

Design and Characterization of Bifunctional Glucocorticoid Ligands Capable of
Producing Novel Transcriptional Profiles

by

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Dedication

For Jim and Paddy

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Abstract

The process of transcription underlies the expression of all gene profiles. Normal expression is caused by carefully coordinating the assembly of transcriptional proteins at specific genes, a procedure mediated through transcription factors that bind specific sequences of DNA. The glucocorticoid receptor (GR) is a transcription factor that influences the expression of genes involved in inflammation pathways. Drug-like molecules targeting GR are capable of stimulating GR to bind DNA; however, these molecules are unable to exert complete control over the members of the transcriptional complex recruited by GR. As such, we are limited in our control of GR activity. My dissertation focuses on addressing this with new GR ligands designed to recruit specific transcriptional proteins to produce novel, desired expression profiles.

We first conjugated a GR molecule to a ligand of the protein FKBP. This bifunctional ligand was capable of localizing GR to DNA, recruiting FKBP-fusions to the GR-regulated gene, and producing transcriptional activity dictated by the recruitment. This was achieved with the recruitment of both a transcriptionally activating protein and a repressing protein, demonstrating the adaptability of this system to toggle the output of a gene of interest.

The design of our system benefits from its inherent modularity; expanding to new targets is simply achieved through synthetic conjugation to an alternative ligand. In a first application of this strategy, we conjugated an agonistic GR ligand to a selective inhibitor of the transcriptional protein BRD4. Recruitment of BRD4 to GR resulted in the suppression of transcription at select genes, and this selectivity drives a novel profile of activated and suppressed genes. In a second application, we conjugated a GR antagonist to the BRD4 inhibitor, allowing for

the further production of novel transcriptional profiles with potential pharmacological utility.

This dissertation also includes a study aimed at introducing undergraduate students to the scientific principles of modern chemical biology research. To enhance student learning, we have developed a new guided-inquiry experimental module for biochemistry laboratory courses. This has been well received, and assessment metrics indicate that the incorporation into the University of Michigan's biochemistry course has raised student cognitive abilities in analysis and application.

Chapter 1

Small molecule regulation of protein complex assembly

1.1 Abstract

Cellular growth and maintenance are accomplished by multiprotein complexes assembled via protein-protein interactions. Often these are dynamic assemblies, with the formation of protein-protein interactions governed by post-translational modifications and ligand-induced allosteric changes. Anomalous formation and abnormal interactions within such multiprotein complexes is implicated in countless disease states, including several cancers and neurological disorders. There is thus significant effort aimed at identifying small molecules that could regulate the assembly of complexes such as the transcriptional machinery. A particular challenge in that regard has been the discovery of molecules that induce the assembly of specific protein complexes. The focus of this introductory chapter is to outline the challenges associated with developing small molecule activators of protein complex assembly, with a particular focus on transcription. Successful examples, including therapeutic applications, are also described.

1.2 Introduction

The assembly of multiprotein complexes is an elaborate process that plays a key role in numerous physiological activities^{1,2}. Proteasomal degradation, gene transcription, protein synthesis, and chaperone-mediated folding, for example, are tasks accomplished by multi-protein machines that are dynamically assembled via protein-protein interactions (PPIs) (represented in Fig. 1.1). The

number of distinct, binary PPIs in a cell has been estimated at $\sim 130,000^3$, and thus PPIs represent an attractive target for modulating the assembly and, thus, function of multiprotein complexes. While many efforts are currently being undertaken to develop small molecules that disrupt PPIs, there have been far fewer devoted to the chemical induction of PPIs. Here, several natural and engineered systems are described wherein PPIs are produced in response to or created through the use of small molecules.

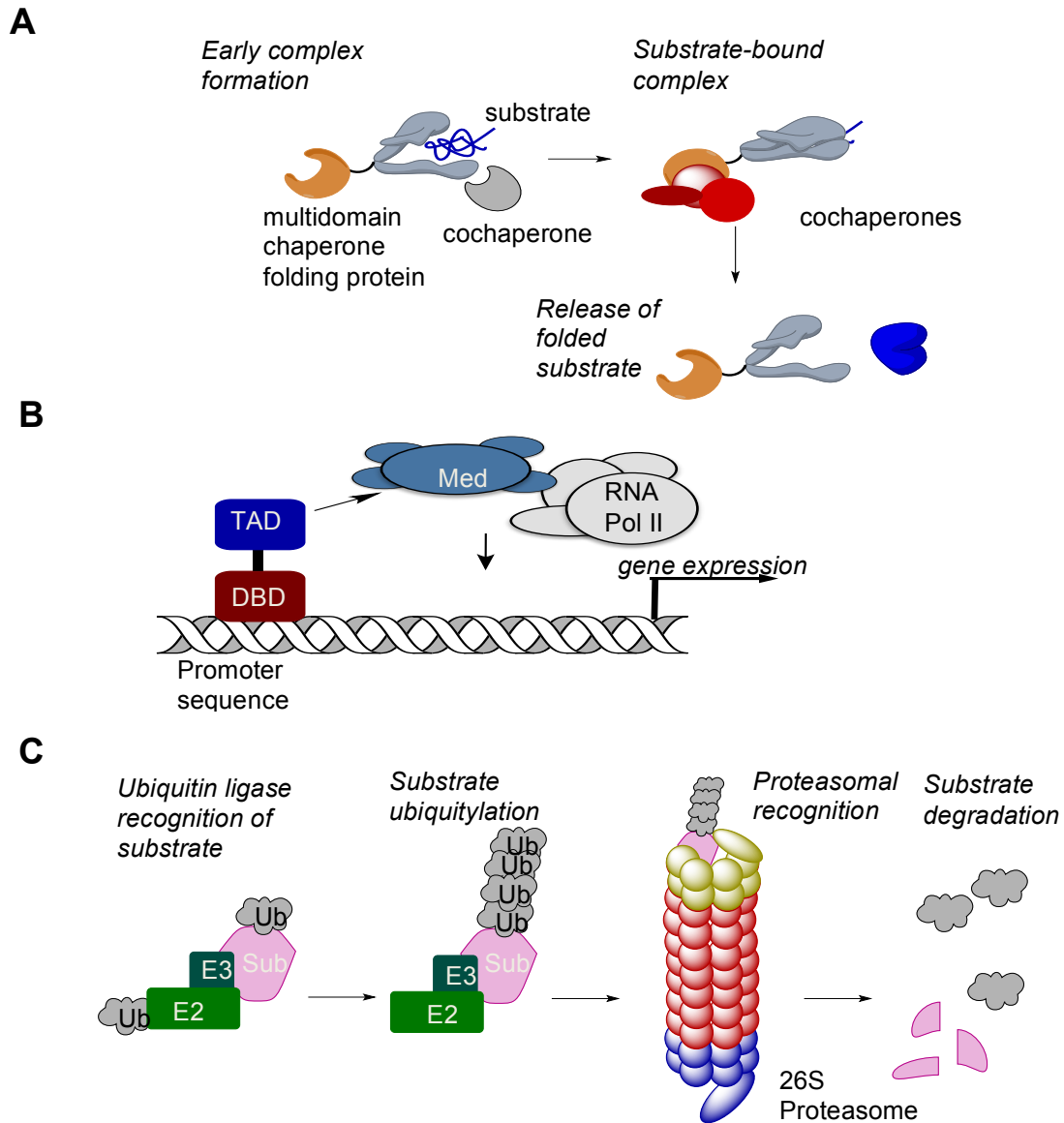


Figure 1.1 Biological processes are driven by multiprotein complexation. **A:** The function of many proteins is dependent on proper folding through chaperone complexes. Chaperone proteins, in concert with various cochaperone partners, facilitate the proper folding of many substrates. **B:** Transcriptional regulation is mediated through extensive protein-protein interactions. Transcription factors, recognizing target DNA sequences through a DNA-binding domain (DBD), use a transcriptional activation domain (TAD) to recruit members of the transcriptional machinery, such as the Mediator complex (Med) and RNA polymerases, to activate the expression of genes. **C:** Proteasomal degradation is carried out through interactions of many proteins. Ubiquitin ligases recognize degradation targets and facilitate the enzymatic attachment of ubiquityl groups to the substrate. Recognizing polyubiquityl signals, the proteasome facilitates the degradation of the substrate target.

1.3 Naturally occurring molecular modulators of protein interactions

Allosteric PPI modulators

In eukaryotic systems, protein complexation often occurs as a result of allosteric ligand binding, such as in GPCR signaling⁴ or chaperone-mediated protein folding⁵. Allosteric induction of protein complexation enjoys many advantages relative to stimuli relying on enzymatic activity; for example, this allows protein partners to dictate interactions not through highly conserved active sites but instead through alternative binding sites, providing a mechanism for promiscuously binding proteins to form specific interactions in a context-dependent manner⁴. Though many allosteric processes rely on protein ligand-induced activities, hormonal signaling and gene regulation are notable for being particularly dependent on small molecules for the construction of correct protein complexes.

For example, in the case of the nuclear receptors (NRs), activity is largely dependent on small molecule hormonal ligands^{6,7}. Members of the NR family are transcription factors that effect changes in gene expression through the nucleation of the transcriptional machinery at specific genes. Ligand binding triggers an allosteric shift in the NR, allowing for DNA binding and subsequent recruitment of several coregulator proteins to form a transcriptional complex that modulates gene transcription. Proper NR action is contingent on being able to correctly interact with specific coregulators out of potential partners numbering in the hundreds^{8,9}. Aberrant NR interactions with coregulators has been implicated in the pathogenesis of several diseases, spurring numerous pharmacological efforts to produce synthetic hormones that produce desired transcriptional profiles by inducing 'correct' transcriptional complexes¹⁰.

An important target of this strategy is the glucocorticoid receptor (GR), owing to its role in important processes including inflammation and metabolism¹¹. Similar to other NRs, GR is an allosterically regulated transcription factor dependent on the binding of endogenous corticosteroids for stimulated activity. When bound to the promoter of a GR-regulated gene, the receptor adopts a conformation capable of making specific contacts with a host of potential coregulators^{9,12}, each capable of subsequent recruitment of transcriptional machinery. A complex transmittance of signals through GR, including properties of the bound ligand and promoter sequence, influence the specific conformation adopted and the resulting identity of recruited coregulators. Through this, a potentially promiscuous coregulator-binding interface¹³ is shaped to allow for only distinct complex formation and resultant transcriptional activity.

Plant hormonal signaling has been less characterized than its mammalian counterpart, but recent discoveries have revealed that allostery plays a role in the transcriptional activity of many plant hormones, though in a fashion significantly different than for their mammalian counterparts. In the case of gibberellin hormones, Ueguchi-Tanaka and co-workers¹⁴ determined that the interaction of these molecules with their recently isolated receptor, GAD1¹⁵, results in transcriptional modulation, but not through the formation of transcriptional complexes. Unlike in NR models, the gibberellin-GAD1 interaction does not cause DNA localization. Rather, gibberellins cause a conformational shift within GAD1 that triggers interaction with the constitutively active transcriptional repressor SLR1. The formation of this complex instigates ubiquitylation of SLR1, resulting in the indirect upregulation of gibberellin-induced genes¹⁴.

PPI stabilizers

An alternative mechanism of small molecule PPI modulation occurs in scenarios wherein ligands act to stabilize transient PPIs. Inositol tetraphosphate (IP₄) has

been known to play a key role in chromatin remodeling, but until recently the mechanism of action has been unclear¹⁶. Watson and coworkers¹⁷ identified IP₄ in a co-crystal structure of the histone deacetylase HDAC3 and the corepressor SMRT. IP₄ acts as an 'intermolecular glue,' neutralizing electrostatic repulsions between several basic residues in the HDAC3-SMRT interaction cleft.

Naturally occurring polyamines such as spermine, spermidine, and putrescine have also been shown to act as PPI stabilizers. Investigating the interactions between mitochondrial cytochrome P45011A1 and two electron transport partners, adrenodoxin (Adr) and adrenodoxin reductase (Adx), Berwanger and co-workers¹⁸ demonstrated a significant increase in association rate and binding strength between Adr and Adx in response to polyamine. Specific negatively charged residues in the Adr-Adx binding interface were identified as potential polyamine interaction points through docking studies and confirmed in subsequent mutagenesis experiments.

Plant and fungal metabolites have shown to be a source of cytotoxics that act by stabilizing PPIs in non-native systems. Forskolin, a diterpene first isolated from *Coleus Forskohlii*¹⁹, has been used in studies as a potent activator of adenylyl cyclase (AC), raising intracellular cyclic AMP levels²⁰. Upon obtaining a crystal structure of the AC catalytic site, Zhang and coworkers²¹ identified a solvent-exposed hydrophobic cavity as the forskolin binding site, allowing for a more energetically favorable dimerization and activation of AC, triggering the potentially cytotoxic event. A similar story unfolded as researchers probed the mechanism of action of brefeldin A, a fungal macrolactone commonly used to study intracellular protein transport²². Biochemical²³ and structural studies²⁴ revealed that brefeldin A stabilizes the interaction between members of the ARF family of G proteins and the associated exchange factors (GEFs). The ARF-brefeldin A-GEF complex is functionally compromised, triggering the movement of Golgi-associated membrane proteins to the endoplasmic reticulum.

Novel PPI inducers

A number of natural products cause an extreme version of PPI stabilization, inducing the dimerization between two non-interacting proteins by first binding a target and then presenting a molecular surface to bind a second target. These are termed chemical inducers of dimerization (CIDs). Cyclosporine A (CsA) is a non-ribosomal cyclic peptide that has been used as an immunosuppressant for decades²⁵. CsA effects this immunomodulatory action through the inhibition of calcineurin, a phosphatase involved in T cell activation²⁶. Interestingly, CsA does not directly inhibit the calcineurin active site but instead binds a distal surface – inhibition occurs because CsA simultaneously binds cyclophilin A and recruitment of this second target render the calcineurin catalytic domain inaccessible^{27,28}.

Rapamycin²⁹ and FK506 (tacrolimus)^{30,31} are two fungal macrolactones that act as immunosuppressants through similar mechanisms of action as CsA^{27,28,32}. Owing to a highly similar scaffold (Fig. 1.2), each binds the prolyl isomerase FKBP12 with extremely high affinity (FK506 K_d : 0.4 nM; rapamycin K_d : 0.2 nM)³³. As with CsA, each inhibits a secondary target in addition to FKBP12. Despite limited structural resemblance between the two, FK506 likewise binds several of the same residues in calcineurin²⁸. This FK506-induced interaction prevents calcineurin enzymatic activity³⁴. Rapamycin, however, does not affect calcineurin activity – mechanistic studies have revealed that it targets the kinase mTOR, an alternative target in immunomodulatory signaling (Figure 1.2). Free energy binding analysis revealed that the recruitment of FKBP to mTOR is entirely directed through protein-rapamycin interactions, with very limited interactions occurring directly between the two proteins³⁵. The ability of these two metabolites to induce the heterodimerization of distinct targets bears high utility, though limitations concerning off-target interactions necessitated further refinement for nuanced applications.

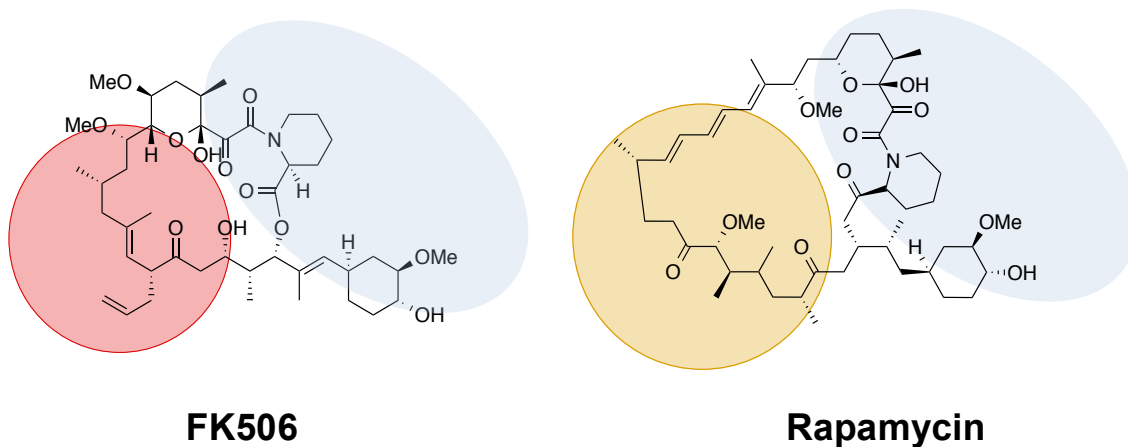


Figure 1.2 Illustrating the similarities and differences between PPI inducers. FK506 and rapamycin share a common, highly similar FKBP-binding domain (blue), while differing in other portions of the scaffold. This is highlighted calcineurin-binding portion of FK506 (red) and the mTOR-binding portion of rapamycin (orange).

1.4 Engineering systems for chemically-inducing protein interactions

Homodimerizing Molecules

Following the elucidation of the mechanisms of action for FK506 and rapamycin, collaborators in Gerald Crabtree's and Stuart Schreiber's laboratories worked to develop a system wherein separate proteins could be localized through a dimerizing synthetic ligand. By connecting two molecules of FK506 through a hydrocarbon linker, they produced a dimeric "bifunctional" molecule, termed FK1012³⁶. In the first example of the utility of this strategy, Spencer and co-workers³⁶ stimulated cell surface receptor signaling in T-cells by expressing the receptors as FKBP-fusion proteins and treating with FK1012; the induced proximity of the receptors acted as a surrogate for natural dimerization. In designing FK1012, the point of conjugation was chosen to be at a key contact with calcineurin, interfering with its recruitment to the FKBP-FK1012 complex and lowering associated toxicity.

Given the ease of expressing chimeric FKBP-fusion proteins in cellular systems, this development spurred the investigation into the role of protein localization in many cellular processes; this has been described in several reviews³⁷⁻⁴². Despite its broad utility, a limitation to this system is that FK1012 interacts with endogenous FKBP proteins, interfering with signaling. Researchers at Ariad Gene Therapeutics employed a two-pronged approach to this issue, developing a number of alternative synthetic ligands for FKBP⁴³ and engineering mutated forms of the protein with unique binding profiles^{44,45}.

An alternative method for avoiding undesirable protein-ligand interactions, such as FK1012 with endogenous FKBP, is to express non-native dimerization targets in cellular systems. Many high affinity inhibitors of bacterial proteins have been described, and several have been cleverly repurposed as dimerizers. A particularly attractive example involves the aminocoumarin family of antibiotics. Investigations with this class of natural products revealed an inhibition of *E. coli* DNA gyrase B activity (gyrB)^{46,47}. Importantly, researchers in the Maxwell laboratory discovered that coumermycin, a member of the family bearing a natural symmetry (Fig. 1.3), is capable of binding two molecules of the gyrB N-terminal domain, inducing dimer formation^{48,49}. Though coumermycin does not bind gyrB with the same high affinity as FK506 for its target (K_d : ~ 24 nM), Farrar and coworkers⁵⁰ were able to utilize its ability to dimerize gyrB in an engineered signal transduction system, activating the MAP kinase cascade through the coumermycin-mediated dimerization of Raf1 kinase-gyrB fusion proteins.

An additional limitation of FK506-based homodimerization systems is the non-trivial nature of FK1012 synthesis and modification. Kopytek and coworkers⁵¹ recognized the potential for a methotrexate-based dimerization system given its high affinity for dihydrofolate reductase (DHFR) (K_d : 8.5 nM)⁵². A bifunctional version of methotrexate, termed BisMTX, was synthetically accessible in three steps and, somewhat unexpectedly, promoted a cooperative binding event

between DHFR monomers, effecting a higher rate of complex formation than predicted⁵¹.

Heterodimerizing Molecules

The systems described heretofore provide the capability for inducing protein self-association. However, these methods are ill equipped to localize dissimilar proteins. For example, in a system where proteins A and B have been expressed as FKBP-fusions, the introduction of FK1012 would yield a heterogeneous population of A–A, A–B, and B–B dimers. The identification of a small, 90 amino acid sub-domain capable of binding the FKBP-rapamycin complex⁵³ aided the design of a system wherein rapamycin is capable of inducing gene transcription, allowing for a fusion moiety less intrusive than full length mTOR. Rapamycin potently triggered the dimerization of a DNA-binding domain fused to FKBP and a transcriptional activation domain-FRB chimera, stimulating the expression of a target gene in both cellular and in vivo mice studies⁵⁴. The rapamycin-induced co-localization of alternative targets has been greatly expanded and has been reviewed recently⁵⁵.

Building off of the success of FK1012 (and in need of alternatives to the potentially toxic rapamycin), additional work on developing heterodimerizing systems focused on producing new bifunctional molecules that bear divergent chemical moieties. Following the establishment of CsA as an alternative to FK506 for building a bifunctional molecule⁵⁶, a synthetic molecule linking FK506 to CsA was constructed, termed FKCsA⁵⁷. Employing appropriate fusion proteins, Belshaw and coworkers were able to use FKCsA to induce a multitude of cellular processes, including protein localization and transcriptional activity.

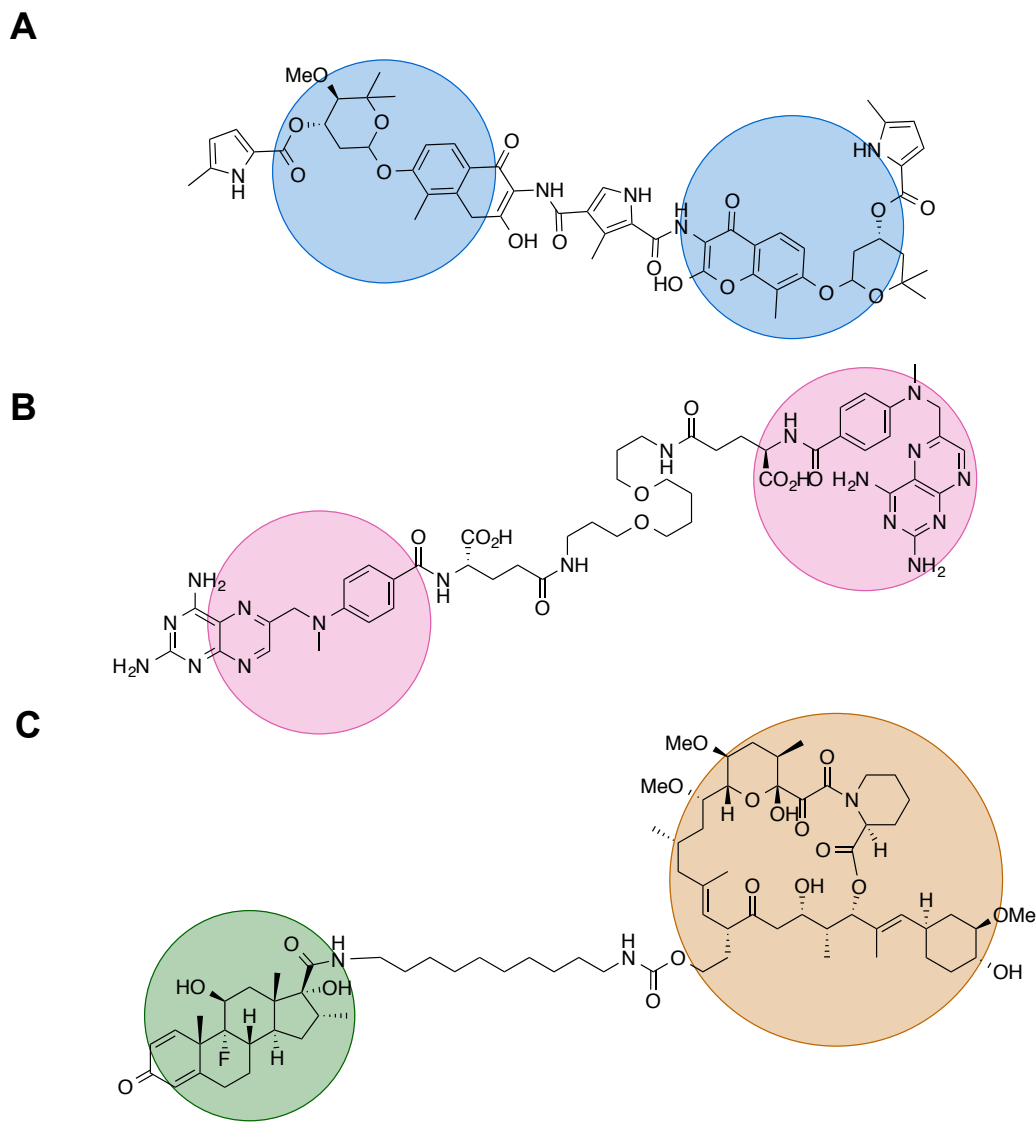


Figure 1.3 Chemical homo- and heterodimerizers. A: Coumermycin contains two DNA gyrase B-binding moieties (blue). The symmetry of this molecule allows for simultaneous binding of two gyrase molecules, causing effective dimerization^{45,46}. B: The designed molecule Bis-MTX⁴⁸ takes advantage of methotrexate's (pink) potent binding of dihydrofolate reductase (DHFR) to localize two reductase molecules, causing the effective dimerization of proteins fused to DHFR. C: An asymmetrical chemical dimerizer allows for heterodimerization. Linking dexamethasone (green) to FK506 (orange) produced Dex-FK506⁷², allowing for the cellular localization of glucocorticoid receptor ligand-binding domain (LBD) to FKBP, causing the functional effects of dimerizing any potential fusion partners of these proteins.

