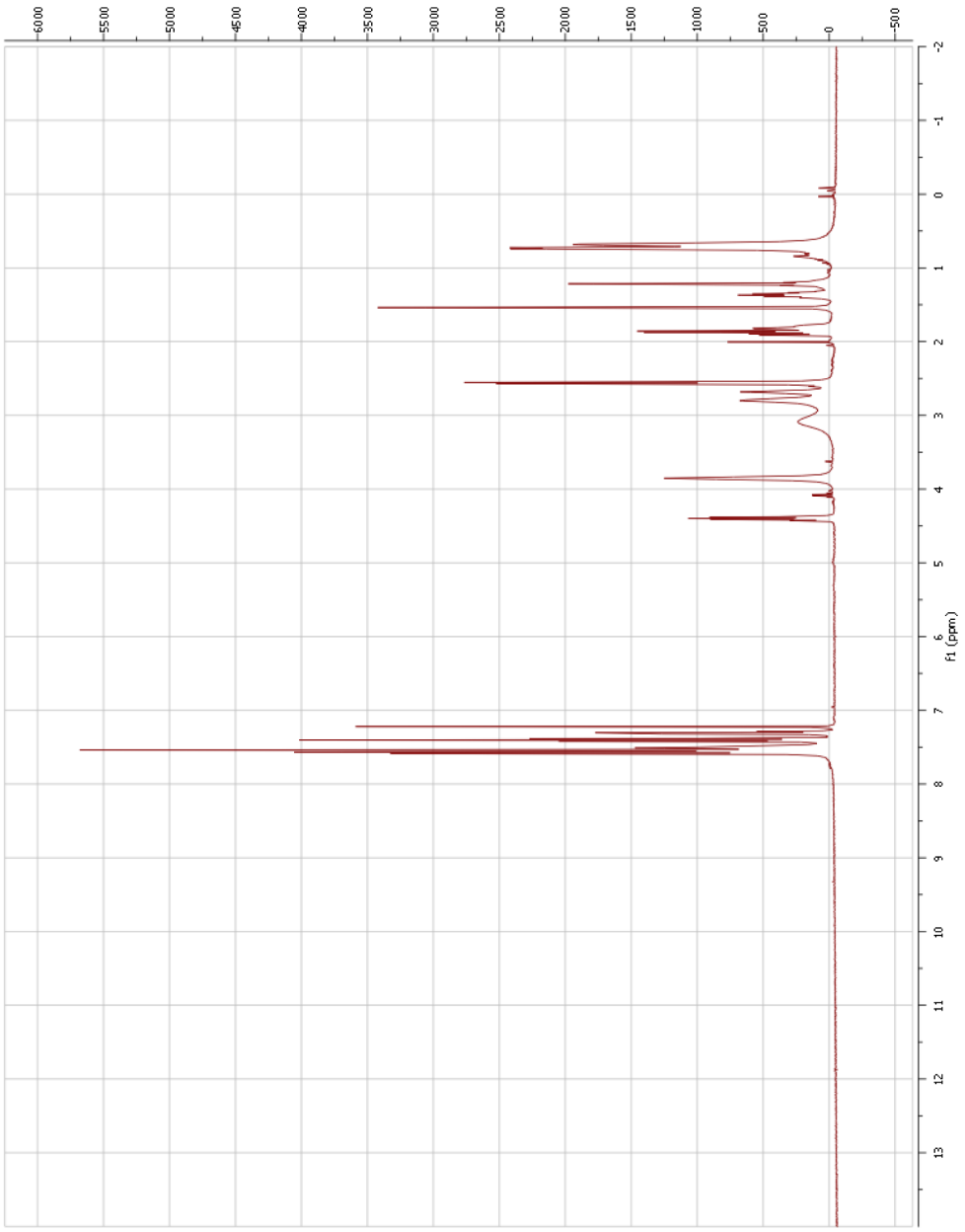
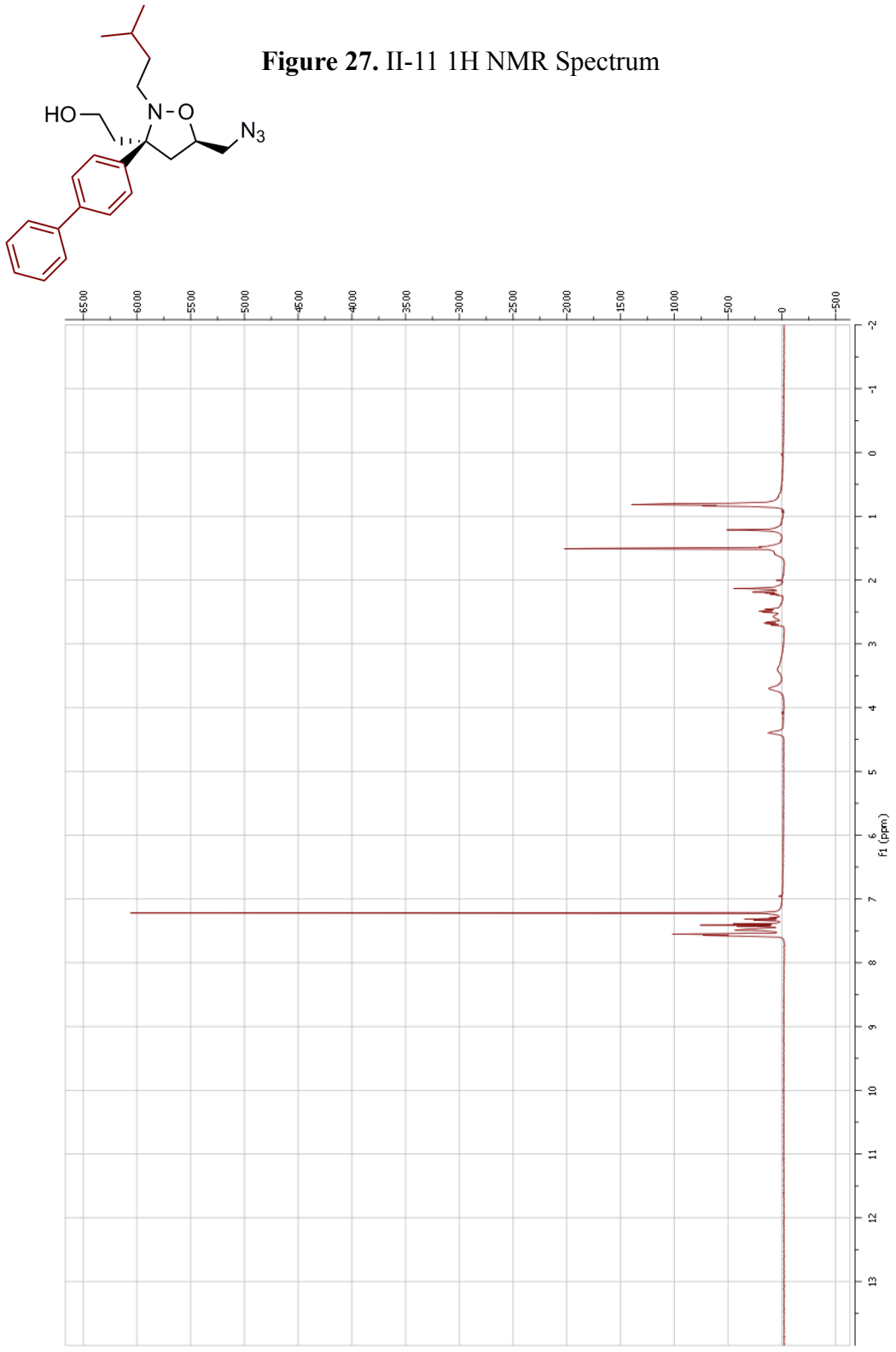


Figure 27. II-11 1H NMR Spectrum



G. References

(1) Lee, L. W.; Mapp, A. K. Transcriptional Switches: Chemical Approaches to Gene Regulation. *Journal of Biological Chemistry* **2010**, *285*, 11033-11038.

(2) Mapp, A. K.; Ansari, A. Z. A TAD Further: Exogenous Control of Gene Activation. *ACS Chem. Biol.* **2007**, *2*, 62-75.

(3) Arndt, H.-D. Small Molecule Modulators of Transcription. *Angew. Chem. Int. Ed.* **2006**, *45*, 4552-4560.

(4) Emami, K. H. Nguyen, C. Ma, H. Kim, D. H. Jeong, K. W. Eguchi, M. Moon, R. T. Teo, J.-T. Oh, S. W. Kim, H. Y. Moon, S. H. Ha, J. R.; Kahn, M. A small molecule inhibitor of B -catenin /CREB-binding protein transcription. *Proceedings of the National Academy of Sciences* **2004**, *101*, 12682–12687.

(5) Kung, A. L. Zabludoff, S. D. France, D. S. Freedman, S. J. Tanner, E. A. Vieira, A. Cornell-Kennon, S. Lee, J. Wang, B. Wang, J. Memmert, K. Naegeli, H.-U. Petersen, F. Eck, M. J. Bair, K. W. Wood, A. W.; Livingston, D. M. Small molecule blockade of ranscriptional coactivation of the hypoxia-inducible factor pathway. *Cancer Cell* **2004**, *6*, 33-43.

(6) Best, J. L. Amezcua, C. A. Mayr, B. Flechner, L. Murawsky, C. M. Emerson, B. Zor, T. Gardner, K. H.; Montminy, M. Identification of small-molecule antagonists thatinhibit an activator:coactivator interaction. *Proceedings of the National Academy of Sciences* **2004**, *101*, 17622–17627.

(7) Fuxreiter, M. Tompa, P. Simon, I. Uversky, V. N. Hansen, J. C.; Asturias, F. J. Malleable machines take shape in eukaryotic transcriptional regulation. *Nat Chem Biol* **2008**, *4*, 728-737.

(8) Langlois, C. Mas, C. Di Lello, P. Jenkins, M. Legault, P.; Omichinski, J. G. NMR Structure of the Complex between the Tfb1 Subunit ofTFIIH and the Activation Domain of VP16: StructuralSimilarities between VP16 and p53. *Journal of the American Chemical Society* **2008**, *130*, 10596–10604.

(9) Rowe, S. P. Casey, R. J. Brennan, B. B. Buhrlage, S. J.; Mapp, A. K. Transcriptional Up-regulation in Cells Mediated by a Small Molecule. *J. Am. Chem. Soc.* **2007**, *129*, 10654-10655.

(10) Minter, A. R. Brennan, B. B.; Mapp, A. K. A Small Molecule Transcriptional Activation Domain. *Journal of the American Chemical Society* **2004**, *126*, 10504-10505.

(11) Buhrlage, S. J. Bates, C. A. Rowe, S. P. Minter, A. R. Brennan, B. B. Majmudar, C. Y. Wemmer, D. E. Al-Hashimi, H.; Mapp, A. K. Amphipathic Small Molecules Mimic theBinding Mode and Function of EndogenousTranscription Factors. *ACS Chemical Biology* **2009**, *4*, 335-345.

(12) Chang, C.-H. Scott, G. K. Kuo, W.-L. Xiong, X. Suzdaltseva, Y. Park, J. W. Sayre, P. Erny, K. Collins, C. Gray, J. W.; Benz, C. C. ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. *Oncogene* **1997**, *14*, 1617 - 1622.

(13) Allander, S. V. Illei, P. B. Chen, Y. Antonescu, C. R. Bittner, M. Ladanyi, M.; Meltzer, P. S. Expression Profiling of Synovial Sarcoma by cDNA Microarrays.

American Journal of Pathology **2002**, *161*, 1587-1595.
(14) Asada, S. Choi, Y. Yamada, M. Wang, S.-C. Hung, M.-C. Qin, J.; Uesugi, M. External control of Her2 expression and cancer cell growth by targeting a Ras-linked coactivator. *Proceedings of the National Academy of Sciences* **2004**, *99*, 12747–12752.
(15) Ptashne, M.; Gann, A. *Genes and Signals*; 1st edition (November 13, 2001) ed. Cold Spring Harbor Laboratory Press.
(16) Hall, D. B. The VP16 Activation Domain Interacts with Multiple Transcriptional Components as Determined by Protein-Protein Cross-linking in Vivo. *Journal of Biological Chemistry* **2002**, *277*, 46043-46050.
(17) Hermann, S. How Transcriptional Activators Bind Target Proteins. *Journal of Biological Chemistry* **2001**, *276*, 40127-40132.
(18) Biddick, R. K. Law, G. L. Chin, K. K. B.; Young, E. T. The Transcriptional Coactivators SAGA, SWI/SNF, and Mediator Make Distinct Contributions to Activation of Glucose-repressed Genes. *Journal of Biological Chemistry* **2008**, *283*, 33101-33109.
(19) Taatjes, D. J. The human Mediator complex: a versatile, genome-wide regulator of transcription. *Trends in Biochemical Sciences* **2010**, *35*, 315-322.
(20) Dames, S. A. Martinez-Yamout, M. De Guzman, R. N. Dyson, H. J.; Wright, P. E. Structural basis for Hif-1a/CBP recognition in the cellular hypoxic response. *Proceedings of the National Academy of Sciences* **2002**, *99*, 5271.
(21) Shimogawa, H. Kwon, Y. Mao, Q. Kawazoe, Y. Choi, Y. Asada, S. Kigoshi, H.; Uesugi, M. A Wrench-Shaped Synthetic Molecule that Modulates a Transcription Factor−Coactivator Interaction. *J. Am. Chem. Soc.* **2004**, *126*, 3461-3471.
(22) Kopp, J. L. Wilder, P. J. Desler, M. Kinarsky, L.; Rizzino, A. Different Domains of the Transcription Factor ELF3 Are Required in a Promoter-specific Manner and Multiple Domains Control Its Binding to DNA. *Journal of Biological Chemistry* **2006**, *282*, 3027-3041.
(23) Eckel, K. L. Tentler, J. J. Cappetta, G. J. Diamond, S. E.; Gutierrez-Hartmann, A. The Epithelial-Specific ETS Transcription Factor ESX/ESE-1/Elf-3 Modulates Breast Cancer-AssociatedGene Expression. *DNA and Cell Biology* **2003**, *22*, 79-94.
(24) Slamon, D. J. Godolphin, W. Jones, L. A. Holt, J. A. Wong, S. G. Keith, D. E. Levin, W. J. Stuart, S. G. Udove, J. Ullrich, A.; Press, M. F. Studies of the HER-2/neu Proto-Oncogene in Human Breast and Ovarian Cancer. *Science* **1989**, *244*, 707-712.
(25) Burstein, H. J. The distinctive nature of HER2-positive breast cancers. *New England Journal of Medicine* **2005**, *353*, 1652–1654.
(26) Hirsch, F. R. Varella-Garcia, M.; Cappuzzo, F. Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. *Oncogene* **2009**, *28*, S32-S37.
(27) Slamon, D. J. Clark, G. M. Wong, S. G. Levin, W. J. Ullrich, A.; McGuire, W. L. Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene. *Science* **1987**, *235*, 177-182.

(28) Yarden, Y. Biology of HER2 and its importance in breast cancer. *Oncology* **2000**, *61*, 1–13.
(29) Garrett, T. P. J. McKern, N. M. Lou, M. Elleman, T. C. Adams, T. E. Lovrecz, G. O. Kofler, M. Jorissen, R. N. Nice, E. C. Burgess, A. W.; others The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Molecular cell* **2003**, *11*, 495–505.
(30) Hendriks, B. S. Quantitative Analysis of HER2-mediated Effects on HER2 and Epidermal Growth Factor Receptor Endocytosis: DISTRIBUTION OF HOMO- AND HETERODIMERS DEPENDS ON RELATIVE HER2 LEVELS. *Journal of Biological Chemistry* **2003**, *278*, 23343-23351.
(31) Stern, D. F. ERBB3/HER3 and ERBB2/HER2 Duet in Mammary Development and Breast Cancer. *J Mammary Gland Biol Neoplasia* **2008**, *13*, 215-223.
(32) Sergina, N. V. Rausch, M. Wang, D. Blair, J. Hann, B. Shokat, K. M.; Moasser, M. M. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature* **2007**, *445*, 437-441.
(33) Kong, A. Calleja, V. Leboucher, P. Harris, A. Parker, P. J.; Larijani, B. HER2 Oncogenic Function Escapes EGFR Tyrosine Kinase Inhibitors via Activation of Alternative HER Receptors in Breast Cancer Cells. *PLoS ONE* **2008**, *3*, e2881.
(34) Amin, D. N. Sergina, N. Ahuja, D. McMahon, M. Blair, J. A. Wang, D. Hann, B. Koch, K. M. Shokat, K. M.; Moasser, M. M. Resiliency and Vulnerability in the HER2-HER3 Tumorigenic Driver. *Science Translational Medicine* **2010**, *2*, 16ra7-16ra7.
(35) Chen, J.-S. Lan, K.; Hung, M.-C. Strategies to target HER2/neu overexpression for cancer therapy. *Drug Resistance Updates* **2003**, *6*, 129-136.
(36) Drebin, J. A. Link, V. C. Stern, D. F. Weinberg, R. A.; Greene, M. I. Down-Modulation of an Oncogene Protein Product and Reversion of the Transformed Phenotype by Monoclonal Antibodies. *Cell* **1985**, *41*, 695-706.
(37) Menendez, J. A. Vellon, L. Mehmi, I. Oza, B. P. Ropero, S. Colomer, R.; Lupu, R. Inhibition of fatty acid synthase (FAS) suppresses HER2/ neu (erbB-2) oncogene overexpressionin cancer cells. *Proceedings of the National Academy of Sciences* **2004**, *101*, 10715–10720.
(38) Mapp, A. K.; Ansari, A. Z. A TAD Further: Exogenous Control of Gene Activation. *ACS Chem. Biol.* **2007**, *2*, 62-75.
(39) Volkman, H. M. Rutledge, S. E.; Schepartz, A. Binding Mode and Transcriptional Activation Potential of High Affinity Ligands for the CBP KIX Domain. *J. Am. Chem. Soc.* **2005**, *127*, 4649-4658.
(40) Rowe, S. P.; Mapp, A. K. Assessing the Permissiveness of Transcriptional Activator Binding Sites. *Biopolymers* **2008**, *89*, 578-582.
(41) Buhrlage, S. J. Brennan, B. B. Minter, A. R.; Mapp, A. K. Stereochemical Promiscuity in Artificial Transcriptional Activators. *Journal of the American Chemical Society* **2005**, *127*, 12456-12457.
(42) Brockhoff, G. Heiß, P. Schlegel, J. Hofstaedter, F.; Knuechel, R. Epidermal Growth Factor Receptor, c-erbB2 and c-erbB3 Receptor Interaction, and Related Cell Cycle Kinetics of SK-BR-3 and BT474 Breast Carcinoma Cells. *Cytometry* **2001**,

44, 338-348.
(43) Wan, J. Sazani, P.; Kole, R. Modification of HER2 pre-mRNA alternative splicing and itseffects on breast cancer cells. *International Journal of Cancer* **2009**, *124*, 772-777.

Chapter III

A Multi-Pronged Approach to Targeting Oncogenic Transcription

A. Chapter Overview

The aberrant function of a growing number of transcriptional activators is associated with the development and progression of human diseases such as cancer.[1,2,3,4] Molecules that interfere with the ability of transcriptional activators to control expression of their target genes have great promise as biochemical tools and therapeutics. Activators regulate transcription through a complex network of interactions with transcriptional coactivator proteins. Blocking these interactions inhibits transcription. However, the affinity of these activator-coactivator interactions is modest (high nano- to low micro-molar), possibly owing to the conformational flexibility of activation domains and the highly promiscuous nature of activator binding sites.[5] In light of these issues, it is unsurprising that small- and large molecule inhibitors of these interactions require micromolar concentrations to exert their effects.[6,7,8,9] It also highlights potential selectivity issues that may arise from blocking interactions with shared coactivator targets.

The previous chapter described the development of a new class of small molecule transcriptional inhibitors that mimic transcriptional activation domains; this includes **II-2**

(figure III-1), an isoxazolidine that mimics the activation domain of the transcription factor ESX, and interferes with transcription of the ESX-regulated oncogene erbB2 at micromolar concentrations. Here we present a strategy that mitigates the potency and selectivity concerns of transcriptional inhibitors through a multi-pronged intervention against the erbB2 regulatory pathway. Specifically, **II-2** is tested in combination with three other agents that target cellular events related to erbB2 activity, as shown in figure III-1. Two of these combinations exhibit synergistic increases in activity and selectivity, and correspondingly, reduce the doses of both agents required to cause 50% of maximal inhibition of mitochondrial viability in erbB2+ cancer cells. For example, combinations of **II-2** and geldanamycin allow dose reductions of greater than 15-fold in both agents, while increasing selectivity for erbB2+ cells by as much as 30 fold when compared to individual agents.

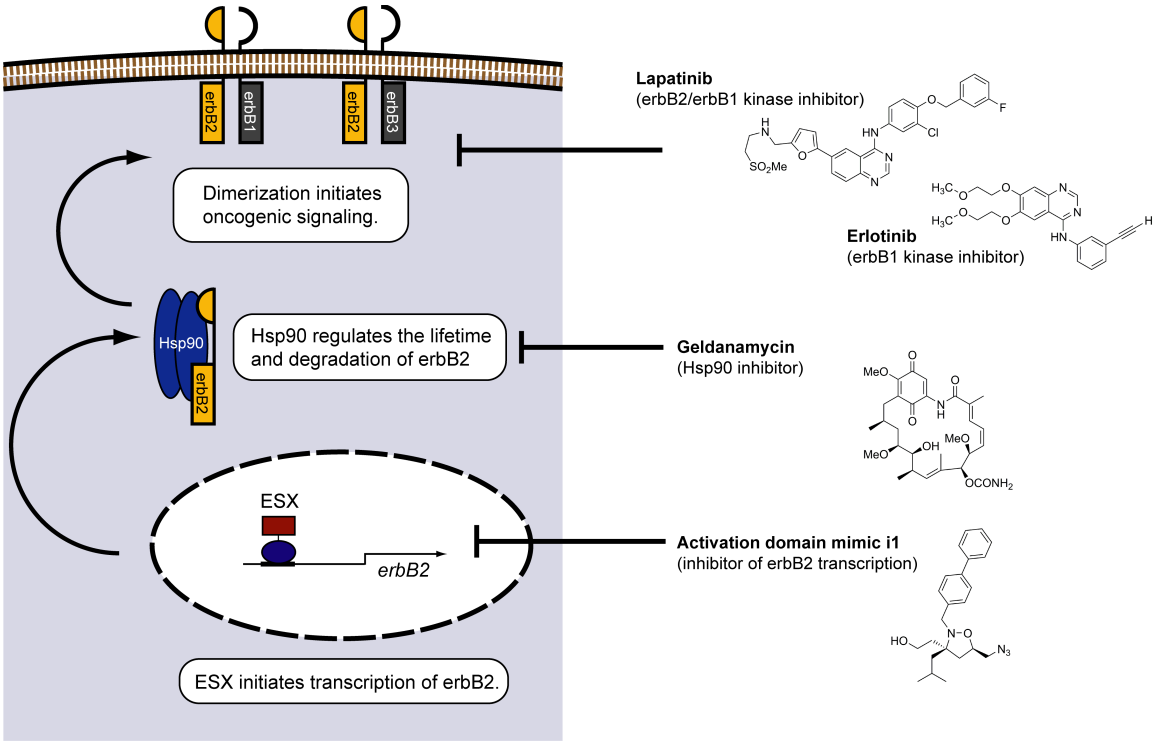


Figure III-1. Rationale for synergy. Combinations of agents that target the transcription of erbB2, as well as the lifetime of the mature protein (Geldanamycin), or the ability of erbB2 containing dimers to initiate signaling (such as Lapatinib or Erlotinib) may be synergistically more effective that any of their individual components.

B. Background

B.1. The Activitiy and Selectivity Concerns that Apply to Transcriptional Inhibitors

Although transcriptional inhibitors have been designed using a variety of scaffolds, from small molecules,[7,8,9] to conformationally constrained and proteolytically resistant peptides,[10,11] there are two recurring issues with these inhibitors – their activity and selectivity. Even in cases where peptides that directly mimic activation domains are used, such the HIF-1α mimic developed by the Arora lab, micromolar concentrations are

required for cellular activity. Because activators initiate transcription through a complex series of low affinity protein-protein interactions with regions of coactivators that are designed to accomodate a variety of non-homologous activation domains,[12,13] there may be a limit to the affinity and biological activity that can be achieved using small molecules or short peptidomimetics.

There are two potential selectivity issues for transcriptional inhibitors – the selectivity for a particular inhibitor-coactivator interaction, and selectivity for a effects on a particular gene. The first is an endemic problem for small molecule inhibitors of protein-protein interactions.[14] Because the surface area of these interactions is typically much greater than the surface area of any reasonably sized small molecule it is likely that small molecule inhibitors will be less specific for a given target than the evolutionarily designed protein interaction partner. Additionally, because transcriptional activators interact with coactivator sites that are also targeted by other transcriptional activators,[6,12] inhibitors that interfere with the ability of one activator to bind to these sites are likely to have effects on the activity of other activators that target that same site. Furthermore, individual transcriptional activators regulate the expression of many different genes, which can include both related programs within the cell or feedback mechanisms that counteract and limit the primary function of the transcriptional activator.[15] All of these factors indicate that even if it were possible to design transcriptional inhibitors which perfectly mimicked a single activator, such inhibitors would still have broad effects on cellular transcription.

B.2. Rationale for the Use of Multiple Agents in Combination

The regulation of biological systems – especially those which are critical to the survival – is often complex and robust. Interventions that target these processes, such as the uncontrolled growth or suppression of apopotosis that characterize cancer cells, can be made irrelevent through redundant systems within the cell.[16] A prime example of this is oncogenic signalling by erbB2 containing dimers on the cell surface. The kinase erbB2 is the preferred dimerization partner for ligand receptors erbB1 and erbB3.[17,18] In cell types where erbB1/erbB2 heterodimers are the primary signaling pair, kinase inhibitors that target erbB1 activity can initially reduce erbB2 signaling, but the cancer cells respond by increasing the steady state levels of erbB3 allowing pro-growth and anti-apoptotic signals to continue in spite of kinase inhibition.[19,20] In these studies, it has been shown that a combination of erbB2 knockdown and kinase inhibition is dramatically more effective than kinase inhibition alone. Similar results, in which the use of multiple agents to target related events within a cell achieve a 'synergistic' increase in effect, have been reported for other systems such as the use of Ribavirin and Disulfiram to target related processes in MRSA.[21]

B.3. Methods for Evaluating Synergy in Multi-Component Treatments

There are two accepted and quantitative measures of synergy. One is an effect based method developed by Bliss and the other is the isobolographic method for

measuring dose reduction developed by Loewe and elaborated by Chou and others.[22] The Bliss measurement is based on calculation of the statistically expected additive effects of two seperate agents (A and B) at particular doses. When combined, these two doses of A and B are expected to have an effect equal to (eA+eB)-(eA*eB), where eA and eB are the effects of A and B converted to a value between 0 and 1. For example, if a given dose of A kills 50% of a cell population (effect = 0.5), and a given dose of B also kills 50% of the same cell population, the statistically expected outcome of a combination of A and B at these same doses, would be to kill 75% of the cell population (50% of the whole population from A, and 50% of the remaining population from B). An example of the use of this method is a series of high throughput screen by Borisy and coworkers that identified a combination of fluconazole and phenazopyridine that is synergistically effective against fungal infection.[23]

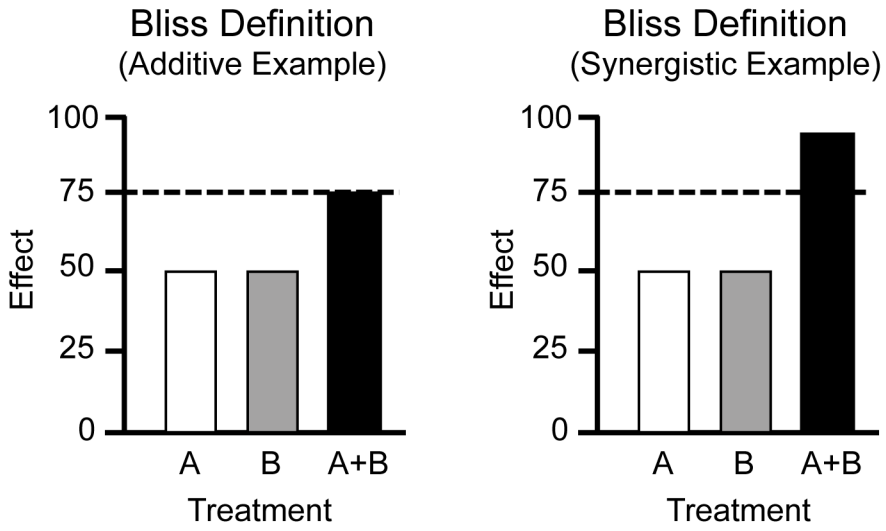


Figure III-2. Bliss definition of synergy. A graphical representation of hypothetical examples being evaluated for synergy using the Bliss (multiplicative) definition. In both examples, the individual agents have an approximately 50% effect on their own. In the additive example, the combination of A and B, at the same doses which they were tested at individually, has a 75% effect. In the synergistic example, the combination has an effect greater than 75%.