1.5 Refining new systems for chemical dimerization

Expanding available heterodimerizers

The utility of the dimerizing systems described stimulated further investigations into refinement and discovery of more selective and effective dimerizers (several depicted in Fig. 1.3). The use of natural product-based dimerizers is restricted by synthetic barriers, including high cost and difficulty of manipulation, and limited bioorthogonality, evidenced by the shared FK506/rapamycin target. Looking to address these issues, researchers in the Crabtree laboratory have recently identified a plant phytohormone as a new inducer of dimerization⁵⁸. (S)Abscisic acid (ABA) targets the pyrabactin resistance (PYR)/PYR1-like (PYL)/regulatory component of ABA receptor (RCAR) and protein phosphatase type 2Cs (PP2Cs), members of a signaling pathway not present in mammalian systems^{59,60}. Liang and coworkers⁵⁸ identified minimal ABA-binding domains of RCAR and PP2Cs for protein fusion and demonstrated ABA-induced gene transcription, protein localization, and signal transduction. Compared to an analogous rapamycin-induced system, ABA-mediated activity was more readily reversed through the removal of compound, and higher doses of ABA were also typically required to produce signal.

Researchers in the Inoue lab^{61,62} similarly looked to plant biology for inspiration in crafting new dimerization systems. Taking advantage of gibberellin-induced interaction of GAD1 with SLR1^{14,15}, they discovered that acetoxymethylated gibberellin (GA₃-AM) is cell permeable and capable of rapidly inducing the interaction of GAD1 and SLR1 expressed in mammalian cells. More germane to this discussion, a series of systematic truncations yielded uncovered minimal domains that maintained similar dimerization efficiency. These researchers further demonstrated that the GA₃-AM system could be used simultaneously and orthogonally to a rapamycin-inducible system, allowing for the construction of a simple and rapid “genetic” cellular logic gate^{61,62}.

Irreversible Heterodimerizer Interactions

Reversible ligand binding, as has been described above, presents certain challenges in the design of heterodimerizing molecules. Disparate ligand-binding kinetics between the constituent members of a bifunctional molecule can make dimerization efficiency challenging to predict or measure^{63,64}, and certain cellular signals can be difficult to detect when induced by transient dimerization. Attempts to address these issues have centered on developing tool proteins that covalently link to their targets, providing a method for irreversible induction of a PPI.

Members of the Johansson laboratory pioneered early work in this field. Initially seeking to provide a method for covalent fluorophore labeling of proteins, they identified the human DNA repair protein O⁶-alkylguanine-DNA alkyltransferase (hAGT) as a potential tool for controlled covalent bond formation between a unique ligand and a designed protein⁶⁵. hAGT readily recognizes O⁶-benzylguanine derivatives and forms a covalent linkage with the benzylic carbon; as a result, hAGT is labeled with any chemical moiety linked to the substrate. Subsequent directed evolution of hAGT produced a mutant, commercially marketed as the 'SNAP-tag,' that displays a 50-fold enhanced activity relative to wild type hAGT⁶⁶. Homodimers of the hAGT substrate were produced that demonstrated the ability of this technology to allow for extended, 'permanent' induced dimerization of target proteins⁶⁷, and a further extension of this technology displayed the ability to modulate gene transcription through covalent dimerization of transcription factor domains⁶⁸. Mimicking the efforts of previous researchers, members of Covalys biosciences quickly moved to develop orthogonal labeling systems that can be used in combination with the SNAP-tag. Termed the 'CLIP-tag,' they engineered a SNAP-tag mutant that recognizes and covalently links to O²-benzylcytosine derivatives. Notably, the CLIP-tag displayed a higher substrate turnover than the SNAP-tag, and the two systems were

successfully used in tandem for fluorophore protein-labeling⁶⁹ and selective heterodimerization⁷⁰.

This methodology was even further expanded with development of the HaloTag, a modified dehalogenase enzyme that recognizes chloroalkane moieties⁷¹. Though this fusion tag is larger in size than the SNAP- or CLIP-tags, with a higher potential for interfering with normal protein function through its attachment, its use has displayed lowered toxicity and minimized interactions with off-target proteins, in addition to requiring a synthetically trivial chemical tag. Demonstrating its use as an orthogonal tool to the SNAP-tag, Chidley and coworkers⁷² showed that the tandem use of bifunctional molecules bearing either the Halo or SNAP substrate could be used to localize target proteins to a SNAP-Halo fusion protein. Members of the Wymann laboratory⁷³ have designed and developed a series of bifunctional molecules that bear both substrates, termed HaXS molecules, allowing for an alternative method of protein heterodimerization through the expression of two fusions and introduction of a single HaXS. Recognizing the potential utility of covalent dimerizers with induced reversible activity, they developed a HaXS that incorporates a nitroveratryl group in its linker (MeNV-HaXS). While retaining a potent ability for protein heterodimerization, MeNV-HaXS is quickly degraded in the presence of near-UV light, allowing for a rapid reversal of induced protein localization⁷⁴.

1.6 Heterodimerizing molecules as tools

Three-hybrid Studies

One of the most powerful and widely used applications of chemical heterodimerizing systems is the three-hybrid assay. Using the two-hybrid assay for detecting protein-protein interactions as a model, in its initial application Licitra and Liu⁷⁵ took advantage of the modular nature of transcription factors to build a model wherein a DNA-binding domain (DBD) and transcriptional activation

domain (TAD) are linked through a heterobifunctional molecule (a FK506-glucocorticoid conjugate). Successful chemical heterodimerization of a DBD and TAD results in the transcription of a reporter gene. The application of this technology has been widespread and has been reviewed extensively^{37,41,76,77}.

Protein Localization

Inducing protein localization is the focal point of the majority of induced dimerization systems. From this standpoint, the possibilities have been versatile and well described^{5,41,42}. However, the systems described heretofore rely on the utilization of genetically fusing protein tags to localization targets. Recent work by Ishida and coworkers has focused on developing 'self-localizing' bifunctional molecules that recruit target molecules to specific cellular locations⁷⁸. In preliminary experiments, DHFR was successfully localized to microtubules, the Golgi, and the nucleus, by synthetically conjugating the DHFR-inhibitor trimethoprim to various localizing moieties. This strategy is limited to protein and localization targets with defined ligands, but with recent efforts to unearth molecular modulators of non-enzymatic targets, this methodology is promising for future work on chemically induced protein localization.

Temporal Control Over Enzymatic Activity

The emergence of split-protein systems as reliable tools for detecting PPIs⁷⁹ has led to a surge in the number of available models for enzyme reassembly. This has opened up opportunities for using chemically induced dimerization for the induction of enzymatic activity. Building off of a similar idea originally pioneered in the Muir laboratory^{80,81}, Camacho-Soto and coworkers⁸² have recently developed dimerizer-inducible kinases and phosphatases, relying on rapamycin, ABA, or gibberellic acid to localize split enzyme-fusion proteins and trigger activity. Their initial efforts suffer from a reliance on high levels of dimerizing molecule and a lack of control over enzymatic substrate specificity, but the tools

they have initially described allows an orthogonal and (subsequently temporal) control over cellular phosphorylation activity that is promising.

1.7. Therapeutic utility of dimerizing molecules

Chemical biologists develop nuanced and intriguing tool compounds to aid investigations of biological systems. However, it is important to situate this work in the perspective that the ultimate goal of these efforts is towards the advancement of therapeutics. The necessity for engineered systems, often utilizing large protein tags, precludes much of this work from advancing outside of tool compound research. Despite this, several methodologies and targets appear particularly amenable to using dimerizing molecules as potential tools in therapeutic development.

Inhibiting protein aggregation

Transthyretin, a thyroxine transporter, has been implicated in the pathogenesis of several amyloidoses⁸³⁻⁸⁵. Monomeric dissociation and aggregation of this normally homotetrameric protein is increased in the presence of destabilizing point mutations, with several having demonstrated linkages to clinical pathologies^{84,86,87}. Working to develop a molecular stabilizer of homotetrameric transthyretin, researchers in Jeffrey Kelly's laboratory discovered Tafamidis, a benzoxazole that decreases transthyretin aggregation and amyloid formation. Further studies yielded a crystal structure, detailing how Tafamidis stabilizes the oligomeric species by interacting with two thyroxine-binding cavities⁸⁸. Tafamidis has recently been approved in Europe and Japan for use in the treatment of familial amyloid neuropathy.

Targeted Antibody Recruitment

Triggering selective antibody recruitment to specific cells would be a useful method for instigating a native immune system to eliminate tumorigenic species. Exploring this idea, members of the Kiessling laboratory⁸⁹ established an initial platform for using bifunctional molecules to induce the localization of antibodies to specific cells. Linking a cell surface receptor ligand to a trisaccharide, they demonstrated a recruitment of an antibody recognizing the sugar epitope that was selective for cells displaying the receptor. O'Reilly and coworkers⁹⁰ were able to utilize a similar strategy to target a B-cell surface receptor, CD22, that had proven notoriously difficult to target with small molecule ligands. However, the bifunctional ligand-mediated recruitment of antibodies allowed a weak ligand to overcome this through an avidity-driven assembly of antibody-CD22 complexes. Parker and coworkers⁹¹ used a similar small molecule epitope linked to molecule targeting HIV glycoprotein 20 (gp120). Here they demonstrated that binding of gp120 by the small molecule allowed subsequent antibody recruitment and cytotoxicity to human cells expressing gp120, and have recently demonstrated encouraging success in developing a platform of small molecules capable of triggering antibody recruitment⁹².

Protein Quality Control

A popular alternative to inhibiting a malignant protein's activity is to instigate its degradation. Collaborators in the Crews and Deshaies laboratories have pioneered the use of small bifunctional molecules to target specific proteins for degradations. Reviewed recently⁹³, their approach has centered on the ability to localize ubiquitin E3 ligases to targeted proteins, relying on proximity accompanied by this ternary complex to induce ubiquitylation of the target and subsequent degradation. These molecules, termed proteolysis targeting chimeras (PROTACs), initially utilized short peptidic sequences to signal ligase recruitment⁹⁴ but have recently included a Nutlin-based moiety capable of targeting the E3 ligase MDM2⁹⁵. Attempting to circumvent the poor efficacy of Nutlin-based PROTACs, researchers in the Hashimoto laboratory^{96,97} have

developed an alternative system targeting the E3 ligase cIAP1; however, this system similarly suffers from poor potency. Members of the Crews laboratory have also uncovered an alternative method of selective protein degradation, termed “hydrophobic tagging”⁹⁸. Here, the covalent linkage of an adamantyl group to a HaloTag molecule led to degradation of the protein, though the mechanism of this response is still unclear. Further examinations should reveal if this strategy can be applied to non-covalent systems, utilizing native ligands modified with an adamantyl moiety.

Controlling transcriptional assembly

As stated in section 1.3, nuclear receptors have been well characterized as targets for molecule-induced complex formation⁹⁹. Influenced not just by ligand-binding^{7,10} but also post-translational modifications^{100,101}, promoter sequence and location¹⁰²⁻¹⁰⁴, and cellular coregulator levels^{9,12,105}, NR activity is the result of processing these complex signals and building defined transcriptional machinery. However, the identity of complex members at gene targets is difficult to predict and is often still a mystery^{106,107}. Misregulation of complex assembly is a driver in the pathogenesis of many diseases^{10,11,108} and the importance of developing methods for directed complex assembly is of the utmost importance. However, it is becoming increasingly clear that dynamic control of complex formation is a significant undertaking. Recently, several research groups have begun to break from the paradigm of using NR ligands to target the LBD to effect wanted transcriptional outcomes. These efforts have largely focused on modulating and inhibiting the interaction between NRs and coregulators, either through recruited steric hindrance¹⁰⁹ or through targeting a conserved NR-coregulator interface¹¹⁰⁻¹¹⁸. An unprobed strategy for NR activity modulation is the designed *assembly* of transcriptional machinery by using small molecules to localize specific coregulators to specific NRs. In lieu of inducing desired PPIs, properly designed bifunctional molecules act as PPI surrogates, circumventing allosterically driven interactions in favor of designed, predictable interactions, permitting rationally

provoked transcriptional profiles. The advancement of chemical heterodimerization methods makes this strategy particularly attractive in light of the recent expansion of the chemical ‘toolbox’ of probes targeting epigenetic proteins¹¹⁹⁻¹²¹. With this knowledge, the rational design of selective bifunctional NR ligands will permit a more controlled assembly of transcriptional machinery at NR-regulated genes, providing a method to greatly expand our ability to produce precise transcriptional profiles.

1.8 Research goals

The primary focus of work outlined in this dissertation is to test the idea of using bifunctional ligands as modulators of NR activity in an endogenous context. The first goal in the establishment of this strategy is to validate that bifunctional steroidal molecules can recruit specific proteins to a DNA-bound receptor. In Chapter 2, I discuss the use of several target proteins and evaluate the functional effects of their recruitment. The second goal of this work lies in its application of this strategy; chiefly, I aim to use designed molecules to produce novel, predicted transcriptional outputs dependent on recruitment, and this is the topic of Chapter 3. A final goal of my graduate work has developed outside of the research laboratory, concerning undergraduate biochemistry education at the University of Michigan. In Chapter 4, efforts to rationally redesign portions of this course’s syllabus through the incorporation of guided-inquiry, and the subsequent results thereof, will be discussed.

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Chapter 2

Using Bifunctional Molecules to Recruit Specific Proteins to a DNA-Bound Glucocorticoid Receptor*

2.1 Abstract

Nuclear receptors are ligand-induced transcription factors. This family, including the sub-class of steroid receptors, responds to specific small molecule hormonal stimuli through nuclear translocation and the modulation of regulated-genes. Transcriptional outcome is effected through allostery, where ligand shape and binding affinity influences receptor conformation and subsequent interaction with transcriptional coregulators. Traditional synthetic ligands have sought to take advantage of this by inducing specific conformations to produce desired transcriptional evidence. However, recent discoveries have shed light on the limitations of this approach, including the necessarily constrained suite of conformations accessible by the receptor. We have shown that bifunctional molecules, aiming to bind both a nuclear receptor and a non-native interaction partner, are capable of acting as protein-protein interaction surrogates, localizing each target and producing the functional consequence of interaction. This

* This work represents a collaborative effort, portions of which have been published: Højfeldt, J.W.; Cruz-Rodriguez, O.; Van Dyke, A.R.; Imaeda, Y.; Carolan, J.P.; Mapp, A.K.; Iñiguez-Lluhí, J. *Mol. Endocrinol.* **2014**, *28*, 249-259. Compounds described in Figure 2.5 were synthesized J. Carolan, J. Højfeldt, Y. Imaeda, and A. Van Dyke. Data in Figures 2.6, 2.9, and 2.11 were obtained by O. Cruz-Rodriguez and J. Iñiguez-Lluhí. Data in Figures 2.7 and 2.10 were collected with O. Cruz-Rodriguez. Compounds described in Figure 2.12 were synthesized by A. Van Dyke and J. Qi. Data in Figure 2.13 were collected by A. Van Dyke. All other experiments were performed by J. Carolan. Where indicated, statistical significances are represented as *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

strategy has been applied to the glucocorticoid receptor and the results are described here.

2.2 Nuclear receptors are ligand-induced transcription factors

Nuclear receptors (NRs) are a large class of ligand-dependent transcription factors typically containing a ligand-binding domain (LBD) and a separate DNA-binding domain (DBD) capable of recognizing short, often palindromic recognition sequences¹. Driving the actions of a receptor is a ligand-binding event, wherein a NR will typically respond by undergoing a conformational shift and nuclear localization. The receptor influences the transcription of a gene by inducing the formation of a coregulatory complex at the recognition sequences^{2,3}. Integrating directions from DNA sequences⁴⁻⁶, signaling cascades⁷, and the bound ligand, the identity of the members in the transcriptional complex determines subsequent up- or down-regulation of a gene⁸ (Fig. 2.1A).

NRs play a critical role in the signaling pathways of countless physiological processes, including embryonic development, maintaining homeostasis, and metabolism⁹. Unsurprisingly, dysregulation of NRs is associated with a variety of disease¹⁰ and, thus, this protein family has been the focus of numerous drug discovery efforts. Currently, NR-targeting synthetic ligands comprise 10-15% of marketed pharmaceuticals¹¹.

The canonical mechanism of NR action involves the ligand-induced rearrangement of a key α -helix in the LBD, helix 12¹²⁻¹⁴. It has been believed that the orientation of this dynamic surface plays a key role in determining coregulator interactions. As such, traditional efforts to develop NR molecular probes and drugs have centered on the construction of ligands designed to provoke specific conformational shifts within the LBD as a means of gaining greater control over produced transcriptional profiles¹⁵. Current therapeutics that accomplish this are often limited by undesirable side effects or the development of resistance.

Recent efforts have often focused on opening pathways to alternative mechanisms of transcriptional control¹⁶, such as in the development of selective estrogen receptor modulators (SERMs) that rely on differential receptor isoform expression for selectivity^{17,18}. An alternative, non-canonical method of modulating NR action has centered on developing protein-protein interaction (PPI) inhibitors, identifying the NR-coregulator interface as a potential point of interference. This interaction site has been extensively studied in several NRs, such as the estrogen receptor (ER)¹⁹ and thyroid receptor (TR)²⁰, and elegantly designed peptide probes with high affinity and selectivity for these interactions *in vitro* have been described, termed coactivator-binding inhibitors²¹⁻²⁴ (Fig. 2.1B). However, several thorough attempts to develop small molecule probes²⁵ targeting this interaction have been fraught with the typical difficulties of small molecule inhibition of PPIs, namely selectivity and potency²⁶, alongside the inability to target proteins interaction at other positions.

Accounting for this additional information, it is unsurprising that recently designed ligands have been unable to elicit the nuanced transcriptional outcomes desired by their creators – though desired PPIs at one site are produced, it is difficult to predict or control for PPIs formed outside of the allosterically rearranged LBD. Acknowledging this, an attractive method of NR control would be through the use of ligands capable of recruiting specific coregulators. Designed properly, a ‘bifunctional’ molecule linking a NR molecule to a coregulator ligand could serve as a ‘PPI surrogate,’ bridging the two targets and inducing the functional outcomes of association; accordingly, this may serve as an opportunity to unlock unique transcriptional profiles (Fig. 2.2).

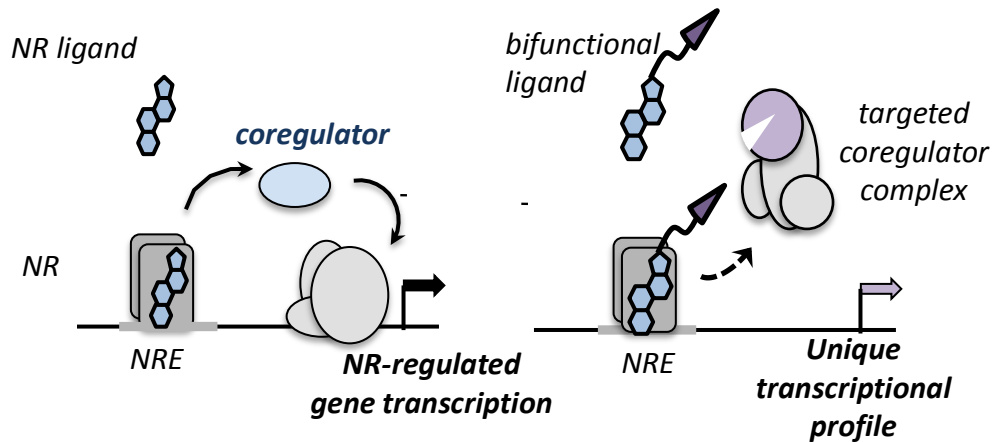


Figure 2.2 Bifunctional NR ligands. Where canonical NR ligands rely on allostery to direct coregulator recruitment and direct transcriptional activity, a bifunctional NR ligand is capable of extrinsic, directed recruitment, producing unique transcriptional profiles.

Here, we investigate the ability of bifunctional NR ligands to recruit specific proteins and the function consequences thereof, utilizing the glucocorticoid receptor (GR) as a representative of this family of transcription factors.

2.3 The glucocorticoid receptor as a target for transcriptional modulation

The glucocorticoid receptor (GR) is a widely expressed transcription factor, and a prototype of the steroid receptor (SR) sub-class of the nuclear receptor family. Members of this family share a modular structure composed of DNA-binding domain (DBD), N-terminal activation domain (AF1), and a C-terminal ligand-binding domain (LBD) bearing an additional, independently-acting activation domain (AF2)²⁹. As with other NRs, GR action is primarily facilitated through the binding of its endogenous agonist, cortisol, a steroidal ligand produced in the adrenal cortex. Cortisol binding by the GR LBD triggers the disassembly of a GR-chaperone complex, allowing for nuclear localization of GR. Primary GR transcriptional activity occurs as the nuclear-bound GR homodimerizes and binds to semi-palindromic, fifteen bp DNA sequences termed glucocorticoid response elements (GREs)⁶. Despite a loose conservation in both GRE sequence and location relative to a modulated gene, this interaction often leads to an increase in gene transcript levels, termed transactivation, though to varying magnitudes³⁰⁻³².

Activated GR can also act to repress the transcription of other genes through several mechanisms. One such mechanism involves GR binding to non-GRE DNA sequences; here, GR interferes by adopting a conformation that favors corepressor recruitment, or, alternatively, by simply interfering with DNA-binding by other transcription factors. A related mechanism is implicated in GR action as a mediator in anti-inflammatory signaling, where ligand-bound GR is believed to physically interact and interfere with the pro-inflammatory transcription factors AP-1³³ and NF- κ B^{34,35}. The use of therapeutic glucocorticoids as anti-inflammatories takes advantage of this GR activity; however, transactivation has been linked to the numerous side effects associated with glucocorticoid use³⁶. While the GR LBD has been utilized as a successful tool for conducting three-hybrid assays³⁷, the use of bifunctional glucocorticoids has yet to be exploited for regulation of the full length, intact receptor. Though the focus of this project is not necessarily to produce dissociated ligands capable of transrepression in the

absence of transactivation, this approach will allow for the general production of new ligands with unique transcription profiles.

2.4 Recruitment of exogenous proteins to the native glucocorticoid receptor

A primary goal of this project is to determine if the principles underlying the three-hybrid assay can be applied to the full length, native GR; chiefly, we aim to test if this approach can be used to recruit specific proteins and direct transcriptional outcomes. To this end, we set to determine if a three-hybrid assay could be constructed utilizing the native GR. While this is conceptually a small step forward, the challenges in this design are considerable. It requires the construction of a molecule that is capable of several activities: binding and localizing the GR to its GRE consensus sequences; recruiting a secondary protein to DNA-bound GR; and localizing that secondary protein in such a fashion that it directs transcriptional outcomes over competing interaction partners through the GR's AF1 and AF2. We identified previous three-hybrid experiments that have successfully linked GR LBD to the prolyl-isomerase protein FK506-binding protein (FKBP) and used these to guide the design of a bifunctional molecule³⁷⁻³⁹.

Synthetic conjugation of a GR- and FKBP-ligand

Dexamethasone (Dex) is a synthetic GR agonist designed to be significantly more potent and efficacious than the natural GR ligand, hydrocortisone⁴⁰, and has been used in the construction of many GR-ligand conjugates⁴¹⁻⁴⁴. Extensive mapping of the Dex structure-activity relationship has identified C₂₁ as an attachment point least perturbed by modification. Though amide-linkage at this point yields the commonly used OxDex compound; however, work surfacing at the time of this synthesis indicated that a thioether linkage at this same point maintains partial agonistic activity and full efficacy of Dex^{43,45}. We prepared a

version of this compound, termed SDex, containing an isothiocyanate moiety, SDex-ITC. This functional group allows for a facile reaction with nucleophilic linkers (Fig. 2.3A).

As described in Chapter 1, FK506⁴⁶ is a high-affinity (0.6 nM) inhibitor of the prolyl-isomerase protein FKBP^{47,48}. FK506 inhibition of FKBP is mediated through the induced dimerization of FKBP with calcineurin, abrogating its ability to interact with substrate proteins or other interaction partners⁴⁹. The first chemical inducer of dimerization, described by Spencer and coworkers, was a homodimer of FK506⁵⁰. In this work, the exocyclic allyl substituent was functionalized with a nucleophilic N-hydroxysuccinimidyl carbonate group. Importantly, this point of modification does not appreciably interfere with the FK506-FKBP interaction, though binding to calcineurin is abrogated. We utilized this synthetic strategy to produce a functionalized version of FK506, termed FK506-NHS (Fig. 2.3C).