Although this method has the advantages of straighforward analysis, and of providing an intuitive readout, it suffers from the limitation that it does not rigorously account for the shape of the dose effect curves of the individual agents.[24] Thus, in a null hypothesis case where agents A and B were the same compound (or enantiomers in a situation where this did not make a difference), and the dose effect curves had a greater than sigmoidal slope, it would be possible to see 'synergistic' effects merely because the total dose of compound had reached the exponential decay portion of the dose effect curve. Although this objection has lead to the development of the isobolographic methods described below, Bliss synergy is still widely used with the caveat that it is capable of producing false postives in some cases.[25]

The isobolographic method for evaluating synergy is based on the principle of

dose equivalence.[24] Tested combinations are compared to the null hypothesis that both components are actually the same agent (the results of which are considered to be additive). Dose-effect curves are evaluated for both individual agents, and for several fixed ratio combinations. Each combination has an IC_{50} for both of its components – because the amount of combination that produces 50% effect will have a particular amount of each component. The combination IC_{50}s are then used to calculate dose fractions for each component. Thus, the dose fraction (of A) for a 1:1 combination of A:B would be the IC_{50} (of A, in the 1:1 combination) divided by the IC_{50} of A as a single agent. Repeating this process for B allows the combinations to be plotted on a isobologram. This chart effectively represents the degree of reduction in the doses required to produce 50% effect caused by combination use. The dotted line drawn between A and B indicates the line that would be produced in the case of the null hypothesis. Combinations which fall inside the region to the left of this line are considered synergistic, because they allow greater dose reduction that would be expected from simply using more of one of the single agents. The degree of synergy is given by the combination index (CI), which is simply the sum of the X and Y coordinates for any given combination. The CI of individual agents and additive combinations will equal one. A CI less than one indicates synergy, while a CI greater than 1 indicates antagonism. An example of this is the synergistic inhibition of TNF-α induction by varying combinations of nortripyline and prednisolone.[21]

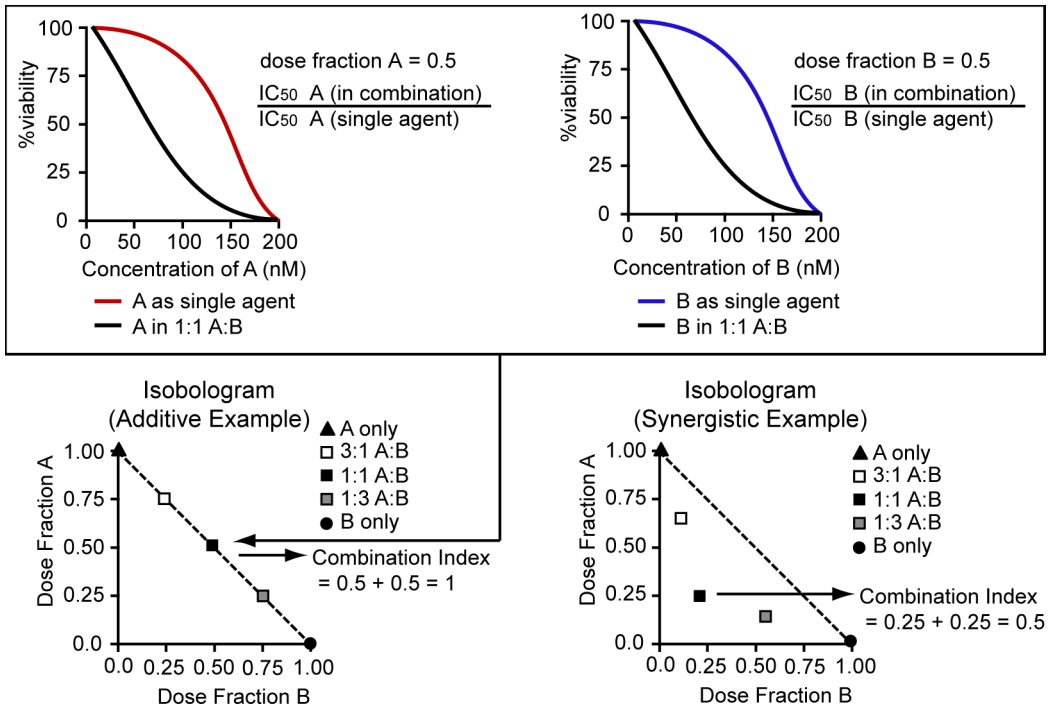


Figure III-3. Loewe definition of synergy. Hypothetical examples of combinations tested for synergy using the Loewe (isobolographic) method. In this method, dose-effect curved are generated for fixed dose ratios of the two agents. Thus, each combination has two IC_{50}s . These IC_{50}'s are normalized to the IC50's of the components on their own, and each combination is plotted on an x,y coordinate graph based on the amount of compound required for 50% effect in the combination divided by the amount of the same agent in isolation required for 50%.

C. The Use of II-2 in Combination with Other Agents that Target ErbB2 Lifetime and Activity

C.1. Rationale for Combination of II-2 and Geldanamycin

Geldanamycin is a naturally occuring antibiotic that binds to the nucleotide pocket of the constituitively expressed chaperone Hsp90, causing degradation of Hsp90 client proteins.[26] In the absence of inhibitor, Hsp90 acts as part of a multi-protein chaperone

complex to promote folding of a number of key pro-growth signalling proteins, including erbB2,[27] into their active forms. Because geldanamycin prevents nucleotide binding and the ATP hydrolysis neccesary for Hsp90's chaperone activity, it prevents maturation of Hsp90 client proteins and traps them in conformations that are targeted for degredation by the cell.[28]

A combination of **II-2,** which inhibits the transcription of erbB2, and Hsp90 was therefore hypothesized to reduce cellular levels of erbB2 more effectively that either individual component, and to have a greater impact on the survival and proliferation of erbB2+ cancer cells (figure III-4). However, geldanamycin has broad activity against the range of Hsp90 client proteins and is known to have substantial toxicity in non-tumorigenic models.[29] We hypothesized that the overlapping effects of **II-2** and geldanamycin on erbB2, but not on other Hsp90 client proteins, would make these combinations more selective than either individual agent.

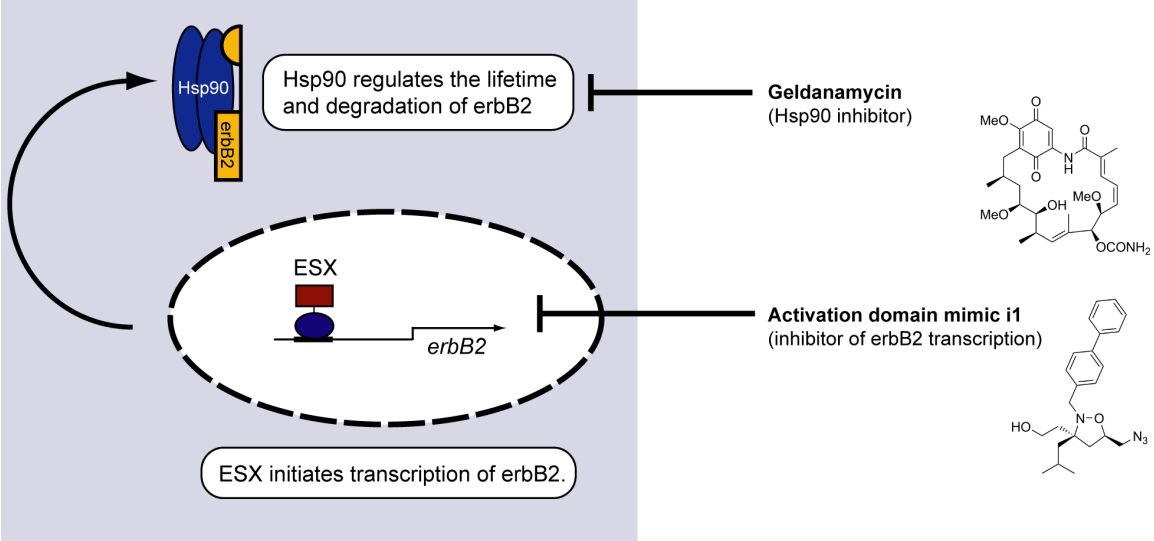


Figure III-4. Rationale for synergy between **II-2** and geldanamycin. Combinations of **II-2** and geldanamcyin simultaneously target the transcription of erbB2, and reduce the lifetime of the mature protein by inhibiting ATPase activity of Hsp90, thereby trapping the erbB2-Hsp90 complex in a conformation that promotes erbB2 degradation.

C.2. Effects of II-2:Geldanamycin Combinations

The hsp90 inhibitor geldanamycin reduces cellular erbB2 levels by preventing the hsp90 complex from acting to maintain erbB2 stability and membrane localization. This combination exhibited strong synergy as evaluated by an isobologram constructed from the dose effect curves of **II-2**, geldanamycin, and a series of fixed ratio combinations (figure II-5). For example, the IC_{50} of a 5:1 ratio of **II-2** to geldanamycin was approximately 30-fold lower in **II-2** and 17 fold lower in geldanamycin when compared to the IC_{50}s of the individual components. Consistent with their modes of action and synergistic effect on the growth of SkBr3 cell, use of a combination of **II-2** and geldanamycin produced a greater reduction in erbB2 levels than use of either agent in isolation.

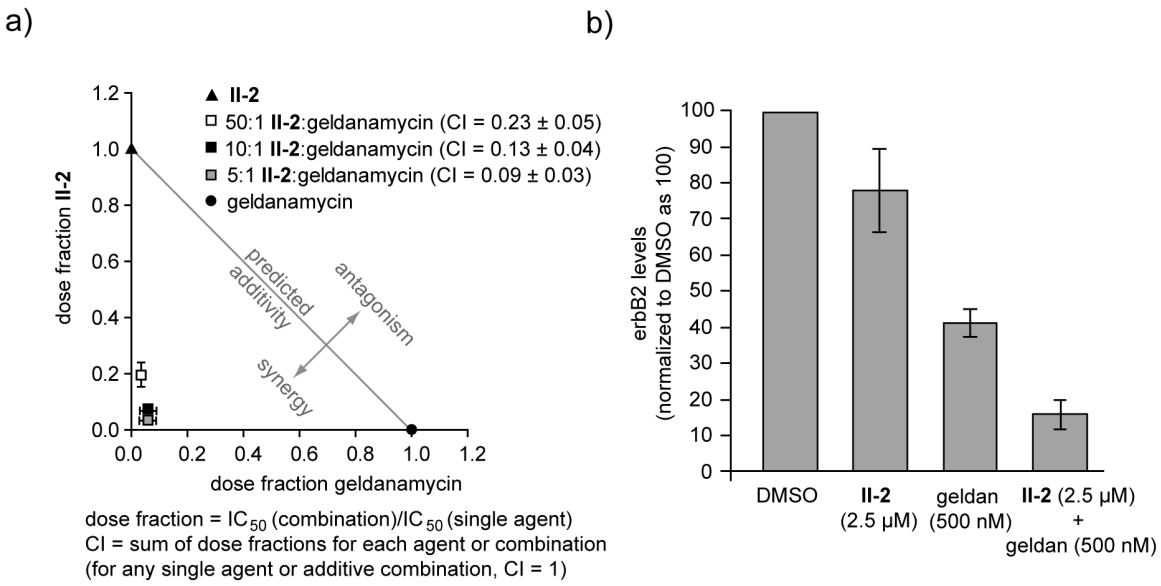


Figure III-5. II-2/geldanamycin combinations (I). a) Comparison of the IC_{50}s (in SkBr3 cells) of **II-2**, geldanamycin, and combinations using an isobologram indicate that combinations provide a synergistic reduction in the required dose of each compound. The combination index, with compounded error from the isobologram, is shown. Error bars on the isobologram represent compounded standard error of the IC_{50}s. b) A combination of **II-2** and geldanamycin is more effective at reducing levels of erbB2 than either agent in isolation ($p < 0.01$). Levels of erbB2 were normalized to the total protein concentration for each well. Error bars represent the standard deviation of this ratio.

This combination was also synergistic according to the Bliss definition. SkBr3 cell dosed with **II-2**, geldanamycin, or a combination of the two were monitored over nine days. As illustrated in figure III-6, the combination was more effective than either individual agent, or then the expected additive effect of the two compounds over the course of the experiment. Concentrations of both compounds below the IC_{50}s were chosen to maximize the dynamic range of the assay, and to highlight the ability of combination treatment to be effective with drastically reduced doses of the individual agents.

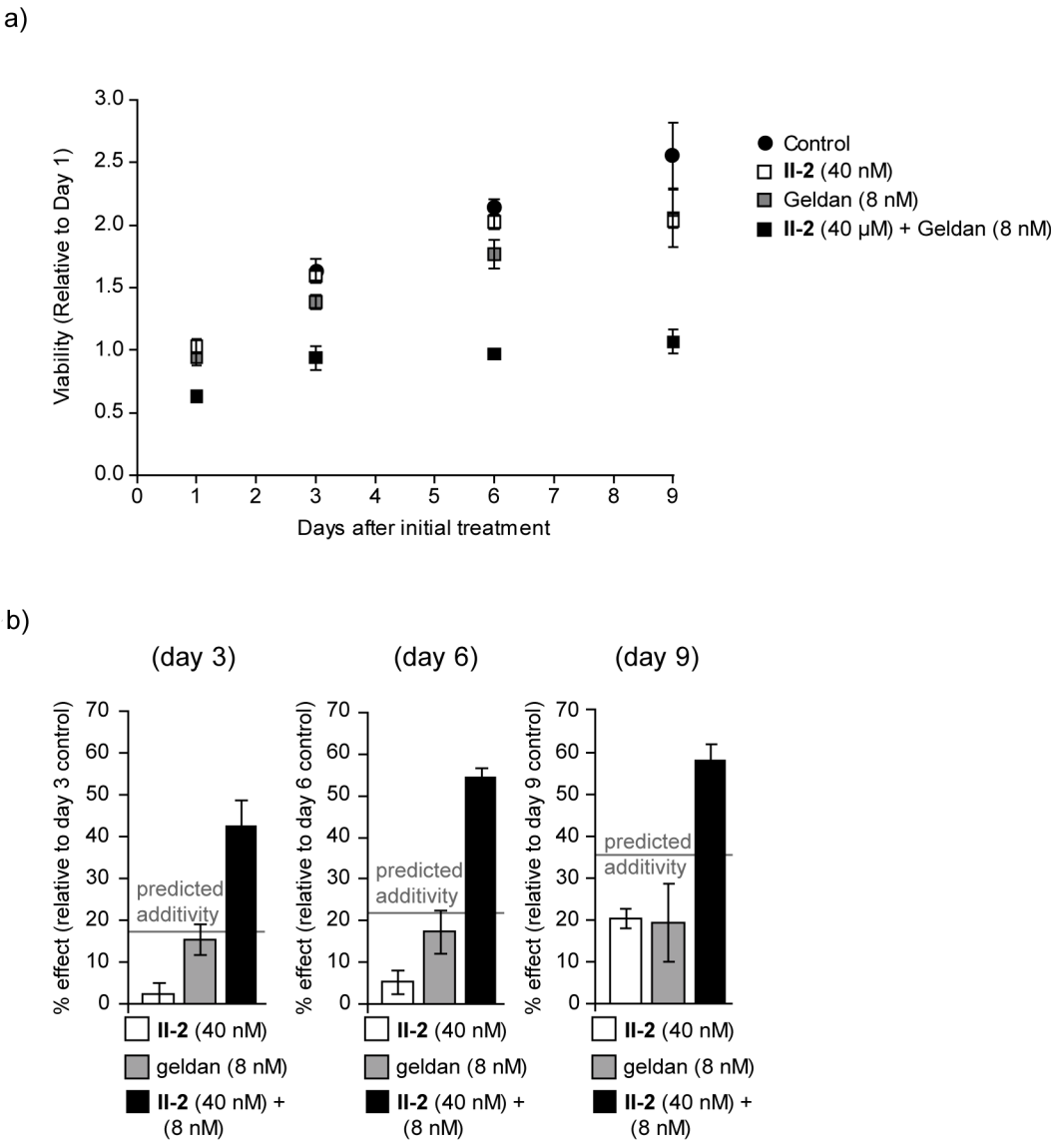


Figure III-6. II-2/geldanamycin combinations (II). The effects of **II-2** (40 nM), Geldanamycin (8 nM) and a combination of the two on ErbB2+ (SkBr3) cells was evaluated over 9 days of treatment. Error bars are 1 standard deviation d) Evaluation of individual timepoints indicates indicate a synergistic increase in effect for the combination. % effect is calculated by normalizing the % viability to that of the control group for each timepoint and subtracting from 100. Predicted additivity is calculated according to the multiplicative method of Bliss as described in the supporting information.

In addition to increased activity, it was hypothesized that the use of two agents

with broad, but overlapping effects such as **II-2** and geldanamycin would provide greater selectivity for their shared targets than either agent individually. For these experiments, the non-tumorigenic fibroblast cell line IMR90 was used as a negative control. The IC_{50}s of each agent and combination for IMR90 cells were divided by the corresponding IC_{50}s for SkBr3 cells to produce a selectivity ratio. The selectivity ratios for the combinations, normalized to each single agent (**II-2** in figure III-7a and geldanamcyin in fig III-7b) indicate statistically significant increases in selectivity for all combinations.

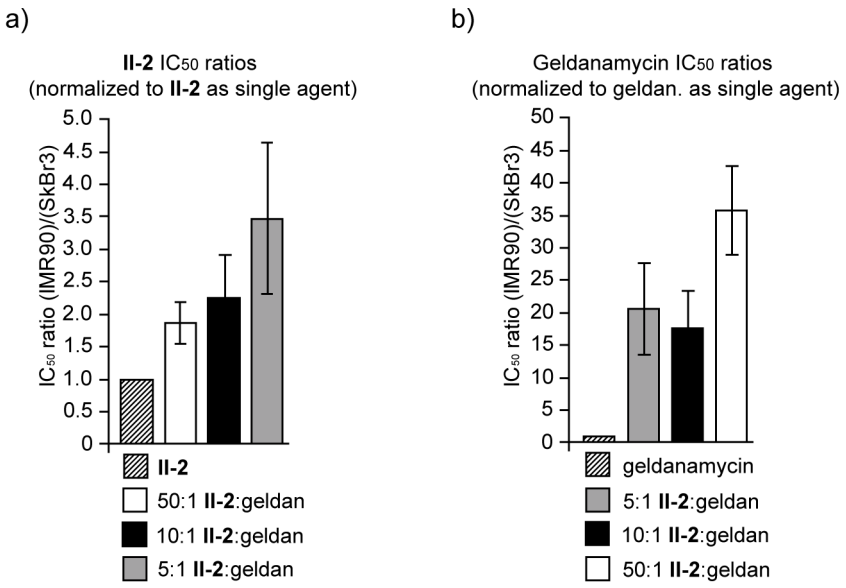


Figure III-7. Selectivity of II-2/geldanamycin combinations (I). The synergy observed for **II-2**:geldanamycin combinations is selective for erbB2+ cells (SkBr3) compared to non-tumorigenic cells (IMR90) as indicated by the ratio of the IC_{50}s for each agent (in isolation and as part of a combination) in the two cell lines. a) The IC_{50} ratios for **II-2** IC_{50}'s, normalized to the ratio of **II-2** as a single agent. b) The IC_{50} ratios for geldanamycin IC_{50}s, normalized to the IC_{50} ratio of geldanamycin as a single agent. Ratios for **II-2** IC_{50}s and geldanamycin IC_{50}s are normalized to those of **II-2**and geldanamycin as single agents. Error bars represent compounded standard error.

To provide an alternative readout that is indicative of the overall selectivity increase provided by the combination, we took the ratio of the CI (combination index) in

non-tumorigenic (IMR90) cells to the CI in erbB2+ (SkBr3) cells. If the combinations were equally synergistic in both IMR90 and SkBr3 cells, this ratio would be 1 as indicated by the dotted line in Figure 3c. The fact that the CI ratio is higher than 1 indicates that the synergy is selective for the target cell population. This can also be seen by comparing the isobologram in Figure 3a, which indicates exceptional synergy in erbB2+ (SkBr3) cells, to the isobologram in nontumorigenic (IMR90) cells (Supplementary Figure S3e) which indicates that the combination is somewhat worse than additive in this non-targeted cell population.

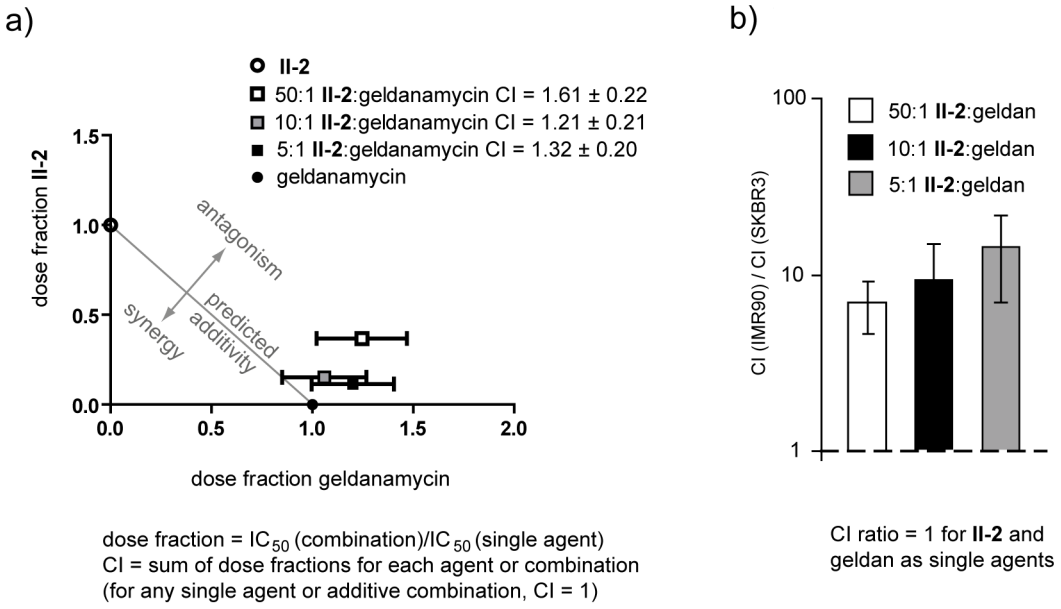


Figure III-8. Selectivity of **II-2**/geldanamycin combinations (II). a) Comparison of the IC_{50}s (in IMR90 cells) of **II-2**, geldanamycin, and combinations using an isobologram indicate that combinations do not provide a synergistic reduction in the required dose of each compound. Error bars represent compounded standard error. The synergy observed for **II-2**:geldanamycin combinations is selective for erbB2+ cells (SkBr3) compared to non-tumorigenic cells (IMR90) as indicated by the ratio of combination indices (CI) in the two cell lines. Error bars represent compounded standard error.

C.3. Rationale for II-2:Erlotinib Combinations

Erlotinib is an ATP competitive inhibitor of the receptor tyrosine kinase erbB1 (EGFR), which is known to form oncogenic heterodimers with erbB2.[30] After ligand induced dimerization via an erbB1 ligand such as EGF, these two kinases undergo a series of auto- and cross- phosphorylation events that lead to the recruitment of signalling adapters and activation of the Ras pathway.[31] Because of the requirement for dimerization and trans-phosphorylation from another erbB protein for erbB2 signaling, we reasoned that partial inhibition of both erbB1 function (erlotinib) and erbB2 production (**II-2**) could prove to be an effective way to curb the growth of erbB2 dependent tumors.

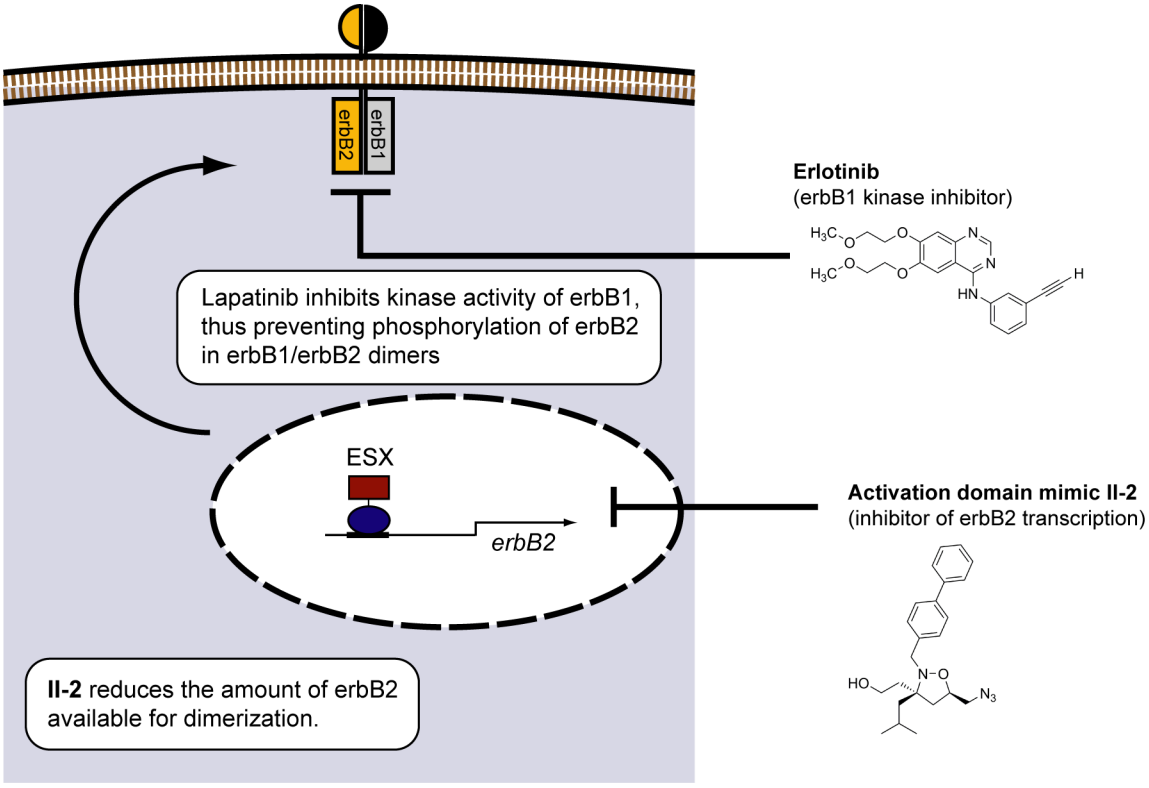


Figure III-9. Rationale for synergy between **II-2** and erlotinib. It was hypothesized that combinations of **II-2** and erlotinib could simultaneously target the transcription of erbB2 and the ability of erbB1/erbB2 heterodimers to trans-phosphorylate and initiate oncogenic signaling.

C.4. Effects of II-2:Erlotinib Combinations.

The combination of **II-2** and erlotinib did not appear to exhibit synergy as evaluated by either dose reduction or increased activity. An isobologram comparing combinations of erlotinib and **II-2** to either agent in isolation indicated minimal or no reduction in the dose required to achieve 50% effect, beyond that predicted by dose equivalent additivity (Figure III-10). This analysis was complicated somewhat by the tendency of erlotinib to precipitate out of solution under our experimental conditions at concentrations near its IC_{50}, which leads to high errors in the isobologram. To verify this lack of synergy, **II-2**:erlotinib combinations were also tested for their combined effect on erbB2 driven SkB3 cells.

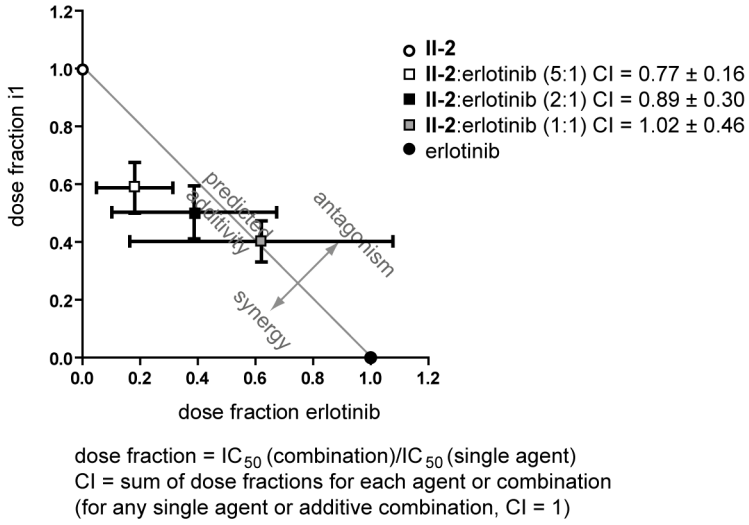


Figure III-10. II-2/erlotinib combinations (I).Comparison of the IC50s after two days of treatment with **II-2**, erlotinib, and combinations using an isobologram indicates that combinations cause an approximately additive reduction in the required dose of each compound, but this analysis is complicated by the tendency of erlotinib to precipitate out of cell culture media at higher concentrations, which are the concentrations at which synergy would be expected. Error bars represent compounded standard error.

No increase in effect was observed by the most effective combination (according to the isobologram) of **II-2**:erlotinib when this was compared to the effects of **II-2** or erlotinib as single agents. Longer treatment did not lead to an increase in effect over the individual agents. The observed lack of synergy, or even of an effect significantly greater than that of the individual components of the combination, strongly supports the lack of synergy indicated by the isobologram.