Linker length, hydrophobicity, and rigidity all affect a bifunctional molecule's ability to chemically dimerize its targets. Though much work has been performed to expand the collective knowledge in this area, it is difficult to determine *a priori* the composition of an ideal linker⁵¹. Polyethylene glycol (PEG) linkers are particularly attractive because of their flexibility and the added solubility imparted by this style of linker. Through a series of optimization experiments, we identified a 10-atom PEG-based linker as ideal for these three-hybrid experiments⁵² and prepared it for reaction with SDex-ITC as described (Fig. 2.3B).

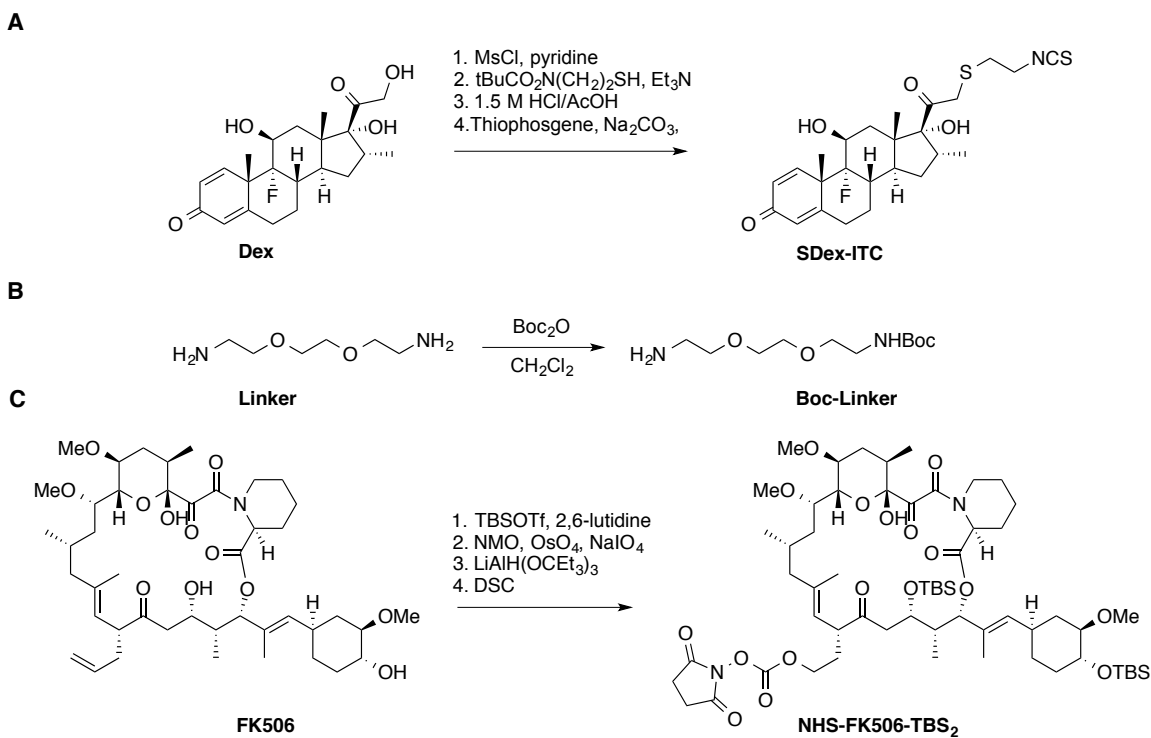


Figure 2.3 Functionalization of constituent ligands. Commercially available dexamethasone (A) and FK506 (C) are functionalized to allow for facile conjugation to a commercially available PEG-based linker (B).

The functionalized glucocorticoid agonist, SDex-ITC, was conjugated to the protected linker compound, Boc-linker, through a simple nucleophilic addition. Following cleavage of the *tert*-butyloxycarbonyl group, mixture with FK506-NHS yielded the reacted conjugate molecule, termed SDex-O2-FK506 (Fig. 2.4).

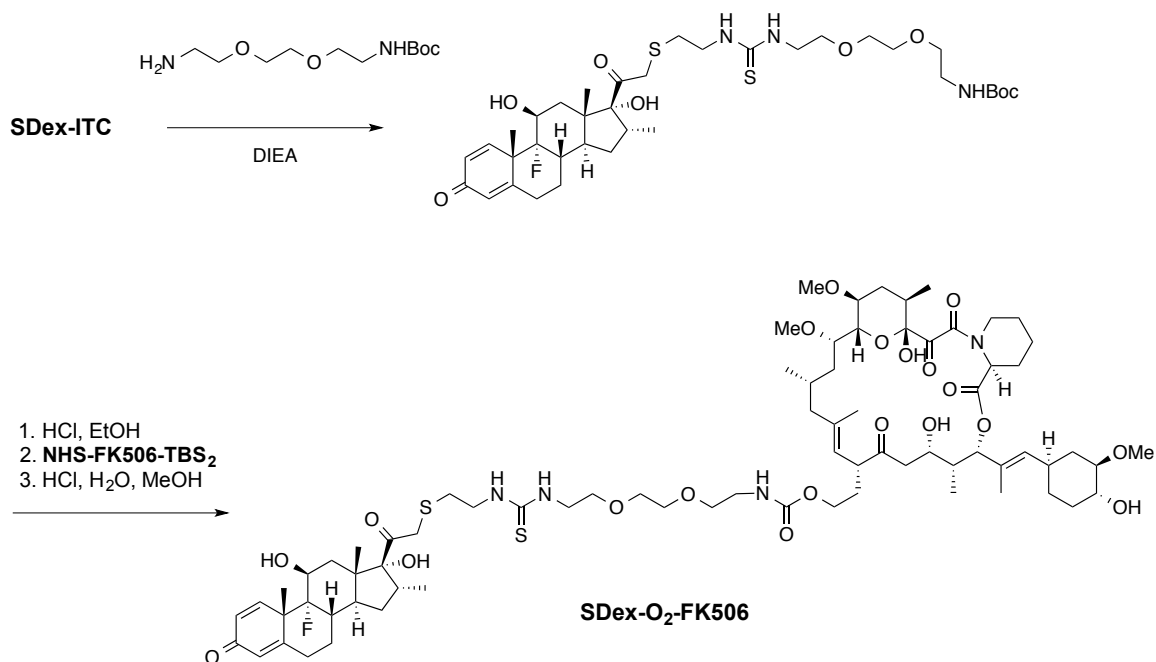


Figure 2.4 Conjugating a GR ligand to FK506. Functionalized Dex (SDex-ITC) is conjugated to a linker through thiourea formation. Subsequent linker deprotection allows for conjugation to functionalized FK506, forming a carbamate linkage.

In addition to the constructed bifunctional molecule, we synthesized a monofunctional, linker-containing compound SDex-O₂-CO₂Me to monitor the effects of modifying Dex, independent of conjugation. The compounds used in subsequent studies have been summarized in Fig. 2.5.

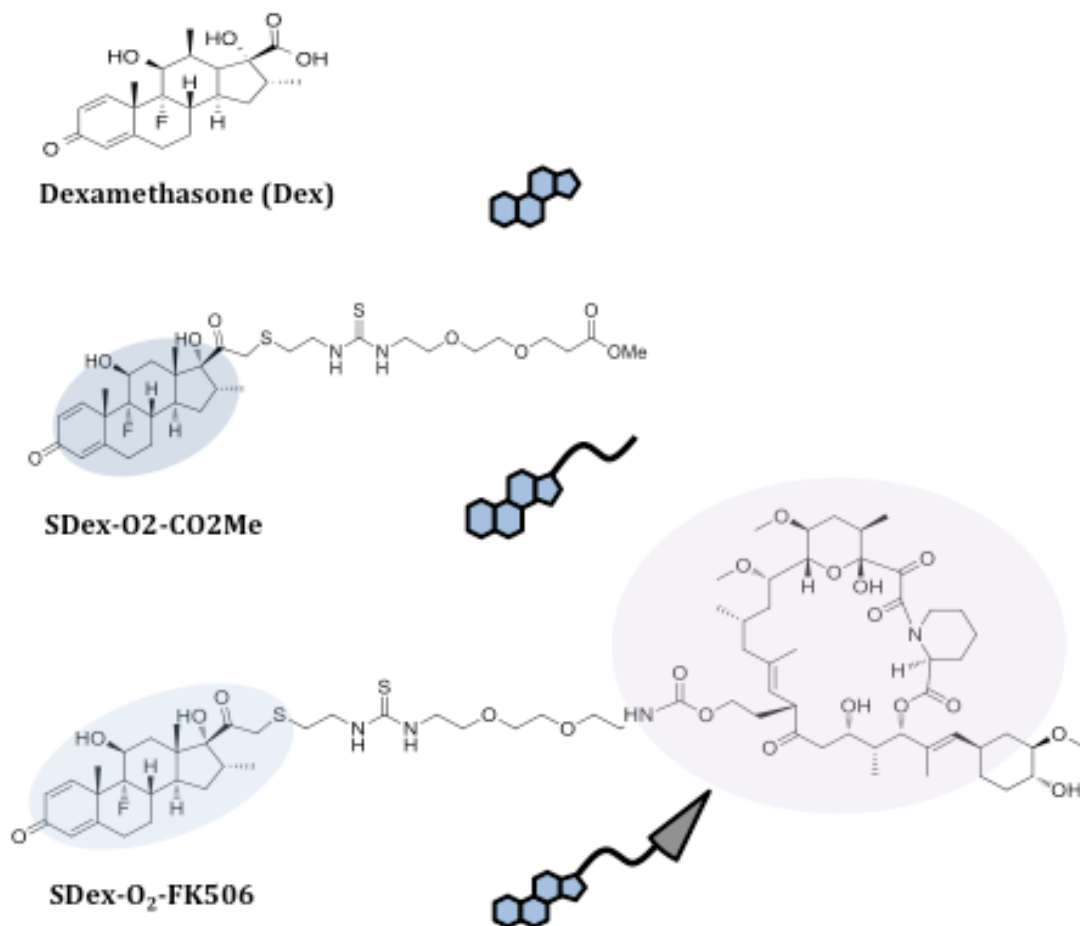


Figure 2.5 Summary of dexamethasone-based ligands. Compounds used in subsequent studies include unmodified dexamethasone, a monofunctional GR ligand-linker compound SDex-O2-CO2Me, and a bifunctional SDex-O2-FK506 linking a GR ligand and FKBP ligand.

In order to confirm our hypothesis that this synthetic strategy will minimally perturb binding to GR, we utilized a competition assay to determine the binding affinity of each described compound for GR. Cellular lysates containing expressed GR were incubated with tritium-labeled dexamethasone ($[^3\text{H}]\text{dex}$) and each indicated compound. Following washing, the concentration of remaining bound $[^3\text{H}]\text{dex}$ was determined and utilized to determine a binding affinity for each compound (Fig. 2.6). Appendage of the synthetic linker resulted in a ~10-fold loss of binding affinity, as the K_d for unmodified dexamethasone was determined to be 4.95 nM while the affinity for the monofunctional compound was

determined to be 48.3 nM. Conjugation to FK506 resulted in a further loss in affinity, as the K_d of binding for SDex-O₂-FK506 for GR was determined to be 104 nM. However, this may be a complicating factor of utilizing lysates that contain FK506-binding proteins.

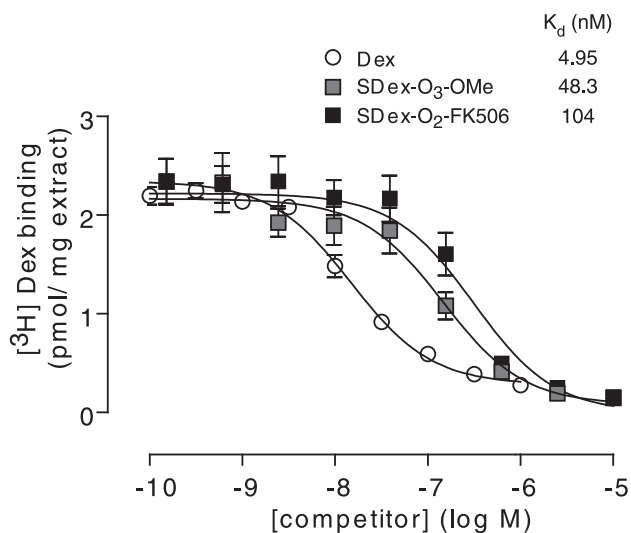


Figure 2.6 Conjugate binding to GR. Binding affinities of the utilized conjugate compounds were determined utilizing a competition assay with radioisotopically labeled dexamethasone. Synthetic modification of dexamethasone mildly perturbs binding to GR.

Utilizing the full length receptor in a three-hybrid assay

In order to determine if a bifunctional glucocorticoid can recruit a protein to the full-length receptor, a modified three-hybrid experiment was constructed, depicted in Fig. 2.7A. In place of a GR-Gal4 hybrid and a luciferase plasmid containing Gal4 DNA binding sites, 293T cells were transfected with full length human GR and a luciferase reporter plasmid containing the endogenous enhancer element from the glucocorticoid-sensitive *FKBP5* gene, which contains two GREs³¹; in this and subsequent experiments, 293T cells were chosen due to this cell line being highly amenable to transfection. Additionally, cells were transfected with either an expression plasmid coding for the VP16-FKBP fusion protein or a non-fusion FKBP protein for control. The activity of dexamethasone

and the two modified compounds is shown in Figure 2.7B-C, where activity is displayed as fold activation over the level expressed in vehicle-treated cells.

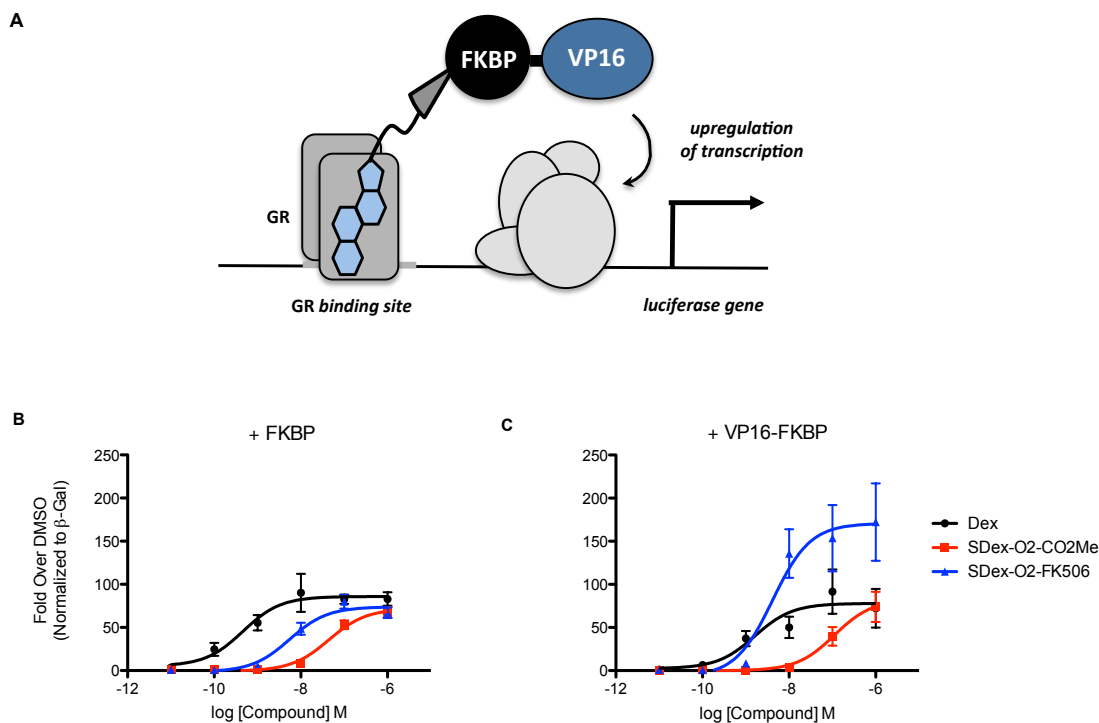


Figure 2.7 Designed ligand activity in a mammalian three-hybrid assay. In A, the bifunctional molecule links GR to a FKBP-fusion protein, driving transcriptional output through the localization of VP16 to the reporter gene. In B, the activity of ligands in the absence of fusion protein is shown. In C, the activity of ligands in the presence of VP16-FKBP is shown. The bifunctional ligand shows fusion protein-dependent activity

In the absence of the VP16-FKBP fusion protein (Fig.2.6B), SDex-O2-CO₂Me and SDex-O2-FK506 acted as partial agonists, showing both reduced potency (by 1-2 orders of magnitude) and efficacy (~ 80%) relative to dexamethasone. This is likely due to a reduced GR binding affinity, while the different activities between the two SDex-compounds may be caused by increased cell permeability by the presence of FK506. The expression of VP16-FKBP has little effect on the activity of SDex-O2-CO₂Me (Fig. 2.7C). However, in the presence of VP16-FKBP, SDex-O2-FK506 activated transcription two-fold relative to the maximal

activation by dexamethasone, while displaying a similar potency (experimental EC_{50} for dexamethasone: 1.6 nM; SDex-O2-FK506: 4.1 nM). The enhanced activation by SDex-O2-FK506 in the presence of VP16-FKBP, but not in its absence, firmly supports the notion that the molecule is both localizing GR to a GRE and recruiting a second protein.

Recruiting VP16-FKBP to native GR-regulated genes

Luciferase reporter experiments provide a platform for rapid screening of multiple treatments; however, a transiently transfected reporter plasmid will differ in structure and accessibility when compared to a native chromatin environment. To demonstrate recruitment of VP16-FKBP to influence the transcription of an endogenous gene, 293T cells were transfected with expression plasmids for the full-length rat GR and either a plasmid coding for the VP16-FKBP fusion protein or a non-fusion FKBP protein for control, then treated with SDex-O2-CO2Me or SDex-O2-FK506. Here, the rat isoform of the receptor was utilized due to previous experience with this variant. Following treatment, total mRNA from each treated sample was isolated. In previous reporter experiments, a reporter plasmid containing a promoter sequence from the glucocorticoid-regulated *FKBP5* gene was used. *FKBP5* is a canonical GR-regulated gene, and this characteristic, in addition to our desire to maintain continuity with the previously performed reporter studies, suggested that it would be an ideal target for monitoring transcript levels in response to our bifunctional ligand^{31,53}. RT-qPCR was utilized to determine relative quantities of *FKBP5* transcript levels in each sample (Fig. 2.8).

FKBP5 Transcript Levels in Transfected 293Ts

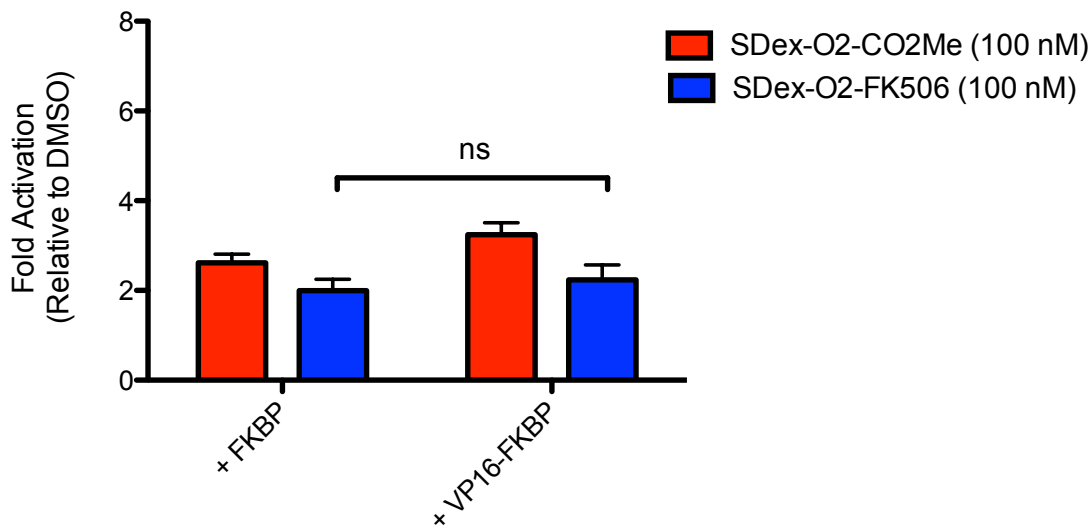


Figure 2.8 Transcriptional modulation of *FKBP5*. The effects of the designed ligands on the transcription of the endogenous gene *FKBP5* are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as a fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method. Statistical significance was determined using a one-tailed student's *t*-test.

In cells expressing the control FKBP protein, SDex-O2-CO2Me and SDex-O2-FK506 both activated transcription to levels twice that of in cells treated with a DMSO vehicle dosing. Unexpectedly, similar activation levels were observed in cells expressing the VP16-FKBP fusion. There are several potential explanations for this; first, while the dosing time was identical in monitoring reporter expression and *FKBP5* transcription, the cellular events involved with each occur on different time scales. Additionally, a benefit to luciferase reporter experiments is the stability of the firefly luciferase protein and its resistance to turnover; however, mRNA transcript stability is variable and often does not compare well. An alternative explanation is that differences in native chromatin architecture are the cause of the disparity in the activity between the two experiments⁵⁴. In the reporter experiment, SDex-O2-FK506 was recruiting VP16-FKBP to a segment of DNA just several hundred base pairs (bp) upstream of the luc gene; however,

when regulating the transcription of *FKBP5*, GR likely binds to a GRE located in an intronic sequence 26 kilobases from the transcriptional start site⁵⁴. It is possible that architectural differences are central to the observed differences³¹.

The low inducibility of the *FKBP5* gene may also play a role in the lack of recruitment-aided activation. In luciferase reporter experiments (Fig. 2.7), activation of transcription by the SDex-based compounds reached levels 1-2 orders of magnitude above those produced in vehicle-treated cells. However, induction of native *FKBP5* transcription was only 2-3 fold. Observing this, we decided to shift focus to an alternative GR-regulated gene. Here, we identified the *S100P* gene^{55,56}; this target codes for a calcium-binding protein that has been shown to be potentially oncogenic in prostate cancer. More importantly, this gene displays comparatively low basal expression in 293T cells but can be stimulated approximately 50-100 fold by dexamethasone in cells expressing GR, indicating a large transcriptional dynamic range. With this observation, we reasoned that this highly inducible gene is an ideal target gene to observe a recruitment-dependent effect on transcription. In cells expressing FKBP, SDex-O2-CO2Me and SDex-O2-FK506 acted as strong activators of transcription to similar levels (Fig. 2.9A). In cells expressing the VP16-FKBP fusion, the bifunctional molecule activated transcription to a level approximately 2-3 fold higher than the monofunctional SDex-O2-CO2Me, with a similarly higher output when compared to the transcript levels measured in cells lacking the fusion protein (Fig. 2.9B).

S100P Transcript Levels in Transfected 293Ts

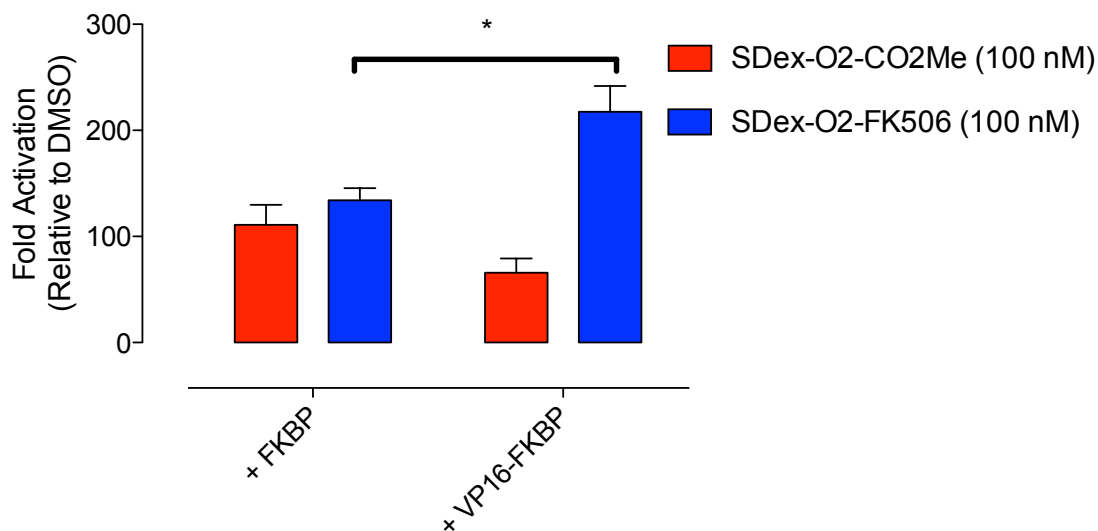


Figure 2.9 Transcriptional modulation of *S100P*. The effects of the designed ligands on the transcription of the endogenous gene *S100P* are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method. Statistical significance was determined using a one-tailed student's *t*-test.

Suppressing transcription through recruitment

A benefit to the system described heretofore is its modularity; investigating alternative bifunctional molecules or recruitment targets is relatively straightforward. NR transcriptional repression is commonly facilitated through the recruitment of corepressor complexes, so we sought to determine the effects of bifunctional molecule-mediated recruitment of a corepressor. Histone deacetylase-1 (HDAC1) acts as a transcriptional repressor both actively, through enzymatic deacetylation of histones lysines, and passively, as a scaffold protein that aids in the formation of corepressor complexes⁵⁷. To this end, an expression plasmid for an HDAC1-FKBP fusion protein was constructed. 293T cells were transfected with a GRE-containing luciferase plasmid, an expression plasmid for the rat GR (rGR), and either an expression plasmid for the HDAC1-FKBP fusion protein or a plasmid coding for FKBP for control. Cells were treated with one of the indicated compounds, and transcriptional activity is displayed in Fig. 2.10 as a function of

luciferase expressed relative to the levels in vehicle-treated cells.

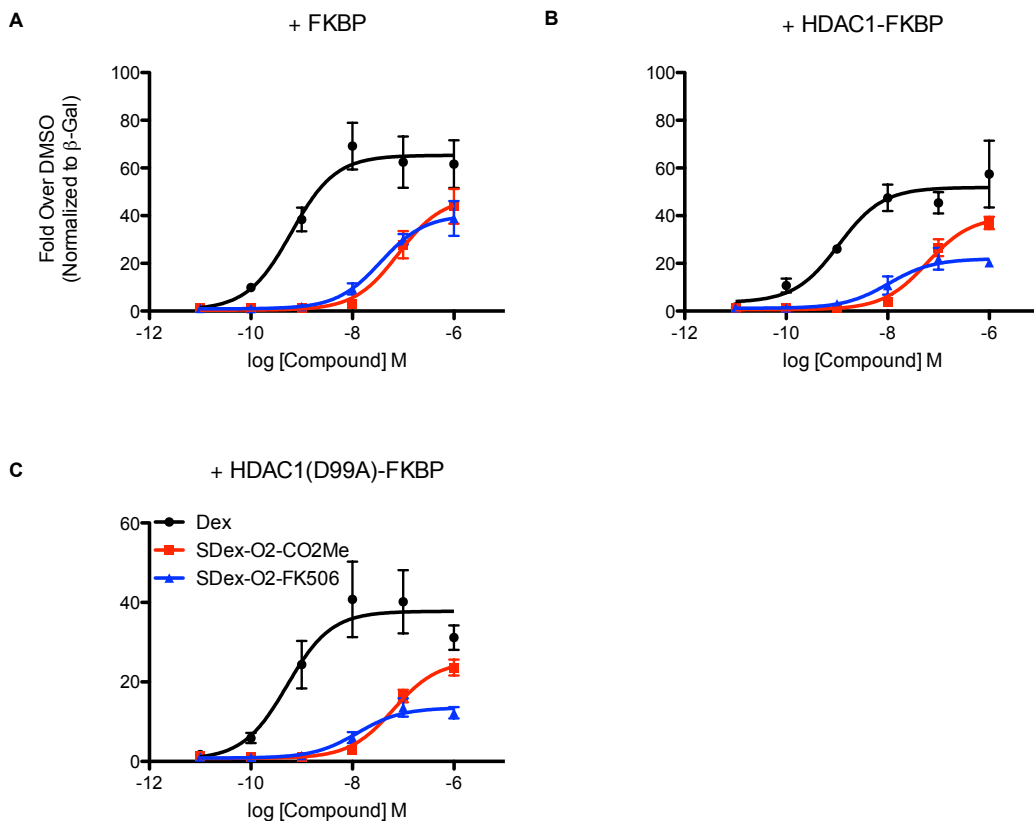


Figure 2.10 Corepressor three-hybrid experiment. A three-hybrid experiment was performed in cells expressing FKBP (A), a HDAC1-FKBP fusion (B), or a fusion containing a catalytically weakened HDAC1 mutant (C). Transcriptional activity of the bifunctional molecule was fusion-protein dependent.

In Fig. 2.10A, SDex-O2-CO2Me and SDex-O2-FK506 activated rGR-mediated transcription as partial agonists, displaying lower potency (two orders of magnitude) and efficacy (achieving ~ 80% maximum activity) relative to dexamethasone in cells only transfected with a FKBP expression plasmid, comparable with the activation of hGR in Fig. 2.7A. The expression of HDAC1-FKBP did not affect the activity of SDex-O2-CO2Me (Fig. 2.10B). However, in cells expressing this fusion, the maximum transcriptional activity induced by

SDex-O2-FK506 was lowered approximately 50%, supporting the notion that bifunctional molecule recruitment of a corepressor protein can inhibit transcriptional activity. It is likely that the residual activation of transcription is from competing allosteric recruitment of coactivators by rGR.

Transcription of the *S100P* gene was affected by the recruitment of VP16-FKBP, suggesting that it would be an ideal system for determining the effects of HDAC1-FKBP recruitment on the expression of an endogenous gene. 293T cells transiently expressing rGR and either FKBP or HDAC1-FKBP were treated with the indicated compounds and subsequently lysed. Following total RNA isolation, the level of *S100P* transcripts was quantified and displayed as fold activation relative to the transcript level in vehicle-treated cells. As seen in Fig. 2.11A, SDex-O2-CO2Me and SDex-O2-FK506 strongly induced the expression of *S100P* in cells expressing FKBP. The expression of HDAC1-FKBP does not interfere with the ability of SDex-O2-CO2Me to activate transcription; however, activity in cells treated with SDex-O2-FK506 is reduced to approximately 50% of the activity level in cells lacking the fusion. Interestingly, whereas activity levels of each compound were similar at the dosing concentration indicated (100 nM) in reporter experiments (Fig. 2.10B), while transcriptional suppression is only seen at high dosing concentration (1 μ M), the bifunctional molecule is capable of recruitment at a lower concentration in this experiment. This disparity illustrates the need for further experimentation monitoring the transcription of alternative GR-targets.

S100P Transcript Levels in Transfected 293T Cells

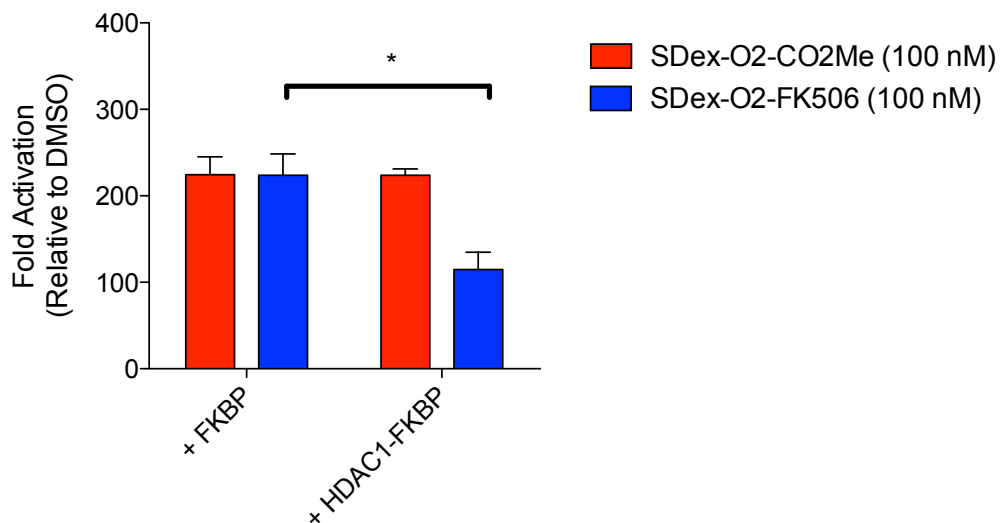


Figure 2.11 Transcriptional modulation of *S100P* by a corepressor fusion. The effects of the designed ligands on the transcription of the endogenous gene *FKBP5* are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method. Statistical significance was determined using a one-tailed student's *t*-test.

HDAC1-mediated transcriptional repression proceeds through a number of mechanisms, as mentioned prior. In order to determine if the transcriptional repression observed as a result of HDAC1-FKBP recruitment is facilitated by HDAC1 deacetylase activity or through an alternative mechanism, a fusion protein expression plasmid was constructed containing a mutant form of HDAC1, HDAC1(D99A)-FKBP. This point mutation severely abrogates HDAC1 enzymatic activity, inhibiting its ability to deacetylate lysine substrates and actively repress open chromatin conformations⁵⁸. 293T cells were transfected with expression plasmids for rGR and the HDAC1(D99A)-FKBP fusion protein and incubated with indicated compounds. As in cells expressing HDAC1-FKBP (Fig. 2.10B), SDex-O2-FK506 activity was suppressed by approximately 50% in the presence of HDAC1(D99A)-FKBP (Fig. 2.10C). It is currently unclear if this suppression is simply a factor of steric hindrance inhibiting the recruitment of transcriptional

machinery, or if mutant HDAC1 is continuing to recruit corepressor complexes; a similar strategy was employed to sterically hinder the formation of transcriptional complexes by ER⁵⁹ but was ineffective, suggesting that it is likely continued contacts between HDAC1 and other corepressors that is mediating suppression. However, the difference in size, concentration, and distribution of our recruitment target relative to that of Moore and coworkers makes direct comparisons difficult, and it is impossible to rule out simple sterics as driving this observation.

2.5 Targeting endogenous coregulators for recruitment

As mentioned previously, a highlight of our system is its modularity. Accordingly, it is relatively straightforward to develop alternative bifunctional molecules capable of recruiting differing targets. In an effort to identify potential ligands for synthetic linkage to dexamethasone, we recognized the potential of (S)-JQ1 (Fig. 2.12) as a secondary targeting moiety in a bifunctional glucocorticoid. (S)-JQ1 is a highly potent inhibitor of the protein bromodomain containing 4 (BRD4)⁶⁰. Bromodomain-containing proteins belong to a diverse family of transcription factors, with 41 human variants having been described^{61,62}. However, each contains at least one conserved bromodomain, a largely helical protein domain capable of recognizing and binding acetylated-lysine molecules. BRD4, as a member of the larger BET family, contains two N-terminal bromodomains, each of which play a role in substrate binding and specificity. BRD4 has been primarily identified for its role as a scaffolding transcription factor, binding specific acetylated histone lysines and aiding in the recruitment of P-TEFb, essential for the proper positioning of RNA Polymerase II^{60,63}, though it has also been demonstrated to bind acetylation marks on alternative transcription factors^{64,65}. These responsibilities, along with recent results indicating that BRD4 plays a critical role in transcriptional elongation, have spurred efforts to develop and molecular modulators of bromodomain-containing proteins such as (S)-JQ1. Structural characterization of the (S)-JQ1-BRD4 complex has revealed that C₆ of the (S)-JQ1 benzodiazepine projects outwards from the protein's binding site^{60,66},

and follow-up studies show that that enantioselective substitution at this point with a synthetic linker is capable with minimal perturbation of ligand-protein binding⁶⁷. Considering these factors, BRD4 is an ideal target for recruitment to DNA-bound GR through a bifunctional molecule. To begin this investigation, we synthesized the following molecules (Fig. 2.12): SDex-O3-CO2Me, a glucocorticoid molecule appended to a synthetic linker; SDex-O3-(S)-JQ1, a bifunctional molecule linking dexamethasone to (S)-JQ1; and SDex-O3-(R)JQ1, an inactive diastereomer with no displayed binding to BRD4.

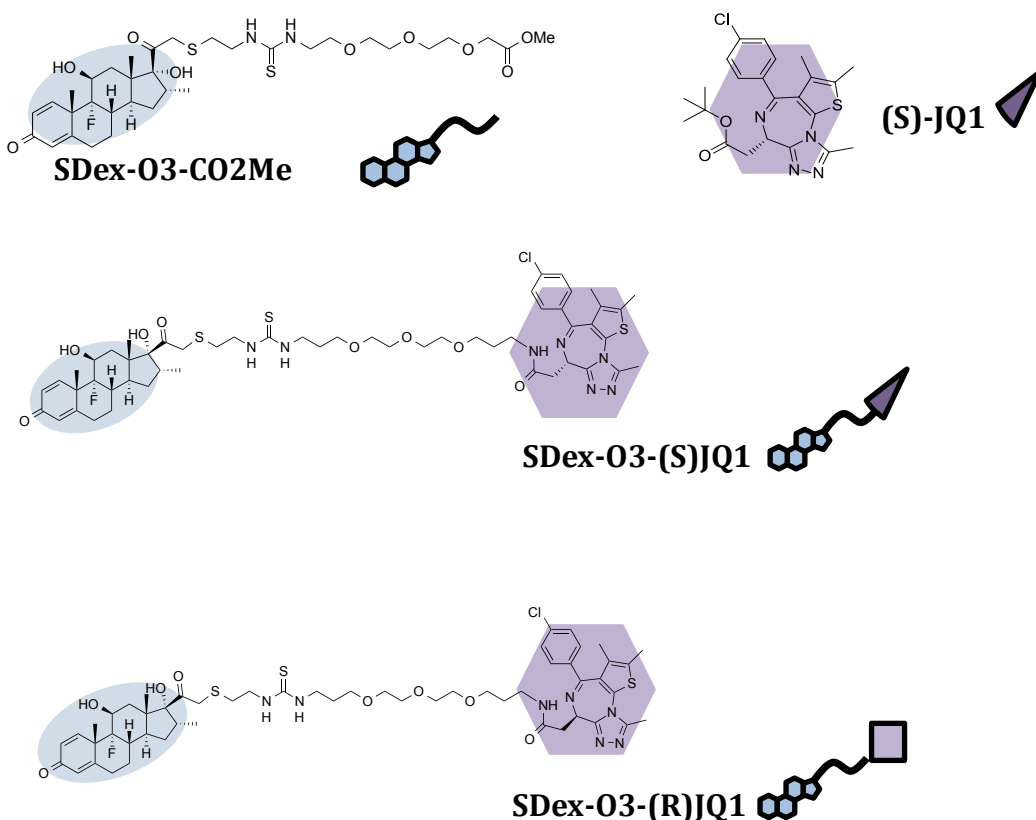


Figure 2.12 Summary of BRD4-targeting ligands. The ligands used in subsequent studies include unmodified (S)-JQ1, a monofunctional GR ligand-linker compound SDex-O3-CO2Me, a bifunctional SDex-O3-(S)JQ1, and a bifunctional molecule conjugated to an inactive enantiomer of JQ1 termed SDex-O3-(R)JQ1.

Dimerization of GR and BRD4

To first determine if the designed SDex-O3-(S)JQ1 is capable of dimerizing GR and BRD4 in the absence of DNA-binding, ligand-induced coimmunoprecipitation experiments were performed. The human osteocarcinoma U2OS cell line lacks detectable GR expression and was chosen for this study to allow for the potential to utilize GR variants in subsequent studies. U2OS cells were transfected with an expression plasmid coding for a multiply myc-tagged version of human GR, hGR-myc₆, and incubated with the indicated compound. Samples were subsequently lysed and incubated with magnetic beads coated in BRD4-recognizing antibody (αBRD4). Bound proteins were eluted and analyzed by Western blot for the presence of hGR-myc₆ using a myc-recognizing antibody (Fig. 2.13).

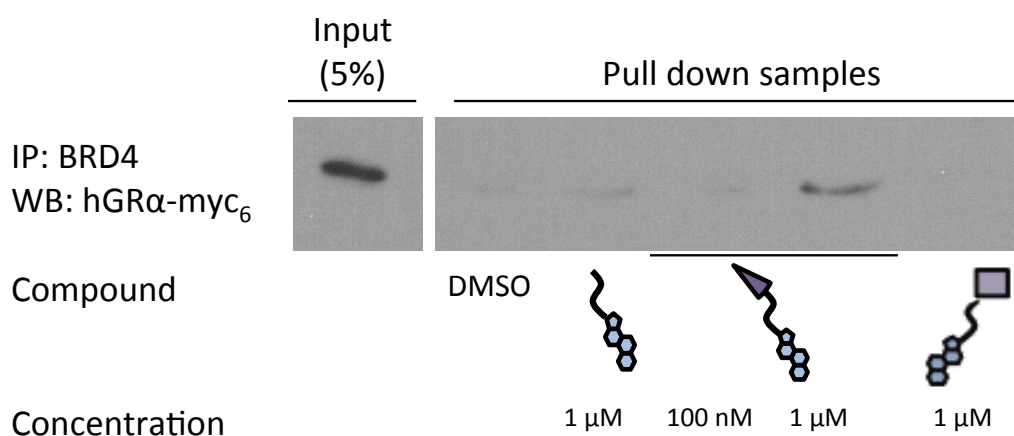


Figure 2.13 Dimerization of hGR and BRD4. Co-immunoprecipitation experiments were performed to detect the ability of the bifunctional molecule to dimerize hGR and BRD4. Cellular lysates were incubated bead support coated in a BRD4 recognition antibody on bead support immobilizing BRD4. Incubation with bifunctional molecule co-immunoprecipitated a tagged version of hGR, hGR-myc₆, as detected by Western blot. Samples, from left to right: DMSO; SDex-O3-CO₂Me (steroid-linker); SDex-O3-(S)JQ1 (steroid-triangle); SDex-O3-(R)JQ1 (steroid-square).

SDex-O3-(S)JQ1 induced the coimmunoprecipitation of GR with BRD4. Neither the vehicle (DMSO) or linker (SDex-O3-CO₂Me) incubated-samples result in the coimmunoprecipitation of hGR-myc₆, precluding the possibility of a non-specific

interaction between GR and BRD4. Additionally, the inactive diastereomer, SDex-O3-(R)JQ1, was incapable of coimmunoprecipitating hGR-myc6, strongly supporting the notion that the observed interaction is being specifically modulated through the interaction of each portion of the bifunctional molecule with its target-binding pocket.

Functional outcome of BRD4 recruitment

BRD4 interacts with acetylation marks with a rapid 'on and off' mode of binding in a preferential manner for specific lysines over others^{68,69}. Moreover, though BRD4 is thought to primarily aid in transcriptional activation through the recruitment of the pTEF-b complex, recent work has shown that BRD4 is capable of forming differing, distinct transcriptional complexes with alternative epigenetic proteins⁷⁰, and it is likely that the neighboring chromatin environment plays a role in the identity of the complex formed. While established precedent allowed for a straightforward prediction of the functional influences of recruiting the activator VP16, these outside factors convolute our ability to foresee what outcome BRD4 recruitment will have on GR-regulated transcription. Further confounding this is the recent discovery that at least one BRD4-transcription factor interaction is dependent on the ability of BRD4 to recognize an acetylation mark on the transcription factor itself, suggesting that recruitment through bifunctionalized JQ1 could interfere with necessary PPIs⁷¹.

To investigate the effects of bifunctional molecule-facilitated BRD4 recruitment on transcription, we initially developed a three-hybrid experiment (depicted in Fig. 2.14A). This arrangement additionally allows us to confirm that SDex-O3-(S)JQ1 (hereafter referred to as SDex-O3-JQ1) is capable of chemically dimerizing GR and BRD4 in a cellular system.

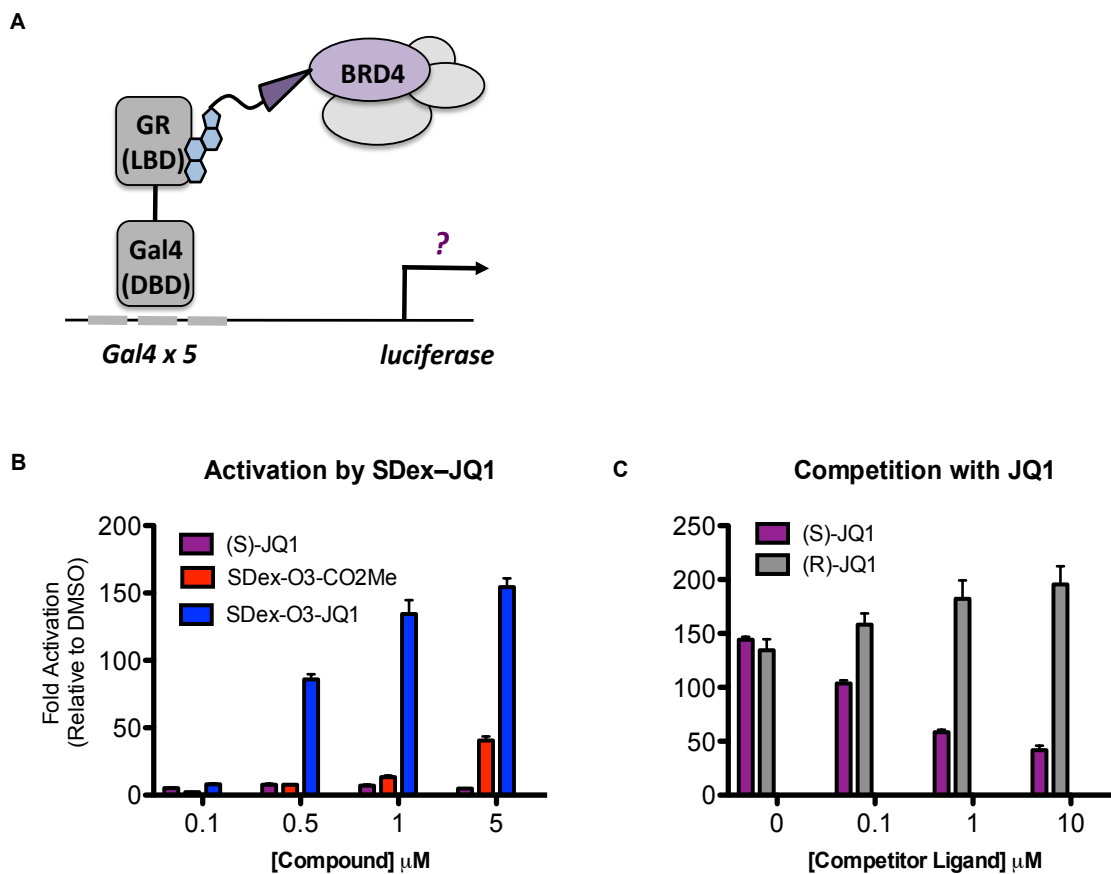


Figure 2.14 Mammalian three-hybrid assay targeting an endogenous protein. A three-hybrid experiment, using a Gal4-GR(LBD) chimera, relying on endogenous BRD4 recruitment to drive activity was constructed (A). B: the monofunctional GR ligand acted as a partial agonist, while bifunctional ligand strongly and potently activated transcription. C: the enhanced activity of the bifunctional molecule was dependent on BRD4 recruitment, as determined by squelching experiments.

HeLa cells expressing endogenous levels of BRD4 were transfected with a luciferase reporter plasmid bearing five Gal4 DNA-binding sites and an expression plasmid for a Gal4-GR(LBD) chimera and treated with the indicated compounds; here HeLa cells were chosen due to their previous usage in a similar model. The resulting activation of transcription is displayed in Fig. 2.13B as a fold activation of luciferase produced over the levels in vehicle-controlled cells. As expected, the monofunctional SDex-O3-CO₂Me acted as an agonist in this system, likely activating transcription through its induced rearrangement of

the GR(LBD) AF2 domain. As was seen with the recruitment of VP16-FKBP, the bifunctional SDex-O3-JQ1 acts as a 'superactivator' of transcription, stimulating the expression of luciferase approximately 3-fold higher than the maximum activity displayed by SDex-O3-CO2Me. Treatment with (S)-JQ1 negligibly affected transcription.

Given (S)-JQ1's high specificity for BRD4⁶⁰, it is unlikely that an alternative protein is being recruited to produce the displayed high activity by SDex-O3-JQ1, though we cannot currently rule out the possibility that other proteins are being recruited. To confirm that the observed effects are at least dependent on a recruitment event, a squelching experiment was performed to determine the observed modified transcriptional activity could be attenuated by competing ligand. HeLa cells were transfected as above and treated with SDex-O3-JQ1 (1 μ M) along with increasing levels of either (S)-JQ1 or inactive (R)JQ1. As seen in Fig. 2.14C, increasing levels of (S)-JQ1 suppressed the transcriptional activity of SDex-O3-JQ1 in a dose-dependent fashion, with high concentrations of (S)-JQ1 (10 μ M) suppressing activity approximately 70%. However, co-treatment with (R)JQ1 did not produce this effect. This observation supports the hypothesis that recruitment is causing the marked difference in activity between SDex-O3-CO2Me and SDex-O3-JQ1; however, (S)-JQ1 is capable of interfering with dexamethasone-induced transcription (unpublished observations) and further experiments are necessary to make this claim with more certainty.

Two- and three-hybrid experiments are excellent at demonstrating the potential for existing or induced PPIs, but they can occasionally be deceiving if counted on for predicting the existence of a functional transcriptional complex⁷². As with the recruitment of FKBP-chimeras, we set out to determine if a bifunctional molecule is capable of recruiting endogenous BRD4 to the native, full-length receptor. To this end, we evaluated SDex-O3-JQ1's activity in the system described in Fig. 2.15A.