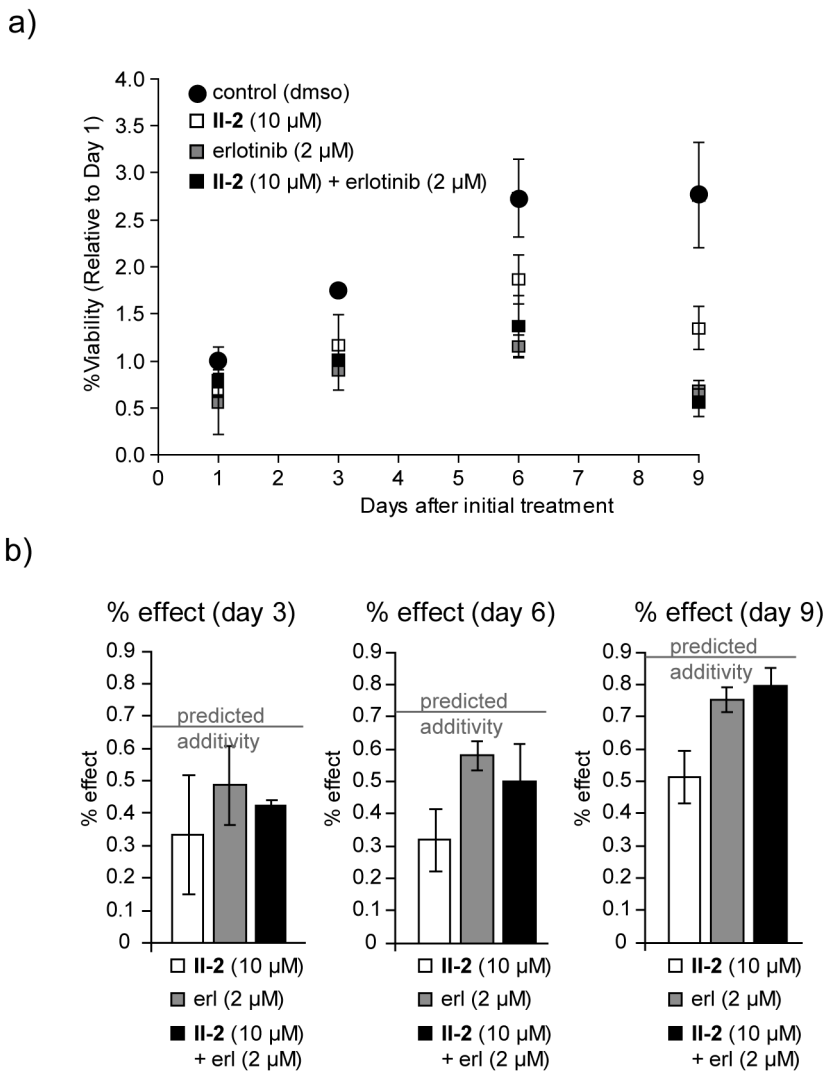


Figure III-11. II-2/erlotinib combinations (II). a) The effects of **II-2** (10 μM), erlotinib (2 μM) and a combination of the two on ErbB2+ (SkBr3) cells was evaluated over 9 days of treatment, with no significant increase in effect. b) Evaluation of individual timepoints indicates do not indicate a synergistic increase in effect for the combination. % effect is calculated by normalizing the % viability to that of the control group for each timepoint and subtracting from 100. Predicted additivity is calculated according to the multiplicative method of Bliss as described in the supporting information.

The lack of synergy observed between **II-2** and erlotinib may indicate that the two interventions are too far removed from each other to effectively combine against the tumor cells compensatory mechanisms. As noted above, one of the ways that erbB2

driven cancers can escape erbB1 kinase inhibition is to increase the amount of erbB3 available for dimerization with erbB2.[19,20,18] Although RNAi knockdown of erbB2 was able to overcome this compensation to a limited degree, first generation small molecule transcriptional inhibitors such as **II-2** are not direct functional mimics of RNAi, and are likely less effective at reducing transcript levels. Furthermore, as SkBr3 cells are thought to rely primarily on erbB2/erbB3 dimer formation as opposed to erbB2/erbB1 dimers, this may simply indicate that targeting erbB1 is not an especially effective tactic in this case – a notion which is supported by comparison of the IC50's of erlotinib and lapatinib, a potent inhibitor of both erbB1 and erbB2.

C. 5. Rationale for Combination of II-2 and Lapatinib.

Like erlotinib, lapatinib is an ATP competitive kinase inhibitor that interferes with a necessary autophosphorylation event that occurs in between dimerization and recruitment of signaling adapters.[32] Unlike erlotinib, lapatinib is a highly effective inhibitor of both erbB1 and erbB2.[33,34] We therefore hypothesized that reduction in erbB2 levels (**II-2**) and inhibition of erbB2 kinase activity (lapatinib) would reduce the overall amount of phosphorylated (active) erbB2 and have a synergistic impact on the viability of cells dependent on erbB2 driven signaling. (figure III-12)

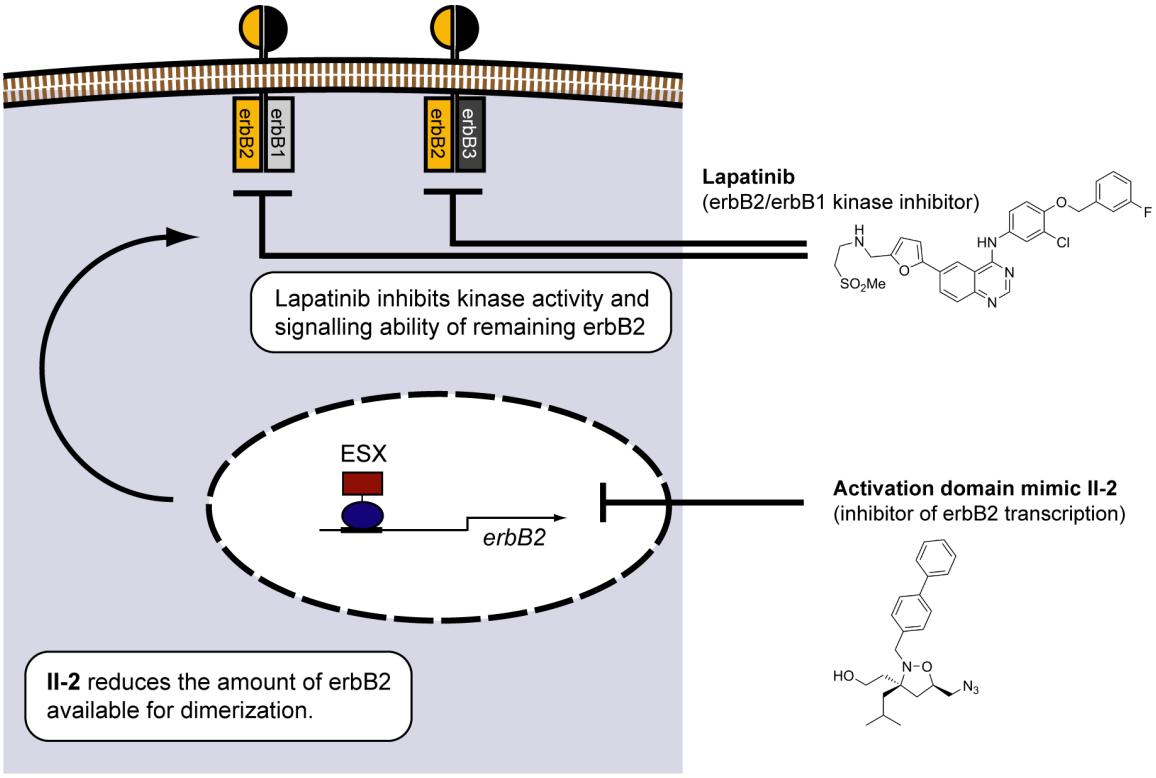


Figure III-12. Rationale for synergy between **II-2** and lapatinib. Combinations of **II-2** and erlotinib simultaneously target the transcription of erbB2 and the ability of heterodimers that include erbB2 to trans-phosphorylate and initiate oncogenic signaling.

C.6. Effects of II-2:Lapatinib Combinations

The effects of this combination were assessed using an ELISA assay to measure levels of erbB2 and phospho-erbB2 in response to treatment (Figure 2b). As expected, **II-2** had moderate effects on both erbB2 and phospho-erbB2 levels. However the **II-2**:lapatinib combination was significantly ($p < 0.05$) more effective at reducing the total amount of active (phosphorylated) erbB2 than equivalent amounts of either **II-2** or lapatinib.

Fixed ratio combinations of II-2 and lapatinib provided a significant reduction in

the doses required for 50% effect on erbB2+ cell viability. As shown in the isobologram in figure III-13 this reduction is greater than that would be predicted from additive dose equivalence. A 500:1 combination of **II-2**:lapatinib was the most synergistic of the combinations employed, and was chosen for followup studies evaluating the possibility of a synergistic increase in effect.

The ability of a combination of this 500:1 combination of **II-2** and lapatinib at sub IC_{50} doses to affect the viability of SkBr3 (erbB2+) tumor cells was compared to the effects of **II-2** and lapatinib in isolation. A dramatic increase in effect was observed for the combination when compared with either individual agent, and this combination effect also exceeded the predicted additive effects of the two individual components, as shown in figure III-13.

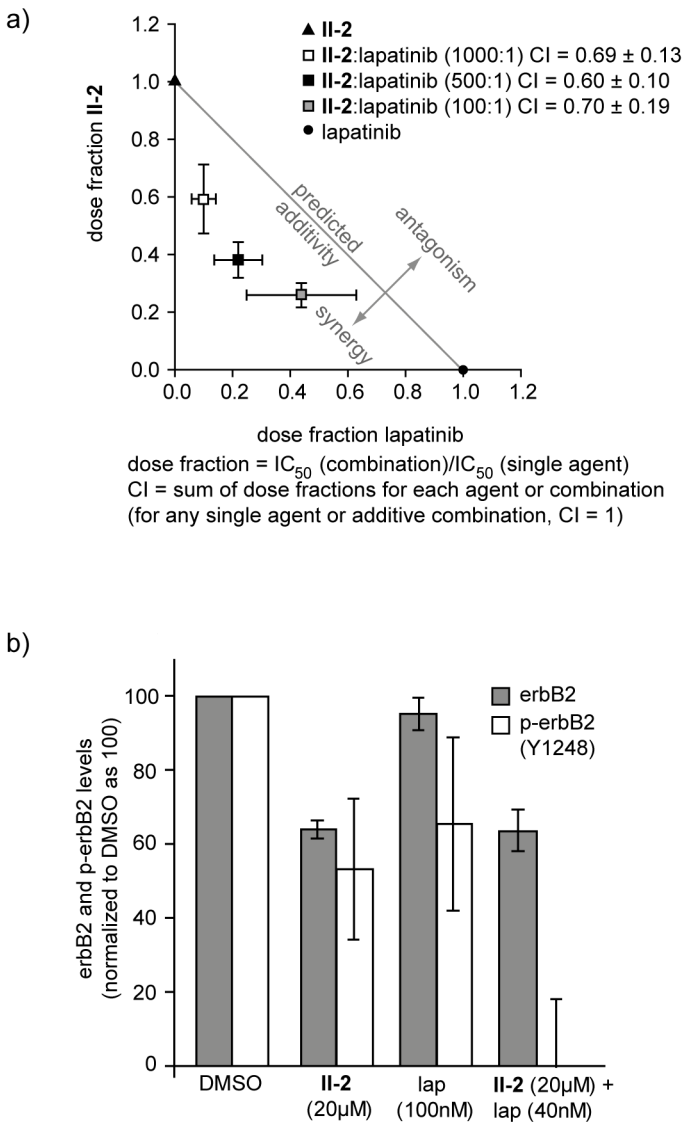


Figure III-13. II-2/lapatinib combinations (I). a) After 1 day, a combination of **II-2** and lapatinib is more effective at reducing levels of active (phosphorylated) erbB2 than either agent in isolation ($p < 0.05$). Levels of erbB2 and p-erbB2 were normalized to the total protein concentration for each well. Error bars represent the standard deviation of this ratio. b) Comparison of the IC50s after two days of treatment with **II-2**, lapatinib, and combinations using an isobologram indicates that combinations provide a synergistic reduction in the required dose of each compound. Error bars represent compounded standard error.

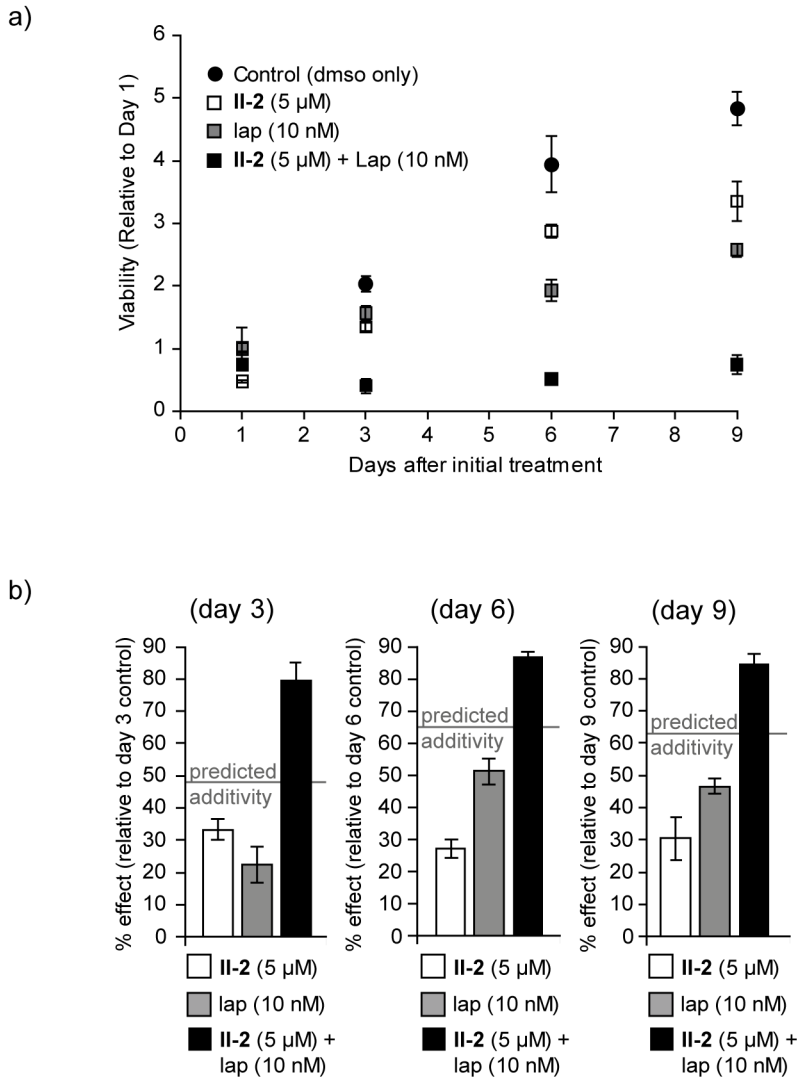


Figure III-14. II-2/lapatinib combinations (II). a) The effects of **II-2** (5 μM), lapatinib (10 nM) and a combination of the two on ErbB2+ (SkBr3) cells was evaluated over 9 days of treatment, with a drastic increase in effect observed for the combination. b) Evaluation of individual timepoints indicates a synergistic increase in effect for the combination. % effect is calculated by normalizing the % viability to that of the control group for each timepoint and subtracting from 100. Predicted additivity is calculated according to the multiplicative method of Bliss as described in the supporting information. All experiments were carried out in triplicate and error bars represent standard deviation unless otherwise indicated.

As with the **II-2**:geldanamycin combinations, the effects of **II-2**, laptinib and various combinations were assessed in IMR-90 cells to expore the possibility of increased

selectivity from combination use. Selectivity compared to **II-2** as a single agent is clearly increased by use of **II-2**:lapatinib combinations. However, lapatinib by itself was not toxic to IMR-90 cells at the concentrations tested, thus precluding the construction of an isobologram for the effects of these combinations in IMR-90 cells, or the evaluation of selectivity when compared to both agents. The increased selectivity observed in comparison to **II-2** remains a useful piece of data, as it indicates that these compounds are not synergistically toxic and that combinations allow greater effect to be achieved before the dose limiting toxicity of the more toxic component becomes prohibitive.

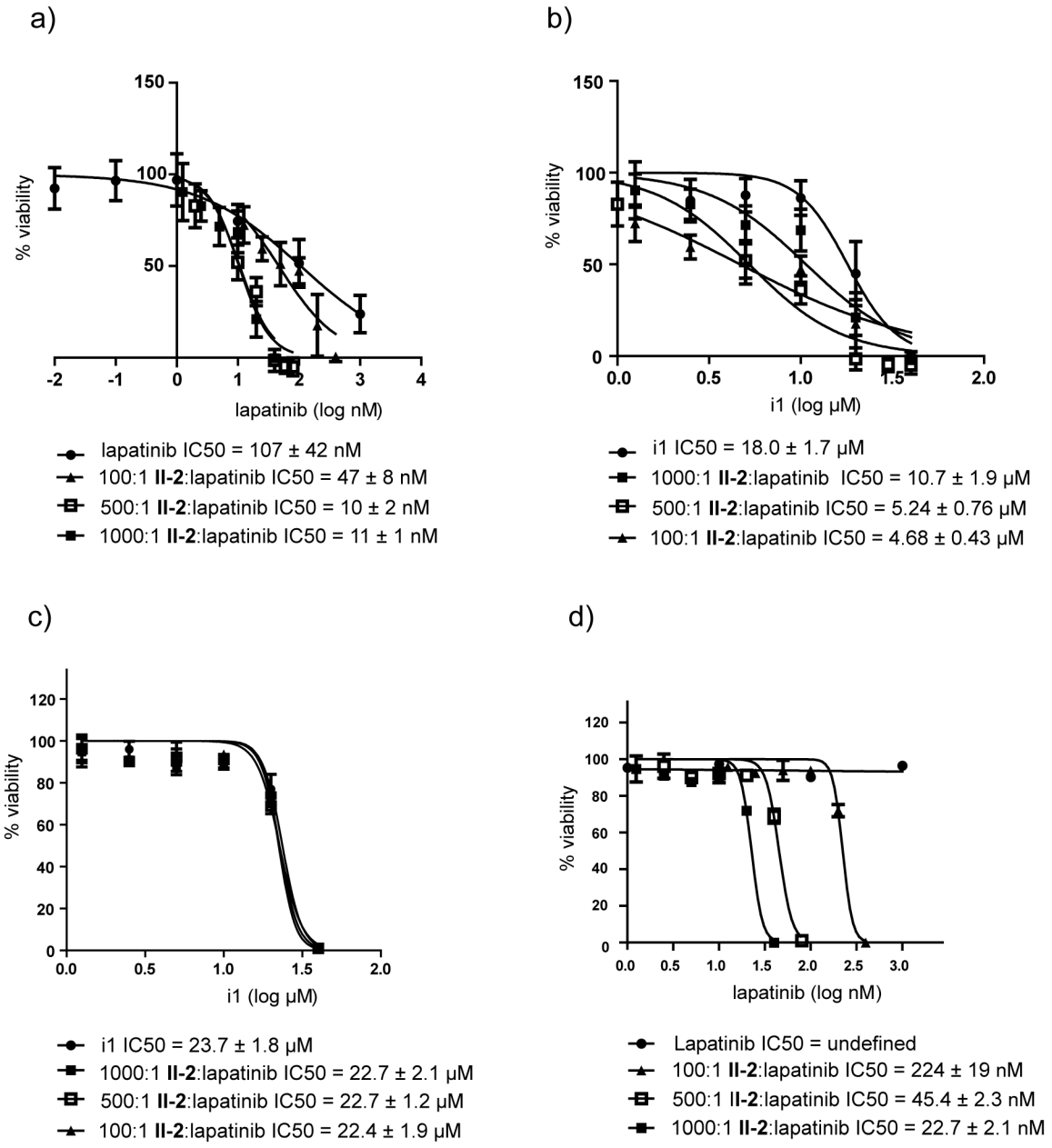


Figure III-15. Dose effect curves for **II-2**/lapatinib combinations. a) Dose-effect curves used to generate isobolograms for **II-2**:lapatinib concentrations, displayed as a function of lapatinib concentration. 500:1 **II-2**:lapatinib is from a seperate plate in which the lapatinib IC_{50} was 48 ± 17 nM. b) Dose-effect curves used to generate isobolograms for **II-2**:lapatinib concentrations. 500:1 **II-2**:lapatinib is from a seperate plate in which the **II-2** IC50 was 13.7 ± 1.0 μM. c) Dose-effect curves used to generate isobolograms for **II-2**:lapatinib concentrations in IMR90 cells. d) Dose-effect curves used to generate isobolograms for **II-2**:lapatinib concentrations in IMR90 cells, displayed as a function of lapatinib concentration. All experiments were carried out in triplicate and error bars represent standard deviation unless otherwise indicated.

D. Conclusions

Combinations of II-2 and either the Hsp90 inhibitor geldanamycin or the erbB1/erbB2 kinase inhibitor lapatinib were shown to be synergistically more effective at curbing the proliferation of erbB2 driven SkBr3 cells than any of the individual components, or the predicted additive effects of these components. These effects lead to greater selectivity for combinations, particularly in the case of II-2:geldanamycin combinations, as judged by their relative effects on SkBr3 cells (erbB2+) compared to IMR-90 cells (non-tumorigenic).

By using a combination that simultaneously curbs expression of the genetic driving force behind a diseased state while also targeting related cellular processes we achieve a synergistic increase in effect that is specific to the target cell population. Taken as a whole, these data demonstrate a generalizable strategy for overcoming the activity and selectivity limitations of transcriptional inhibitors. This approach promises to improve the utility of transcriptional inhibitors as both biochemical tools, and potential therapeutics. By demonstrating the potential of this approach, these results also suggest that this strategy could be successfully applied to other systems that are regulated by complex networks of protein-protein interactions.

E. Supporting Information.

E.1. Materials

Isoxazolidine **i1** was prepared as described in chapter 2 Lapatinib ditosylate and erlotinib were purchased from AK scientific, and geldanamycin was a generous gift from Dr. Brian Blagg. The identity and purity of all compounds were verified by NMR analysis and HPLC. Antibodies were purchased from Santa Cruz Biotechnology. Absorbance data was collected on a Tecan GENios Pro.

E.2. Calculation of synergy

IC_{50}s were calculated in Graphpad Prism v 5.0. All other calculations were performed in excel. Isobolograms were generated by computing dose fractions directly from the IC_{50}s. Dose fraction is defined as the dose of one component in a combination required to exert a given effect (usually IC_{50}, as in this case) divided by the dose of that component in isolation required to exert the same effect. Thus each combination reported has two dose fraction measurements (eg. dose fraction **i1** and dose fraction lapatinib) that define the combinations x/y coordinates on the isobologram.

Dose fraction of A for combination AB = IC_{50}(A in AB) / IC_{50}(A in isolation)

Dose fraction of B for combination AB = IC_{50}(B in AB) / IC_{50}(B in isolation)

The sum of these x/y coordinates are the combination index. By definition, the CI for either agent in isolation is 1. For the null hypothesis (in which both agents act as though they are equivalent doses of the same agent) the CI will be 1. For combinations where the CI < 1, synergy is present.

CI (combination index) = dose fraction A + dose fraction B.

Bliss additivity for a given combination was calculated by multiplying the fractional effect of the two components, according to the formula given below for combination AB whose components have effects eA and eB (which are expressed as fractional effects between 0 and 1) in isolation.

Predicted effect of AB = (eA+eB) - (eA*eB)

E.3. Mammalian Cell Culture

SkBr3 and IMR90 cells were purchase from ATCC and cultured in RPMI 1640 (SkBr3) or DMEM (IMR90) with 10% FBS and no antibiotics.

For the experiments used to generate dose-effect curves for the isobolograms and selectivity experiments, cells were plated at 3000 cells per well in 96 well plates. After adhering overnight, media was changed to 2.5% FBS and compound (as a solution in DMSO) was added. New media and compound were added for each additional day of treatment (2 days in the case of lapatinib and 3 days in the case of geldanamycin). The day after the final treatment, cell viability was measured using WST-1 reagent (Roche) in

accordance with the manufacturer's instructions.

For the 9 day cell growth assays cells were plated at 15000 cells per well in 24 well plates (one for each timepoint to minimize potential for contamination). Doses at or below the IC50's from the above experiments were chosen to maximize the dynamic range of the assay. These experiments were otherwise run in the same way as those done in 96 well plates.

E.4. ErbB2 and p-erbB2 ELISA:

ELISA assays were performed based on those published elsewhere.[35] SkBr3 cells were plated in 10% FBS at 15000 cells per well in 24 well plates. After adhering overnight, media was changed to 2.5% FBS and compound was added. After 24 hours, media was removed carefully and cells were fixed and permeabilized with cold (-20º C) methanol. The cells were then washed twice with TBST, blocked for 2 hours at room temperature with superblock (TBST solution from Pierce), incubated with the primary antibody overnight at 4º C (as a 1:500 solution in superblock). Cells were then washed twice more with TBST and incubate with secondary antibody for 2 hours at room temperature (as a 1:1000 solution in superblock). After being washed 3 times with TBST, Slow TMB (Pierce) was added to measure antibody levels in accordance with the manufacturer's instructions and the absorbance at 370 nm was measured. The cells were washed 3 more times with TBST and total protein levels were measured at 560 nm using BCA reagent (Pierce). ErbB2 and p-erbB2 levels were normalized to total protein

concentration by calculating (A370-A370blank)/(A560-A560 blank), where the A370 blank was from TMB treated cells not treated with a primary antibody, and the A560 blank was BCA reagent in wells that did not have cells plated in them. The resulting ratios were then normalized to cells treated with DMSO.

F. References

(1) Vogelstein, B.; Kinzler, K. W. Cancer genes and the pathways they control. *Nat Med* **2004**, *10*, 789-799.

(2) Albertson, D. G. Gene amplification in cancer. *Trends in Genetics* **2006**, *22*, 447-455.

(3) Semenza, G. L. Evaluation of HIF-1 inhibitors as anticancer agents. *Drug Discovery Today* **2007**, *12*, 853-859.

(4) Annunziata, C. M. Davis, R. E. Demchenko, Y. Bellamy, W. Gabrea, A. Zhan, F. Lenz, G. Hanamura, I. Wright, G. Xiao, W. Dave, S. Hurt, E. M. Tan, B. Zhao, H. Stephens, O. Santra, M. Williams, D. R. Dang, L. Barlogie, B. Shaughnessy, J. D. Kuehl, W. M.; Staudt, L. M. Frequent Engagement of the Classical and Alternative NF-κB Pathways by Diverse Genetic Abnormalities in Multiple Myeloma. *Cancer Cell* **2007**, *12*, 115-130.

(5) Rowe, S. P.; Mapp, A. K. Assessing the permissiveness of transcriptional activator binding sites. *Biopolymers* **2008**, *89*, 578-581.

(6) Lee, L. W.; Mapp, A. K. Transcriptional Switches: Chemical Approaches to Gene Regulation. *Journal of Biological Chemistry* **2010**, *285*, 11033-11038.

(7) Emami, K. H. Nguyen, C. Ma, H. Kim, D. H. Jeong, K. W. Eguchi, M. Moon, R. T. Teo, J.-T. Oh, S. W. Kim, H. Y. Moon, S. H. Ha, J. R.; Kahn, M. A small molecule inhibitor of B -catenin /CREB-binding protein transcription. *Proceedings of the National Academy of Sciences* **2004**, *101*, 12682–12687.

(8) Best, J. L. Amezcua, C. A. Mayr, B. Flechner, L. Murawsky, C. M. Emerson, B. Zor, T. Gardner, K. H.; Montminy, M. Identification of small-molecule antagonists thatinhibit an activator:coactivator interaction. *Proceedings of the National Academy of Sciences* **2004**, *101*, 17622–17627.

(9) Shimogawa, H. Kwon, Y. Mao, Q. Kawazoe, Y. Choi, Y. Asada, S. Kigoshi, H.; Uesugi, M. A Wrench-Shaped Synthetic Molecule that Modulates a Transcription Factor−Coactivator Interaction. *J. Am. Chem. Soc.* **2004**, *126*, 3461-3471.

(10) Henchey, L. K. Kushal, S. Dubey, R. Chapman, R. N. Olenyuk, B. Z.; Arora, P. S. Inhibition of Hypoxia Inducible Factor 1—Transcription Coactivator Interaction by a Hydrogen Bond Surrogate α-Helix. *J. Am. Chem. Soc.* **2010**, *132*, 941-943.

(11) Moellering, R. E. Cornejo, M. Davis, T. N. Bianco, C. D. Aster, J. C. Blacklow, S. C. Kung, A. L. Gilliland, D. G. Verdine, G. L.; Bradner, J. E. Direct inhibition of the NOTCH transcription factor complex. *Nature* **2009**, *462*, 182-188.

(12) Fuxreiter, M. Tompa, P. Simon, I. Uversky, V. N. Hansen, J. C.; Asturias, F. J. Malleable machines take shape in eukaryotic transcriptional regulation. *Nat Chem Biol* **2008**, *4*, 728-737.

(13) Goodman, R. H.; Smolik, S. CBP/p300 in cell growth, transformation, and development. *Genes & development* **2000**, *14*, 1553.

(14) Crews, C. M. Targeting the Undruggable Proteome: The Small Molecules of My Dreams. *Chemistry & Biology* **2010**, *17*, 551-555.

(15) Ptashne, M.; Gann, A. *Genes and Signals*; 1st edition (November 13, 2001) ed. Cold Spring Harbor Laboratory Press.