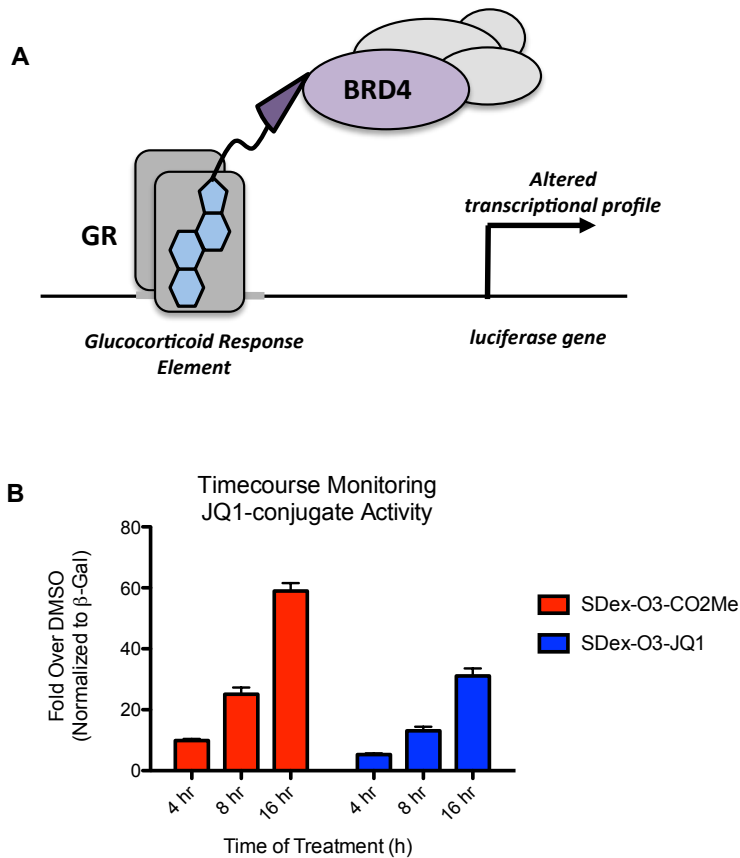


Figure 2.15 Recruitment of BRD4 to full-length GR. A reporter experiment was designed utilizing full-length GR (A). B: the activity of each GR-ligand was time dependent, with maximal observed activity occurring at 16 hours of treatment.

Initially, U2OS cells were transfected with a reporter plasmid bearing a consensus GRE immediately upstream of a promoter driving luciferase expression and an expression plasmid coding for human GR. Cells were treated with either SDex-O3-CO₂Me or SDex-O3-JQ1 (100 nM) for the indicated time. Transcriptional activity was determined by monitoring luciferase expression and displayed as fold activation over the levels of luciferase in vehicle-treated cells (Fig. 2.15B). Both the monofunctional and bifunctional ligands acted as agonists of GR-mediated transcription, stimulating activity that increased with increasing incubation time. A 16-hour treatment produced the strongest transcriptional response and was chosen for further investigations into the activity of SDex-O3-JQ1.

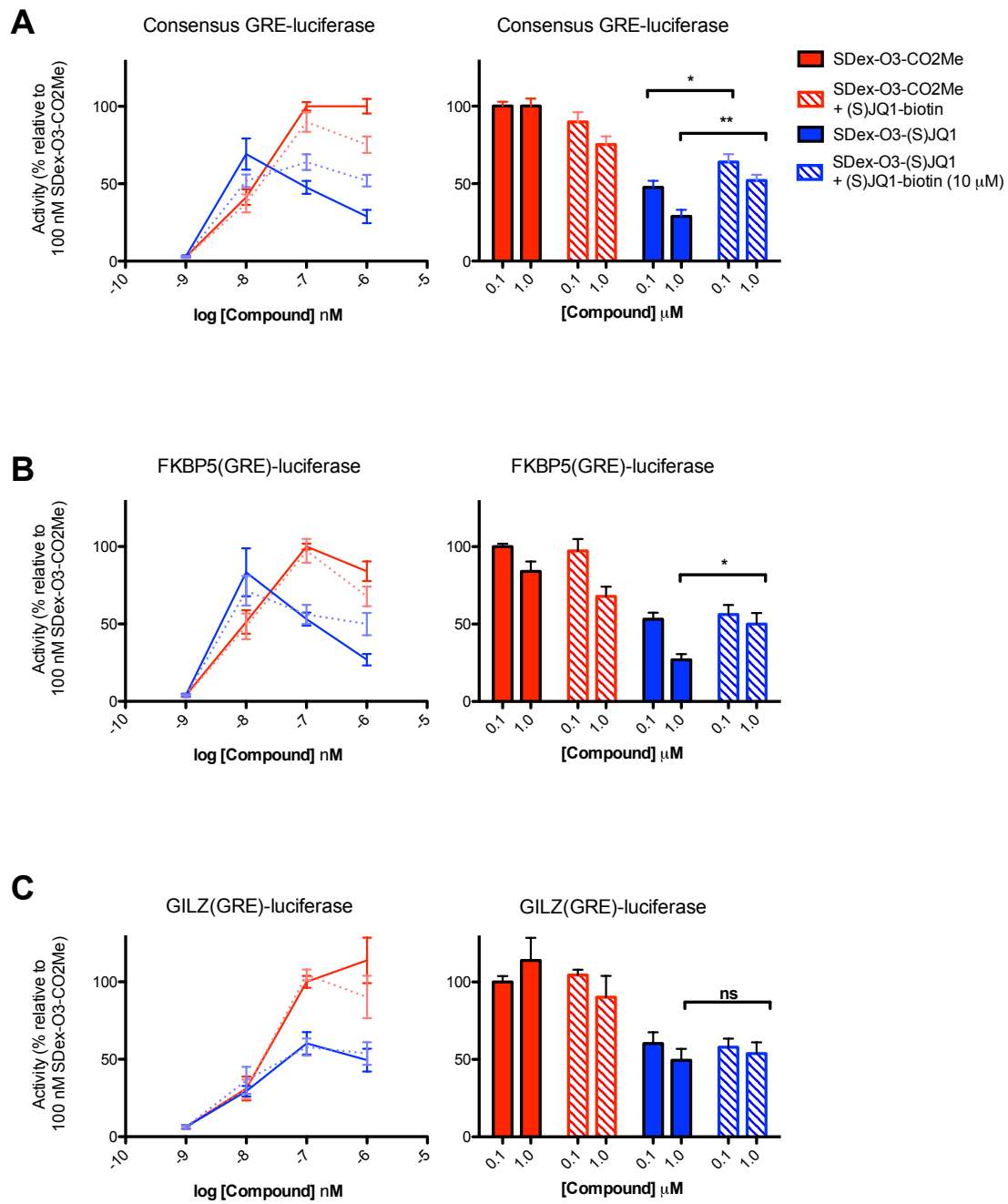


Figure 2.16 Utilizing alternative GREs in reporter experiments. Full-length GR was utilized in a transcriptional reporter experiment, with a reporter plasmid using a consensus GRE (A), *FKBP5* GRE (B), or *GILZ* GRE (C). Statistical significances were determined using a two-tailed student's *t*-test.

U2OS cells were transfected as described earlier and incubated with the indicated compound(s) for 16 hours. This measurement of transcriptional activity is displayed as a relative percentage normalized to the activity produced by dosing with SDex-O3-CO₂Me (100 nM) in Fig. 2.16A; data are presented as a traditional dose-response curve (left panel) alongside a focused presentation of the response to specific doses, including statistical significance analysis (right panel). In addition to doses with the monofunctional SDex-O3-CO₂Me and bifunctional SDex-O3-JQ1, a 'trans' addition dosing of equimolar SDex-O3-CO₂Me and a biotinylated form of (S)-JQ1 [(S)-JQ1-biotin] and a 'squelch' dosing including SDex-O3-JQ1 and an excess (10 μM) of (S)-JQ1-biotin were included. The trans addition components were chosen to ensure that transcriptional outcomes caused by SDex-O3-JQ1 are not simply additive or synergistic responses provoked independently by each moiety in the bifunctional molecule, while the squelch dosing is included to confirm that effects are induced through recruitment. As expected, SDex-O3-CO₂Me acts agonistically to activate GR-driven transcription, while co-dosing with (S)-JQ1-biotin results in a suppression of transcription at high (1 μM) concentration, but otherwise minimally affects the activity of SDex-O3-CO₂Me. Increasing concentrations of SDex-O3-JQ1 result in a bell-shaped activity curve, initially increasing at low concentration but declining with higher concentrations of compound; at the highest dose (1 μM), activity levels are approximately 50% of the maximum stimulation level (produced at 10 nM). The co-addition of excess (S)-JQ1-biotin increases activity in a significant manner, albeit not to the same level as seen with the trans addition treatment. The suppression of activity seen with SDex-O3-JQ1, paired with the observation that free (S)-JQ1-biotin competes with suppression, strongly suggests that the recruitment event is interfering with GR-mediated transcription.

As noted earlier, GREs are loosely conserved sequences, and even single base pair differences can effect large differences in the identity of GR binding partners and subsequent transcriptional activity⁶. Given the disparity in activity produced by SDex-O3-JQ1 when comparing the results described above, we sought to

determine if altering the reporter GRE sequence would result in an altered activity profile. U2OS cells were transfected and treated as described earlier; however, in lieu of the reporter plasmid bearing a consensus GRE, cells were transfected with a reporter plasmid bearing a GRE taken from the *FKBP5* gene (differing in spacer sequence and at two positions) or a GRE from the *GILZ* gene (differing in spacer sequence and at three positions) immediately upstream of a promoter driving luciferase expression. Data are presented as above. As seen in Fig. 2.16B, the altered sequence of the *FKBP5* GRE was well tolerated, with the bifunctional molecule producing a very similar pattern of activity. A similar suppression of activity was produced with a high loading of SDex-O3-JQ1, while the presence of free (S)-JQ1-biotin again enhanced activation. However, utilizing a *GILZ* GRE sequence (Fig. 2.16C) resulted in a distinct activation pattern. As the cellular concentration of SDex-O3-JQ1 is increased, a canonical agonistic dose-response curve is produced, and the presence of free (S)-JQ1-biotin does not alter transcriptional activation by SDex-O3-JQ1. Despite this altered pattern, SDex-O3-JQ1 continues to act as a weaker activator of the full-length GR than SDex-O3-CO2Me, in contrast to experiments utilizing Gal4-GR(LBD), suggesting an additional mechanism for this lowered transcriptional response.

Recruiting BRD4 to endogenous genes

As displayed earlier, reporter experiments can be poor predictors of the ligand-induced transcriptional response at endogenous genes. Indeed, even knowledge of the chromatin environment surrounding a GRE can be misleading when attempting to predict transcriptional responses. To investigate the effects of bifunctional molecule treatment on endogenous gene expression, U2OS cells were transfected with an expression plasmid for the human GR and treated with the indicated compound(s). Following treatment, cells were lysed, total RNA isolated, and the indicated transcript was quantified relative to the levels in vehicle-treated cells. The transcriptional activity is displayed as fold activation in Fig. 2.17.

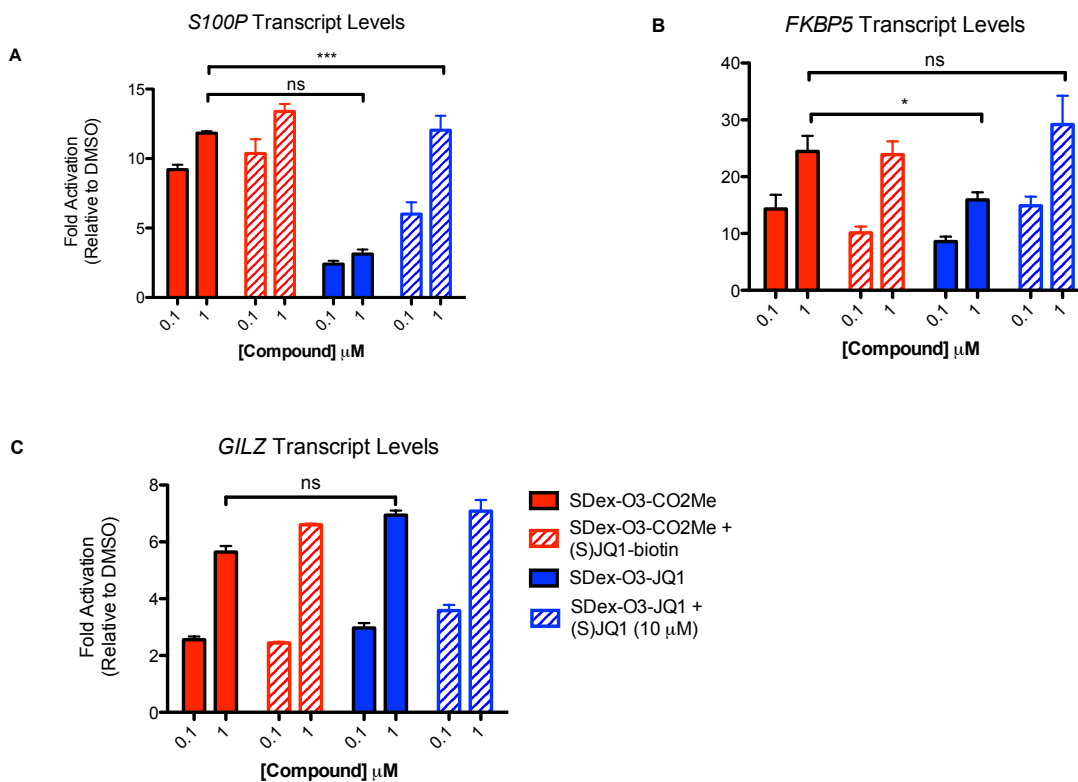


Figure 2.17 Effects of BRD4 recruitment on endogenous gene transcription. The effects of the designed ligands on the transcription of *S100P* (A), *FKBP5* (B), and *GILZ* (C) in transfected U2OS cells are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method. Statistical significance was determined using a one-tailed student's *t*-test.

The relative transcript levels of *S100P* (Fig. 2.17A), *FKBP5* (Fig. 2.17B), and *GILZ* (Fig. 2.17C) were determined^{31,52}. SDex-O3-CO2Me treatment activated the transcription of each these three GR-target genes. Unlike in reporter experiments, co-dosing with (S)-JQ1-biotin did not appreciably alter the activity of SDex-O3-CO2Me; this may be a byproduct of a shorter dosing time in RNA quantification studies, though the transcriptional response to both glucocorticoids⁷³ and (S)-JQ1⁷⁴ is rapid. In comparison to SDex-O3-CO2Me, SDex-O3-JQ1 weakly activated transcription of the *S100P* gene (Fig. 2.17A), raising levels to approximately 25% of the maximum level induced by SDex-O3-

CO₂Me. Consistent with the hypothesis that BRD4 recruitment inhibits GR activity, the addition of excess (S)-JQ1 allows SDex-O3-JQ1 to activate *S100P* transcription to similar levels as SDex-O3-CO₂Me. SDex-O3-JQ1 induced a similar, but less pronounced, effect in activating transcription of the *FKBP5* gene to approximately 60% of the level induced by SDex-O3-CO₂Me (Fig. 2.17B). Again, addition of excess (S)-JQ1 squelches the suppressed agonism of SDex-O3-JQ1, raising activity to a level comparable with SDex-O3-CO₂Me. Interestingly, the transcriptional response at the *GILZ* gene was even more disparate; treatment with SDex-O3-JQ1 alone or in tandem with (S)-JQ1 produced a near identical activation of *GILZ* transcription. While further analysis of additional targets is necessary prior to making conclusions, the wide range in activity induced by the bifunctional glucocorticoid may point to the possibility for selectivity in gene expression.

The human adenocarcinoma lung epithelial cell line A549 expresses endogenous GR and is commonly used as a model line for the study of GR actions and activity³¹. It has previously been shown that expression levels of GR vary between A549 cells and transfected U2OS cells, and this likely influences the observed differences in gene expression profiles produced by treatment with glucocorticoids². The effects of SDex-O3-JQ1 treatment on transcriptional activity in A549 cells was investigated to determine if similar patterns are displayed in cells expressing endogenous levels of GR. Comparing the transcription of *S100P* and *GILZ* provided the starkest difference in activity mediated by SDex-O3-JQ1 and provided a template for further studies in A549 cells. Following treatment with the indicated compounds, A549 cells were lysed, total RNA was isolated, and the indicated transcripts were quantified. Transcriptional activity is displayed as fold activation relative to transcript levels in vehicle-treated cells (Fig. 2.18).

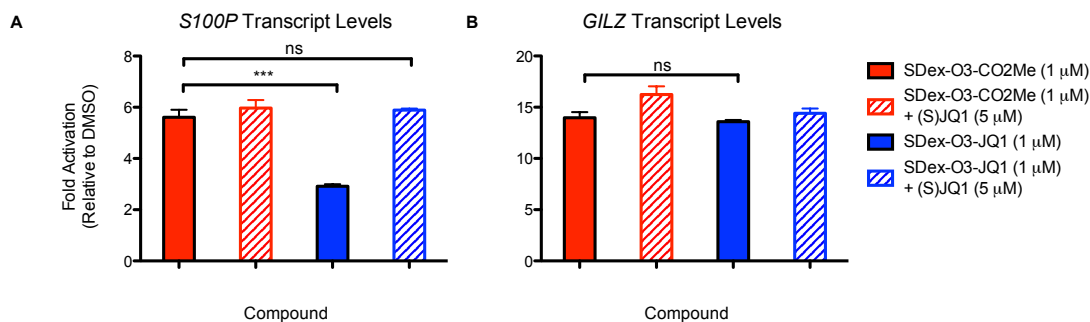


Figure 2.18 Transcriptional modulation in A549 cells. The effects of the designed ligands on the transcription of *S100P* (A) and *GILZ* (B) in A549 cells are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method. Statistical significance was determined using a one-tailed student's *t*-test.

The activation patterns of *S100P* (Fig. 2.17A) in A549 cells resemble the profile produced in transfected U2OS cells. Treatment with SDex-O3-CO2Me in the absence or presence of (S)-JQ1 produced a strong agonistic response, while treatment with the bifunctional SDex-O3-JQ1 activated transcription to approximately 50% of the level induced by the monofunctional glucocorticoid. As observed before, co-treatment of SDex-O3-JQ1 with an excess of (S)-JQ1 produces transcriptional activity nearly identical to SDex-O3-CO2Me, implying that (S)-JQ1 is capable of competing away BRD4 recruitment and causing SDex-O3-JQ1 to act as its parent, monofunctional ligand. Similarly, the pattern of *GILZ* transcription (Fig. 2.17B) resembled the activities produced in transfected U2OS cells, wherein each of the described compounds and combinations produced nearly identical responses. These initial observations indicate that the activity patterns of the bifunctional molecule may be similar in different cell lines. Currently, efforts are underway to expand the list of monitored genes to aid in establishing an activity pattern.

Targeting a ligand-sensitive cell line

(S)-JQ1 has emerged as a potent downregulator of *MYC* expression, spurring investigations into the role it may play in inhibiting the growth of pathogenic species driven by c-Myc related oncogenic activity⁷⁴. Multiple myeloma (MM) is a hematological malignancy that has been established as an apt model for c-Myc dysregulation^{53,74,75}. Currently, glucocorticoids are commonly used as a chemotherapeutic treatment to induce apoptosis in MM cells, but this treatment is limited by the rapid development of resistance. Given the dual sensitivity of MM cells to both (S)-JQ1 and glucocorticoids, we identified the myeloma cell line MM.1S as an ideal model for further evaluation of SDex-O3-JQ1 activity. Unlike the related multiple myeloma line MM1.R, a similar line that is believed to have suppressed GR expression, we hypothesized that this line may be acutely sensitive to modulation with our designed ligand.

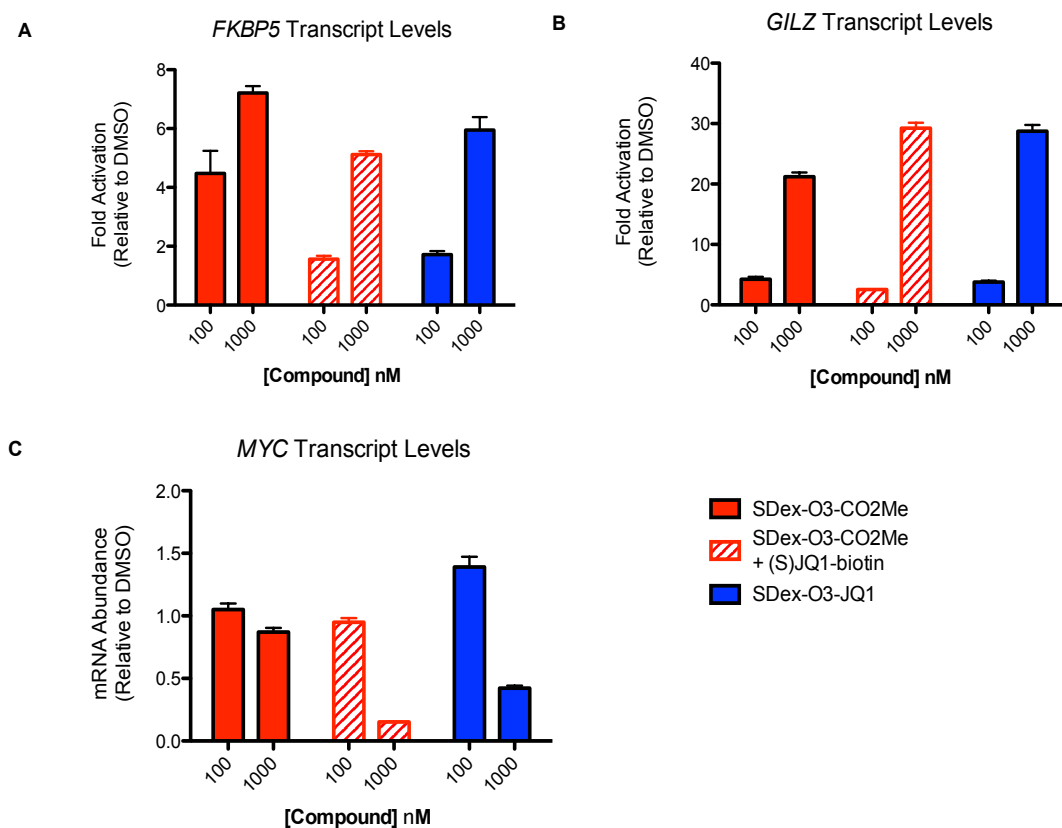


Figure 2.19 Transcriptional modulation in a multiple myeloma model. The effects of the designed ligands on the transcription of *FKBP5* (A), *GILZ* (B), and *MYC* (C) in MM.1S cells are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method.

MM.1S cells were incubated with the compounds indicated prior to total RNA isolation. The expression of the target genes *FKBP5*, *GILZ*, and *MYC* was monitored in response to treatment; *S100P* expression in MM.1S cells was not detectable, even in response to glucocorticoid. Transcriptional activity is displayed as fold activation (Fig. 2.19A and B) or relative mRNA abundance (Fig. 2.19C) relative to transcript levels in vehicle-treated cells. Induced levels of both *FKBP5* and *GILZ* were relatively similar with all treatments, unlike in A549 cells and transfected U2OS cells. This convolutes the hypothesis that BRD4 recruitment is allowable at the *FKBP5* GRE but not the *GILZ* GRE, though a

more detailed analysis of GR and BRD4 levels in each line is necessary. The repression of *MYC* transcription (Fig. 2.19C) was not observed by SDex-O3-CO2Me, though SDex-O3-JQ1 retained the ability of its parent moiety (S)-JQ1 to knockdown the expression of *MYC*. A trans addition of SDex-O3-CO2Me and (S)-JQ1-biotin was more effective at *MYC* repression; biotinylation will interfere with (S)-JQ1's ability to repress *MYC* transcription, while this indicates that conjugation to SDex may further inhibit that ability. Despite this, the observations in Fig. 2.19 indicate that SDex-O3-JQ1 is capable of influencing both GR and c-Myc signaling pathways. Accordingly, collaborative efforts are currently being undertaken to monitor the effects of SDex-O3-JQ1 on multiple myeloma proliferation

Effects of a bifunctional molecule on GR transrepression

A key target of GR transrepression is Nuclear Factor kappa-B (NF-κB) pathway^{34,35,76,77}, believed to be a primary mechanism of glucocorticoid-induced anti-inflammatory responses. Ligand-bound GR acts to physically interact with the transcription factor NF-κB, interfering with its ability to recruit the pTEF-b and subsequently RNA Polymerase II. To study the effects of BRD4 recruitment to a transrepressing GR molecule, a luciferase reporter experiment was conducted utilizing a reporter plasmid regulated by NF-κB.