(16) Fitzgerald, J. B. Schoeberl, B. Nielsen, U. B.; Sorger, P. K. Systems biology and combination therapy in the quest for clinical efficacy. *Nat Chem Biol* **2006**, *2*, 458-466.
(17) Yarden, Y. Biology of HER2 and its importance in breast cancer. *Oncology* **2000**, *61*, 1–13.
(18) Stern, D. F. ERBB3/HER3 and ERBB2/HER2 Duet in Mammary Development and Breast Cancer. *J Mammary Gland Biol Neoplasia* **2008**, *13*, 215-223.
(19) Sergina, N. V. Rausch, M. Wang, D. Blair, J. Hann, B. Shokat, K. M.; Moasser, M. M. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature* **2007**, *445*, 437-441.
(20) Kong, A. Calleja, V. Leboucher, P. Harris, A. Parker, P. J.; Larijani, B. HER2 Oncogenic Function Escapes EGFR Tyrosine Kinase Inhibitors via Activation of Alternative HER Receptors in Breast Cancer Cells. *PLoS ONE* **2008**, *3*, e2881.
(21) Lehár, J. Krueger, A. S. Avery, W. Heilbut, A. M. Johansen, L. M. Price, E. R. Rickles, R. J. Short III, G. F. Staunton, J. E. Jin, X. Lee, M. S. Zimmermann, G. R.; Borisy, A. A. Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nat Biotechnol* **2009**, *27*, 659-666.
(22) Berenbaum, M. C. What is Synergy? *Pharmacological Reviews* **1989**, *41*, 93-141.
(23) Borisy, A. A. Elliott, P. J. Hurst, N. W. Lee, M. S. Lehar, J. Price, E. R. Serbedzija, G. Zimmermann, G. R. Foley, M. A. Stockwell, B. R.; Keith, C. T. Systematic discovery of multicomponent therapeutics. *Proceedings of the National Academy of Sciences* **2003**, *100*, 7977–7982.
(24) Chou, T. C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. *Pharmacological Reviews* **2006**, *58*, 621-681.
(25) Goldoni, M.; Johansson, C. A mathematical approach to study combined effects of toxicantsin vitro : Evaluation of the Bliss independence criterionand the Loewe additivity model. *Toxicology in Vitro* **2006**, *21*, 759-769.
(26) Roe, S. M. Prodromou, C. O'Brien, R. Ladbury, J. E. Piper, P. W.; Pearl, L. H. Structural Basis for Inhibition of the Hsp90 Molecular Chaperone by the Antitumor Antibiotics Radicicol and Geldanamycin. *J. Med. Chem.* **1999**, *42*, 260-266.
(27) Xu, W. Sensitivity of Mature ErbB2 to Geldanamycin Is Conferred by Its Kinase Domain and Is Mediated by the Chaperone Protein Hsp90. *Journal of Biological Chemistry* **2000**, *276*, 3702-3708.
(28) Isaacs, J. S. Xu, W.; Neckers, L. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer* **2003**, *3*, 213-218.
(29) Chiosis, G. Huezo, H.; Rosen, N. 17AAG: Low Target Binding Affinity and Potent Cell Activity—Finding an Explanation. *Molecular Cancer Therapeutics* **2003**, *2*, 123-129.
(30) Diermeier, S. Horváth, G. Knuechel-Clarke, R. Hofstaedter, F. Sz\öllosi, J.; Brockhoff, G. Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation. *Experimental cell research* **2005**,

304, 604–619.
(31) Hendriks, B. S. Quantitative Analysis of HER2-mediated Effects on HER2 and Epidermal Growth Factor Receptor Endocytosis: DISTRIBUTION OF HOMO- AND HETERODIMERS DEPENDS ON RELATIVE HER2 LEVELS. *Journal of Biological Chemistry* **2003**, *278*, 23343-23351.
(32) Spector, N. L. Study of the Biologic Effects of Lapatinib, a Reversible Inhibitor of ErbB1 and ErbB2 Tyrosine Kinases, on Tumor Growth and Survival Pathways in Patients With Advanced Malignancies. *Journal of Clinical Oncology* **2005**, *23*, 2502-2512.
(33) Kim, J. W. Kim, H.-P. Im, S.-A. Kang, S. Hur, H. S. Yoon, Y.-K. Oh, D.-Y. Kim, J. H. Lee, D. S. Kim, T.-Y.; Bang, Y.-J. The growth inhibitory effect of lapatinib, a dual inhibitor of EGFR and HER2 tyrosine kinase, in gastric cancer cell lines. *Cancer Letters* **2008**, *272*, 296-306.
(34) LoRusso, P. M. Jones, S. F. Koch, K. M. Arya, N. Fleming, R. A. Loftiss, J. Pandite, L. Gadgeel, S. Weber, B. L.; Burris, H. A. Phase I and Pharmacokinetic Study of Lapatinib and Docetaxel in Patients With Advanced Cancer. *Journal of Clinical Oncology* **2008**, *26*, 3051-3056.
(35) Huezo, H. Vilenchik, M. Rosen, N.; Chiosis, G. Microtiter Cell-Based Assay for Detection of Agents that Alter Cellular Levels of Her2 and EGFR. *Chemistry & Biology* **2003**, *10*, 629-624.

Chapter IV

Design and Implementation of a Peer-Led Discussion Module for Teaching Research Ethics to Incoming Graduate Students.[a]

A. Chapter Overview

The need for explicit discussion of research ethics and professionalism outside of the student-adviser relationship has been well established.[1,2,3,4,5,6] As researchers who are responsible for generating and publishing original research, graduate students are a key audience for these discussions. However, at the time this module was designed, there was no formal medium for these issues to be discussed within the department. In collaboration with a fellow graduate student – Amy Danowitz – I designed and taught a short module to address this deficiency. The goal of this module was to provide incoming students with the tools and awareness to deal productively with the ethical issues that they are likely to encounter in their roles as researchers and graduate students. We used small and large group discussions and case studies designed to be both believable in scale and relevant to the concerns of incoming graduate students. Based on student responses to the module, and feedback from the chemical community, this module was an effective tool for

a Parts of this chapter appear in Danowitz, A. M.; Taylor, C. E. Integrating a Peer-Taught Module on Practical Research Ethics into the Graduate Student Orientation Curriculum. *Journal of Chemical Education* **2011**, *88*, 1090–1093.

increasing student's ability to formulate and identify constructive solutions to ethical problems, and a valuable contribution to the literature on teaching research ethics.

B. Background

B.1. Definitions of Scientific Misconduct

Science, as a cumulative and often cooperative undertaking, relies on a reasonable amount of trust between different researchers. In the absence of such trust, the exchange of information and the dissemination of results that are essential to the present research paradigm become suspect and ultimately useless. Accordingly, funding agencies actively discourage research misconduct. The Office of Research Integrity, which is also responsible for investigating alleged misconduct and administering penalties to offenders, defines research misconduct in terms of falsification, fabrication, and plagiarism.[7]

> Research misconduct means fabrication, falsification, or plagiarism in proposing, performing, or reviewing research, or in reporting research results. (a) Fabrication is making up data or results and recording or reporting them. (b) Falsification is manipulating research materials, equipment, or processes, or changing or omitting data or results such that the research is not accurately represented in the research record. (c) Plagiarism is the appropriation of another person's ideas, processes, results, or words without giving appropriate credit. (d) Research misconduct does not include honest error or differences of opinion.

Although these definitions are generally used when misconduct is discussed, they are not comprehensive. They are not generally interpreted to include activities such as sabotage,

the misrepresentation of experimental protocols, or the use of research designs that are known to be inadequate or inappropriate.

B.2. Occurrence of Scientific Misconduct

There are numerous well-document and highly publicized cases of research misconduct. These include extensive forgeries such as those of Hendick Schon at Bell labs,[8] in which Dr. Schon was found to have fabricated the data used in a series of papers published in several journals, including both Nature and Science. A more recent example of misconduct comes from a research lab at Columbia University,[9,10] which involved some 21 counts of, fabrication and falsification by a graduate student and led to the retraction of 6 papers. Another recent example, albeit one that does not fit the technical definition of misconduct, took place at the University of Michigan.[11] In this case, it was determined that a post-doctoral researcher had been sabotaging a graduate students research for a period of several months, with total damages estimated at approximately $70,000. There is an emerging consensus in the literature that these cases represent only the 'tip of the iceberg' and that there are many instances that go unreported.[12,13,14]

The prevalence of research misconduct has been evaluated by administering anonymous surveys, in which researchers are asked about their own conduct and that of their colleagues. Although there is variation in the estimated frequency of misconduct depending on the definitions and methods used, the data from surveys consistently indicate a surprisingly high rate of questionable or inappropriate conduct. In an analysis

of approximately 3,000 NIH funded researchers, Martinson et al.[14] determined that although only 0.3% of their respondents admitted to 'Falsifying or cooking research data,' 15% admitted to 'dropping observations or data points from analyses based on a gut feeling that they were incorrect,' and 13.5% admitted to using inadequate or inappropriate research designs. A meta analysis of the literature on misconduct[12] concludes that the falsification rates reported by Martinson are probably artificially low, in part because the language used asks specifically about falsification and fabrication (which researchers are reluctant to admit to) rather than particular behaviors that fall under these categories. The overall results from the meta-analysis indicate that approximately 2% of respondents admit to having falsified or fabricated at least one piece of data themselves, while 15% had knowledge of a coworker who has done so. Frequency of other questionable practices (such as using inappropriate research designs, or reporting intentionally incomplete methodological data) were approximately 30% for self-admission, and 70% for observed behavior of coworkers. At this point, the causes of the discrepancy between the self-reported rates of misconduct and the rates of known or observed misconduct are largely speculative. In addition to the obvious explanation – that researchers are reluctant to admit to misconduct even under anonymous conditions – it is possible that the rates of known or observed misconduct include events that are suspected but unproven, or multiple reportings of single events or persons known to many researchers. Despite this caveat, data such at these indicate that researchers often act in ways that damage the implicit trust and openness that is part of the ethos of modern science.

A Format for Ethical Decision Making
Adapted from a handout written by Professor Michael Davis, for the Teaching Research Ethics conference in 2009. Used with permission.

1) Determine the problem.
The initial problem may be an explicitly ethical one (e.g. 'I think this data was fabricated') or it may not be (e.g. 'I am going to miss a research deadline').

2) Determine/check facts.
As much as possible, try to separate objectively verifiable facts from suspicions or rumors. In many cases, the issue may change or disappear upon careful consideration.

3) Consider limitations and objectives.
There will probably be many goals involved in a situation – maintaining personal integrity, advancing your career, making a favorable impression with your advisor or other faculty members, your relationship with your peers, etc.

4) Develop a list of 2-3 options.
Be specific about courses of action – who would you talk to, how would you discuss the issues, etc.

5) Test Options. The following are good examples of tests that are not necessarily dependent on specific ethical theories.

Harm test – does this option do less harm than any of the other alternatives?

Publicity test – would I want my choice of this option known throughout the department?

Defensibility test – could I defend this choice in front of faculty members or other authorities? If so, what would I say?

Reversibility test – would I still think this was the best option if I were one of the people adversely affected by it.

6) Choose an option based on steps 1-5.

7) Consider what you can do to make it less likely that this issue will come up again.

Box IV-1. A Format for ethical decision making. A handout given to students to provide common basis for discussions of ethics and problem solving strategies.

B.3. Addressing the Causes of Scientific Misconduct

There is not a single explanation for the root cause of scientific misconduct. One view[13] holds that the vast majority of science is conducted in accordance with ethical norms, but that a few 'bad apples' are responsible for gross violations of these norms and that these cases receive a disproportionate amount of publicity. Other views[13] hold that misconduct is driven by flaws in the research culture of either particular institutions, or that of science as a whole. It is plausible that these explanations are all true to one degree or another – that there are (rarely) unscrupulous researchers who will commit fraud out of hand, but that much of the driving force for misconduct and lesser offenses comes from an inability on the part of researchers to cope with the pressures placed on them by their environment. This view of misconduct as a failed coping strategy is implicit in the instutional and cultural explanations for misconduct. It also indicates that education on the responsible conduct of research should address 'survival skills' in addition to simply defining various sorts of misconduct.

This expanded view of research ethics education closely parallels recent findings by the National Academy of Science,[15] which offered a general critique of many current research ethics courses that amount to 'another box for institutions to check.' In particular, the council's findings emphasized the need for interactivity, the use of case studies or an equivalently engaging method, and that ethics training will only be effective to the extent that researchers can simultaneously be both ethical and successful.

C. Pedagogical Rationale for Module Design

C.1. Module Goals and Approach

In line with the recommendations made by the National Academy of Science[15] as well as the ideas presented by Coppola[3] and Rytting and Schowen[2] that the drive for personal success and desire to comply with external pressures can outweigh moral concerns, we designed the module around the idea that most students do not start out with the intention of committing morally dubious acts; rather, they might do so in response to the pressures that they face as graduate students. This line of reasoning indicates that ethical decision-making and the ability to navigate the conflicts and hazards of graduate school are closely intertwined.

In designing the content of this module, we had three major goals. First, we wanted to provide students with examples of potential moral dilemmas that they were likely to face as first and second year graduate students. As noted by Kovac,[5]

> "It is essential that students be able to relate to both the context and the characters [of a given situation]. If the people and the scenario are too distant, students will tend to see the situation in simplistic right-or-wrong terms."

In order to provide the students with examples they could relate to, we constructed a series of case studies based on problems that arose among our peer group during their first and second years.

We also designed these studies to serve as a vehicle for students to practice their ethical reasoning skills. In particular, we wanted them to base their interpretations on facts rather than suspicions or rumors, identify stakeholders and propose solutions that would benefit (or at least minimize harm to) these parties, and to evaluate the immediate and long term effects of potential actions. These criteria were given in detail in a handout titled 'A Format for Ethical Decision Making,' which was adapted from material presented at the Teaching Researching Ethics conference (Box IV-1). Another purpose of these discussions was to illustrate that it is often possible to mitigate external pressures and thus alleviate the temptation to commit ethically dubious acts. In the course of working through a case study, students were asked to commit to an appropriate course of action prior to discussion. We did this in order to give them practice in making such a commitment in a relatively safe and low-stakes setting.

Second, we aimed to give students practice in not only identifying potential dilemmas, but also in addressing their concerns about ambiguous situations in a professional and respectful manner. This was emphasized because of the possibility that, even if students lead exemplary research careers, there may be questions about the work of a collaborator or coworker. The way in which these questions are pursued is of great importance, as can be seen by examples such as the unresolved case in Baltimore,[16] which including raids by the Secret Service and in which the original finds of scientific misconduct by the Office of Research Integrity were later overturned by a Health and Human Services appeals panel. Given the highly collaborative nature of science, and the possible outcome of a poorly handled false accusation of misconduct on the part of a

coworker or collaborator, the ability to voice concerns about potential misconduct in a calm and appropriate manner is essential.

The idea of sharing concerns and discussing potential conflicts brings us to our final goal: giving students a detailed over-view of the personnel and resources that can be involved in conflict resolution (for example the Graduate Committee and the Ombud's office) and the types of issues that are appropriate for each resource. It has been our experience that graduate students are often unaware of resources other than their coworkers and adviser. By providing students with this list and helping them to decide whom they should approach with a given problem, we placed potential solutions to moral dilemmas within their grasp.

In order to meet these goals, we developed six case studies and a data interpretation exercise that we used as vehicles to discuss issues such as authorship, plagiarism, resolution of intra-lab conflict, and data integrity from the perspective of a first or second year graduate student.

C.2. Rationale for Peer-Leadership

Senior graduate students are uniquely suited to lead discussions about the challenges and ethical dilemmas that face incoming graduate students at their institution. Advanced graduate students work in the same departmental environment that the incoming students will, and often have recent first or second hand experiences that they can draw on in designing and discussing case studies. Senior students only have to look

back a year or two in order to empathize with the concerns of new students and are in a unique position to address the current concerns in a rapidly changing research climate. It must be noted, however, that senior graduate students lack a firsthand faculty perspective on these issues. For this reason, we found it extremely useful to consult with faculty members in determining discussion points and potential solutions for each case study.

Additionally, the fact that graduate students are leading discussions on ethics indicates to incoming graduate students that the subject matter is important to their peer group, and by extension, should be important to them. Furthermore, we think it likely that students are more candid around other students than they would be if faculty members were leading the sessions. This is particularly true in the case of new graduate students who may worry about making a good impression on a potential adviser or committee member.

D. Module Layout and Implementation

We implemented this module in two, two-hour blocks over the course of two days. The sequence began with us giving a brief presentation on the resources that are available to graduate students in dealing with conflicts, particularly those that could either lead to misconduct on their part or stem from the misconduct of other researchers. Students participated in a brainstorming session that addressed different sorts of conflicts that can arise in graduate school. The peer leaders (the authors) then guided a large group discussion about the most appropriate venues for resolving these problems. This

discussion included potential steps for escalation of unresolved problems, and of alternative courses of action for cases where the usual resources, such as the student's adviser, were not appropriate.

Most of the module was based on case studies that focused on dilemmas that students might face as first or second year graduate students. Students discussed cases in small groups (4-6 students) with the discussion leaders moving from group to group in order to facilitate discussion**.** The entire group then discussed the case, with students volunteering solutions or feedback on other students' solutions. At this stage, the discussion leaders acted as moderators and guided the group towards a general consensus on the best courses of action for each situation. In both the small and large group discussions, students were encouraged to consider the long term consequences of each proposed action and to think about other stakeholders who may be affected by their actions. Peer leaders and students also discussed the differences between professional and unprofessional ways of handling conflicts.

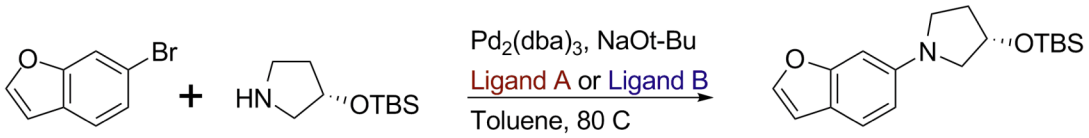
The module ended with an 'off the record' question and answer session, in which students could ask the discussion leaders questions about graduate school, or broach any unaddressed concerns that they had. Student had the opportunity to ask questions during a period in which an advising faculty member was present, or after the faculty member left.

E. Module Content

E.1. Example Data Interpretation Exercise.

To give students concrete examples of how data analysis can lead to questions that affect the integrity of the researcher, we used an exercise and group discussion that dealt with interpreting and presenting ambiguous data to illustrate the ethical dimensions of this process. After a brief presentation on what qualifies as fabrication, falsification and plagiarism, students were presented with examples of data such as synthetic yields obtained when testing the efficacy of two different ligands (Box IV-2). The students were also given a list of potential conclusions that could be drawn from the data. They then discussed circumstances under which a given conclusion would or would not be valid. In the latter case, students also considered whether inappropriate conclusions represented poor science or misconduct, and discussed the factors that determine this distinction.

The following reaction was run with the reported yields:



Ligand A

Run#	Yield
1	95%
2	26%
3	33%
4	29%

Ligand B

Run#	Yield
1	56%
2	48%
3	47%
4	0%

Which of the following conclusions would you accept/amend/reject?

a) Using the most effective ligand (Ligand A) the reaction occurred smoothly in 95% yield.

b) The reaction proceeded in higher yield with Ligand B (48% vs. 33% with Ligand A).

c) Ligand A (mean yield 46%) was more effective at facilitating the reaction than ligand B (mean yield 38%).

d) Ligand B (median yield 48%) was more effective at facilitating the reaction than ligand A (median yield 31%)

Are there circumstances under which any of these are ethical problems?

Box IV-2. Sample data interpretation exercise. Data interpretation exercise based on the evaluation of two ligands for an organometallic reaction. Students are asking to evaluate the scientific and ethical reasonability of several potential conclusions, and to propose alternatives.

E.2. Example Case Studies

The case study in Box IV-3 presents students with a plausible complication in what is commonly considered one of the most stressful experiences of the early years of

graduate school. This case study is designed to present students with a situation in which there is an obvious short term gain from an inappropriate but seemingly minor action. The discussion of this case study dealt primarily with evaluating the potential long term complications for ethically dubious actions, such as knowingly glossing over problems with one's research (as in a or b), and with finding alternative solutions that helped the student highlight their research efforts and deliver a compelling candidacy exam in spite of these difficulties.

Case Study - "Key Result"

You are a second year student, and you have been working in your thesis lab for several months. You are working on the document that you will submit to your candidacy committee. Your thesis research is quite ambitious and your advisor has warned you that your committee members are likely to be skeptical. Nevertheless, you are confidant that it will work and by hard work and luck, have acquired an early result from one of your key experiments that demonstrates proof of concept for your work. While you are writing the paper, a fact comes to light that casts doubt on the veracity of your results (see below). There is not time to repeat the experiment before the deadline for your candidacy document.

In which of these situations (and with what qualifiers) is it permissible to include the questionable experimental results in your document and presentation.

Situation A)
You realize that you did not perform a necessary control to rule out a likely confounding factor in your key experiment.

Situation B)
A serious technical problem is brought to your attention (e.g. the instrument you collected data on was incorrectly calibrated, etc).

Situation C)
You find a paper with an experiment very similar to yours, but which has a significantly different result, and from which the authors draw conclusions which indicate that your idea should not work.

Box IV-3. Case Study - "Key Result."A case study in which the student faces a potential crises before candidacy and must determine how best to solve the problem in a way that meets both academic and ethical standards.

Many of the student responses addressed issues from the 'Format for Ethical Decision Making' handout that we had previously discussed with them. Several students brought up the potential effects of their actions on their adviser or collaborators (i.e. additional stakeholders). Other responses included assessment of potential long-term

effects, for example, that feigning ignorance of the needed control in situation A could set the student up for further acts of fabrication or falsification if they are faced with the invalidation of their 'results' later on.

In addition to recognizing the ethical issues embedded in the case study, students proposed solutions that allowed them to meet the demands of graduate school without committing misconduct or setting themselves up to do so in the future. For example, in situation B, the possibility was raised that students may be able to postpone candidacy until the systematic error has been fixed, rather than present suspect data. For situation A, students discussed ways to present the limitations of the data (i.e. "absent this control we see…") that might allow them to discuss their data without misrepresenting their results. These responses indicate that students are seeing beyond the simplistic binary dilemma of committing a morally dubious act or failing to meet imposed standards.

Box 2: Case Study - "Continuing a Project"

You have inherited a project from a 6th year graduate student who has left the lab. The student's work was published as a communication, and your thesis research will be a series of followup studies based on their initial results. Your advisor suggests that you repeat some of their experiments to "test your hands" at the technique they had been using. After running their experiments as written up in the experimental section of their communication for several weeks, you are unable to reproduce their data. You go through their notebooks and contact them via email in order to try and determine what is going wrong with your experiments.

Propose two situations/results from your actions (emailing and looking through notebooks) which would cause you be concerned about misconduct, and two situations which would not.
(For example, if the student emails back that there is a minor technical issue that she forgot to record, and once you this into account, your experimental results are consistent with those in the paper, misconduct is unlikely).

For one of the situations in which misconduct may be an issue, propose both a reasonable course of action (who would you talk to, what would you say) and a less reasonable course of action for dealing with the situation.

Box IV-4. Case Study - "Continuing a Project."A case study in which students propose potential situations that would meet the criteria for either a case in which suspicion of misconduct is warranted, or which can likely be ascribed to honest mistakes by a collaborating researcher.

For another case study (Box IV-4), students discussed the possibility of misconduct on the part of a coworker. These sorts of problems may arise when multiple scientists contribute to a project. It is therefore important for students to think about how to determine if misconduct has occurred. Students should also practice professional and courteous ways of broaching these types of concerns with other scientists.

For this exercise, students proposed specific details that would lead them to believe that either (a) misconduct had been committed by the previous student or (b) the

previous student had not committed misconduct. This gave students the opportunity to become creatively engaged with the material, and opened up the discussion to a wider variety of circumstances and issues than if they were given a single, completely defined case study. In one instance, a student proposed a detailed situation based directly on their previous research experience, and asked the group for their interpretation. This illustrated both the plausibility of these sorts of situations and the importance of the resulting discussion.

The second portion of this case study centered on appropriate responses to situations where a student believed misconduct may have occurred. Specifically, students proposed professional ways of dealing with this conflict and noted the importance of presenting the facts of the case, rather than their suspicions or opinions about the former student. During these discussions, students again brought up the possible affects on stakeholders in this situation and suggested courses of action would entail the least harm to those involved. For example, some students initially found it reasonable to first take these concerns to a party outside the lab for a second opinion (in particular, a former adviser or other trusted faculty member). Upon further discussion, they realized that this option has potential consequences for both themselves and their current adviser. They also pointed out that this solution fails the reversibility test, as they would be displeased with the adviser or former student broaching concerns about their work to one of their peers rather than to them.

F. Response by Students and the Larger Chemical Community

Responses to the module from both the students who have taken it, and the broader community have been positive. During both the years it was offered, students indicated that they found the case studies and resulting discussions to be both useful and eye-opening. The only substantive critique of the module offered by students was that too much time was being given to the individual case studies. In reponse to this, we incorporated additional details into each case rather than holding them back for the large group discussion. The time for small group discussions was reduced, which allowed us to implement the data interpretation exercise described above.

After the first year in which the module was presented, the ACS invited us to deliver a presentation about our experiences. This presentation was very well received, with several members of the audience commenting that they thought we should write this up for publication. The resulting publication was accepted with enthusiastic reviews and minimal revisions in the Journal of Chemical Education.

G. Conclusions

The purpose of this module was to introduce incoming graduate students to the methods and tools that they can use to navigate the ethical hazards of graduate research. Students practiced these skills by working through a number of exercises based on scenarios that they may face in the next couple of years. Students were also advised on

potential resources that they can call on to help resolve ethical issues that arise in the course of their research.

We used the nature of the responses given by the new graduate students to asses the effectiveness of the module. These responses indicate that students were able assimilate the lessons and identify the larger moral dimensions of the situations presented in the case studies. They were able to propose solutions that allowed them to maintain both their ethical and professional integrity, without failing to meet the demands of graduate school. The students' ability to articulate the issues in these scenarios and propose reasonable solutions to them indicates that they are beginning to appreciate the nuances of ethical decision making. Student responses also show an understanding of the need to consider the long term effects of decisions on both themselves and others.

Recently, Rackham has mandated that all departments, including Chemistry, implement credited courses on the responsible conduct of research and that these course be taught by faculty. Although the implementation of this course will make the module redundant, the rationale behind the module's construction, and much of the materials generated for it are being adapted to this new course. Additionally, one of the graduate students who designed and implemented this module – Amy Danowitze – will be teaching the department's responsible conduct of research course in collaboration with a full time member of the faculty.

H. Course Materials

Case Study: Authorship

As part of your rotation, you run an experiment to identify an unknown protein by MS/MS. You perform the technical aspects of the experiment, under the supervision of your rotation mentor. Your mentor also helps you work up and analyze the data. Based on these results, the protein is tentatively identified. After your rotation ends, you hear that the group is writing up a paper in which the identification of this protein is used as supporting evidence for a proposed regulatory mechanism.

Under what circumstances is your omission from the authorship list reasonable? Under what circumstances is it a problem? If it is a problem, how could you go about addressing this?

One of the key questions is how significant of a contribution you made to the experiments resulting in the initial identification of this protein. Propose factors that (1) would clearly indicate that you made a significant contribution to the project, (2) would clearly indicate that you did not make a significant contribution, or (3) fall somewhere in between.

Box IV-5. A case study in which students evaluate the different criteria for authorship on a paper, and propose ways of addressing situations in which they believe correct attribution has not been made.

Case Study: Safety Issue

You are rotating in the lab you wanted to join. Your rotation project is going well, and you get along well with most of your labmates. However, the post-doc who works next to you often leaves flasks of the chemicals she is working with uncapped and on the bench. You have politely asked her to deal with them in an appropriate manner, but she has told you not to worry about it – none of them are especially toxic, and besides part of your job as a chemist is putting up with unpleasant smells.

Propose reasonable and unreasonable courses of action for each of the following conditions:
1) The chemical is not particularly toxic (eg. Acetone, Ethyl Acetate).
2) The chemical is something potentially toxic or carcinogenic (eg. Hexanes, Dichloromethane).
3) The chemical readily decomposes to Hydrogen Cyanide (Volatile and Lethal).
4) Changes to any of the above, if the lab's advisor is out of town for an extended period of time.

Box IV-6. A case study in which students propose different ways of dealing with a potential safety hazard caused by one of their senior coworker. A key part of the resulting discussion is resolving the situation in a manner which makes the research environment safe for the student, but which does not alienate coworkers or the adviser.

Case Study: Rotation Project

You are several weeks into a rotation in the research lab that you would like to join. Although the lab's research focus interests you, the senior graduate student you are working with is having you run reactions for his methodology project according to a protocol that he has already developed and using substrates that he has already decided upon.
This lab only will only be accepting a single new student at the end of the year, and has already had three rotators. You do not have much direct interaction with the lab's advisor, and other lab members have told you that whether or not you are selected to join the lab will depend almost entirely on the presentation you give at the end of your rotation. This presentation is given to the entire group, including the advisor, and you will be expected to present and discuss the work you have completed during your rotation. You are concerned that your lack of creative input in your project will negatively affect the quality of your presentation and your chances at being accepted into this lab.

Are there circumstances under which this would not be a problem (describe them)? How would you go about determining whether or not this is the case?

Assuming that it turns out to be a problem, propose both a reasonable and unreasonable solution.

Box IV-7. A case study in which students evaluate a potential intra-lab conflict, in which their rotation mentor may or may not be assigning them projects that will adversely affect their academic goals. A key part of the resulting discussion revolves around the highly variable nature of these sorts of practices between labs, and the fact that what may be a problem in one lab could be standard practice in another.

Case Study: Inspiration

You are finishing up a rotation in the lab you intend to join, and have an upcoming meeting with your future advisor in which the two of you will discuss potential thesis projects. All during your rotation, you had been thinking about a project based on a future direction presented by a graduating PhD student in the lab you worked in as an undergraduate. You think this project fits in nicely with what your grad lab is doing, builds on your current skills and interests, and will be exciting enough to send out as a JACS or perhaps a PNAS paper if it works. You are sure that your new advisor would be as excited about this idea as you are.

What practical and ethical concerns should you have about proposing this project?

For the following situations, propose both a reasonable and unreasonable course of action.

1) Your undergraduate advisor is retiring and you know they are no longer carrying out research.

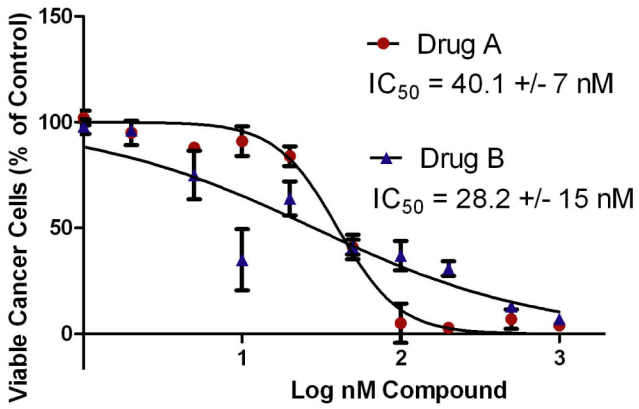
2) You know the project is intended as an REU project for the next summer.

3) The original idea for this project came from either an experimental result of yours (as an undergraduate) or a conversation between you and your undergraduate advisor and/or the graduate student who mentored you.

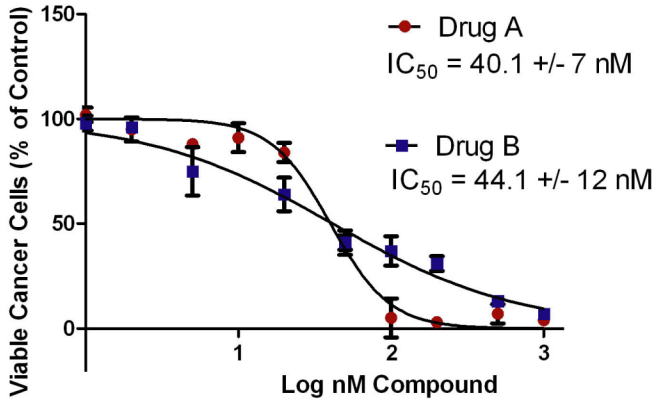
Box IV-8. A case study in which students evaluate the potential for plagiarism of a research idea from an old lab. In particular, students are encouraged to come up with ways in which they can (given appropriate permission) use the idea, without violating academic and ethical standards.

The ability of drugs A and B to kill cancer cells was measured

Graph 1



Graph 2 - with offline point removed



Which of the following conclusions would you accept/amend/reject?

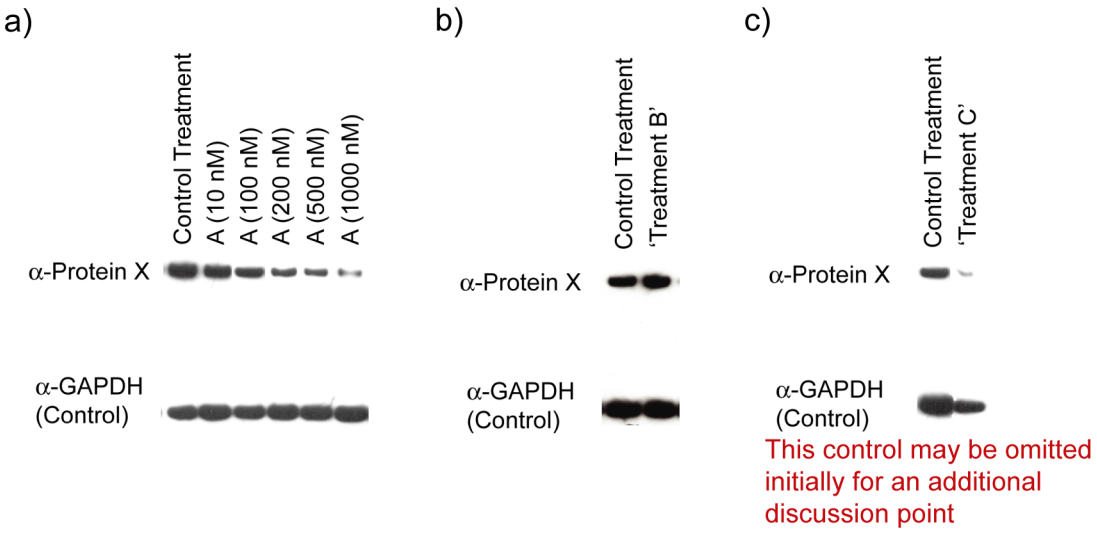
a) Based on graph 1, drug B is more potent than drug A.

b) Removing the off-line data point at X=1 drug B produces graph 2. Graph 2 is morefor appropriate for publication.

Are there circumstances under which any of these are ethical problems?

Box IV-9. A data interpretation exercise in which students are asked to evaluate and amend potential conclusions reached using one or both of the above graphs.

The ability of a 'treatment' (drug, RNAi, etc) to alter expression of protein X was determined by Western Blot



Which of the following conclusions would you accept/amend/reject?

a) Treatment A reduces expression of protein X with an EC50 of approximately 100 nM.

b) Treatment B induces a ~20% increase in expression of protein X.

c) Treatment C reduces expression of protein X.

Are there circumstances under which any of these are ethical problems?

Box IV-10. A data interpretation exercise in which students are asked to discuss, evaluate and amend conclusions based on a series of Western Blot assays. For this particular exercise, some knowledge of the principles and technical considerations of Western Blot analysis is highly beneficial.

I. References

(1) Coppola, B. P.; Smith, D. H. A Case for Ethics. *Journal of Chemical Education* **1996**, *73*, 33-34.
(2) Rytting, J. H.; Schowen, R. L. Issues in scientific integrity: a practical course for graduate students in the chemical sciences. *Journal of chemical education* **1998**, *75*, 1317.
(3) Coppola, B. P. Targeting entry points for ethics in chemistry teaching and learning. *Journal of Chemical Education* **2000**, *77*, 1506.
(4) Sweeting, L. M. Ethics in science for undergraduate students. *Journal of chemical education* **1999**, *76*, 369.
(5) Kovac, J. Scientific ethics in chemical education. *Journal of chemical education* **1996**, *73*, 926.
(6) Treichel, P. M. Ethical Conduct in Science-the Joys of Teaching and the Joys of Learning. *Journal of chemical education* **1999**, *76*, 1327.
(7) Office of Research Integrity Definition of Research Misconduct.
(8) Beasley, M. R. Datta, S. Kogelnik, H.; Kroemer, H. REPORT OF THE INVESTIGATION COMMITTEE ON THE POSSIBILITY OF SCIENTIFIC MISCONDUCT IN THE WORK OF HENDRIK SCHÖN AND COAUTHORS.
(9) Office of Research Integrity Case Summary - Bengu Sezen.
(10) Service, E. Columbia Lab Retracts Key Catalysis Papers. *Science* **2006**, *311*, 1533.
(11) Maher, B. Sabotage! *Nature* **2010**, *467*, 516-519.
(12) Fanelli, D. How many scientists fabricate and falsify research? A systematic review and meta-analysis of survey data. *PLoS One* **2009**, *4*, e5738.
(13) Sovacool, B. K. Exploring Scientific Misconduct: Isolated Individuals, Impure Institutions, or an Inevitable Idiom of Modern Science? *Bioethical Inquiry* **2008**, *5*, 271-282.
(14) Martinson, B. C. Anderson, M. S.; de Vries, R. Scientists behaving badly. *Nature* **2005**, *435*, 737-738.
(15) National Academy of Sciences. *Integrity in Scientific Research: Creating an Environment that Promotes Responsible Conduct*; Washington D.C., 2002.
(16) Department of Health and Human Services Thereza Imanishi-Kari, Ph.D., DAB No. 1582.

Chapter V

Conclusions and Future Directions

A. Inhibition of ErbB2(Her2) Expression with Small Molecule Transcription Factor Mimics

A.1. Conclusions

We set out to determine if a scaffold that can act as synthetic transcriptional activators would could be tailored to inhibit the activity of a particular activation domain. At the outset it was unclear whether or not the promiscuous nature of natural activator-coactivator interactions would pose serious problems for inhibition. The data suggest that isoxazolidine **II-2** is a much improved mimic of the transcriptional activation domain of ESX relative to the generic TAD mimic **II-1,** validating our strategy for using an artificial TAD as a scaffold for the design of transcriptional inhibitors. Treatment of ErbB2+ cancer cells with isoxazolidine **II-2** reduces erbB2 mRNA and protein levels, indicating that the molecule effectively interferes with the ability of ESX to activate transcription of erbB2. This molecule is also effective at attenuating the growth and viability of erbB2 overexpressing cancer cells, which is consistent with the expected effects of an erbB2 transcriptional inhibitor. However, **II-2**, suffers from the same limitations to its potency

and selectivity as other small molecule transcriptional inhibitors.

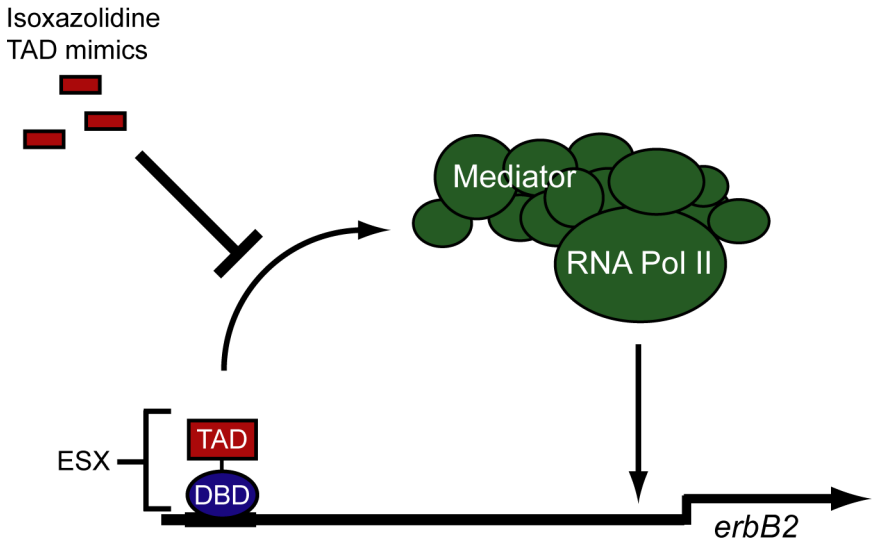


Figure V-1. Rationale for isoxazolidine based inhibitors. Isoxazolidines which are designed to mimic key functionality on the ESX TAD are effective at countering erbB2 overexpression.

A.2. Rationale for the Use of Helical Peptidomimetics in Order to Identify New Ligands for the Transcriptional Machinery

Because amphipathic transcriptional activation domains are believed to have largely helical character when they interact with their coactivator targets,[1] the use of polymeric scaffolds designed to mimic helices may enable the rapid identification of effective inhibitors of transcription. Several examples of this have emerged recently and are discussed below. Such structures could also be used to develop artificial activation domains. If native TADs take on a helical interaction when recruiting coactivators, artificial constrained helices may prove to be extremely potent activation domains.

A.2a. Peptidic Scaffolds

Recent results have shown that L-peptides which have been covalently constrained into short helices are effective inhibitors of transcription. In one example, a constrained helix was used to block the dimerization of transcription factors required to initiate transcription at the NOTCH promoter.[2] In the other, a short constrained helix was designed based on a region of the activation domain of HIF-1α. This peptide was shown to bond to the CH1 domain of CBP, and to inhibit HIF-1α driven gene expression in cell culture.[3]

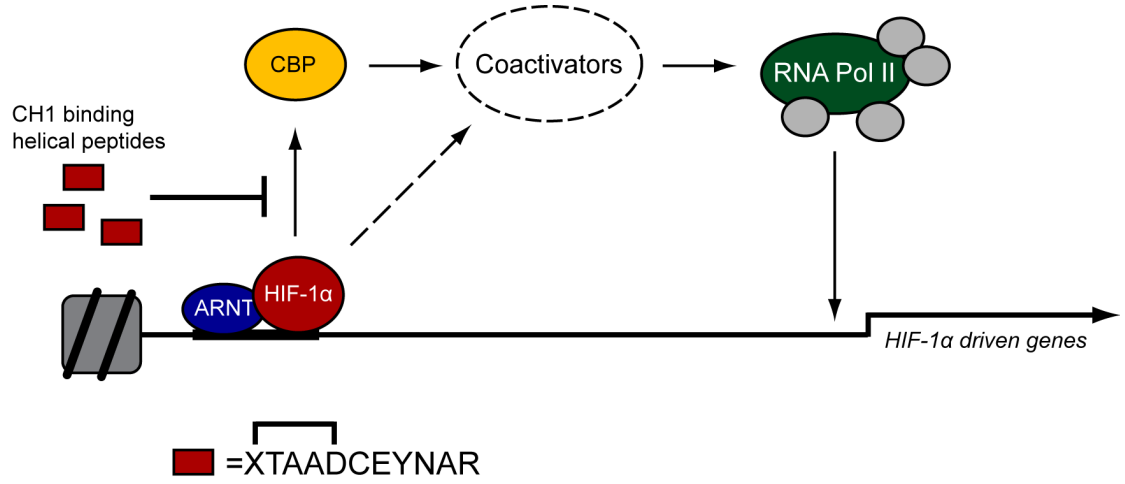


Figure V-2. Helical inhibitor of HIF-1α mediated transcription.Constrained helical peptides such as the one shown here have been used successfully as transcriptional inhibitors. X = pentenoic acid. The covalent tether is the result of an alkene metathesis between the terminal alkene of pentenoic acid, and an allyl group attach to the backbone nitrogen of the aspartic acid. The dotted lines and dashed circle labed 'coactivators' represent steps in the recruitment process that are uncharacterized, but likely given the prevailing model.

The potential uses of peptidic ligands for the transcriptional machinery are

twofold. First, such molecules could be used as activators or inhibitors in their own right. In principle, the ability to rapidly synthesize peptide based molecules that have high affinity and selectivity for a particular target (or that are selective mimics of a particular activation domain) could prove to be a valuable tool for evaluating the consequences of particular inhibition strategies.

On the other hand, such peptidomimetics could also be used to inform the design of small molecules. Constrained helices in particular provide a well-defined interaction surface that can be perturbed in predictable ways by substituting in natural or unnatural amino acids. Once a short but effective peptide sequence is identified, and the most important contacts are known, computational analysis could be used to identify scaffolds that would place functionality in the appropriate places. This could enable structure-based design, even in circumstances where very little was known about the coactivator.

A.2b. Synthetic Scaffolds

An alternative approach would be the use of synthetic polymers as helical mimics. Work by the Hamilton lab identified Terphenyls as approximate mimics of the i, i+3, i+4 positions of an α-helix (Figure V-3).[4] Subsequent studies have focused primarily on altering the scaffold to reduce its hydrophobicity[5] and expand the range of amino acid substitution patterns that can be mimicked.[6] In order to apply these scaffolds to transcription, it would likely be necessary to identify a system in which the positions mimicked by the terphenyl corresponded with important amino acids in the native TAD,

but as these positions fall on one face of the helix that is unlikely to pose a major problem. The primary drawbacks of this method are the size and lipophilicity of the resulting synthetic polymers, and the fact that they are imperfect and less constrained mimics of helical arrangements, so drawing using them to inform the design of smaller synthetic molecules would be less straightforward than for constrained helical peptides.

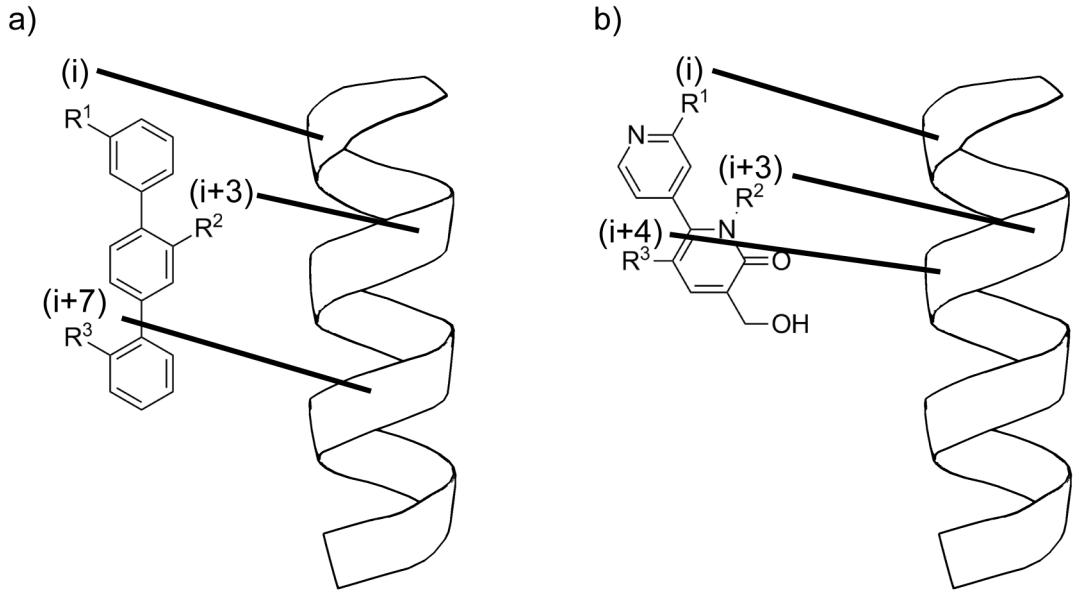


Figure V-3. Non-biomolecule helical mimics. a) The original terphenyl design mimicked the i, i+3 and i+7 positions on a helix. b) A newer indane is designed to mimic the i, i+3, and i+4 positions.

B. A Multi-Pronged Approach to Targeting Oncogenic Transcription

B.1. Conclusions

In spite of their considerable therapeutic potential, the development of highly

potent and selective transcriptional inhibitors has proven elusive. Biological factors such as the promiscuous and low affinity nature of activator-coactivator interactions, and the shared nature of coactivators make these issues difficult to solve by traditional means. However, the use of transcriptional inhibitors such as **II-2** in combination with other agents provides a generalizable strategy for overcoming the activity and selectivity limitations of transcriptional inhibitors. By using a combination that simultaneously curbs expression of the genetic driving force behind a diseased state while also targeting related cellular processes we achieve a synergistic increase in effect that is specific to the target cell population. This approach promises to improve the utility of transcriptional inhibitors as both biochemical tools, and potential therapeutics. By demonstrating the potential of this strategy, these results also suggest that it could be successfully applied to other systems that are regulated by complex networks of protein-protein interactions.

B.2. Application of Synergy to p65 Regulated Transcription

NF-κβ subunit p65 plays a central role in the canonical NF-κβ pathway,[7] where is forms a dimer with the DNA binding subunit p50. The two activation domains of p65 then recruit the machinery needed to initiate transcription. The role of NF-κβ in processes such as inflammation and oncogenesis makes this a system with direct applications to human health.[8] In addition, unlike ESX mediated transcription of erbB2, p65 is part of a well-characterized regulatory network that acts to control p65 release and nuclear transport. Inhibitors have been developed that target several of these events. For

example, several molecules have been identified that inhibit the kinase activity of IKKα and/or IKKβ, which (together with NEMO) form the complex responsible for phosphorylating and triggering the degradation of IKβα, which holds p65 and p50 in an inactive state in the cytoplasm.[9] It is likely that the use of inhibitors that target processes upstream of the transcription factor in question (as in this case) will be an effective alternative to synergy with inhibitors that target downstream processes (as were used in the case of erbB2 expression). Furthermore, this provides a highly complimentary approach, as it will provide selectivity for the activity of a particular activator, while the downstream synergy provides selectivity for one particular target of an activator. It is possible that this broader selectivity will be useful in cases like NF-κβ that regulate several genetic programs which have interrelated cellular effects.

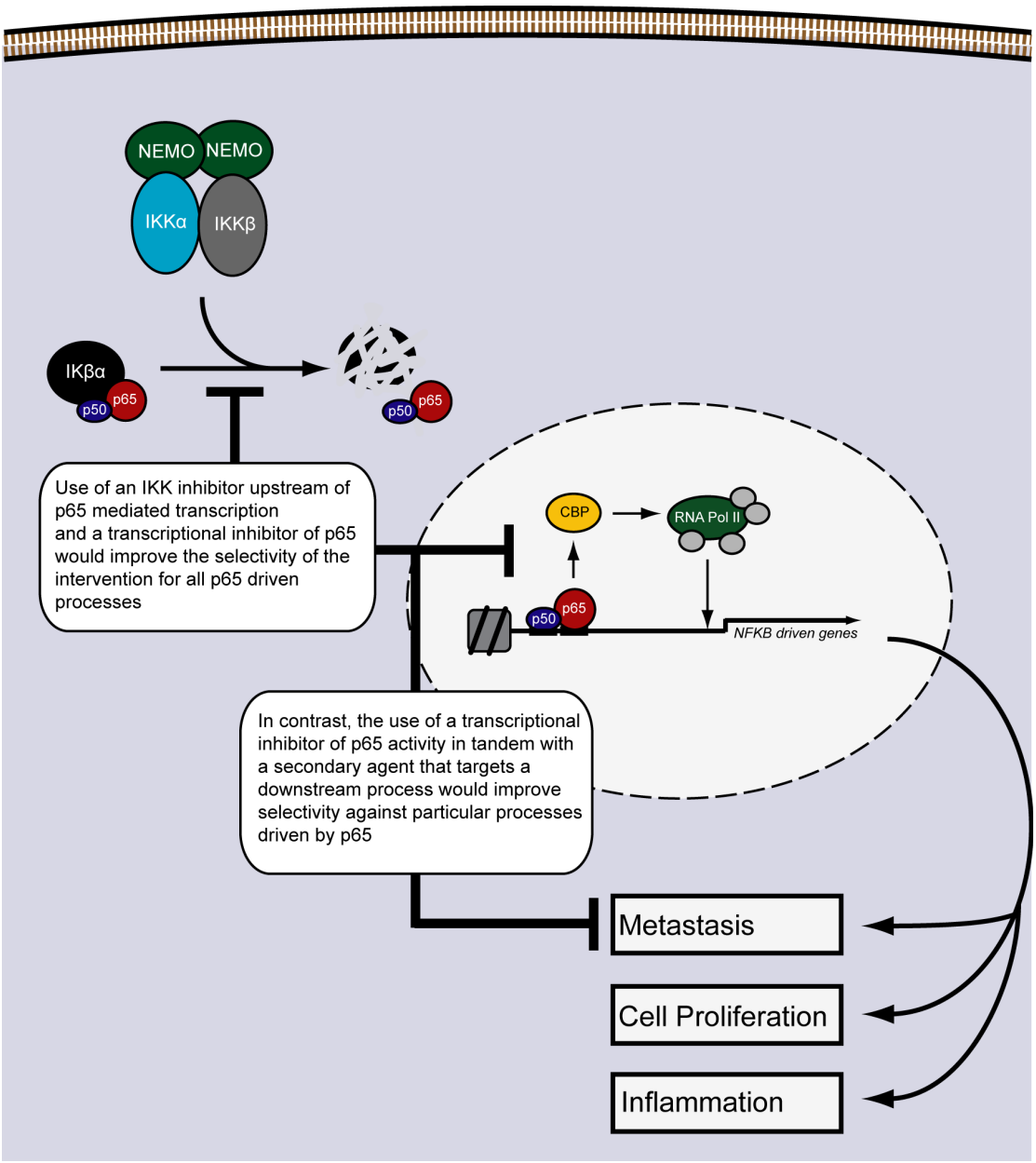


Figure V-4. Upstream vs. downstream synergy. The use of secondary agents that target processes upstream of transcription would increase selectivity for programs initiated by a particular activator (p65 in this case). Combinations of a transcriptional inhibitor with secondary agents that target downstream processes would increase selectivity for a particular subset of the processes driven by a particular activator. In this case, selectivity for metastasis would be increased relative to the effects on proliferation of inflammation.

B.3. The Use of Synergy to Alter the Selectivity of a Transcriptional Inhibitor

An additional application of synergy is the potentual use of two different secondary agents to switch the selectivity of a transcriptional inhibitor from one pathway to another. For example, both p65 and HIF-1α are thought to depend on contact with the CH1 domain of CBP in order to initiate transcription.[10,11] It is therefore likely that inhibitors which bind the CH1 domain of CBP will affect both of these pathways, in addition to the activity of any other transcriptional activator proteins that rely on contact with the CH1 domain.

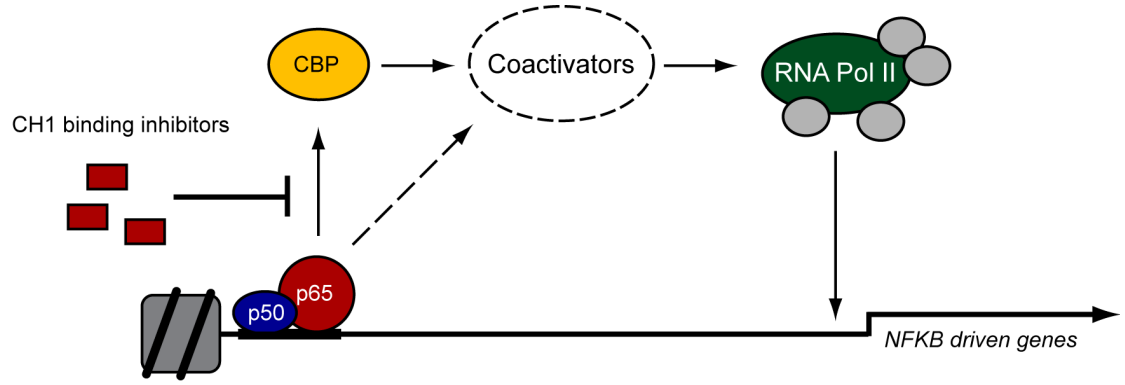


Figure V-5. Rationale for CH1 binding peptides as inhibitors of p65 mediated transcription.CH1 binding peptides can be used to inhibit the interaction between p65 and CBP that is necessary for p65 iniated recruitment of the transcriptional machinery. The dotted lines and dashed circle labed 'coactivators' represent steps in the recruitment process that are uncharacterized, but likely given the prevailing model.

However, if the use of secondary agents that act on processes up- or down- stream of a specific activator produces a selective and synergistic effect on that activator's activity, this can be expanded to produce selectivity for either activator. In this case, the combination of a CH1 targeting transcriptional inhibitor and an IKK inhibitor would be

selective for p65 driven transcription, while the use of the same CH1 targeting transcriptional inhibitor and an inhibitor of HIF-1α DNA binding would be selective for HIF-1α driven transcription.

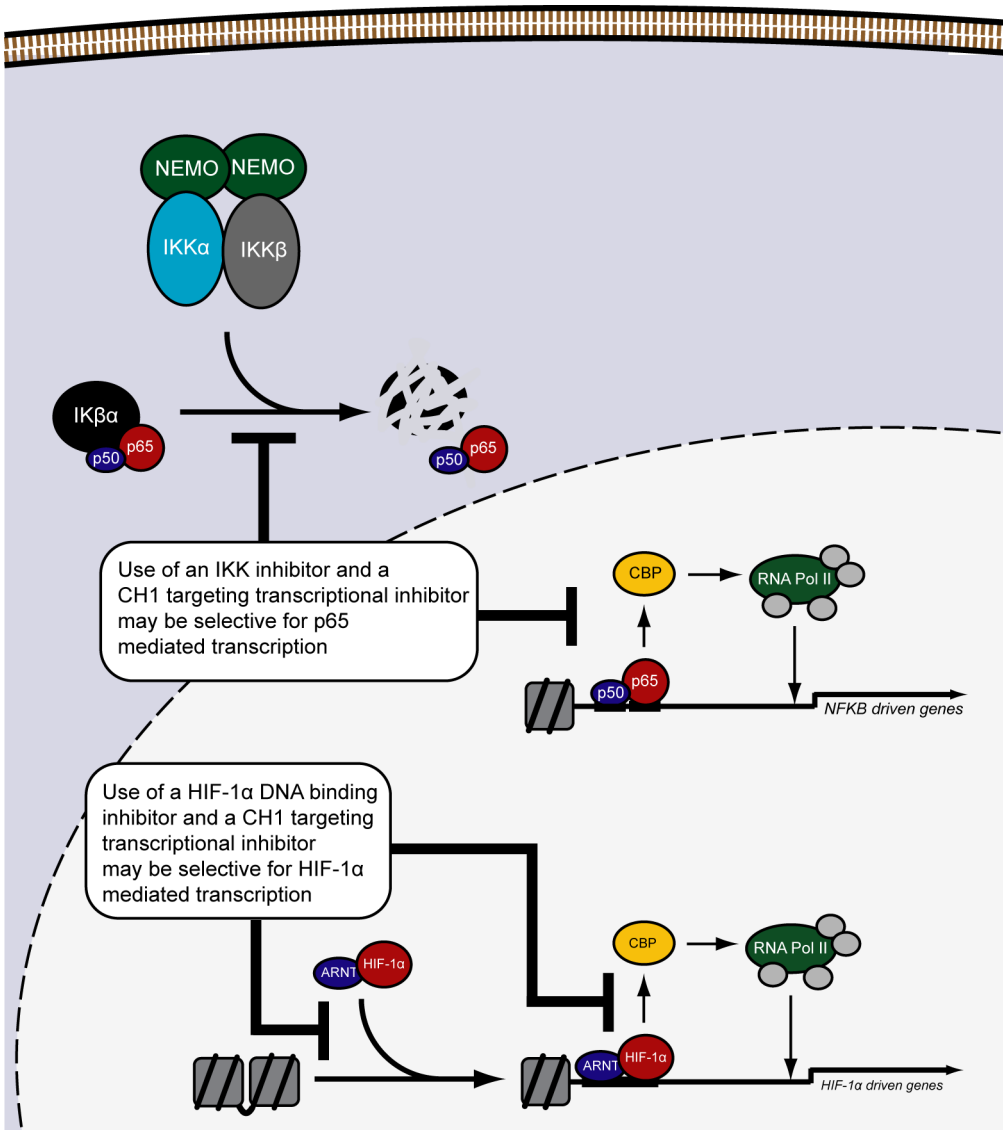


Figure V-6. Complementary approaches to synergy. By using different secondary agents, synergistic combination could allow CH1 binding peptides to act selectively on either the canonical NF-κβ pathway (p65 driven) or HIF-1α driven transcription.