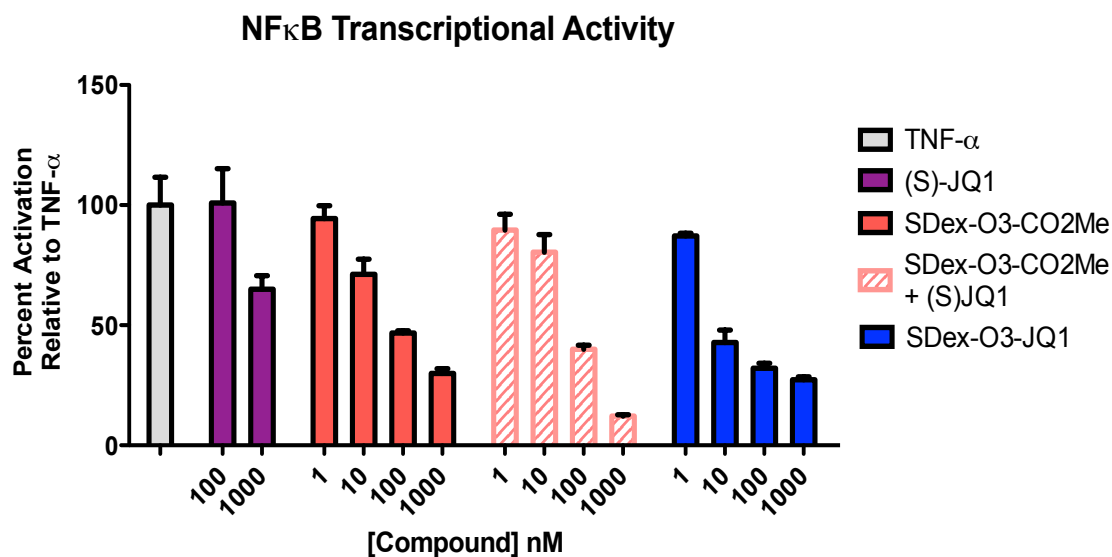


Figure 2.20 GR transrepression of NF- κ B activity. The effects of the designed ligands on NF- κ B activity were tested, where NF- κ B activity was stimulated and the effects of the ligands were determined in a reporter system.

U2OS cells were transfected with this reporter plasmid and an expression plasmid for GR, and NF- κ B activity was stimulated with the protein ligand TNF- α . NF- κ B activity was repressed with the indicated compounds and expressed as a percent of activity in cells stimulated with TNF- α alone (Fig. 2.20). SDex-O3-CO₂Me and SDex-O3-JQ1 repressed NF- κ B activity with similar potency and efficacy. Trans addition of SDex-O3-CO₂Me and (S)-JQ1 was the most efficacious modulator of repression, reducing NF- κ B activity to just 10% of stimulated activity. (S)-JQ1 has recently been shown to be capable of repressing NF- κ B activity as a monofunctional molecule, interfering with the recruitment of BRD4 to NF- κ B to form a coactivator complex, and as such the additive repressive activity by SDex-O3-CO₂Me and (S)-JQ1 is expected. The activity observed by SDex-O3-JQ1, however, would not be expected if BRD4 is being recruited to GR; given that GR transrepression is mediated through a steric hindrance mechanism, recruitment of additional proteins to this complex would be expected to further suppress transcription. The similarities between monofunctional and bifunctional molecule treatment is consistent with NF- κ B

transrepression by SDex-O3-JQ1 is only occurring through the glucocorticoid moiety of the molecule.

2.6 Conclusions and discussion

Synthetic linkage of the glucocorticoid agonist dexamethasone to FK506 provides a method for the recruitment of FKBP-fusion proteins to DNA-bound GR. Localization of an activator-FKBP fusion to GR raised the transcriptional activation potential past the level produced by a full glucocorticoid agonist. This effect was most potently demonstrated at a reporter gene, though it translated to the transcription of an endogenous gene modulated by GR. However, recruitment is influenced by GRE environment and enhanced activation does not occur universally at GR-regulated genes. Alternatively, recruitment of the repressor fusion protein HDAC1-FKBP facilitated the downregulation of GR transcriptional activity. Investigations into this effect were limited to one endogenous gene and, as with the recruitment of VP16-FKBP, this may transpire in a gene-specific manner. The transcriptional suppression of a reporter gene was only evident at a higher (1 μ M) concentration of bifunctional molecule, and lower concentrations produced a near identical transcriptional response as the monofunctional parent molecule. This may be a confounding factor native to all chemical dimerization systems, stemming from differences in ligand-protein affinities for each moiety of the bifunctional molecule. As such, dimeric GR-ligand complexes may be more prevalent than the trimeric FKBP-ligand-GR complex at low concentrations, allowing for the glucocorticoid portion of SDex-O2-FK506 to act as a simple partial agonist. Alternatively, this effect may be caused by GR's AF1; recruitment of HDAC1-FKBP likely interferes with the recruitment of coactivators through AF2, though the independent action of AF1 may overwhelm the ability of HDAC1-FKBP to repress transcription at lower concentrations.

The modular design of SDex-O2-FK506 allows for a facile replacement of FKBP as a recruitment partner for other targets. Substituting FK506 with (S)-JQ1, the

bifunctional SDex-O3-JQ1 demonstrated an ability to recruit BRD4 to GR in lysates, at the promoter of a reporter gene, and to an endogenous GRE. BRD4 binding of acetylated histone lysines is typically associated with upregulation of genes in close proximity⁶⁸; however, SDex-O3-JQ1 was not capable of triggering an enhanced level of transcription as was observed with the recruitment of VP16-FKBP. VP16 is an extremely potent viral activator, and this likely plays a factor in the lack of enhanced activation. Another likely explanation is that SDex-O3-JQ1 is unable to recruit BRD4 in a manner that allows for a transcriptionally favorable conformation. Accordingly, this is in line with observations that BRD4 recruitment actively inhibits the formation of a favorable transcriptional complex. In luciferase reporter studies, the transcriptional response to SDex-O3-JQ1 resembled SDex-O2-FK506 activity in the presence of HDAC1-FKBP. As seen with the recruitment of FKBP-fusions, it appears that the ability of a bifunctional glucocorticoid to recruit secondary proteins, or at very least for those proteins to influence transcriptional activity, may be influenced by the chromatin environment surrounding a GRE. Our ability to make comparisons between the effects of SDex-O3-JQ1 at different genes and in different cell lines is currently limited, but a few preliminary conclusions can be made. The *S100P* gene is responsive to glucocorticoid treatment likely due to the presence of a GRE in the *S100P* promoter in close proximity (218 bp) from the gene's transcriptional start site⁵⁵. This resembles the environment utilized in reporter experiments, wherein each GRE was incorporated in close proximity to the luciferase gene; accordingly, this may explain the similarity in transcriptional patterns of luciferase and *S100P* expression. This differs starkly from the landscape surrounding the *FKBP5* gene, where a GRE located in an intronic enhancer 26,000 bp downstream from the transcription start site is the putative cause for glucocorticoid responsiveness, and the *GILZ* environment, where transcriptional activity is directed by two GREs located 3000 bp upstream of the start site³¹. Predicting DNA-protein interactions in a three-dimensional environment is difficult, and it is unclear currently if the location of these GREs allow for a scenario in which recruitment partners can be properly positioned. These observations may allow us to predict the ability of

bifunctional molecules to differentially regulate genes with GREs located immediately upstream of a transcriptional start site; however, it would be premature to make this generalization. Current collaborative efforts are being undertaken to profile the effects of SDex-O3-JQ1 on a genomic scale and will undoubtedly help guide further conclusions.

The SDex-O3-JQ1 bifunctional molecule maintained the ability of its parent ligands to potently inhibit the expression of a NF- κ B-regulated gene. Paired with a reduced ability to mediate transactivation, this is a desired outcome in anti-inflammatory signaling. While GR inhibition of NF- κ B activity is mediated through a direct PPI, an enhanced transrepression was not observed by SDex-O3-JQ1 relative to a trans addition of SDex-O3-CO₂Me and (S)-JQ1. Given that both glucocorticoids and (S)-JQ1 are independently capable of transrepressing NF- κ B activity, discerning if BRD4 recruitment is occurring in this scenario is difficult. Currently, SDex-O3-JQ1 is being evaluated for its ability to suppress AP-1 signaling, an additional participant in the inflammation response that has no documented response to (S)-JQ1 treatment.

The developments described in Chapter 2 establish the full length GR as a viable target of transcriptional modulation by bifunctional ligands. This strategy has been used to both enhance activation past full agonist-induced levels and to suppress GR from fully activating transcription in manners dependent on recruitment. Moreover, this work has demonstrated that much of this activity is dependent on the structural environment of a modulated gene, hinting at the possibility for selective gene modulation and the production of further unique transcriptional outputs. This idea is first progressed in the research described in Chapter 3. As more selective, potent ligands of epigenetic proteins continue to be developed, the lessons learned here will hopefully guide future efforts to chemically induce PPIs and build novel transcriptional complexes.

2.7 Materials and Methods

Cell culture and transfections

HeLa (CCL-2), U2OS (HTB-96), and A549 (CCL-185) cells were purchased from ATCC. 293T cells were a gift from J. Iñiguez-Lluhí. Cells were cultured in DMEM with 10% FBS at 37°C under 5% CO₂. MM1.S cells were a gift from J. Bradner and were cultured in RPMI-1640 with 10% FBS under otherwise identical conditions. Transient transfections were done using Lipofectamine or Lipofectamine 2000 (Invitrogen) with conditions optimized using GFP expression plasmid and fluorescent analysis or by using a β -gal expression plasmid and activity assay.

Plasmids

All oligonucleotides for construction of plasmids were purchased from Integrated DNA Technologies.

pCMV- β -gal is a β -galactosidase coding plasmid driven by a CMV promoter and was generously provided by J. Iñiguez-Lluhí.

pCDNA3 hGR (human GR coding plasmid) and p6R GR (rat GR coding plasmid) were gifts from J. Iñiguez-Lluhí.

pVP16-FKBP is a mammalian expression plasmid that encodes a fusion of Gal1-11 (for efficient expression), NLS from SV40 large T antigen (nuclear localization signal), VP16(411-456) (potent activation domain, activation domain 1 from the herpes simplex virion protein 16), and human FKBP1A(2-107) (FK506 binding protein 1A, 12kDa). It was constructed by cloning of FKBP1A into pAct. The coding sequence is shown below, with the elements listed above marked with capital letters in brackets:

[ATGAAGCTACTGTCTTCTATCGAACAAGCATGC][CCAAAAAAGAAGAGAAA
GGTAGAT]gaattcccgggg[ATCTCGACGGCCCCCGACCGATGTCAGCCTGG
GGGACGAGCTCCACTTAGACGGCGAGGACGTGGCGATGGCGCATGCCGA
CGCGCTAGACGATTTGATCTGGACATGTTGGGGGACGGGGATTCCCCGG
GTCCGGGA]tcgccaggatcc[GGCGTGCAGGTGGAGACTATCTCCCCAGGAGAC
GGGCGCACCTTCCCCAAGCGCGGCCAGACCTGCGTGGTGCCTACACCGG
GATGCTTGAAGATGGAAAGAAATTTGATTCCTCCCGGGACAGAAACAAGCC
CTTTAAGTTTATGCTAGGCAAGCAGGAGGTGATCCGAGGCTGGGAAGAAGG
GGTTGCCCAGATGAGTGTGGGTCAGAGAGCCAAACTGACTATATCTCCAGA
TTATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCAC
TCTCGTCTTCGATGTGGAGCTTCTAAACTGGAAAGATCTGTGCGACTTGACG
CGT]

pLIC-FKBP is a cloning vector designed for making fusions to FKBP (and Gal4(1-11) and SV40 large T NLS). This was made by cutting VP16 out of pVP16-FKBP with EcoRI and BamH1, and ligating in an LIC cassette in the form of annealed oligos: 5'- AAT TGG GAA GCA CCG GTT CTG GTG ATC -3' and 5'- GAT CGA TCA CCA GAA CCG GTG CTT CCC -3'. To prepare the vector for LIC cloning, it was digested with AgeI and processed with T4 DNA polymerase (Novagen) with dTTP added as the only nucleotide. Genes to be cloned into the vector were PCR amplified with primers that have sequences appended to 5' end of gene specific sequence. These appendages are: Sense-primer 5'- GGGAAGCACCGGT; Antisense-primer 5'- CACCAGAACCGGT.

pLIC-myc₆ is a LIC cloning⁷⁸ vector to facilitate fusion of genes to six myc-tags in a mammalian expression vector. It was prepared by performing site-directed mutagenesis to pCS2+MT with the primers: 5'- CAA GCT ACT TGT TCT TTT TGC ACC ATG GGA AGC ACC GGT TCT GGT GAG ATG GAG CAA AAG CTC ATT TCT G -3' and 5'- CAG AAA TGA GCT TTT GCT CCA TCT CAC CAG AAC CGG TGC TTC CCA TGG TGC AAA AAG AAC AAG TAG CTT G -3'. To prepare the vector for LIC cloning, it was digested with AgeI and processed with T4 DNA polymerase (Novagen) with dTTP added as the only nucleotide. Genes to be cloned into the vector were PCR amplified with primers that have sequences appended to 5' end of gene specific sequence. These appendages

are: Sense-primer 5'- GGGAAGCACCGGT; Antisense-primer 5'- CACCAGAACCGGT.

pGR-myc₆ was prepared with LIC of human GR into pLIC-myc₆.

pHDAC1-FKBP was constructed by inserting the sequence from mouse histone deacetylase-1 into pLIC-FKBP.

pHDAC1(D99A)-FKBP was constructed by single primer site-directed mutagenesis using the following primer:

F-Pr 5'- TGTTGGTGAGGCCTGTCCGGTATTTGATGG -3'

pConsensus GRE-luc, pFKBP5(GRE)-luc, and pGILZ(GRE)-luc were generously provided by K. Yamamoto (UCSF).

pNF-κB-luc was provided generously by G. Nuñez (Univ. of Michigan).

Mammalian three-hybrid assay (Figs. 2.7 and 2.10)

3,000,000 293T cells were plated in a 10 cm dish in 10 mL DMEM. The following day they were transfected by replacing media with transfection mix: 400 ng pGBR 6.1 luc, 200 ng pCMV-β gal, 50 ng of p6rGR or pCDNA3 hGRα (as indicated), 200 ng of indicated FKBP expression plasmid, 7.1 μg pBSKS, and 16 μL Lipofectamine in 5 mL serum-free DMEM. After 6 hours, the transfection mix was replaced with DMEM + 5% charcoal stripped FBS and allowed to recover overnight. The following day, cells were removed by trypsinization and replated at a density of 10,000 cells per well in a 96-well dish. Individual wells were treated with the indicated compounds to a final DMSO concentration of 0.01%. After 16 hours, media was removed and cells were lysed using a passive-lysis buffer. β-gal activity was assayed as described previously using a Molecular Devices Spectramax plate reader. Luciferase activity was determined as

described previously using a Molecular Devices Spectramax L luminometer. Standard error from triplicate samples is represented by error bars on graph.

Transcriptional reporter assay (Fig. 2.15 and 2.16)

U2OS cells were seeded at a density of 250,000 cells per well in a 6-well plate with 2 mL DMEM + 10% FBS. The following day, cells were transfected with 1 µg of indicated pGRE-luc, 0.5 µg of pCDNA3 hGR α , 0.5 µg of pCMV- β gal, and 5 µL of Lipofectamine 2000. After 6 hours, the transfection mix was replaced with DMEM + 5% charcoal stripped (CS) FBS and allowed to recover overnight. The following day, cells were removed by trypsinization and replated at a density of 10,000 cells per well in a 96-well dish. Individual wells were treated with the indicated compounds to a final DMSO concentration of 0.01%. After 16 hours, media was removed and cells were lysed using a passive-lysis buffer. β -gal activity was assayed as described previously using a Molecular Devices Spectramax plate reader. Luciferase activity was determined as described previously using a Molecular Devices Spectramax L luminometer. Standard error from triplicate samples is represented by error bars on graph.

RT-qPCR (Figs. 2.10, 2.17, 2.18, and 2.19)

50,000 transfected U2OS, A549, or MM1.S cells were plated in 24-well plate with 0.5 mL DMEM + 5% CS FBS. Next day cells were treated with compounds or DMSO (0.2% final DMSO for all samples). Total RNA was isolated with Qiagen RNeasy Plus mini kit. cDNA synthesis was done with iScript RT Mastermix (Biorad). 20 µL qPCR reaction mix: 2 µL of produced cDNA, 0.2 µL CXR, 0.4 µL forward primer (200 nM final), 0.4 µL reverse primer, 7 µL H₂O, 10 µL GoTaq qPCR master mix (Promega). qPCR was done on ABI StepOne Plus. Products were analyzed with melt curve for quality control. Transcript levels were normalized to the levels of a housekeeping gene, *RPL19*. Activation was displayed relative to transcript levels in DMSO-treated cells and determined using the $\Delta\Delta C_T$ method. Primer pairs used:

FKBP5: F-Pr 5'- GGAATGGTGAGGAAACGCCG -3'
FKBP5: R-Pr 5'- CTCTCCTTTCTTCATGGTAGCCACC -3'
GILZ: F-Pr 5'- CGAACAGGCCATGGATCTGGTGAA -3'
GILZ: R-Pr 5'- AGAACCACCAGGGGCCTCGG -3'
S100P: F-Pr 5'- CGGAACTAGAGACAGCCATGGGCAT -3'
S100P: R-Pr 5'- AGACGTGATTGCAGCCACGAACAC -3'
MYC: F-Pr 5'- AAACACAAACTTGAACAGCTAC -3'
MYC: R-Pr 5'- ATTTGAGGCAGTTTACATTATGG -3'
RPL19: F-Pr 5'- ATGTATCACAGCCTGTACCTG -3'
RPL19: R-Pr 5'- TTCTTGGTCTCTTCCTCCTTG -3'

Co-immunoprecipitation (Fig. 2.13)

α -BRD4 was purchased from Bethyl laboratories (A301). α -myc-HRP was purchased from Santa Cruz Biotechnology (9E10). 3,000,000 293T cells were seeded in a 10-cm dish in 10 mL of DMEM + 10% FBS. The following day, cells were transfected with 4 μ g of pCDNA3 and 4 μ g of phGR-myc₆ with 16 μ L of Lipofectamine 2000 in 5 mL DMEM + 5% CS FBS. One day following transfection, cells were lysed in RIPA buffer including HALT protease inhibitor cocktail (Life Technologies), sonicated, and cleared. Lysate protein concentration was quantified by BCA assay. 6 μ g of α -BRD4 was incubated with 30 μ L of Protein G Dynabeads and subsequently washed. Antibody-coated beads were incubated 100 μ g of lysate and the indicated compound, at a final DMSO concentration of 0.4% for 2 hours at room temperature. Beads were washed and bound proteins were eluted in Laemmli buffer. The presence of hGR-myc₆ in eluent was detected by Western blot using α -myc-HRP.

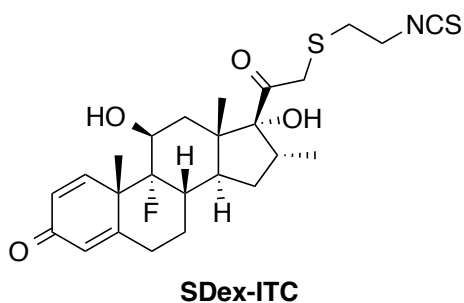
Transrepression reporter assay (Fig. 2.20)

U2OS cells were seeded at a density of 250,000 cells per well in a 6-well plate with 2 mL DMEM + 10% FBS. The following day, cells were transfected with 1 μ g

of indicated pNF- κ B-luc, 0.5 μ g of pCDNA3 hGR α , and 0.5 μ g of pCMV- β gal. After 6 hours, the transfection mix was replaced with DMEM + 5% charcoal stripped FBS and allowed to recover overnight. The following day, cells were removed by trypsinization and replated at a density of 10,000 cells per well in a 96-well dish. Individual wells were treated with the indicated compounds to a final DMSO concentration of 0.01%, along with hTNF- α (Roche) at a final concentration of 5 ng/mL. After 16 hours, media was removed and cells were lysed using a passive-lysis buffer. β -gal activity was assayed as described previously using a Molecular Devices Spectramax plate reader. Luciferase activity was determined as described previously using a Molecular Devices Spectramax L luminometer. Standard error from triplicate samples is represented by error bars on graph.

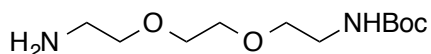
Chemical synthesis

FK506 was purchased from LC Laboratories. Dexamethasone was purchased from Enzo Life Sciences. Commercially available reagents and solvents were used as received. Chromatographic separations were carried out on silica gel 60 (230-400 mesh, E. Merck) or by reverse phase HPLC on C18 column using the indicated eluents. Yields are unoptimized. ESI-MS spectra were obtained on Micromass LCT TOF mass spectrometer. High-resolution mass spectra (HRMS) were obtained on Micromass AutoSpec Ultima Magnetic sector mass spectrometer. 1 H-NMR spectra were obtained at 400MHz on a Varian MR-400 spectrometer. Chemical shifts are given in δ (ppm) values.



SDex-ITC was synthesized by J. Carolan and J. Højfeldt following a published procedure⁴⁵.

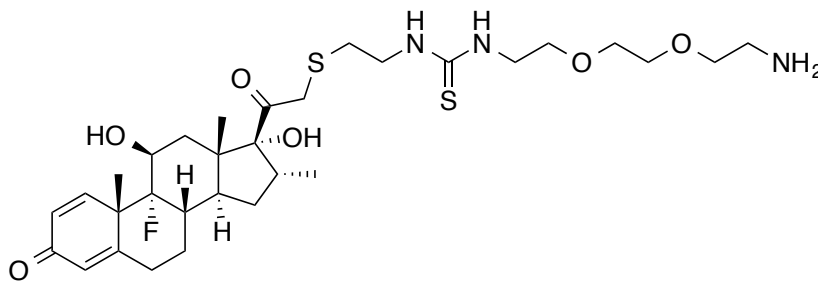
Dexamethasone (1.00 g, 2.5 mmol) was dissolved in pyridine (7 mL) and cooled to 0°C. Methanesulfonyl chloride (0.26 mL, 3.3 mmol) was added to the mixture dropwise over 15 min. After stirring at 0°C for 30 min, the reaction mixture was poured into water (300 mL) at 0°C. The precipitation was collected by filtration and washed with water. The obtained product and tert-butyl 2-sulfanylethylcarbamate (1.24 mL, 7.3 mmol) were dissolved in acetone (24 mL). Triethylamine (2.05 mL, 7.3 mmol) was added to the solution and then the resulting mixture was stirred at room temperature for 15 h. The reaction mixture was diluted with water and extracted with ethyl acetate. The extract was washed with water and brine, dried over sodium sulfate and concentrated in vacuo. The residue was crystallized from ethyl acetate-hexane to give a colorless powder. The obtained powder was dissolved in 1.5 M hydrochloric acid in acetic acid (1 mL) and then the mixture was stirred at room temperature for 15 min. The mixture was diluted with water and chloroform and basified with saturated aqueous sodium carbonate solution and the slurry was stirred at room temperature for 15 min (pH of the slurry should be over 10). Thiophosgene (0.18 mL, 2.4 mmol) was added to the slurry, and then the resulting mixture was stirred at room temperature for 1.5 h. The chloroform layer was separated, and the layer was washed with water and brine, dried over sodium sulfate and concentrated in vacuo. The residue was purified by silica gel chromatography (ethyl acetate/hexane = 1/4 to 1/1) to give product (150 mg, 18%) as a pale yellow powder. ¹H NMR (400MHz, CDCl₃): δ 0.88 (3H, d, J = 7.2 Hz), 1.02-1.80 (10H, m), 2.01-2.57 (6H, m), 2.81 (2H, t, J = 6.8 Hz), 3.04-3.07 (1H, m), 3.24 (1H, d, J = 13.6 Hz), 3.58 (1H, d, J = 14.0 Hz), 3.71 (2H, t, J = 5.8 Hz), 4.34 (2H, d, J = 7.6 Hz), 6.08 (1H, s), 6.29 (1H, d, J = 8.4 Hz), 7.14 (1H, d, J = 10 Hz). ESI-MS calculated for [C₂₅H₃₂FNO₄S₂ + H]⁺: 494.1, found 494.0.



Boc-Linker

Boc-linker was synthesized by J. Carolan and A. Van Dyke.

To a solution of 2-[2-(2-aminoethoxy)ethoxy]ethanamine (5.00 g, 33.7 mmol) in ethanol (50 mL) was added a solution of di-tert-butyl dicarbonate (2.45 g, 11.2 mmol) in dichloromethane (10 mL) dropwise at 0 °C over 2 h. After stirring at room temperature for 15 h, the mixture was concentrated *in vacuo*. The residue was diluted with ethyl acetate and acidified with 10% aqueous citric acid solution. The aqueous layer was collected, washed with ethyl acetate twice, basified with 3 M NaOH and extracted with dichloromethane. The extract was dried over Na₂SO₄ and concentrated *in vacuo* to give Boc-linker (2.02 g, 73%) as a colorless oil. ¹H NMR (500MHz, CDCl₃): d 1.45 (9H, s), 2.89 (2H, t, *J* = 5.5 Hz), 3.32-3.34 (2H, m), 3.51-3.57 (4H, m), 3.61-3.64 (4H, m), 5.15 (1H, br s). ESI-MS calcd for [C₁₁H₂₄N₂O₄ + H]⁺: 249.1, found 249.1.