C. Design and Implementation of a Peer-Led Discussion Module for Teaching Research Ethics to Incoming Graduate Students

C.1. Conclusions

The purpose of this module was to introduce incoming graduate students to the methods and tools that they can use to navigate the ethical hazards of graduate research. Students practiced these skills by working through a number of exercises based on scenarios that they may face in the next couple of years. Students were also advised on potential resources that they can call on to help resolve ethical issues that arise in the course of their research. The students' ability to articulate the issues in these scenarios and propose reasonable solutions to them indicates that they are beginning to appreciate the nuances of ethical decision making. Student responses also show an understanding of the need to consider the long term effects of decisions on both themselves and others.

C.2. Future Opportunities for the Discussion of Research and Data Interpretation.

Recently, Rackham has mandated that all departments, including Chemistry, implement credited courses on the responsible conduct of research and that these course be taught by faculty. Although the implementation of this course will make the module redundant, the rationale behind the module's construction, and much of the materials generated for it are being adapted to this new course. Additionally, one of the graduate

students who designed and implemented this module – Amy Danowitz – will be teaching the department's responsible conduct of research course in collaboration with a full time member of the faculty. The materials in this module could also be expanded and adapted for use as in research group meetings. In this way, it would provide a vehicle for individual advisers to discuss and establish group standards for conflict resolution, safety, and data interpretation.

D. References

(1) Lee, L. W.; Mapp, A. K. Transcriptional Switches: Chemical Approaches to Gene Regulation. *Journal of Biological Chemistry* **2010**, *285*, 11033-11038.

(2) Moellering, R. E. Cornejo, M. Davis, T. N. Bianco, C. D. Aster, J. C. Blacklow, S. C. Kung, A. L. Gilliland, D. G. Verdine, G. L.; Bradner, J. E. Direct inhibition of the NOTCH transcription factor complex. *Nature* **2009**, *462*, 182-188.

(3) Henchey, L. K. Kushal, S. Dubey, R. Chapman, R. N. Olenyuk, B. Z.; Arora, P. S. Inhibition of Hypoxia Inducible Factor 1—Transcription Coactivator Interaction by a Hydrogen Bond Surrogate α-Helix. *J. Am. Chem. Soc.* **2010**, *132*, 941-943.

(4) Becerril, J.; Hamilton, A. D. Helix Mimetics as Inhibitors of the Interaction of the Estrogen Receptor with Coactivator Peptides. *Angew. Chem. Int. Ed.* **2007**, *46*, 4471-4473.

(5) Volonterio, A. Moisan, L.; Rebek, J. Synthesis of Pyridazine-Based Scaffolds as α-Helix Mimetics. *Org. Lett.* **2007**, *9*, 3733-3736.

(6) Kim, I. C.; Hamilton, A. D. Diphenylindane-Based Proteomimetics Reproduce the Projection of the i , i +3, i +4, and i +7 Residues on an α-Helix. *Org. Lett.* **2006**, *8*, 1751-1754.

(7) Hoffmann, A. Natoli, G.; Ghosh, G. Transcriptional regulation via the NF-κB signaling module. *Oncogene* **2006**, *25*, 6706-6716.

(8) Bassères, D. S.; Baldwin, A. S. Nuclear factor-κB and inhibitor of κB kinase pathways in oncogenic initiation and progression. *Oncogene* **2006**, *25*, 6817-6830.

(9) Scheidereit, C. IκB kinase complexes: gateways to NF-κB activation and transcription. *Oncogene* **2006**, *25*, 6685-6705.

(10) Zhong, H. Voll, R.; Ghosh, S. Phosphorylation of NF-kB p65 by PKA Stimulates Transcriptional Activity by Promoting a Novel Bivalent Interaction with the Coactivator CBP/p300. *Molecular Cell* **1998**, *1*, 661–671.

(11) Dames, S. A. Martinez-Yamout, M. De Guzman, R. N. Dyson, H. J.; Wright, P. E.

Structural basis for Hif-1a/CBP recognition in the cellular hypoxic response. *Proceedings of the National Academy of Sciences* **2002**, *99*, 5271.

Appendix I

The Design and Evaluation of d-Peptide Inhibitors of p65 Mediated Transcription

A. Overview

The misregulation of transcription is associated with a wide variety of human diseases, such as cancer.[1,2] In spite of this, generalizable methods to modulate transcription remain elusive. An archetypal example of a transcription factor that drives pathological gene expression is the NF-κB subunit p65, which plays a key role in processes such as inflammation and oncogenesis.[3,4] P65 is known to make contacts with the KIX and CH1 domains of the coactivator CBP,[5] and the TA1 and TA2 activation domains of p65 are likely responsible for mediating other protein-protein contacts necessary for the initiation of transcription.[6,7] D-peptide mimics of the TA1 transcriptional activation domain of p65, and a d-peptide version of known CH1 and KIX binding peptides were tested for their ability to inhibit the transcriptional activity of p65. Although results indicate that the CH1 binding peptide (A1-7) may be effective at reducing p65 activity in response to TNFα stimulation, further investigation is needed to confirm the mechanism of this effect, and the development of more effective inhibitors would be highly beneficial.

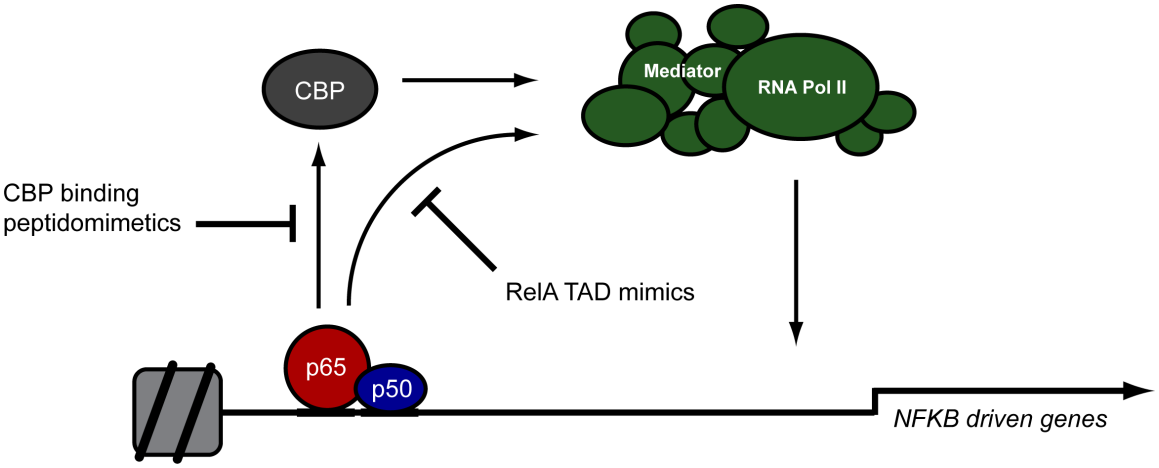


Figure A1-1. Rationale for inhibition of p65 mediated transcription. It is possible that p65 mediated transcription could be inhibited by peptidomimetics that are designed to bind to known activator binding regions of CBP – thus inhibiting the interaction between p65 and CBP, or by peptidomimetics designed to mimic the activation domain of p65, thereby blocking probable interactions between p65 and components of the mediator complex.

B. Background

B.1. NF-kB subunit P65

The NF-kB family of proteins includes the transcriptional activators RelA (p65), RelB, and C-rel, in addition to the proteins p50 and p52.[8] A heterodimer of p65 and p50 is the active transcription factor commonly referred to as NF-kB, and the primary driver of NF-kB activity under the canonical NF-kB signaling cascade.[9]

The TAD of p65 is generally divided into two regions TA1 (521-551) and TA2 (428-521).[6] The transcriptional activity of both Gal4-TA1 and Gal4-TA2 fusion proteins

suggests that these domains are able to function individually as transcriptional activation domains. However, either TA1 or TA2 is sufficient to squelch activity of RelA(286-551) in COS cells.[10] The activity of the TA1 domain appears to be dependent on the presence of two phenylalanines (F534 and F542).[11] Repeats of a sequence containing one of these regions of the TA1 domain were seen to activate transcription, and a putatively 'TA1-like region' has been identified in TA2.[10] On the whole, results from the literature suggest the existence of 2 or more potentially redundant domains that are both capable of squelching each other when not fused to a DNA binding domain. This in turn suggests that a peptide mimic of any one of these regions may be effective at reducing the activity of DNA bound p65.

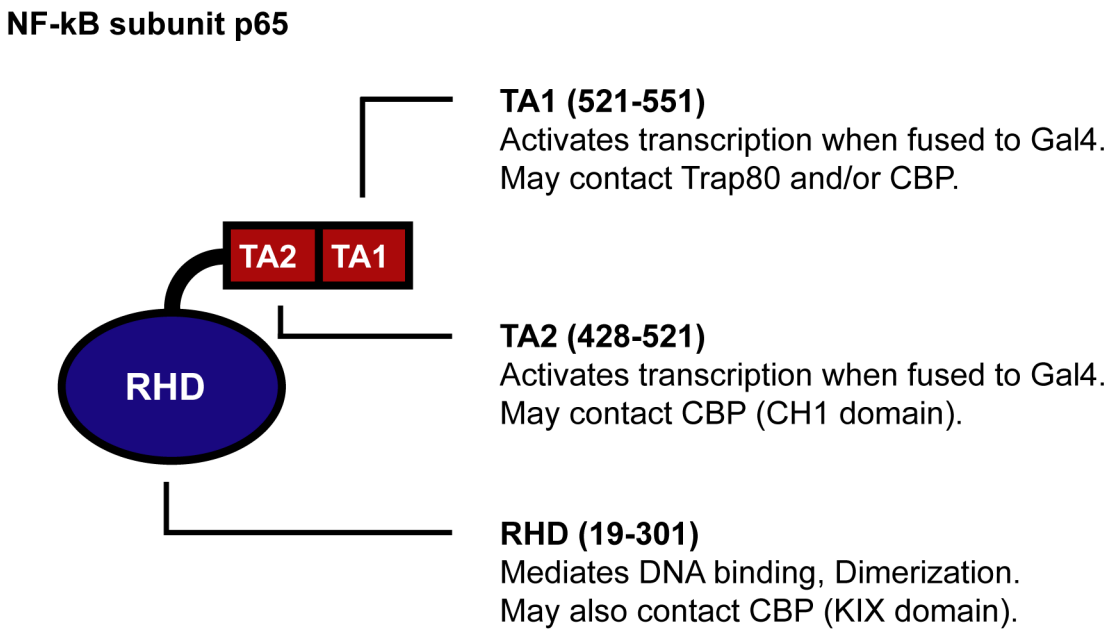


Figure A1-2. Major domains of p65.

B.2. CBP

Creb-Binding Protein (CBP) and its paralog p300 act as coactivators for hundreds of transcriptional activation proteins.[12,13] Although the details of their role in faciliting transcription appears to be complex and heavily context dependent, they may act to localize Histone Acetyl Transferases to open up the chromatin structure near the promotor, or to recruit general transcription factors such as TFIIB and TFIIB to aid in formation of the pre-initiation complex at the promoter. [14,15] CBP and p300 have several domains which are known to bind to overlapping sets of transcriptional activators. In particular, the KIX and CH1 domains of CBP are known to interact with a variety of transcriptional activation domains, including p65.[5,12]

B.3. P65-CBP interactions

It has been established that transcription via p65 is mediated by the homologous coactivators CBP and p300.[16,17] The C-terminus of p65 contains 2 activation domains (TA1 and TA2) either of which can activate transcription when fused to a DNA binding domain.[6] Early indications of the interaction between CBP/p300 and p65 came from the Nabel lab,[17] where p300 was found to coprecipitate with p65-CDK complexes, and to enhance the activation of p65 by the cell cycle regulatory protein p21. Work from Collins and co-workers[16] supports that both CBP and p300 can potentiate p65-driven transcription of a reporter gene. The results of these experiments also indicated

interactions between p65 286-551 (which contains both the TA1 and TA2 domains) and the N-terminal regions of both CBP and p300. The results of a series of experiments by Zhong and coworkers[5] indicate the presence of an interaction between the KIX domain of CBP and p65, which is dependent on p65 S276 phosphorylation, and a phosphorylation-independent interaction between the C terminal region (TA1 and TA2) of p65 and the N terminal region (1-450) of CBP, which contains the CH1 domain.

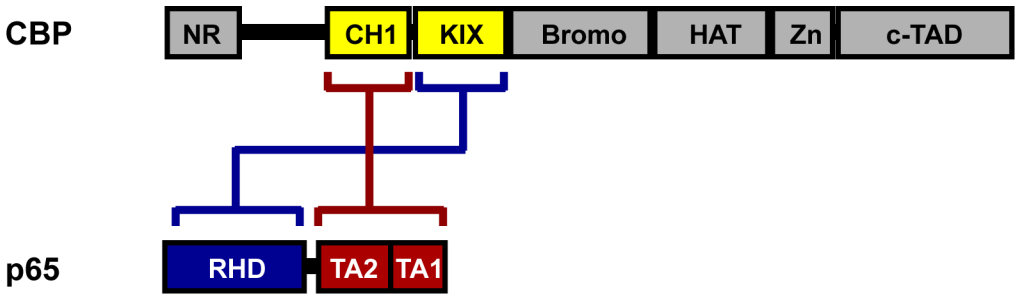


Figure A1-3. Putative interactions between p65 and the coactivator CBP. The interaction between the Rel Homology Domain (RHD) of p65 and the KIX domain of CBP is shown in blue. The interaction between the activation domains of p65 (possibly either TA1, TA2, or a combination) and the CH1 domain is shown in red.

Taken together, these experiments indicate that CBP and/or p300, particularly the N terminal domains such as KIX and CH1, play a key role in mediating p65 dependent transcription. This makes sense, given that both the KIX and CH1 domains are well-validated targets for many other transcription factors.[12,14] However, the functional significance of these potential interactions is not clear.

C. Design of d-peptide inihibitors of p65 transcriptional activity

Enantiomeric d-peptides were used to increase the proteolytic stability of the peptides, thereby making degradation by cellular proteolysis less of a factor in their activity. Peptides were tagged with a fluorescein isothiocyante (FITC) label at the N-terminus to aid in quantification, and to allow cellular uptake to be assessed by fluoresence microscopy. An 8-Amino-3,6-Dioxaoctanoic Acid ('MiniPeg') linker was used to attach the fluorescein label to the N-terminus of the d-peptide, in order to prevent the label from interfering with the peptides ability to interact with CBP or other coactivators. Although some of the peptides, such as the CH1 binding d-peptide were expected to be cell permeable, most were given a C-terminal hexaarginine tag to increase cell uptake.

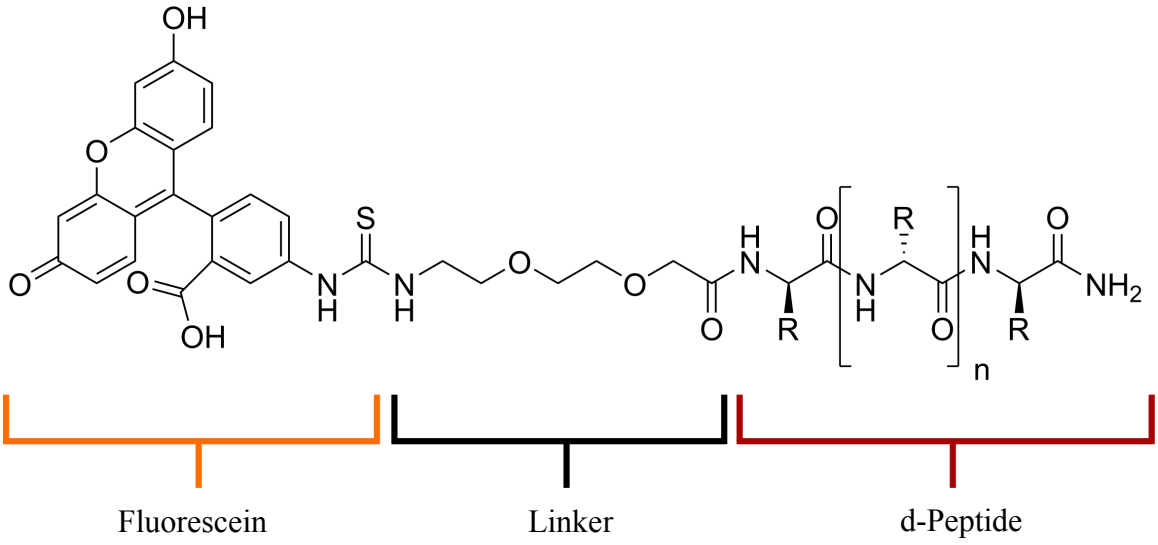


Figure A1-4. General schematic of the d-peptide inhibitors designed to curb p65 activity.

D-peptide mimics of the TA1 domain focused on the activator sequence DFSALL. This six-residue sequence has been shown to activate transcription when repeated and fused to a DNA binding domain, and is thought to be one of two necessary portions of the TA1 domain.[11] As shown in Table IV-1, d-peptides incorporating additional positively charged amino acids (A1-1 to A1-4) were synthesized to test alternatives to hexaarginine cell permeability tags.

The CH1 binding d-peptide (A1-7) was based on a 'hydrogen bond surrogate' developed by the Arora lab,[18] in which a hydrocarbon bridge was used to mimic the hydrogen bonding pattern of an alpha-helix and hold the peptide in a helical conformation. The KIX binding d-peptide (A1-6) was based on earlier work in the Mapp lab, in which peptidic ligands for the KIX domain of CBP were evaluated by fluorescence polarization.[19]

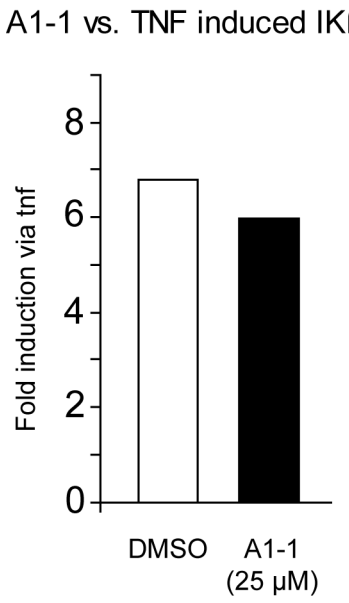
A1-1	FITC-MiniPEG-d-ADMDFSALLR	p65 TA1 mimic with C-term R
A1-2	FITC-MiniPEG-d-ARMRFSALL	p65 TA1 mimic with D to R
A1-3	FITC-MiniPEG-d-DFSALLRRRRRR	p65 TA1 mimic with C-term R6
A1-4	FITC-MiniPEG-d-DFSALLRRRK	p65 TA1 mimic with C-term RK
A1-5	FITC-MiniPEG-d-RRRRRR	R6 peptide control
A1-6	l-K(FITC)-d-SWAVYELLFGS	known KIX binding d-peptide
A1-7	FITC-MiniPEG-d-TAADCEYNAR	d-peptide based on CH1 HBS

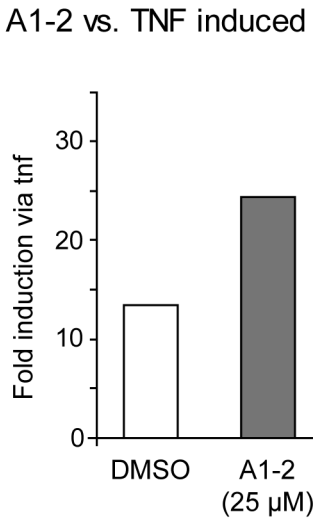
Table A1-1. D-peptides designed to inhibit p65 mediated transcription.

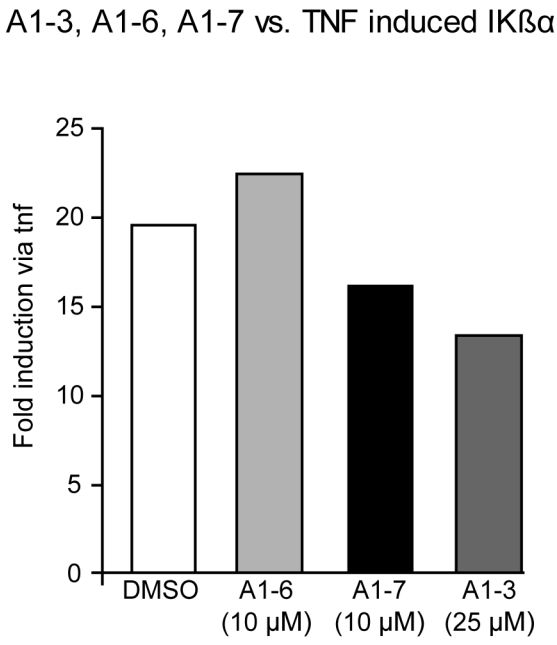
D. Activity of d-Peptide Inhibitors of p65 Mediated Transcription

D.1. Activity Against TNFα Induced p65 Driven Gene Expression

The initial readout for peptide activity was their effect of the induction of NFKBIA (Iκβα) by TNFα treatment. Iκβα is a well-known target of p65 activity in this context, forming part of a negative regulatory loop that then serves to reduce p65 activity back to basal levels.[20,21,22] The CH1 binding peptide A1-7 and potentially the TA1 mimic with the hexaarginine tag (A1-3) appear to reduce p65 mediated activation of Iκβα. Other peptides, including the TA1 mimic with a single arginine (A1-1), and the KIX binding peptide (A1-6) did not appear to have an effect. The hexarginine tag by itself (A1-5) appears to cause an increase in Iκβα induction, possibly as a side effect of the toxicity attributed to highly cationic molecules.[23,24] Although this effect is likely attentuated in longer peptides which contain the hexaarginine sequence, it is still a potential confounding factor. This complication highlights the importance of finding an acceptable balance between cell permeability and excess positive charge.







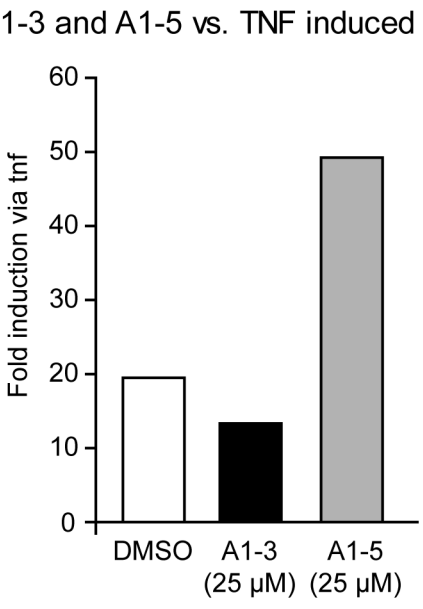


Figure A1-5. Results from initial screen of d-peptides. Initial results of quantitative PCR (qPCR) experiments used to evaluate the effects of d-peptides on tnf induced, p-65 mediated expression of Iκβα. All data represent the median of experiments from a single biological sample run in triplicate, normalized to matching tnf negative controls.

A more thorough test of the CH1 binding peptide A1-7 and the A1-3 indicates that the former is likely to have a significant impact on p65 activity, while the latter may not. A1-7 was also tested for its ability to interfere with transcription of a second p65 target, the oncogene ICAM-1. Although these results lack statistical significance, it does suggest an effect by A1-7.

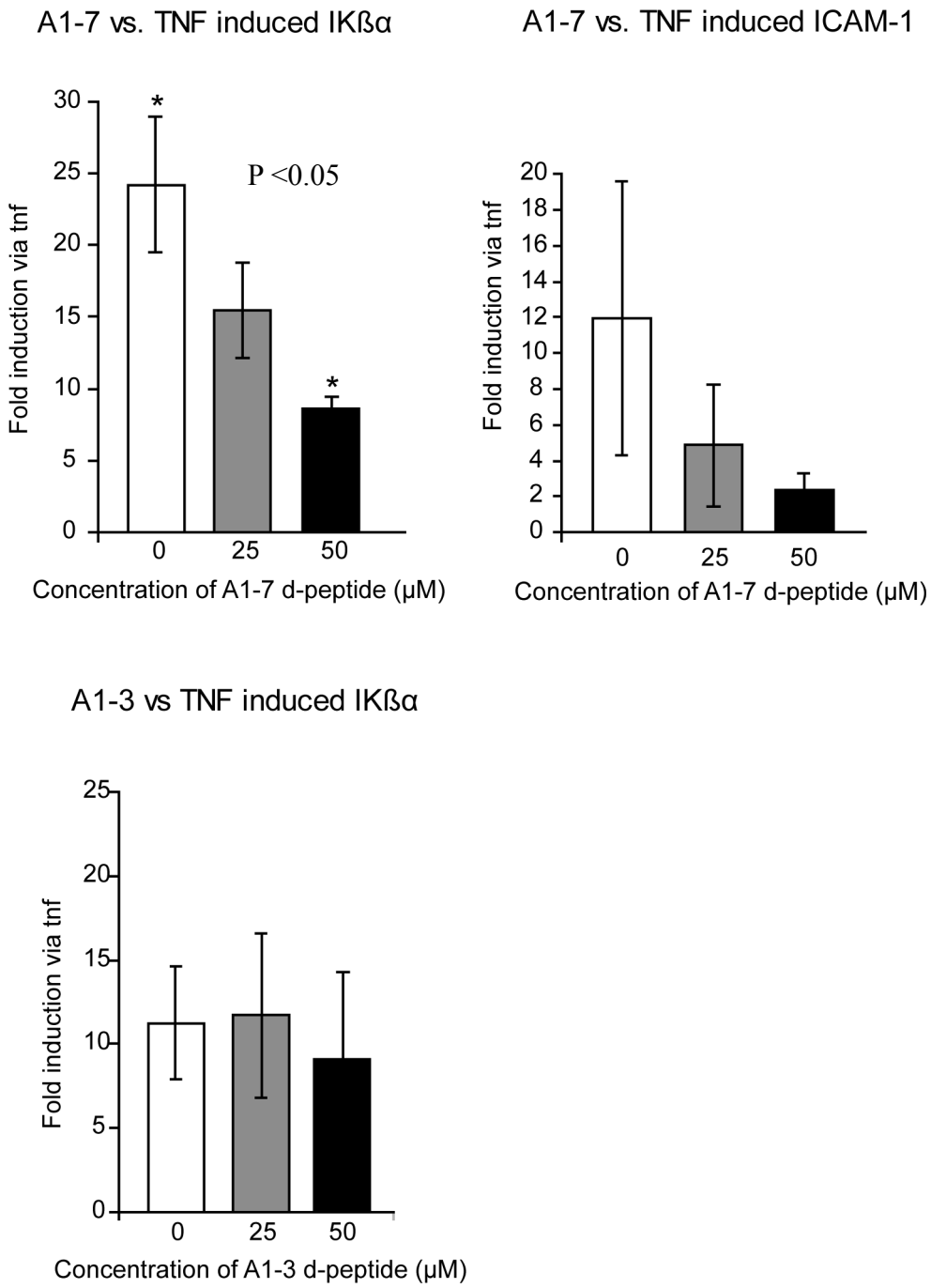


Figure A1-6. Results from followup evaluation of A1-3 and A1-7. Results of qPCR experiments used to evaluate the effects of d-peptides on tnf induced, p-65 mediated expression of Iκβα. All data represent the median of experiments from 3 biological samples, each run in triplicate, normalized to matching tnf negative controls.

D.2. Cellular Uptake of d-Peptides

In order to deconvolute the effects of potentially limited cellular uptake on the activity of the peptides, we employed fluorescence microscopy to analyze the ability of the peptides to enter cells. Results indicate uptake of both A1-7 and A1-3. However, nuclear permeability appears to be limited at best. This may be a limiting factor on the activity of these peptides, though the effectiveness of the structurally similar HBS peptides[18] suggests that these peptides might have effects in spite of limited nuclear permeability. An additional TA1 mimic A1-4, using a nominal nuclear localization sequence (RRRK) was synthesized by Paul Bruno, but a significant increase in nuclear permeability was not seen. More effective strategies to induce nuclear uptake of these peptides may increase their activity substantially.

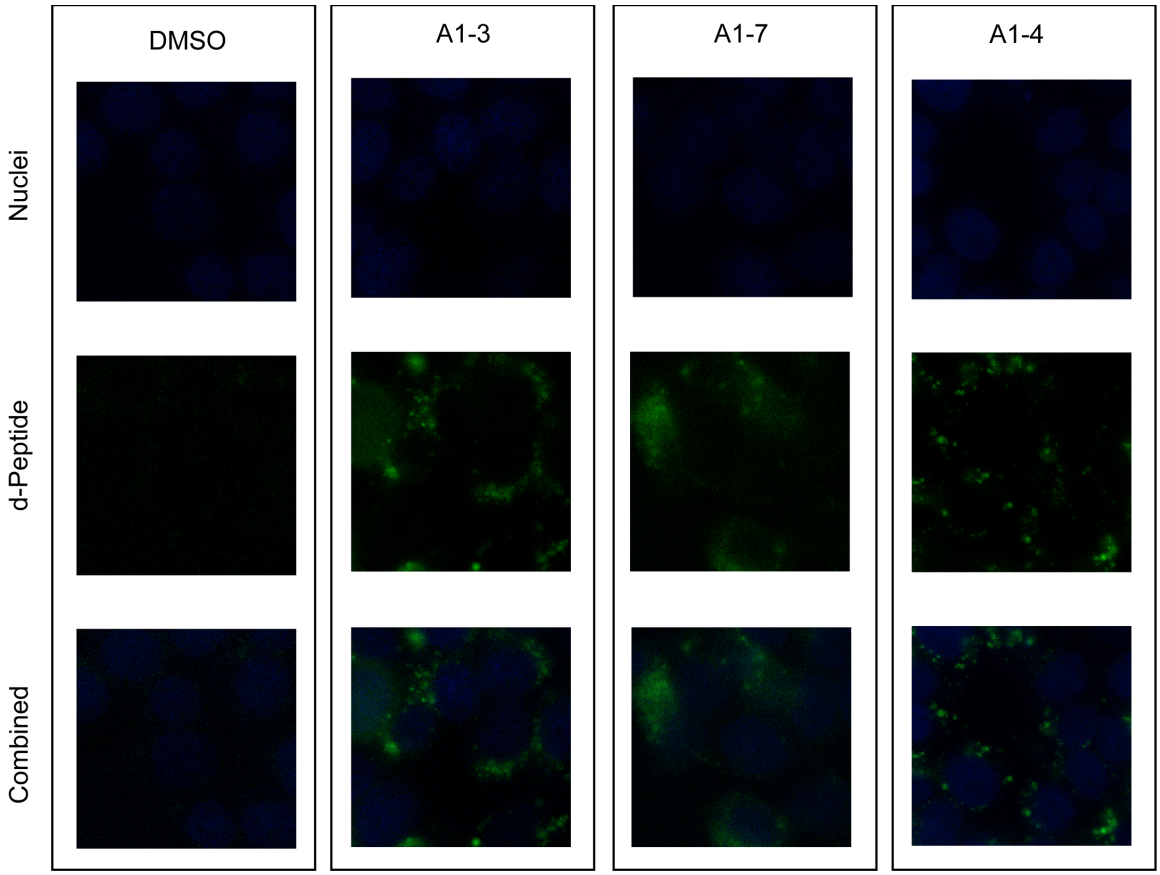


Figure A1-7. Results of fluorescent microscopy experiments. These were carried out to determine cellular permeability of promising d-peptide inhibitors of p65 activity. Results shown are for compounds at 50 μM, 24 hours after addition of compound. Hoechst stain was used to visualize nuclei and the fluorescein tag was used to visualize peptides.

D.3. Effects on the Activity of Gal4-p65 Mediated Transcription of a Luciferase Reporter

In an effort to validate potential mechanism, and to assess their effects under more controlled settings, peptides A1-3, A1-4, and A1-7 were tested for their ability to curb the activity of a Gal4-p65 construct. Results indicate that none of the peptides was effective at concentrations up to 50 μM. This is not suprising in the case of peptides such as A1-3, which were not observed to be effective against p65 driven transcription of Iκβα. The

failure of A1-7 to inhibit Gal4-p65 activity suggests that CBP may not be neccesary under the assay conditions, perhaps due to the reduced chromatinization of the reporter gene environment. Additionally, as the construct utilized for these assays included both the TA1 and TA2 activation domains of p65, it may be more useful to test peptides against the individual domains initially, and then optimize promising leads to affect the activity of a construct containing both domains.

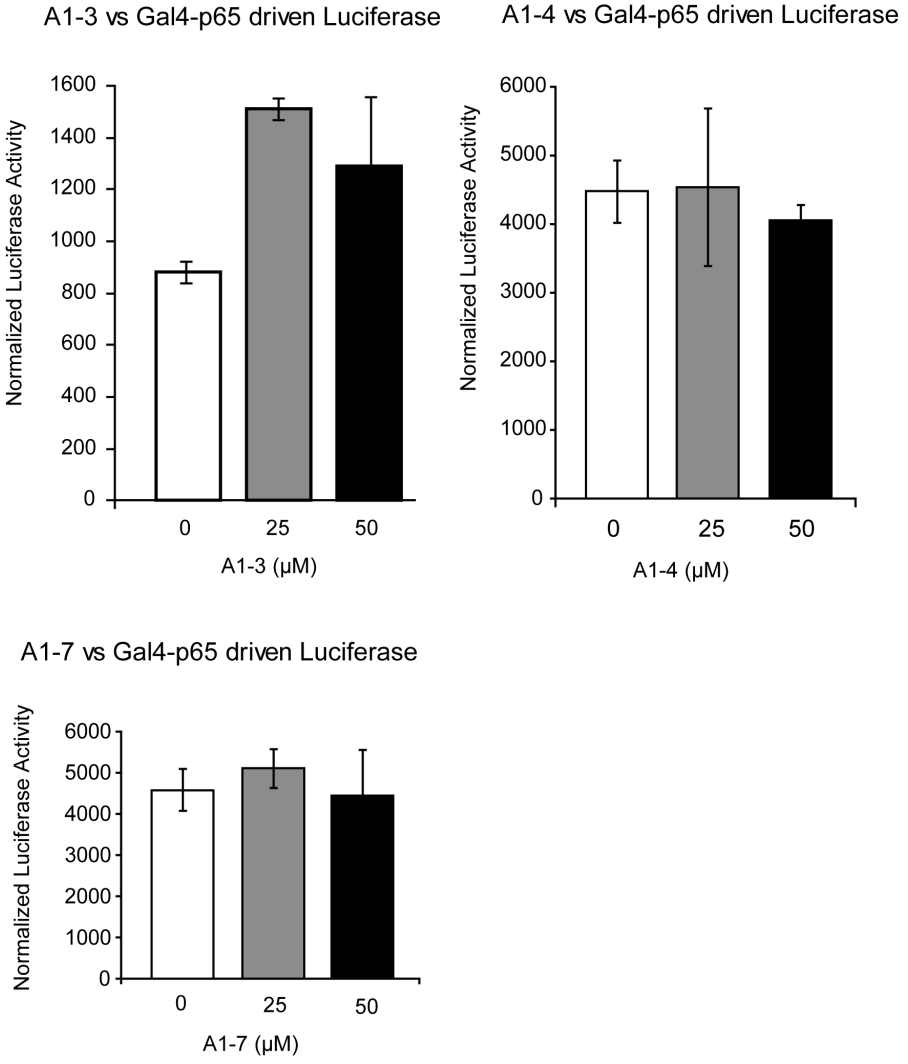


Figure A1-8. Results of luciferase reporter assays. These were designed to evaluate the ability of d-peptides to inhibit the activity of the TA1 and TA2 domains of p65.

E. Conclusions

A series of proteolytically resistant d-peptides were designed to inhibit p65 mediated activation. A peptide based on a known CH1 binding motif (A1-7) appears to interfere with p65 mediated transcripion at low to mid micromolar concentrations. Cellular uptake of this peptide is reasonable, though it may suffer from poor nuclear localization. Peptides designed to mimic the TA1 domain (A1-1 through A1-4) did not appear to be effective in either reducing p65 mediated response to TNFα, or to attenuating the ability of a Gal4-p65 construct to activate transcription of a reporter gene.

Confirmation of the proposed mechanism of action for the A1-7 would lend additional results more weight. A direct measurement of the peptide's ability to interfere with the interaction between P65 and the CH1 domain would be particularly useful. Potential methods for this include binding curves based on the fluorescence polarization of a tagged peptide based on the p65 TA1 or TA2 domains interacting with the CH1 domain of CBP in the presence or absence of inhibitor. Surface plasmon resonance, luciferase complemenation, or isothermal calorimetry provide alternative methods for examining this interaction.

One of the primary future directions for this project include the identification and application of peptides that inhibit p65 mediated transcription. If the activity of A1-7 can be reliably reproduced, combinations of this peptide and other agents that target up- or down- stream points on the canonical p65 signaling pathway may prove synergistic. Furthermore, such combinations have the potential to offer a form of switchable

selectivity, in which a CH1 binding peptide and an agent that targets the HIF-1α pathway have very different phenotypic selectivity and effects that the same CH1 binding peptide and an agent that targets the canonical p65 pathway. Additionally, the identification of more effect nuclear localization elements would be extremely useful to the development of this and other peptidomimetics that act on nuclear processes.

F. Supplementary Information

F.1. Materials

Peptides were synthesized using standard solid phase synthesis techniques from Fmoc protected d-amino acids purchased from Peptides International. The identity and purity of peptides was determined by mass spectrometry and HPLC analysis. Peptides were quantitated using by taking their A280 in a 1:1 solution of water:methanol. Peptides were then concentrated to white powder using a lyophilizer. Taqman probes for RT-qPCR were purchased from Applied Biosystems.

F.2. Cell Culture and qPCR assays

HEK293T cells were grown in DMEM with 10% Fetal Bovine Serum without antibiotics. Cells were plated at approximately 50,000 cells per well in 24 well plates. After adhering overnight, media was replaced and cells were dosed with d-peptides in

DMSO (1% final concentration). After 24 hours, TNFα (10ng/mL final concentration) was added to half the wells to induce p65 release and activity. After 4 hours, RNA was harvested using an RNAeasy kit from Qiagen, according to the manufacturer's instructions. RNA was quantified by UV/Vis analysis, and 1 ug of each sample were subjected to a reverse transcription reaction in accordance with the manufacturer's instructions (Applied Biosystems). qPCR reactions were then carried out in triplicate from single samples of cDNA using TaqMan probes. Data was analysed using the ΔΔCT method, comparing TNFα treated cells to untreated cells and using Ribosomal Subunit p0 as an internal control for each cDNA sample.

F.3. Luciferase Assays

HEK293T Cells were plated at 3,000 cells per well in white 96 well plates. After adhering overnight, cells were transfected for 4 hours (optimem and lipofectamine according to the manufacturer's instructions) with 250 ng/mL of a plasmid coding for a fusion of Gal4(1-100) and p65(286-551), as well as 500 ng/mL of a plasmid containing 5 Gal4 binding sites upstream of a luciferase reporter (PG5Luc) and 40 ng/mL of a plasmid that constituitively expresses Renilla (PRLSV40). After transfection, media was changed back to DMEM+10% FBS and cells were dosed with d-peptides in DMSO (1% final concentration). After 24 hours, Luciferase and Renilla activity were measured using the Dual-Glo assay kit (Promega) in accordance with the manufacturer's instructions.

F.4. Fluorescence Microscopy

HEK293T cells were plated in glass microscopy slides at a concentration of approximately 400,000 cells per slide in 1 mL of media and allowed to adhere overnight. Cells were then dosed with d-peptides in DMSO (1% final concentration) for 24 hours. Hoechst stain (0.5 μL in an additional mL of media for a final concentration of 0.25 μL/mL) was added and incubated for 15 minutes. After this, the cells were rinsed very carefully with cold, sterile PBS, and then 2 mL DMEM + 10% FBS but without phenol red was added. Cells were then observed using filters for Hoechst stain and fluorescein. Images were worked up and combined in Photoshop CS. This protocol produced somewhat faint nuclear staining, and the concentration of Hoechst stain should probably be doubled in future experiments.

G. References

(1) Albertson, D. G. Gene amplification in cancer. *Trends in Genetics* **2006**, *22*, 447-455.

(2) Vogelstein, B.; Kinzler, K. W. Cancer genes and the pathways they control. *Nat Med* **2004**, *10*, 789-799.

(3) Bassères, D. S.; Baldwin, A. S. Nuclear factor-κB and inhibitor of κB kinase pathways in oncogenic initiation and progression. *Oncogene* **2006**, *25*, 6817-6830.

(4) Burstein, E.; Duckett, C. S. Dying for NF-κB? Control of cell death by transcriptional regulation of the apoptotic machinery. *Current Opinion in Cell Biology* **2003**, *15*, 732-737.

(5) Zhong, H. Voll, R.; Ghosh, S. Phosphorylation of NF-kB p65 by PKA Stimulates Transcriptional Activity by Promoting a Novel Bivalent Interaction with the Coactivator CBP/p300. *Molecular Cell* **1998**, *1*, 661–671.

(6) O'shea, J. M.; Perkins, N. D. Regulation of the RelA (p65) transactivation domain. *Biochem. Soc. Trans* **2008**, *36*, 603.

(7) Perkins, N. D. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene* **2006**, *25*, 6717-6730.
(8) Hoffmann, A. Natoli, G.; Ghosh, G. Transcriptional regulation via the NF-κB signaling module. *Oncogene* **2006**, *25*, 6706-6716.
(9) Annunziata, C. M. Davis, R. E. Demchenko, Y. Bellamy, W. Gabrea, A. Zhan, F. Lenz, G. Hanamura, I. Wright, G. Xiao, W. Dave, S. Hurt, E. M. Tan, B. Zhao, H. Stephens, O. Santra, M. Williams, D. R. Dang, L. Barlogie, B. Shaughnessy, J. D. Kuehl, W. M.; Staudt, L. M. Frequent Engagement of the Classical and Alternative NF-κB Pathways by Diverse Genetic Abnormalities in Multiple Myeloma. *Cancer Cell* **2007**, *12*, 115-130.
(10) Schmitz, M. L. dos Santos Silva, M. A.; Baeuerle, P. A. Transactivation Domain 2 (TA2) of p65 NF-kB. *Journal of Biological Chemistry 270*, 15576-15584.
(11) Schmitz, M. L. dos Santos Silva, M. A. Altmannl, H. Czischl, M. Holakl, T. A.; Baeuerle, P. A. Structural and Functional Analysis of the NF-KB p65 C Terminus. *Journal of Biological Chemistry* **1994**, *269*, 25613-25620.
(12) Dames, S. A. Martinez-Yamout, M. De Guzman, R. N. Dyson, H. J.; Wright, P. E. Structural basis for Hif-1a/CBP recognition in the cellular hypoxic response. *Proceedings of the National Academy of Sciences* **2002**, *99*, 5271.
(13) Goodman, R. H.; Smolik, S. CBP/p300 in cell growth, transformation, and development. *Genes & development* **2000**, *14*, 1553.
(14) Vo, N.; Goodman, R. H. CREB-binding protein and p300 in transcriptional regulation. *Journal of Biological Chemistry* **2001**, *276*, 13505.
(15) Karamouzis, M. V. Konstantinopoulos, P. A.; Papavassiliou, A. G. Roles of CREB-binding protein (CBP)/p300 in respiratory epithelium tumorigenesis. *Cell Research* **2007**, *17*, 324–332.
(16) Gerritsen, M. E. Williams, A. J. Neish, A. S. Moore, S. Shi, Y.; Collins, T. CREB-binding protein/p300 are transcriptional coactivators of p65. *Proceedings of the National Academy of Sciences* **1997**, *94*, 2927-2932.
(17) Perkins, N. D. Felzien, L. K. Betts, J. C. Leung, K. Beach, D. H.; Nabel, G. T. Regulation of NF-κB by Cyclin-Dependent Kinases Associated with the p300 Coactivator. *Science* **1997**, *275*, 523-527.
(18) Henchey, L. K. Kushal, S. Dubey, R. Chapman, R. N. Olenyuk, B. Z.; Arora, P. S. Inhibition of Hypoxia Inducible Factor 1—Transcription Coactivator Interaction by a Hydrogen Bond Surrogate α-Helix. *J. Am. Chem. Soc.* **2010**, *132*, 941-943.
(19) Rowe, S. P.; Mapp, A. K. Assessing the Permissiveness of Transcriptional Activator Binding Sites. *Biopolymers* **2008**, *89*, 578-582.
(20) Scheidereit, C. IκB kinase complexes: gateways to NF-κB activation and transcription. *Oncogene* **2006**, *25*, 6685-6705.
(21) Wright, C. W.; Duckett, C. S. The Aryl Hydrocarbon NuclearTranslocator Alters CD30-MediatedNF- k B –Dependent Transcription. *Nature* **2009**, *323*, 251-256.
(22) Wright, C. W. Rumble, J. M.; Duckett, C. S. CD30 Activates Both the Canonical and Alternative NF- B Pathways in Anaplastic Large Cell Lymphoma Cells. *Journal of Biological Chemistry* **2007**, *282*, 10252-10262.
(23) Thomas, T. P. Majoros, I. Kotlyar, A. Mullen, D. Banaszak Holl, M. M.; Baker Jr,

J. R. Cationic poly (amidoamine) dendrimer induces lysosomal apoptotic pathway at therapeutically relevant concentrations. *Biomacromolecules* **2009**, *10*, 3207–3214.

(24) Parimi, S. Barnes, T. J. Callen, D. F.; Prestidge, C. A. Mechanistic insight into cell growth, internalization, and cytotoxicity of PAMAM dendrimers. *Biomacromolecules* **2009**, *11*, 382–389.

Appendix II

Applications of a Novel Synthesis of Substituted Alleneamides via a [3,3] Sigmatropic Rearrangement[a]

A. Overview

The identification of new scaffolds for small molecules that interact with transcriptional activators or coactivators is an area of ongoing research. Because of the putatively helical nature of amphipathic activation domains,[2] and the success of isoxazolidines such as II-1[3] and II-2,[4] we sought out new methods for the rapid and facile generation of small molecules that have a dense, radial display of functionality. Alleneamides serve as a precursor to many such scaffolds, however, the generation of asymmetric and densely substituted alleneamides has proven difficult.

Previous research in the Mapp lab identified a rearrangement of allylic phosphorimidates to phosphoramidates that could be used to construct allylic amines.[5,6] Building on these discoveries, we developed a route to alleneamides via the [3,3] rearrangement of phosphorimidates. The starting material for this reaction is synthesized by a one-step functionalization of propargylic alcohols, which in turn are available either

[a] Parts of this chapter are reproduced in Danowitz, A. M.; Taylor, C. E.; Shrikian, T. M.; Mapp, A. K. Palladium-Catalyzed [3, 3]-Rearrangement for the Facile Synthesis of Allenamides. *Organic Letters* **2010**, *12*, 2574–2577.

commercially or through well known reactions. This method is suitable for the generation of protected mono-, di-, or tri- substituted alleneamides, and the use of chiral propargylic alcohols allows the stereoselective synthesis of asymmetric alleneamides. Finally, the alleneamides generated using this method are unusually stable, and undergo reactions similar to those of allenes rather than electron rich alleneamides, thus providing a novel building block in the construction of complex and densely functionalized molecules.

B. Background

B.1. The Role of Alleneamides in the Discovery of New Scaffolds for the Design of Small Molecules Ligands of Transcriptional Activator Proteins

Small molecules that regulate transcription by interacting with transcriptional coactivator proteins have great promise as both biochemical tools and therapeutics. However, a very limited number of such molecules have been discovered.[7,2] The identification of additional scaffolds that could be used to design new ligands for the transcriptional machinery would facilitate development of new and potentially more effective ligands. The utility of allenes and alleneamides in the rapid and modular synthesis of complex small molecules makes them attractive building blocks for the contruction of new scaffolds.

B.2. Reactivity of Allenes and Alleneamides

Allenes themselves can act as either nucleophiles or electrophiles, with the reactivity and selectivity being heavily dependent on the nature of the substituents.[8] Alleneamides are generally more nucleophilic than allenes, owing to the electron donating character of the nitrogen (Figure A2-1). Alleneamides are known substrates for a variety of reactions leading to densley functionalized cyclic molecules such as densley functionalized pyrans,[9] oxazolidonyl substituted heterocycles,[10] and dihydrofurans.[11] A facile route for the preparation of alleneamides would therefore lend itself to rapid preparation of libraries of these compounds.

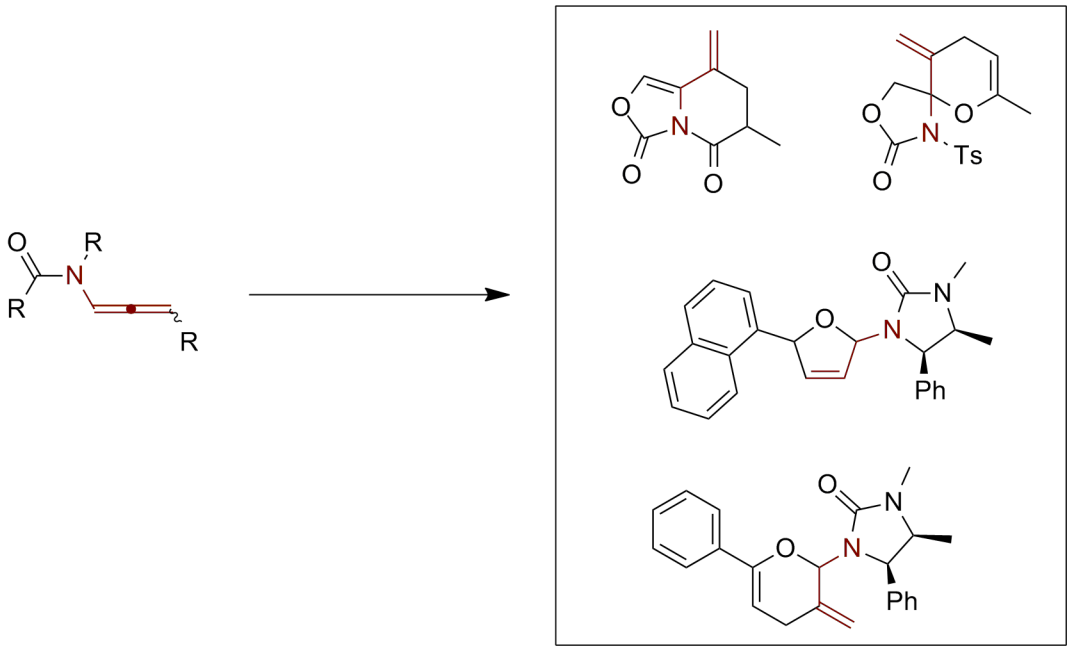


Figure A2-1. Applications for alleneamides. Alleneamide precursors can be used to generate a variety of densely functionalized heterocycles.

B.3. Previously existing routes to alleneamides

Known methods for the preparation of alleneamides have two general limitations: length, and control of stereochemistry. The iterative synthesis of allenamides is commonly performed in the literature, but the installation of each sustituent requires a seperate reaction under strongly basic conditions, and any stereocontrol must come from a chiral auxilliary.[8] Alternative routes exist that use either the copper catalyzed coupling of allenyl halides to amides,[12] or the [2,3]-rearrangement of propargylic sulfides.[13] However, in both cases the selective preparation of asymmetric alleneamides requires the use of chiral starting materials (allenyl halides or propargylic sulfides) that are difficult to prepare.

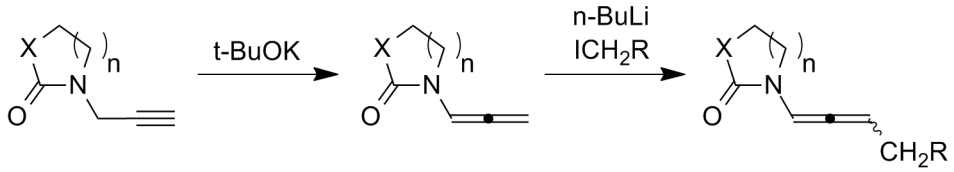


Figure A2-2. Iterative construction of allenamides. One of the existing ways to synthesize functionalized allenamides is the iterative route shown above. After base induced isomerization from a propargylic amide to an allenamide, sequential rounds of deprotonation and treatment with electrophile are used to functionalize the alleneamide.

C. [3,3] Rearrangement of Phosphorimidates

C.1. Substrate Preparation and Optimization of Reaction Conditions

The phosporimidate was generated as described in Figure A2-3 from a propargylic alcohol, a chlorophosphite, and then Staudinger reduction with an azide. Optimization of reaction conditions included screening solvents, concentrations, temperature and phosphorous ligands. The conditions which reliably produced the best yield were the use

of either Dicholormethane or Toluene at room temperature as the solvent, at a substrate concentration of 10 μM, and using diethyl phosphorochloridate as the chlorophosphite. In particular, low substrate concentration was found to be necessary, indicating the potential for unwanted intermolecular reactions.

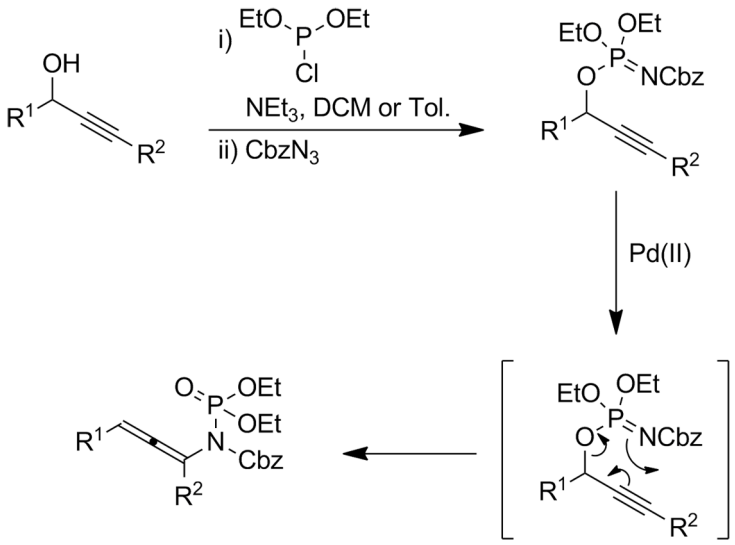


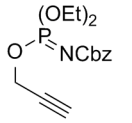
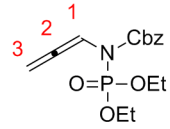
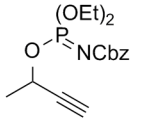
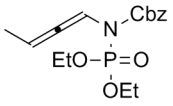
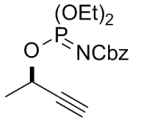
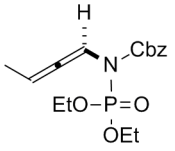
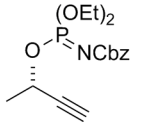
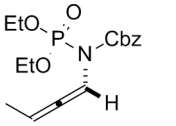
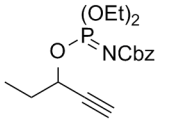
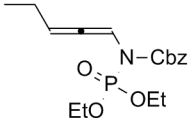
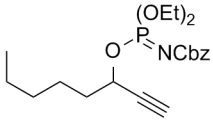
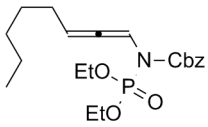
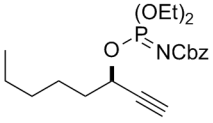
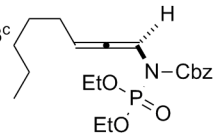
Figure A2-3. General scheme for alleneamide synthesis. The general route developed to synthesize alleneamides is shown above. In brief, a propargylic alcohol is transformed to the corresponding phosphorimidate via treatment with a chlorophosphite, followed by reduction with a protected azide. This phosphormidate undergoes rearrangement to the corresponding alleneamide (a phosphoramidate) in the presence of a palladium catalyst.

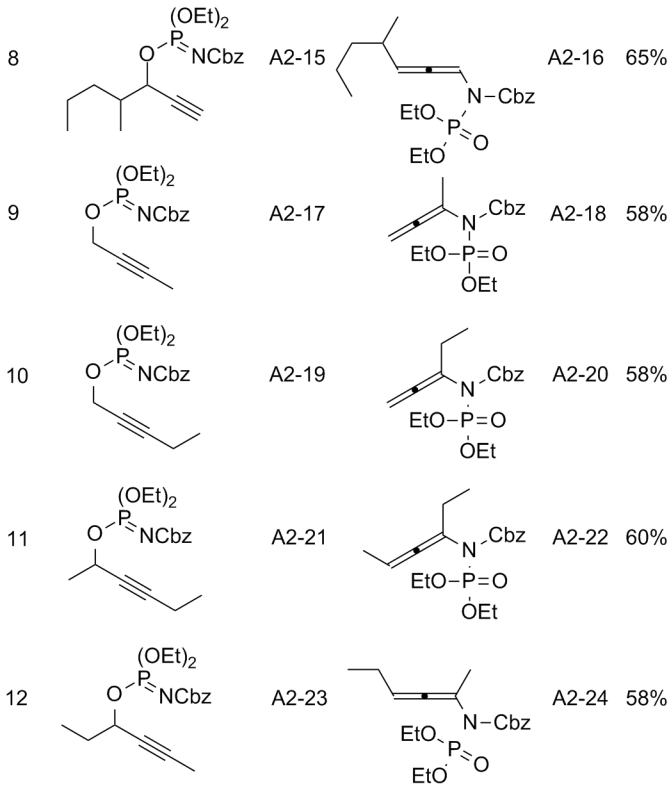
C.2. Evaluation of Reaction Scope

These conditions were subsequently used to test the scope of the reaction, as shown in Table A2-1. The reaction can be used to synthesize alleneamides which have bulky substitutents at either the 1 or 3 position, but does not provide access to 3,3-disubstituted alleneamides, as the corresponding phosphorimidates fail to form. Both branched and straight-chain alkane substituents form the desired alleneamides in

moderate to good yields.