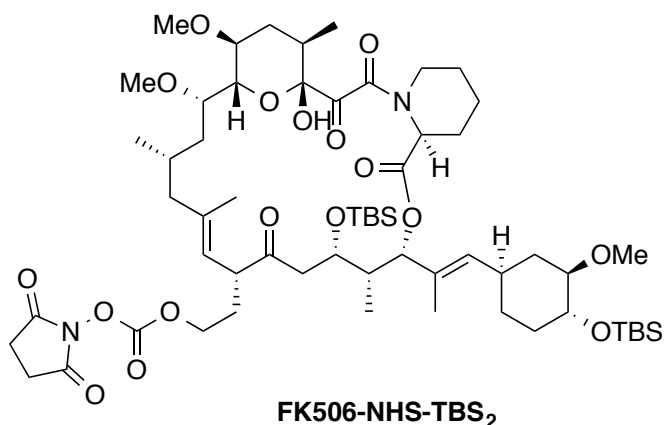


SDex-O₂-NH₂

SDex-O₂-NH₂ was synthesized by J. Carolan and Y. Imaeda.

A solution of Boc-linker (50 mg, 0.20 mmol) and SDex-ITC (100 mg, 0.20 mmol) in dichloromethane (5 mL) was stirred at room temperature for 15 h. After concentration *in vacuo*, the residue was purified by silica gel chromatography (ethyl acetate/hexane = 1/3 to 3/1). The product was treated with concentrated

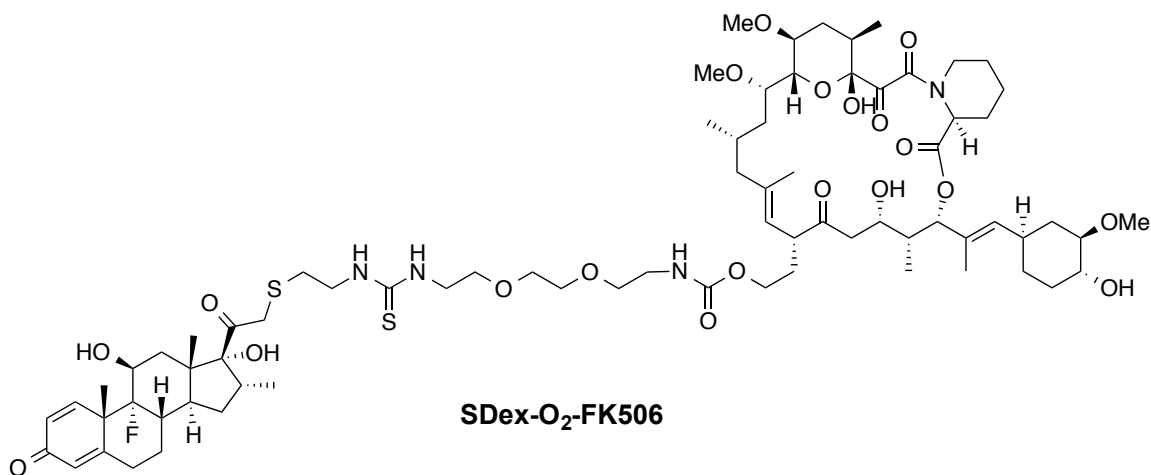
hydrochloric acid (1 mL) and ethanol (2 mL) and then stirred at room temperature for 1 h. The mixture was basified to pH 12 with 3 M aqueous NaOH solution and extracted with dichloromethane twice. The extract was dried over Na₂SO₄ and concentrated in vacuo to give compound **11** (80 mg, 62%) as a pale yellow amorphous powder. ¹H NMR (400MHz, CDCl₃): δ 0.82-1.76 (19H, m), 2.09-2.34 (6H, m), 2.51-2.71 (3H, m), 2.86 (1H, m), 3.03-3.05 (1H, m), 3.41-3.66 (12H, m), 4.27 (1H, d, *J* = 10.0 Hz), 5.23 (2H, s), 6.04 (1H, s), 6.25 (1H, d, *J* = 10.4 Hz), 7.15 (1H, d, *J* = 10 Hz). ESI-MS calcd for [C₃₁H₄₈FN₃O₆S₂ + H]⁺: 642.3, found 642.3.



FK506-NHS-TBS₂ was synthesized by J. Carolan and Y. Imaeda following a published procedure⁵⁰.

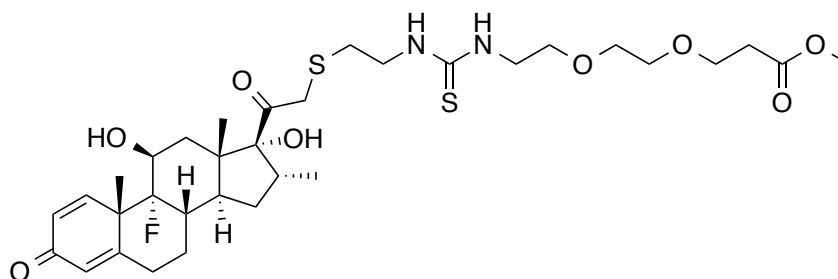
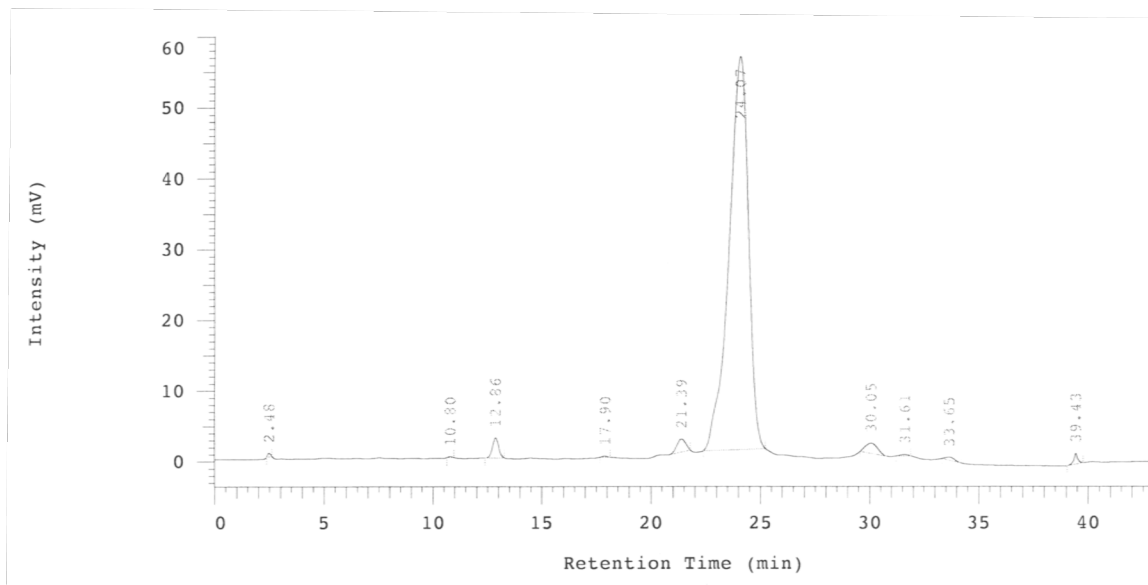
To a solution of FK506 (400 mg, 0.50 mmol) and 2,6-lutidine (0.29 mL, 2.5 mmol) in dichloromethane (10 mL) was added tert-butyldimethylsilyl trifluoromethanesulfonate (TBSOTf; 0.46 mL, 2.0 mmol) dropwise at 0°C under a nitrogen atmosphere. After stirring at 0°C for 1 h, the reaction mixture was diluted with dichloromethane. The solution was washed with water, 10% aqueous citric acid solution and brine then dried over Na₂SO₄ and concentrated *in vacuo*. The oil product was dissolved in tetrahydrofuran (THF; 16 mL) and water (3.2 mL) and charged with 4-methylmorpholine *N*-oxide (4-NMO; 278 mg, 2.4 mmol) and

osmium tetroxide (2.5% wt in *tert*-butanol; 0.97 mL, 2.4 mmol). After stirring at room temperature for 2.5 h, the reaction mixture was diluted with 50% aqueous methanol (4.8 mL). Sodium periodate (1.02 g, 4.8 mmol) was added to the mixture and stirred at room temperature for 1 h. The mixture was extracted with diethyl ether. The extract was washed with brine, dried over Na₂SO₄, and concentrated *in vacuo*. The resulting product was dissolved in THF (16 mL) and treated with LiAlH(OCEt₃)₃ solution (0.5M in THF; 1.42 mL, 0.71 mmol) at -78°C. After stirring for 40 min, the reaction was quenched with saturated aqueous ammonium chloride (1.6 mL), dried over Na₂SO₄ and concentrated *in vacuo*. The product was then dissolved in acetonitrile, 2,6-lutidine (1.11 mL, 9.5 mmol) and *N,N'*-disuccimidyl carbonate were added. After stirring at room temperature for 18 h, the reaction mixture was concentrated *in vacuo*. The residue was diluted with ethyl acetate, washed with water, 5% aqueous citric acid solution, and brine, dried over Na₂SO₄, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (ethyl acetate/hexane = 1/3 to 2/5) to give compound FK506-NHS-TBS₂ (350 mg, 63%) as a pale yellow film. ¹H NMR (400MHz, CDCl₃): δ 0.00-0.12 (12H, m), 0.84-1.06 (22H, m), 1.28-2.37 (44H, m), 2.87-2.89 (3H, m), 2.98 (1H, m), 3.35-3.63 (10H, m), 3.82-5.27 (8H, m). ESI-MS calcd for [C₆₀H₁₀₀N₂O₁₇Si₂ + Na]⁺: 1199.6, found 1199.6.



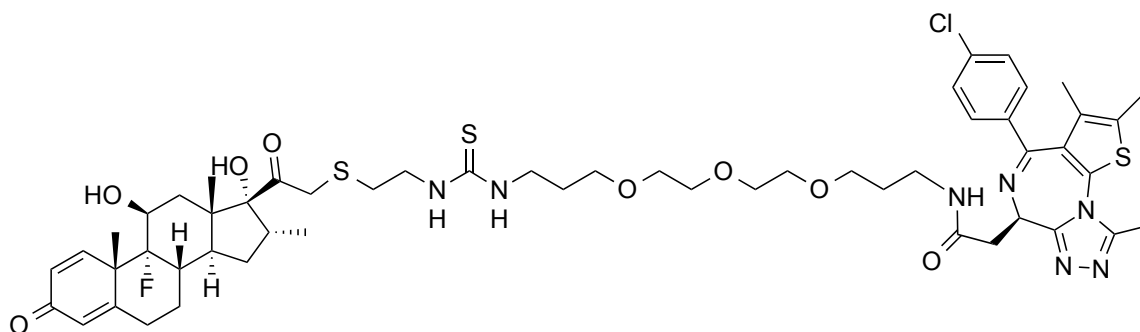
SDex-O₂-FK506 was synthesized by J. Carolan and Y. Imaeda, adapting a published procedure⁵⁰.

A mixture of SDex-O₂-NH₂ (16 mg, 0.025 mmol), FK506-NHS-TBS₂ (30 mg, 0.025 mmol) and *N,N*-diisopropylethylamine (0.009 mL, 0.050 mmol) in dichloromethane (0.5 mL) and DMF (0.5 mL) was stirred at room temperature for 3 days. The mixture was diluted with dichloromethane, washed with water, 5% aqueous citric acid solution, and brine, dried over Na₂SO₄ and concentrated *in vacuo*. The residue was purified by silica gel chromatography (ethyl acetate/hexane = 1/3 to 4/1) to give the TBS protected product (10 mg). The product (5 mg) was treated with 1.5 M HCl in AcOH (0.5 mL) and water (0.1 mL) and then stirred at room temperature for 30 min. The mixture was neutralized with NaHCO₃ powder and suspended in acetonitrile. The mixture was filtered and the filtrate was concentrated *in vacuo*. The residue was purified by HPLC (0.1% aqueous TFA/acetonitrile = 45/55 to 25/75) to give compound SDex-O₂-FK506 (1.8 mg, 42%) as a colorless amorphous powder, calculated to be 95% pure through peak integration. ¹H NMR (400MHz, CD₃OD): δ 0.86-1.12 (19H, m), 1.22-2.35 (50H, m), 2.76 (3H, br s), 3.04-3.15 (3H, m), 3.38-4.25 (33H, m), 5.08-5.22 (3H, m), 6.07 (1H, s), 6.26-6.29 (1H, m), 7.40 (1H, m). HRMS (ESI) calcd for [C₇₅H₁₁₅FN₄O₂₀S₂ + Na]⁺: 1497.7422, found 1497.7415. HPLC chromatogram of purified product is displayed below:



SDex-O₂-CO₂Me

SDex-O₂-CO₂Me was synthesized by A. Van Dyke.



SDex-O₃-(R)JQ1

SDex-O₃-(R)JQ1 was synthesized by A. Van Dyke and J. Qi.

2.8 References

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Chapter 3

Functional Agonism by a Glucocorticoid Antagonist-Based Bifunctional Molecule*

3.1 Abstract

Glucocorticoids, such as dexamethasone and prednisolone, are commonly used anti-inflammatory and immunosuppressive drugs prescribed for the treatment of such maladies as asthma, arthritis, and various allergic disorders. Long-term use of this class of therapeutics is limited by mild to severe side effects, believed to be through alternative receptor actions. Current efforts to produce ligands capable of dissociating desirable from undesirable actions have been unsuccessful. Bifunctional glucocorticoids, acting through mechanisms distinct from classical glucocorticoid ligands, are capable of producing altered transcriptional profiles that may prove useful in this endeavor. Here, we characterize the actions of a bifunctional molecule constructed from a glucocorticoid antagonist, RU486, that instigates novel glucocorticoid receptor activity.

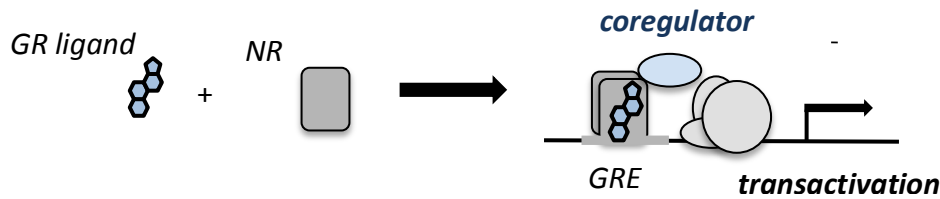
3.2 Limitations of current glucocorticoid ligands

* The work described in this chapter is the product of collaborative efforts. J. Carolan, A. Van Dyke, J. Bradner, and A. Mapp conceived of the experiments described. The compounds described in Figure 3.2 were synthesized by J. Carolan, J. Højfeldt, A. Van Dyke, and J. Qi. The data presented in Fig. 3.3 were collected by A. Van Dyke. All other experiments were performed by J. Carolan. Where indicated, statistical significances are represented as *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

It is well established that the glucocorticoid receptor (GR) regulates the transcription of genes as a response to ligand-interaction^{1,2} through two distinct mechanisms: transactivation, involving the formation of multiprotein complexes at GR-binding sequences (Fig. 3.1A)³⁻⁵; and transrepression, involving the indirect or direct activation disruption by other transcription factors (Fig. 3.1B)⁶⁻¹¹. GR has been identified as central to perturbing the inflammation response, and many have recognized that this proceeds largely through GR transrepression¹²⁻¹⁴. Given this pivotal role in repressing inflammation, innumerable pharmaceutical efforts have been devoted to producing novel glucocorticoid ligands as anti-inflammatory therapeutics. Many potent glucocorticoids have been described¹⁵⁻¹⁸ that differ in pharmacokinetic and pharmacodynamics properties; however, they largely share the same mechanism of action and universally produce similar undesirable side effects. A paradigm has arisen wherein it is believed that many of these effects arise through GR transactivation, and, as such, glucocorticoids with the highest therapeutic indices would be those that best dissociate GR transrepression from transactivation¹⁵. Accordingly, many efforts of the past decade to selectively modulate GR activity have focused on producing novel ligands that induce GR conformations capable of separating these responses¹⁹⁻

²².

A



B

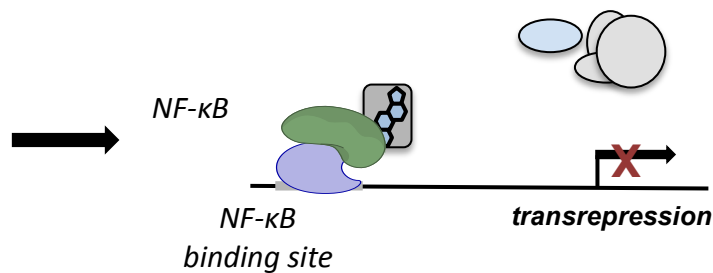


Figure 3.1 Distinct mechanisms of GR action. A: GR transactivation occurs following GR ligand binding, causing DNA-binding and increased transcription of regulated genes. B: GR transrepression occurs through several mechanisms, such as with the NF- κ B pathway, where GR directly interferes with recruitment of the transcriptional machinery to DNA-bound NF- κ B.

There have been successful structural characterizations of individual GR domains, helping guide the synthesis of new ligands^{9,23}. However, structural data has been limited for the full-length receptor²⁴, making it difficult to predict the global allosteric effects of ligand binding. Recent biochemical characterizations have established that, despite its modularity, GR is a structurally plastic protein and its activities are highly dependent on total protein conformation^{2,25,26}. This realization helped guide the landmark discovery that the short, six base pair (bp) half-sequence of a glucocorticoid response element (GRE) is capable of serving as an allosteric ligand in and of itself⁴.

Several additional insights regarding the non-ligand regulation of GR activity have further complicated efforts to rationally design GR modulators. GR is subject to varying post-translational modifications (PTMs)^{27,28}, including phosphorylation²⁹⁻³¹, acetylation³², sumoylation³³, and ubiquitylation^{34,35}, alongside varying, cell-specific levels of modification, that influence resultant GR activity^{36,37}. Moreover, the long-held belief that GR activity is modulated through a single splice isoform, GR α , has recently been disputed; transcriptional activities have begun to be attributed to alternative GR splice isoforms^{2,38}. It has also been discovered that the GR coding gene, *NR3C1*, harbors multiple non-traditional transcriptional start sites, resulting in the production of alternative GR α protein products, each with similar ligand-binding capabilities though producing distinct transcriptional profiles^{2,4,38,39}. Proceeding from these discoveries, over 350 coregulators have been detected as potential interaction-partners with members of the GR family^{40,41}.

This collected knowledge reveals insights into why there has been limited success in producing ligands that produce desirable therapeutic outcomes solely through allosteric rearrangement of the GR ligand-binding domain (LBD). Importantly, this may expose limitations into the transactivation/transrepression dissociation model as the gold standard for GR modulation, a realization compounded by recent reports of certain anti-inflammatory gene products being produced through GR transactivation^{42,43}. Accordingly, we need new methods for targeting GR, not new ligands.

In Chapter 2, we demonstrated our ability to produce novel GR transcriptional profiles by synthetically modifying an existing glucocorticoid molecule. Learning from recent insights into GR function and inflammation, we reason that the *selective* activation of GR-regulated genes is of high importance, and this may be accomplished through the use of a ligand ordinarily incapable of activation. Here, we describe work to develop an antagonist-based bifunctional molecule to accomplish this goal.

3.3 Developing an antagonist-based bifunctional ligand

As mentioned above, pharmaceutical efforts have been fruitful in developing synthetic glucocorticoid agonists. Surprisingly, there is only one clinically prescribed antiglucocorticoid⁴⁴. RU486 (clinically known as mifepristone) was developed by scientists at Roussel-Uclaf in the 1980's⁴⁵. Maintaining a high binding affinity for GR, this synthetic steroid antagonizes glucocorticoid binding⁴⁴. Interestingly, RU486 is capable of inducing GR nuclear translocation⁴⁶, manifesting in a low transactivation potential⁴⁷ believed to be dependent on context through the GR AF1 domain⁴⁸, though this is disputed⁴⁹. However, RU486 bears a dimethylaniline moiety stemming from C₁₁ of the steroidal core that projects from the GR LBD, disrupting helix 12 from forming an active coactivator-recruiting conformation⁹; limited evidence supports the notion that this disruption may induce helix 12-facilitated recruitment of corepressor proteins⁴⁸.

Owing to its antiglucocorticoid activity, RU486 is frequently used to treat Cushing's syndrome, a set of symptoms that typically emerge in response to long-term glucocorticoid use or adrenal gland hyperactivity causing elevated cortisol-release⁵⁰. As a therapeutic, RU486 suffers limitations in use because of pan-steroidal anti-activity; indeed, it was originally developed as an abortifacient through an anti-progestin mechanism, antagonizing progesterone receptor (PR) activity, and it maintains weak androgen receptor (AR) activity⁵¹. While this promiscuity curbs therapeutic RU486 use, it marks it as a potentially appealing tool to investigate the effects of bifunctional molecule-mediated recruitment on a variety of NRs while also expanding our collective ability to modulate GR activity through alternative mechanisms.

The amino group of the dimethylaniline moiety has previously been used as a conjugation point for the synthetic linkage of RU486 to a variety of targeting

groups, including glucuronic acid⁵² and bile acid^{53,54}, as a method for cell-specific localization of anti-glucocorticoid activity. Despite this chemical modification, RU486-conjugates maintain a high affinity for GR⁵³. Accordingly, we developed a similar strategy to synthetically link RU486 to FK506. While we noted a marked (~10-fold) decrease in binding affinity for the receptor following conjugation, with the K_d of binding GR for RU486 to be 13 nM whereas conjugates displayed K_d 's determined to be 83.6-93.9 nM, the bifunctionalized RU486 molecule was able to recruit FKBP-fusion proteins to GR, resulting in an effect on the transcription of both reporter and endogenous genes⁵⁵. Encouragingly, when recruiting VP16-FKBP, this effect manifested as an activation of transcription; in essence, synthetically converting RU486 allowed for a reprogramming of its intrinsic, antagonistic nature, initiating its actions as an agonist-like glucocorticoid.

This prior work established that the intrinsic properties of a GR ligand can be dissociated from consequent functional effects through extrinsic recruitment, and, more importantly, established RU486 as a building block for additional bifunctional ligands. If we can effect a similar antagonist-to-agonist reprogramming through the recruitment of an endogenous cellular target, this exponentially increases the potential matrix of modules available for bifunctional molecule design and, subsequently, the potential for unique transcriptional profiles.

3.4 Recruitment of BRD4 by an antagonist-based bifunctional ligand

In the work described in Chapter 2, we established that synthetically tethering a glucocorticoid agonist to the bromodomain inhibitor (S)-JQ1⁵⁶ allows for the targeted recruitment of BRD4, though this appeared to be a gene-specific event. Moreover, the functional effects of BRD4 recruitment appeared to significantly vary based on the nature of the utilized receptor; whereas recruitment to a truncated GR containing only the GR led to robust transcriptional activation, the recruitment of BRD4 to a full length GR resulted in the production of a more

nuanced transcriptional profile. These observations made it difficult to predict the functional outcomes of recruiting BRD4 to antagonist-bound GR, compounded by the unclarity regarding whether a disrupted helix 12 will accommodate chemical dimerization with BRD4. To begin this investigation, we synthesized the following molecules (Fig. 3.2): RU-O3-N3, a glucocorticoid antagonist appended to a synthetic linker; and RU-O3-JQ1, a bifunctional molecule linking RU486 to (S)-JQ1.