Table A2-1. Scope of the rearrangement.

entry[a]	phosporimidate		allenamide		yield[b]
1		A2-1		A2-2	76%
2		A2-3		A2-4	68%
3		A2-5[c]		A2-6	70% 92% ee[d]
4		A2-7[c]		A2-8	67% 92% ee[d]
5		A2-9		A2-10	68%
6		A2-11		A2-12	70%
7		A2-13[c]		A2-14	70% 80% ee[d]

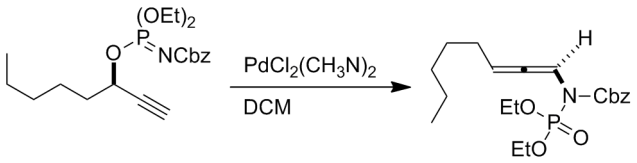


[A] Conditions as described above. See section F for additional details. [B] Isolated yields. [C] ee of commercially available starting alcohol found to be <95% in each case. [D] ee determined by chiral HPLC.

C.3. Synthesis of Asymmetric Alleneamides

Synthesis of the enantiomers A2-6 and A2-8 was achieved with good enantioselectivity (92% in both cases). In the case of the bulkier alleneamide A2-14, enantioselectivity was reduced (80% ee). This was unusual, given that a rearrangement would be expected to proceed in a highly stereoselective manner. In order the determine whether the problem was reduced enantioselectivity of the rearrangement, or subsequent

racemization of the product, the alleneamide A2-14 was re-subjected to the reaction conditions as shown in A2-4. The time dependent reduction in enantioselectivity indicates that the alleneamide undergoes racemization under the reaction conditions. This racemization was likely more pronounced with alleneamide A2-14 than A2-6 or A2-8, because of the longer reaction times needed for its formation.



A2-13 A2-14

Reaction Time (hr)	ee	% yield
5	80	60
7.5	72	70
7.5 + Additional 5 (12.5 Total)	51	80 [a]

Figure A2-4. Racemization of A2-14. This is indicated by both the apparant time dependence of racemization, and by the fact that resubjecting A2-14 to the reaction conditions causes the loss of enantiopurity. [A] Yield recovered based on the amount resubjected to the reaction conditions.

D. Applications of Alleneamides in Organic Synthesis

The usual reactivity pattern for alleneamides or allenamines is dominated by the electron donating character of the nitrogen. This is in contrast to allenes, where steric considerations tend to control reactivity. Initial attempts to use alleneamides such as A2-2 in known reactions for alleneamides or allenamines[8] resulted in decomposition, or the

recovery of starting material. Based on the presence of two electron withdrawing groups on the nitrogen, and the observed benchtop stability of these alleneamides, which contrasts with the rapid degradation that has been observed for allenamines and some other allenamides, we hypothesized that electron donation into the allenic system by the nitrogen was significantly reduced. Given this, we tested the utility of these diprotected alleneamides in reactions generally used for allenes. In addition to traditional reactions such as Diels-Alder or 2+2 cycloadditions,[14] we also wanted to determine if the allene could be used for organometallic reactions with reasonable selectivity for the less hindered olefin. Based on conditions described in the literature,[15] we were able to effect a palladium catalyzed tandem acylation/borylation to form A2-26, which is primed for further reactions.

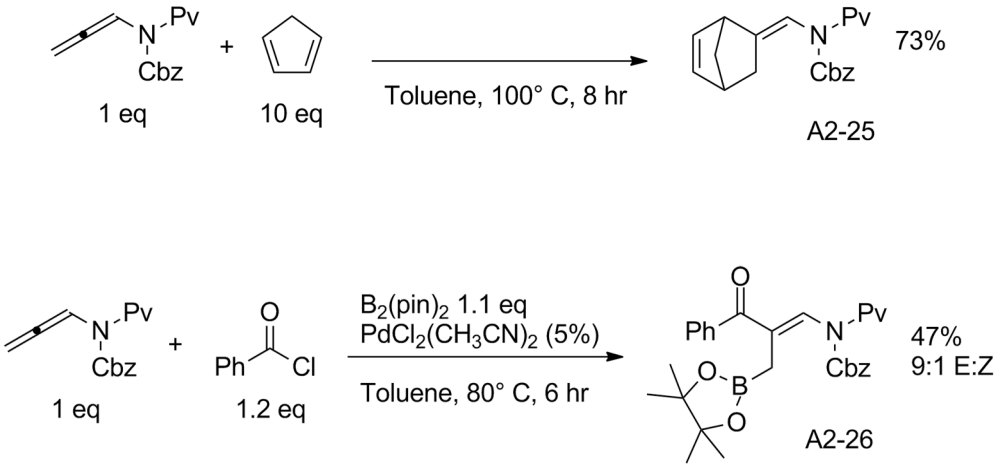


Figure A2-6. Applications of A2-1. The relatively electron-neutral character of the di-protected alleneamides allows them to be used in reaction previously unknown for alleneamides, such the one used to generate A2-26.

E. Conclusions

A novel synthesis of di-protected alleneamides from phosphorimidates was developed. This method provides several advantages over existing routes to alleneamides, including the facile synthesis of substituted alleneamides and the unusual reactivity of the products. By incorporating an additional protecting, the resulting alleneamides are more stable than those generated by other routes, and their reactivity is complementary, with reactions occuring at the distal olefin rather than the proximal olefin, as is common with other alleneamides.

Future lines of investigation include extending the functional group tolerance of this method, and developing a palladium free variation that avoids racemization of the product under the reaction conditions. Although attempts at thermal catalysis have been unsuccessful, there is evidence in the literature that gold catalysis may be a useful alternative to palladium in this case. Finally, the success of this project provides a tool for the rapid synthesis of complex molecules that may act as ligands or inhibitors for parts of the transcriptional machinery.

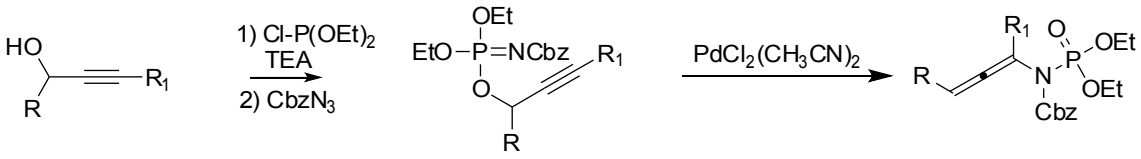
F. Supplementary Information.

Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. DCM, Et_2O, and toluene were dried by passage though activated alumina columns and degassed by stirring under a dry nitrogen

atmosphere. Purification by flash chromatography was carried out with E. Merck Silica Gel 60 (230-400 mesh) according to the procedure of Still, Kahn, and Mitra. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere. Cbz azide was prepared according to literature procedure.

3-Phenylprop-2-yn-1-ol, 3-(4-chlorophenyl)prop-2-yn-1-ol, 3-(3-methoxyphenyl)prop-2-yn-1-ol, and 3-(4-methoxyphenyl)prop-2-yn-1-ol were prepared according to literature procedures. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 400 or 500 MHz and 125 MHz. IR spectra were measured as thin films on NaCl plates.

General procedure for allenamide preparation via rearrangement:



To Et_2O (10 mL) cooled in an ice/H_2O bath was added the propargylic alcohol (1.3 mmol, 1.6 eq) and Et_3N (1.0 mmol, 1.3 eq), followed by dropwise addition of diethyl chlorophosphite (1.0 mmol, 1.3 eq). The solution was allowed to stir for 20 min and the precipitated $Et_3N{\cdot}HCl$ was removed by filtration. The Et_2O was removed under vacuum and the residue dissolved in 1.7 mL DCM. Cbz azide (0.77 mmol, 1.0 eq) was added dropwise at RT. The reaction was then allowed to stir for 2 h. The DCM was removed under vacuum and the crude mixture was subjected to flash chromatography (gradient 1:5 hexanes/EtOAc to 1:2.5 hexanes/EtOAc). The phosphorimidate decomposed upon standing and was thus used immediately upon isolation.

To a solution of DCM (20 mL) was added the phosphorimidate (0.2 mmol, 1.0 eq) and 3 mol% $PdCl_2(CH_3CN)_2$. The reaction was stirred at room temperature with monitoring by TLC (1:1 hexanes/EtOAc) for 5-7h, at which time starting material was no longer present. The DCM was then removed under vacuum and the crude mixture was *immediately* subjected to flash chromatography (1:5 hexanes/EtOAc to 1:2.5 hexanes/EtOAc to 1:1 hexanes/EtOAc) to provide the product allenamide. Note that prolonged exposure to palladium salts leads to decomposition and epimerization.

Benzyl diethoxyphosphoryl(propa-1,2-dienyl)carbamate (A2-2): Prepared via the general procedure in 76% yield (57 mg) from the corresponding phosphorimidate (82% yield) as a colorless oil. ^{1}H NMR: δ 1.27 (t, J = 6.6 Hz, 6H), 4.00-4.15 (m, 4H), 5.19 (dd, J = 3.5, 6.3 Hz, 2H), 5.22 (s, 2H), 6.33 (dd, J = 6.4, 13.3 Hz, 1H), 7.29-7.39 (m, 5H); ^{13}C NMR: δ 15.93 (d, J = 8.6 Hz), 64.19 (d, J = 7.6 Hz), 68.6, 83.6, 95.6, 135.1, 153.4, (d, J = 9.5 Hz), 206.4 (d, J = 5.8 Hz); IR (film): 2984, 1731, 1455, 1441, 1383, 1283, 1026 cm^{-1}; HRMS (ESI) calcd for $[C_{15}H_{20}NO_5P+Na]^+$: 348.0977, found: 348.0972.

Benzyl buta-1,2-dienyl(diethoxyphosphoryl)carbamate (A2-4): Prepared via the general procedure in 68% yield (44 mg) from the corresponding phosphorimidate (71% yield) as a colorless oil. ^{1}H NMR: δ 1.29 (t, J = 7.5 Hz, 6H), 1.72 (dd, J = 2.5, 7.2 Hz, 3H), 4.08-4.22 (m, 4H), 5.25 (s, 2H), 5.57-5.60 (m, 1H), 6.22- 6.24 (m, 1H), 7.33-7.42 (m, 5H); ^{13}C NMR δ: 14.3 (d, J = 2.0 Hz), 16.0 (d, J = 3.4 Hz), 64.1 (d, J = 3.4 Hz), 64.1 (d, J = 3.4), 68.5, 94.6 (d, J = 3.4 Hz), 94.9, 128.2, 128.4, 128.5, 135.3, 153.7 (d, J = 9.6

Hz), 202.1 (d, J = 5.4 Hz); IR (film): 2984, 1728, 1456, 1381, 1276, 1024 cm^{-1}; HRMS (ESI) calcd for $[C_{16}H_{22}NO_5P+Na]^+$: 362.1133, found: 362.1126.

(S)-Benzyl buta-1,2-dien-1-yl(diethoxyphosphoryl)carbamate (A2-6): Prepared via the general procedure in 70% yield from the corresponding phosphorimidate (56% yield) as a colorless oil. ^1H and ^{13}C data identical to that outlined for compound **4** $[\alpha]_D^{23}$= -53.7 (c = 1, CH_2Cl_2) . 92% ee (Chiral HPLC Chiralcel OJ column, flow rate 1 mL/min, 0.4 % hexanes in IPA, detected at 240 nm. R_t = 16.63 (maj), 19.58 (min), see supplementary HPLC traces.

(R)-Benzyl buta-1,2-dien-1-yl(diethoxyphosphoryl)carbamate (A2-8): Prepared via the general procedure in 67% yield from the corresponding phosphorimidate (72% yield) as a colorless oil. ^1H and ^{13}C data identical to that outlined for compound **4**. 92% ee (Chiral HPLC Chiralcel OJ column, flow rate 1 mL/min, 0.4 % hexanes in IPA, detected at 240 nm. R_t = 16.30 (min), 18.44 (maj), see supplementary HPLC traces.

Benzyl diethoxyphosphoryl(penta-1,2-dienyl)carbamate (A2-10): Prepared via the general procedure in 68% yield (54 mg) from the corresponding phosphorimidate (25% yield) as a pale yellow oil. ^1H NMR: δ 1.01 (t, J = 7.0 Hz, 3H), 1.27 (t, J = 7.0 Hz, 6H), 2.03-2.10 (m, 2H), 4.04-4.21 (m, 4H), 5.22 (s, 2H), 5.59-5.65 (m, 1H), 6.22-6.26 (m, 2H), 7.30-7.41 (m, 5H); ^{13}C NMR: δ 12.9, 16.0 (d, J = 6.9 Hz), 22.2 (d, J = 1.5 Hz), 64.0 (d, J = 6.1 Hz), 68.4, 95.5 (d, J = 2.3 Hz), 101.6, 128.1, 128.3, 128.4, 135.3, 153.7 (d, J

= 7.7 Hz), 201.0 (d, *J* = 4.6 Hz); IR (film): 2967, 1728, 1380, 1278 1024 cm^{-1}; HRMS (ESI) calcd for $[C_{17}H_{24}NO_5P+Na]^+$: 376.1290, found: 376.1281.

Benzyl diethoxyphosphoryl(octa-1,2-dien-1-yl)carbamate (A2-12): Prepared via the general procedure in 70% yield (55 mg) from the corresponding phosphorimidate (67% yield) as a pale yellow oil. ^1H NMR: δ 0.86 (t, *J* = 7.2 Hz, 3H), 1.22-1.30 (m, 10H), 1.40 (m, 2H), 2.04 (m, 2 H), 4.02-4.21 (m, 4H), 5.21 (s, 1H), 5.53-5.59 (m, 1H), 6.18-6.22 (m, 1H), 7.30-7.40 (m, 5H); ^{13}C NMR: δ 14.0, 16.0 (d, *J* = 8.8 Hz), 22.4, 28.4, 28.8 (d, *J* = 1.3 Hz), 31.3, 64.1 (d, *J* = 7.3 Hz), 68.4, 95.0 (d, *J* = 3.4 Hz), 100.1, 128.1, 128.3, 128.5, 135.3, 153.7 (d, *J* = 10.1 Hz), 201.3 (d, *J* = 5.8 Hz); IR (film): 2957, 1732, 1456, 1380, 1277, 1026 cm^{-1}; HRMS (ESI) calcd for $[C_{20}H_{24}NO_5P+Na]^+$: 418.1759, found: 418.1755.

(S)-Benzyl diethoxyphosphoryl(octa-1,2-dien-1-yl)carbamate (A2-14): Prepared via the general procedure in 70% yield (44 mg) from the corresponding phosphorimidate (81% yield) as a pale yellow oil. ^1H and ^{13}C data identical to that outlined for compound **A2-12**.

$[\alpha]_D^{23}$= -50.9 (c = 1 CH_2Cl_2). 80% ee (Chiral HPLC Chiralcel OJ column, flow rate 1 mL/min, 0.3% hexanes in IPA, detected at 254 nm. R_t = 10.97 (maj), 12.68 (min). The yield increased with extended reaction times but epimerization was observed. See supplementary HPLC traces.

Benzyl diethoxyphosphoryl(4-methylhepta-1,2-dienyl)carbamate (A2-16): Prepared

via the general procedure, with the exception that the rearrangement took 12 h for depletion of starting material, in 65% yield (51 mg) from the corresponding phosphorimidate (77% yield) as a pale yellow oil. The reported product is a mixture of 2 diastereomers derived from the 2 diastereomers present as a 1:1 mixture in the starting alcohol. ^{1}H NMR: δ 0.86 (t, *J* = 6.8 Hz, 3H), 1.00 (d, *J* = 6.8 Hz, 3H) 1.22-1.36 (m, 8H), 2.19-2.28 (m, 1H), 4.05-4.21 (m, 4H), 5.22 (s, 1H), 5.48-5.55 (m, 1H), 6.22 (dt, *J* = 2.6, 6.2 Hz, 1H), 7.30-7.41 (m, 5H); ^{13}C NMR: δ 14.0, 14.1, 15.9, 16.0, 19.8, 19.9, 20.2, 33.5 (d, *J* = 1.4 Hz), 33.7, 38.3, 38.9, 64.0 (d, *J* = 6.3 Hz), 64.0 (d, *J* = 4.8 Hz), 68.4, 95.5 (d, *J* = 3.3 Hz), 95.6 (d, *J* = 3.4 Hz) 105.7, 105.8, 128.1, 128.2, 128.4, 135.3, 153.7 (d, *J* = 10.0 Hz), 200.4 (d, *J* = 4.6 Hz); IR (film): 2958, 1731, 1456, 1379, 1275, 1026 cm^{-1}; HRMS (ESI) calcd for $[C_{20}H_{24}NO_5P+Na]^+$: 418.1759, found: 418.1749.

Benzyl buta-2,3-dien-2-yl(diethoxyphosphoryl)carbamate (A2-18): Prepared as stated, with the exception that the rearrangement was carried out in toluene at 100 °C for 12h on a 0.6 mmol scale (0.23 g phosphorimidate), in 58% yield (125 mg) from the corresponding phosphorimidate (75% yield) as a pale yellow oil. ^{1}H NMR: δ 1.28 (dt, *J* = 0.8, 7.0, 6H), 1.28 (t, *J* = 7.0 3H), 1.99 (dt, *J* = 1.2, 3.1 Hz 3H), 4.04-4.22 (m, 4H), 5.00-5.04 (m, 2H), 5.22 (s, 2H), 7.32-7.40 (m, 5H); ^{13}C NMR: δ 16.0 (d, *J* = 6.9 Hz), 19.7, 64.0 (d, *J* = 5.4 Hz), 68.2, 80.0 (d, *J* = 2.3 Hz), 105.0, 127.9, 128.2, 128.5, 135.5, 153.7 (d, *J* = 9.2 Hz), 208.4 (d, *J* = 4.7 Hz); IR (film): 2983, 1728, 1497, 1455, 1380, 1271, 1024 cm^{-1};
HRMS (ESI) calcd for $[C_{16}H_{22}NO_5P+Na]^+$: 362.1133, found: 362.1121.

Benzyl diethoxyphosphoryl(penta-1,2-dien-3-yl)carbamate (A2-20): Prepared via the general procedure, with the exception that the rearrangement was carried out in toluene at 100°C, in 58% yield (32.5 mg) from the corresponding phosphorimidate (75% yield) as a colorless oil. ^{1}H NMR: δ 0.99 (t, *J* = 7.6 Hz, 3H), 1.25 (dt, *J* = 0.8, 6.8 Hz, 6H), 2.18-2.28 (m, 2H) 4.00-4.18 (m, 4H), 5.08-5.11 (m, 2H), 5.18 (s, 2H), 7.30-7.40 (m, 5H); ^{13}C NMR: δ 11.0, 16.0 (d, *J*= 8.8 Hz), 25.7, 64.0 (d, *J*= 7.3 Hz), 68.2, 82.1 (d, *J*= 2.4 Hz), 111.0, 127.8, 128.2, 128.4, 135.5, 153.9 (d, *J*= 11.6 Hz), 207.7 (d, *J*= 4.9 Hz); IR (film): 2977, 1727, 1456, 1380, 1278, 1025 cm^{-1}; HRMS (ESI) calcd for $[C_{17}H_{24}NO_5P+Na]^+$: 376.129, found: 376.1277.

Benzyl diethoxyphosphoryl(hexa-3,4-dien-3-yl)carbamate (A2-22): Prepared via the general procedure, with the exception that the rearrangement was carried out in toluene at 100°C on a 0.1 mmol scale (41 mg phosphorimidate), in 60% yield (25 mg) from the corresponding phosphorimidate (48% yield) as a yellow oil. ^{1}H NMR: δ 0.98 (t, *J* = 7.4 Hz, 3H), 1.28 (dt, *J* = 0.8, 7.2 Hz, 6H), 1.69 (d, *J* = 5.4 Hz, 3H), 2.21-2.27 (m, 2H), 4.03-4.21 (m, 4H), 5.37 (dd, *J* = 12.5, 19.5 Hz, 2H), 5.46-5.52 (m, 1H), 7.30-7.38 (m, 5H); ^{13}C NMR: δ 11.1, 14.1 (d, *J* = 2.9 Hz), 16.0 (d, *J* = 7.6 Hz), 26.2, 63.8 (d, *J* = 3.8 Hz), 63.9 (d, *J* = 3.9 Hz), 68.0, 93.4 (d, *J* = 2.9 Hz), 110.0, 127.8, 128.1, 128.4, 135.6, 154.0, 203.0 (d, *J* = 4.9 Hz); IR (film): 2978, 1726, 1456, 1378, 1272, 1024 cm^{-1}; HRMS (ESI) calcd for $[C_{18}H_{26}NO_5P+Na]^+$: 390.1446, found: 390.1444.

Benzyl diethoxyphosphoryl(hexa-2,3-dien-2-yl)carbamate (A2-24): Prepared via the

general procedure, with the exception that the rearrangement was carried out in toluene at 100°C, in 58% yield (42.5 mg) from the corresponding phosphorimidate (50% yield) as a yellow oil. ^{1}H NMR: δ 0.99 (t, *J* = 7.6 Hz, 3H), 1.27 (t, *J* = 7.2 Hz, 6H), 1.97 (dd, *J*= 1.2, 2.8 Hz, 3H), 2.03 (dq, *J*= 2.4, 8.6 Hz, 2H), 4.04-4.19 (m, 4H), 5.20 (s, 2H), 5.40-5.48 (m, 1H), 7.30-7.40 (m, 5H); ^{13}C NMR: δ 13.1, 16.0 (d *J* = 9.1 Hz), 20.3, 22.1 (d, *J* = 3.3 Hz), 63.5 (d, *J*= 5.1 Hz), 68.1, 98.2 (d, *J*= 2.4 Hz), 105.0 (d, *J* = 1.0 Hz), 128.0, 128.2, 128.4, 135.5, 153.9 (d, *J* = 11.9 Hz), 202.7 (d, *J* = 5.3 Hz); IR (film): 2980, 1728, 1456, 1380, 1277, 1026cm^{-1}; HRMS (ESI) calcd for $[C_{18}H_{26}NO_5P+Na]^+$: 390.1446, found: 390.1445.

(Z)-benzyl bicyclo[2.2.1]hept-5-en-2-ylidenemethyl(diethoxyphosphoryl)carbamate (A2-25)

Allenamide A2-2 (70 mg, 0.22 mmol) was combined with freshly cracked cyclopentadiene (0.18 mL, 2.2 mmol) in toluene (0.4 mL) under N_2. The reaction was sealed and heated to 100º C for 8 hours, after which the toluene was removed under reduced pressure and the crude reaction mixture was purified by flash chromatography (25-50% ethyl acetate in hexanes) to yield **23** as a clear oil (63 mg, 0.16 mmol, 73%). Configuration determined by 1D NOESY. ^{1}H NMR: δ 1.24 (dt, J = 0.4, 6.8 Hz, 3H), 1.25 (dt, J = 0.8, 6.8 Hz, 3H), 1.41 (d, J = 8.4 Hz, 1H), 1.58 (d, J = 10 Hz,1H), 1.71*1.75 (m, 1H), 2.16*2.20 (m, 1H), 2.92 (s, 1H), 3.27 (s, 1H), 4.00*4.16 (m, 4H), 5.12*5.17 (m, 2H), 5.95 (d, J = 2.5 Hz, 1H), 5.99*6.00 (m, 1H), 6.12*6.13 (m, 1H), 7.26*7.32 (m, 5H); ^{13}C NMR: δ 16.03 (d, J = 2.3 Hz), 16.10 (d, J = 2.7), 31.93, 41.42, 48.36, 50.36,

64.02*64.13 (m), 68.08, 114.1, 127.9, 128.0, 128.2, 128.4, 133.3 (d, J = 1.9), 135.5, 137.7, 146.8 (d, J = 4.67 Hz), 154.1 (d, J = 10 Hz), IR (film): 3000, 1731, 1455, 1441, 1383, 1283, 1026 cm*1; HRMS (ESI) calcd for [C20H26NO5P+Na]+: 414.1446, found: 414.1444.

(E)-benzyl diethoxyphosphoryl(3-oxo-3-phenyl-2-((4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)methyl)prop-1-enyl)carbamate (A2-26)

To allenamide A2-2 (35 mg, 0.107 mmol) was added bis(pinacolato)diboron (31 mg, 0.12mmol) and Bis(acetonitrile)dichloropalladium(II) (1.4 mg, 0.0055 mmol), and a stir bar. The flask was evacuated and purged with nitrogen 3 times, followed by the addition of Toluene (0.44 mL) and benzoyl chloride (distilled from Calcium Chloride, 15 μL, 0.13 mmol). The reaction was heated to 80º C under nitrogen for 6 hrs after which the solvent was removed under reduced pressure and the crude reaction was purified by flash chromatography (25-50% ethyl acetate in hexanes) to give 24 as a clear oil (28 mg, 0.050 mmol, 47%). Configuration of major isomer determined by 1D NOESY. ^{1}H NMR: δ 1.18 (s, 9H), 1.24 (dt, J = 1, 6 Hz, 6H), 1.97 (s, 2H), 4.06*4.20 (m, 4H), 5.21 (s, 2H), 6.51*6.52 (m, 1H), 6.58 (m, 1H, Z isomer), 7.30*7.39 (m, 7H), 7.44*7.48 (m, 2H), 7.71*7.73 (m, 2H), 8.04*8.06 (m, 2H, Z isomer). ^{13}C NMR: δ 13.48 (d, J = 6.7 Hz), 16.77, 22.21, 62.08 (d, J = 6.23 Hz), 66.26, 80.86, 125.5, 125.7, 125.9, 126.0, 126.0, 127.2, 127.6, 129.4, 131.8, 132.5, 135.5, 137.6 (d, J = 4.8 Hz), 150.3 (d, J = 8.2 Hz), 194.8, IR (film): 2923, 1717, 1685, 1279, 1208, 1142, 1026 cm*1; HRMS (ESI) calcd for [C28H37BNO8P+Na]+: 580.2248, found: 580.2260.

G. References

(1) Danowitz, A. M. Taylor, C. E. Shrikian, T. M.; Mapp, A. K. Palladium-Catalyzed [3, 3]-Rearrangement for the Facile Synthesis of Allenamides. *Organic Letters* **2010**, *12*, 2574–2577.

(2) Lee, L. W.; Mapp, A. K. Transcriptional Switches: Chemical Approaches to Gene Regulation. *Journal of Biological Chemistry* **2010**, *285*, 11033-11038.

(3) Rowe, S. P. Casey, R. J. Brennan, B. B. Buhrlage, S. J.; Mapp, A. K. Transcriptional Up-regulation in Cells Mediated by a Small Molecule. *J. Am. Chem. Soc.* **2007**, *129*, 10654-10655.

(4) Lee, L. W. Taylor, C. E. C. Desaulniers, J. P. Zhang, M. Hojfeldt, J. W.; Mapp, A. K. Inhibition of ErbB2(Her2) expression with small molecule transcriptionfactor mimics. *Bioorganic & medicinal chemistry letters* **2009**, *19*, 6233-6236.

(5) Chen, B.; Anna, K. A phosphorimidate rearrangement for the facile and selective preparation of allylic amines. *Journal of the American Chemical Society* **2004**, *126*, 5364–5365.

(6) Chen, B.; Anna, K. Thermal and catalyzed [3, 3]-phosphorimidate rearrangements. *Journal of the American Chemical Society* **2005**, *127*, 6712–6718.

(7) Mapp, A. K.; Ansari, A. Z. A TAD Further: Exogenous Control of Gene Activation. *ACS Chem. Biol.* **2007**, *2*, 62-75.

(8) Wei, L.-li; Xiong, H.; Hsung, R. P. The Emergence of Allenamides in Organic Synthesis. *Acc. Chem. Res.* **2003**, *36*, 773-782.

(9) Wei, L.-L. Hsung, R. P. Xiong, H. Mulder, J. A.; Nkansah, N. T. First Stereoselective Inverse Demand [4 + 2] Cycloaddition Reactions of Novel Chiral Allenamides with Heterodienes. Preparation of Highly Functionalized 2-Arylpyranyl Heterocycles. *Org. Lett.* **1999**, *1*, 2145-2148.

(10) Kimura, M. Wakamiya, Y. Horino, Y.; Tamaru, Y. Efficient Synthesis of 4-Ethenylidene-2-oxazolidinones via Palladium- Catalyzed Aminocyclization of 2-Butyn-l,4-diol Biscarbamates. *Tetrahedron Letters* **1997**, *38*, 3963-3966.

(11) Berry, C. R. Hsung, R. P. Antoline, J. E. Petersen, M. E. Challeppan, R.; Nielson, J. A. Syntheses of 2,5-Disubstituted Dihydrofurans from γ-Substituted Chiral Allenamides. *J. Org. Chem.* **2005**, *70*, 4038-4042.

(12) Trost, B. M.; Stiles, D. T. Synthesis of Allenamides by Copper-Catalyzed Coupling of Allenyl Halides with Amides, Carbamates, and Ureas. *Org. Lett.* **2005**, *7*, 2117-2120.

(13) Yin, G. Zhu, Y. Zhang, L. Lu, P.; Wang, Y. Preparation of Allenephosphoramide and Its Utility in the Preparation of 4,9-Dihydro-2 *H* -benzo[*f*]isoindoles. *Org. Lett.* **2011**, *13*, 940-943.

(14) Kimura, M. Horino, Y. Wakamiya, Y. Okajima, T.; Tamaru, Y. Pronounced Chemo-, Regio-, and Stereoselective [2+ 2] Cycloaddition Reaction of Allenes toward Alkenes and Alkynes. *Journal of the American Chemical Society* **1997**, *119*, 10869–10870.

(15) Yang, F.-Y. Wu, M.-Y.; Cheng, C.-H. Highly Regio- and Stereoselective Acylboration of Allenes Catalyzed by Palladium Complexes: An Efficient Route to a New Class of 2-Acylallylboronates. *J. Am. Chem. Soc.* **2000**, *122*, 7122-7123.