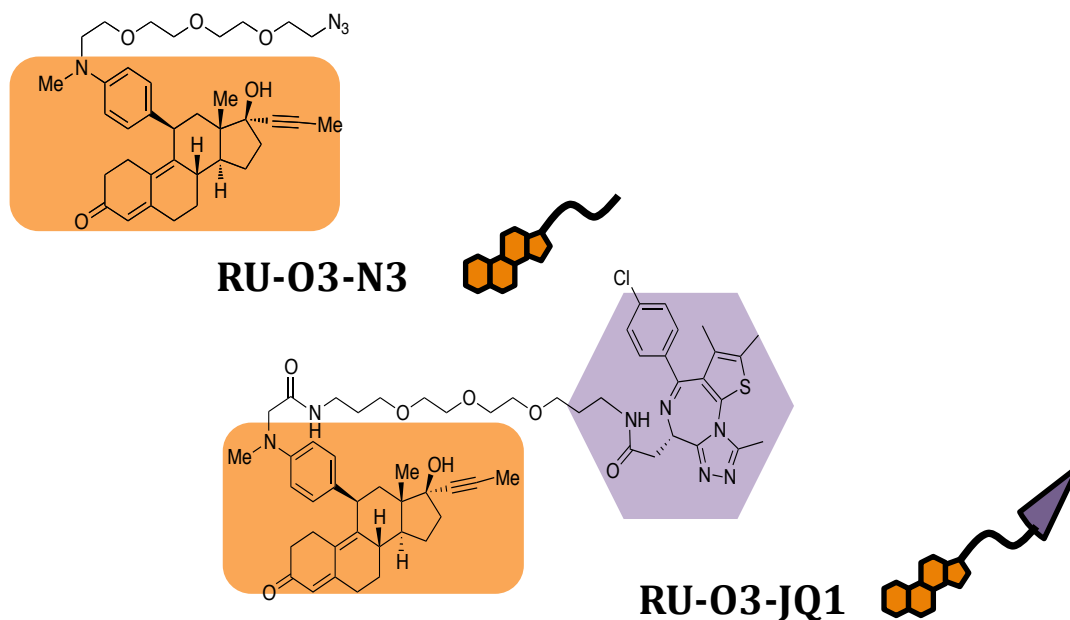


Figure 3.2 Bifunctional antagonist-based molecules. A monofunctional GR antagonist-linker molecule, RU-O3-N3, was constructed, along with the bifunctional RU-O3-JQ1.

The synthesized bifunctional molecule was demonstrated to display a modest (~10-fold) loss in binding affinity for BRD4 in an *in vitro* assay (unpublished observations, collected by Jun Qi).

BRD4 recruitment in a traditional three-hybrid experiment

To investigate the effects of antagonist-facilitated BRD4 recruitment on transcription, we employed a traditional three-hybrid experiment (depicted in Fig. 3.3A). This arrangement additionally allows us to confirm that RU-O3-JQ1 is capable of chemically dimerizing GR and BRD4 in a cellular system.

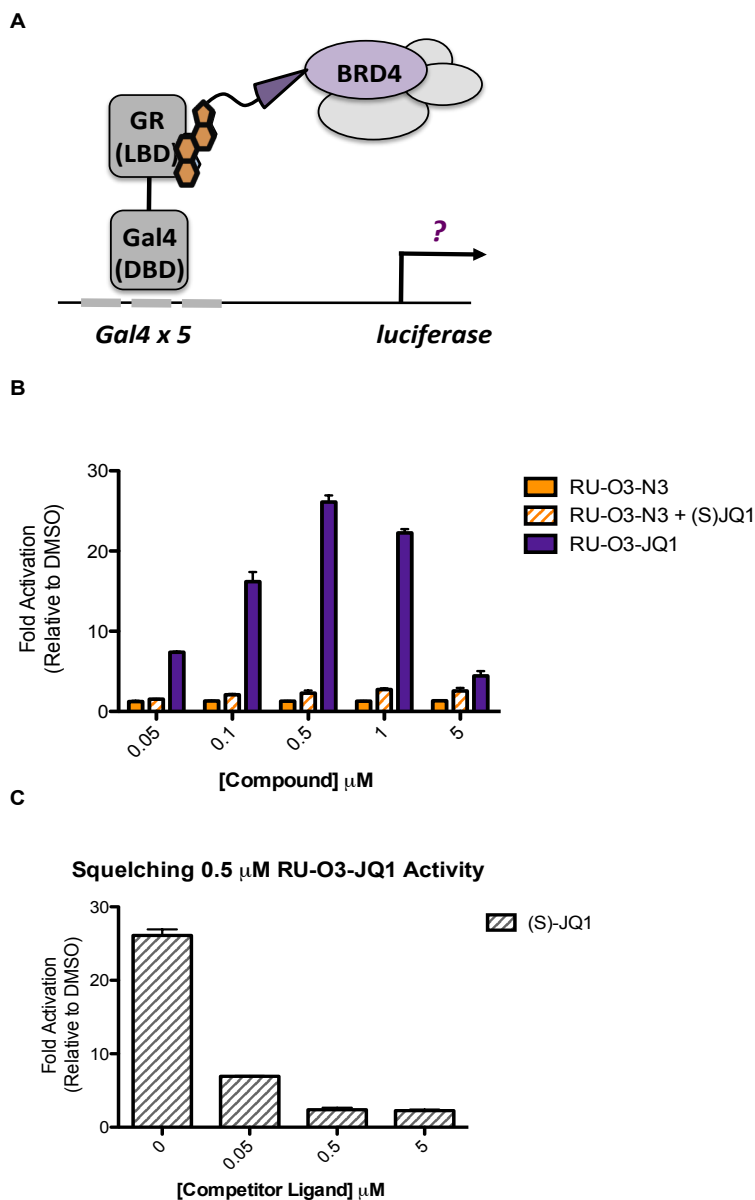


Figure 3.3 Recruitment of endogenous BRD4 in a traditional three-hybrid experiment. A: a traditional three-hybrid design, using a Gal4-GR(LBD) chimera, was modified to be responsive to the recruitment of endogenous BRD4. B: In this three-hybrid experiment, the monofunctional molecule negligibly activated transcription, while the bifunctional molecule acted as a strong activator of transcription. C: Co-addition of the bifunctional molecule and free (S)-JQ1 in a squelching experiment attenuated the activation capability of RU-O3-JQ1, supporting the belief that the bifunctional molecule's activity was dependent on BRD4 recruitment.

HeLa cells expressing endogenous levels of BRD4 were transfected with a luciferase reporter plasmid bearing five Gal4 DNA-binding sites and an

expression plasmid for a Gal4-GR(LBD) chimera and treated with the indicated compounds; here, HeLa cells were chosen owing to previous experience with this cell line while conducting three-hybrid experiments. The resulting activation of transcription is displayed in Fig. 3.3B as a fold activation of luciferase produced over the levels in vehicle-controlled cells. As expected, the monofunctional RU-O3-N3 minimally affected GR activity relative to the DMSO control treatment; the lack of an appreciable partial agonistic effect can likely be attributed to the lack of the GR AF1 domain in this system, believed to play a large role in RU486-induced transactivation. Interestingly, the ‘trans’ addition of RU-O3-N3 and unconjugated (S)-JQ1 produced a small increase in activation; this was unexpected, as the co-treatment of (S)-JQ1 with a glucocorticoid agonist has been shown to suppress GR activity (data not shown). Treatment of these transfected cells with the bifunctional RU-O3-JQ1 led to a sharp, dose-dependent increase in transcription, similar to the effect produced by the agonist-based SDex-O3-JQ1. At high concentrations, this effect dissipates; this observation would be in accordance with BRD4 recruitment facilitating the activation of transcription, as high levels of bifunctional molecule serve to saturate each protein binding partner, favoring dimeric species over trimeric complexes and lowering the effective concentration of BRD4 localized to DNA. However, cellular viability was lowered at high (5 μ M) dosing of RU-O3-JQ1, confounding this conclusion.

As an alternative means of confirming that BRD4 recruitment is occurring and is instigating transactivation, a squelching experiment was performed (Fig. 3.3C). HeLa cells were transfected as above and treated with RU-O3-JQ1 (0.5 μ M) along with increasing levels of (S)-JQ1. In the absence of competing inhibitor, RU-O3-JQ1 treatment produced a 30-fold activation of transcription relative to levels in DMSO-treated cells. However, co-treatment with (S)-JQ1 (0.05 μ M) resulted in attenuated activity with 7-fold activation relative to levels in control-treated cells, approximately 25% of the activity produced in the absence of competing ligand. Equimolar treatment with unconjugated (S)-JQ1 produced a

nearly full attenuation of activity. These data firmly support the premise that RU-O3-JQ1 is acting as a protein-protein surrogate to induce an interaction between GR and BRD4, and, as was seen in three-hybrid experiments evaluating SDex-O3-JQ1 activity, this results in transcriptional activation.

Recruitment to the full-length receptor

The data presented in Fig. 3.3B demonstrates that RU-O3-JQ1 is capable of bridging the GR LBD and BRD4, and the localization of BRD4 to a reporter gene in this context results in strong activation. However, this does not reveal any insights into whether RU-O3-JQ1 maintains its parent ligand's ability to induce GR nuclear translocation, or if the presence of the GR DBD and AF1 will interfere with BRD4-facilitated upregulation. We developed a luciferase reporter assay in order to investigate this, depicted in Fig. 3.4A.

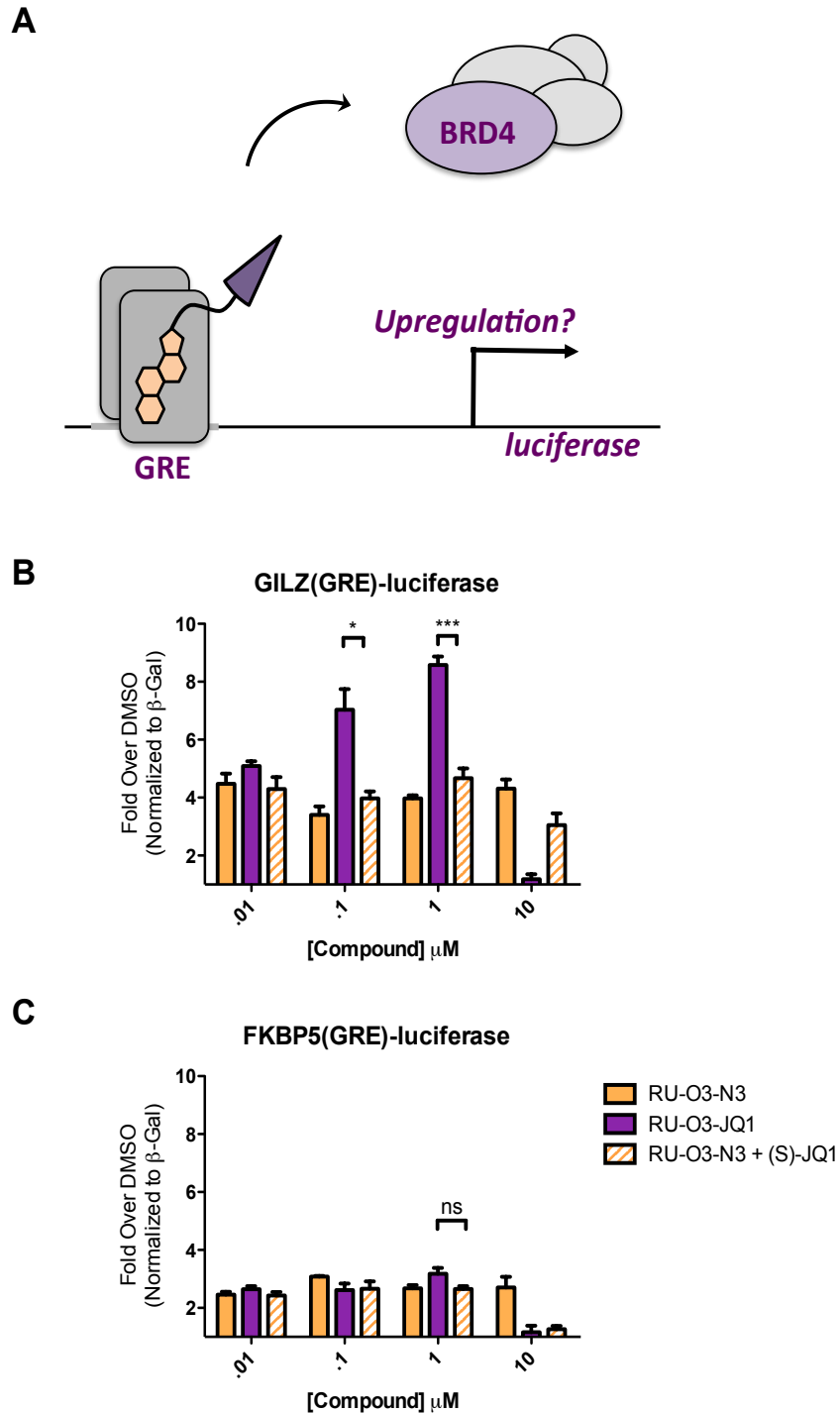


Figure 3.4 Recruitment of BRD4 to full-length rat GR. A: A transcriptional reporter assay was designed to monitor the effects of BRD4 recruitment on transcriptional activity by full-length GR. The activity of the indicated ligands was measured utilizing a reporter plasmid containing a GRE from the *GILZ* gene (B) and the *FKBP5* gene (C).

U2OS cells were transfected with a reporter plasmid bearing one of the indicated glucocorticoid response elements (GREs)⁴ immediately upstream of a promoter driving luciferase expression and an expression plasmid coding for rat GR (rGR), utilizing rGR due to previous experience with this GR variant. In this and subsequent experiments, U2OS cells were utilized due to their negligible expression of GR, allowing for the introduction of GR variants through transfection. Cells were treated RU-O3-N3, RU-O3-JQ1, or a trans addition of RU-O3-N3 and (S)-JQ1 at the indicated concentrations. Transcriptional activity was determined by monitoring luciferase expression and displayed as fold activation over the levels of luciferase in vehicle-treated cells. The data displayed in Fig. 3.4B reflects cells transfected with a reporter plasmid bearing a GRE sequence from the *GILZ* gene. In this system, the monofunctional ligand acted as weak agonist in the presence and absence of unconjugated (S)-JQ1. The bifunctional ligand, however, activated transcription in a dose-dependent fashion, inducing activity levels 8-fold higher than DMSO-treated cells and approximately 200% of the activity levels induced by RU-O3-N3. High levels of (S)-JQ1, either unconjugated or as a part of RU-O3-JQ1, result in a loss of viability. In contrast, cells transfected with a reporter plasmid bearing a GRE from the *FKBP5* gene (Fig. 3.4C) were weakly stimulated to equivalent transcriptional levels by all three dosing combinations. This data suggests that the role of the GRE may determine if BRD4 recruitment is sterically permissible, or if BRD4 recruitment serves to activate transcription in the presence of other, GRE-determined complexation partners. As mentioned above, GRE sequence plays a determining role in GR transactivation, acting as an allosteric ligand that aids in dictating GR binding partners and, subsequently, the magnitude of transcriptional activation. The *GILZ* and *FKBP5* GREs share an identical AGAACA half sequence, identical to the most consistently present GR binding sequence⁵⁷. However, they differ in both the spacers between half sequences and, more importantly, in the second half sequences. Whereas the *FKBP5* GRE bears a second half sequence that is perfectly palindromic with its first half sequence (TGTTCT), the *GILZ* GRE is a loosely conserved semi-palindrome, utilizing the nucleotide sequence GGTTCC

as its second GR binding site. This imperfect palindromic sequence may disfavor GR dimerization at the GRE, allowing for the recruitment of BRD4 and successive transcriptional upregulation. However, a more nuanced analysis of GR in complex with this is necessary in order to determine the mechanism of the activation selectivity observed here.

As stated above, several variables were introduced when transitioning from a traditional three-hybrid system to a reporter system using the full-length receptor; GRE sequence is accountable for solely one of these variables. In Chapter 2, it was hypothesized that the presence of AF1 in the full-length receptor, and the additional protein contacts present therein, may play a fundamental role in producing the disparate activity profiles observed when comparing SDex-O3-JQ1 activity in each reporter system²⁶. To better explore the responsibilities borne by AF1 and AF2, two mutant rGR expression plasmids were constructed, introducing mutations into the AF1 (E219K/F220L/W234R) or AF2 (E773R) that have been demonstrated to attenuate the activation capability of each respective domain^{4,36,58} (Fig. 3.5A).

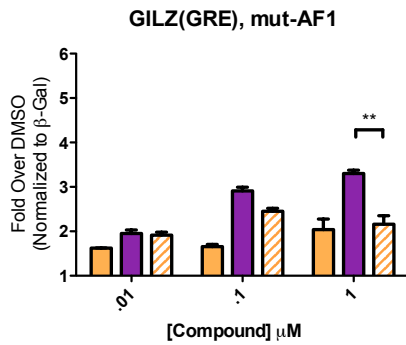
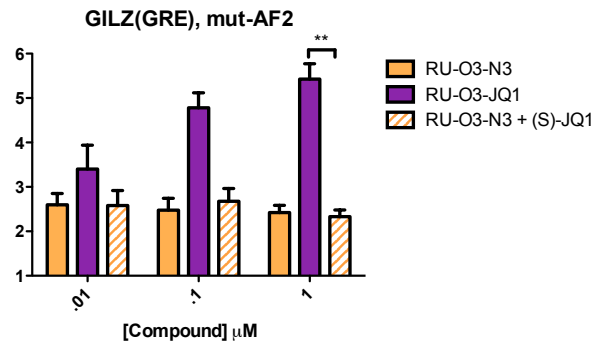
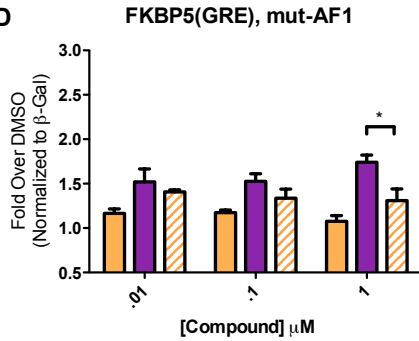
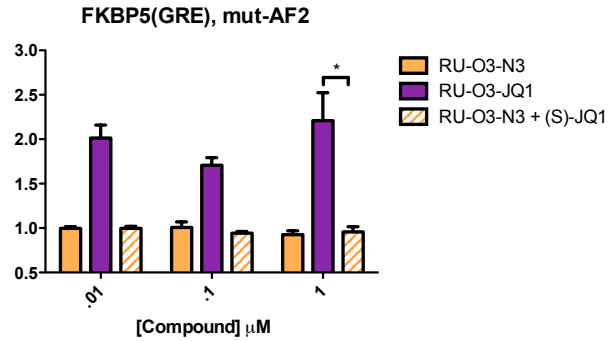
A**B****C****D****E**

Figure 3.5 Effects of GR mutations on bifunctional molecule activity. A: GR is a multidomain protein, including two activating functions, AF1 and AF2, and a DBD. The indicated mutations abrogate the activation capability of the indicated AF domain. The effects of the indicated compounds on the activity of mutant full-length rGR were monitored under several conditions: mutant AF1 at reporter plasmid using a *GILZ* GRE (B); mutant AF2 activity at the *GILZ* GRE (C); mutant AF1 activity at the *FKBP5* GRE (D); and mutant AF2 activity at the *FKBP5* GRE (E). Statistical significance is determined using a one-tailed student's *t*-test.

U2OS cells were transfected as above with the indicated GRE-containing reporter plasmid and a mutated AF-1 rGR (mut-AF1) or AF-2 rGR (mut-AF2)

expression plasmid. Following transfection recovery, cells were treated as indicated above and assessed for levels of produced luciferase, displayed as transcriptional activity relative to a treatment control. In contrast to cells expressing the wild type receptor, cells expressing mut-AF1 did not display appreciable activity levels in response to the monofunctional glucocorticoid antagonist at either the *GILZ* (Fig. 3.5B) or *FKBP5* (Fig. 3.5D) GREs, supporting the theory that RU486-facilitated transactivation occurs largely as a result of AF-1 activity. Despite the attenuated AF-1 activity, RU-O3-JQ1 is able to still act as a dose-dependent transcriptional activator of the *GILZ*-based reporter, triggering luciferase production levels 3-fold higher than in DMSO-treated cells and approximately 150% of the levels produced in cells treated with the monofunctional compound. Notably, RU-O3-JQ1 similarly triggered a response 150% the level of that produced in response to RU-O3-N3 at the *FKBP5*-based reporter, contrasting with the response produced by the native rGR; however, AF-1 mutation attenuated activity universally at this promoter, confounding interpretation of the significance of this data. Mutation of AF-2 appeared to negligibly affect the activities of RU-O3-N3 or RU-O3-JQ1 at the *GILZ* promoter (Fig. 3.5C); the monofunctional compound continued to act as a weak transcriptional agonist, while RU-O3-JQ1 produced a dose-dependent activation with a maximal response approximately 200% that of the RU-O3-N3 response. The GR conformation induced by binding the *GILZ*(GRE) likely does not favor the interaction with coregulators that would impede BRD4 recruitment, evidence by the near identical responses of rGR and mut-AF2 to RU-O3-JQ1 treatment. Fascinatingly, RU-O3-N3 was incapable of inducing mut-AF2 transactivation (Fig. 3.5E) of the *FKBP5*(GRE)-driven reporter, though RU-O3-JQ1 produced a weak agonistic response; this provide an example of activation that is both sequence- and coregulator-context specific, and may provide a path to investigating if RU-O3-JQ1 acts as a more potent activator in systems expressing low levels of AF-2-recruited coregulators.

Modulating human GR activity

The rat glucocorticoid receptor has been extensively characterized, providing the opportunity to exploit this expanded knowledge base to conduct nuanced mechanistic investigations. The 795 amino acid rGR shares a high level of sequence homology (88%) with the dominant human glucocorticoid receptor, the 777 amino acid isoform α (hGR α). However, though the two receptors are frequently used interchangeably in the literature, they often display different activities and produce different transcriptional profiles when evaluated in identical systems, likely due to differences in post-translational modifications²⁹ and the presence of an expanded polyglutamine tract in the rGR AF1.

Acknowledging the potential for species-specific differences in response, the activities of the antagonist-based molecules were evaluated in a reporter system utilizing the human receptor.

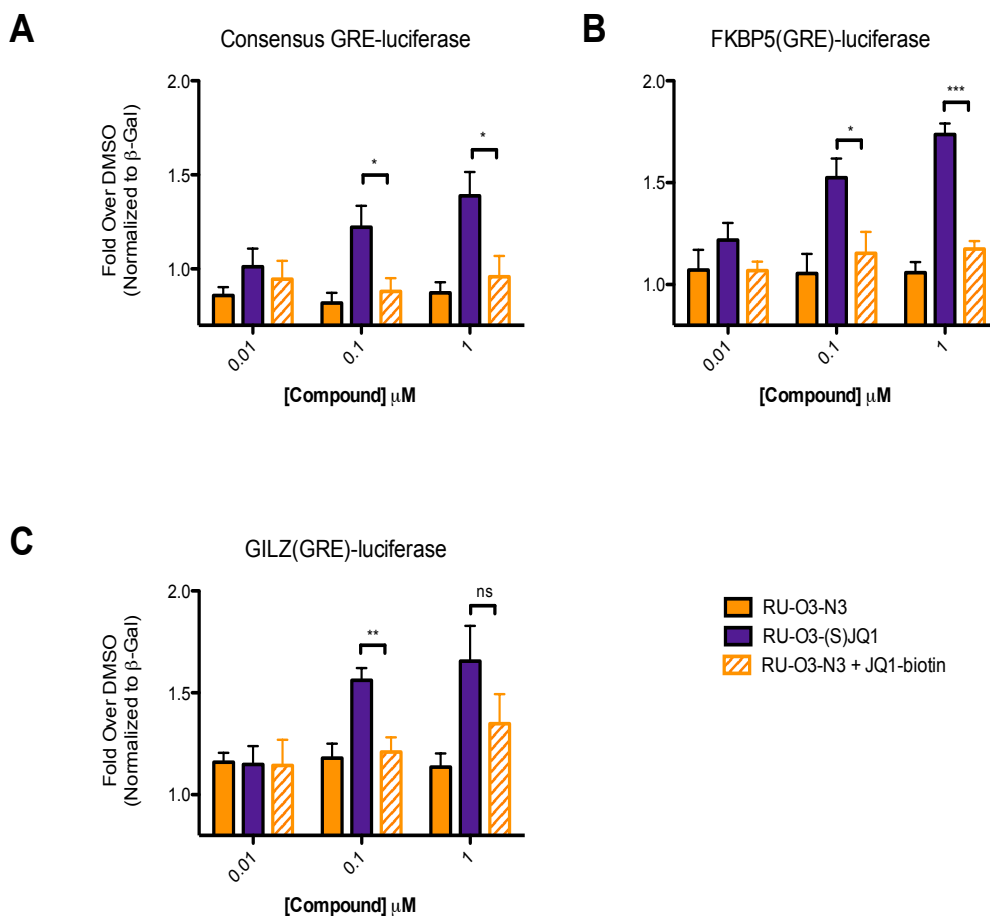


Figure 3.6 Transcriptional activity of full-length hGR. The effects of the indicated compounds on the transcriptional activity of hGR were measured at reporter plasmids containing several different GREs, including a consensus GRE (A), a *FKBP5* GRE (B), and a *GILZ* GRE (C). Statistical differences were determined using a one-tailed student's *t*-test.

U2OS cells were transfected with an expression plasmid coding for hGR α and a luciferase reporter plasmid driven by a promoter containing a consensus GRE (Fig. 3.6A), a GRE from the *FKBP5* sequence (Fig. 3.6B), or a GRE from the *GILZ* promoter (Fig. 3.6C). Signifying a brief departure from previous experiments, the 'trans' addition employed in this set of trials contained a biotinylated version of (S)-JQ1, acknowledging its utility as a better mimic of the effects of synthetic conjugation on (S)-JQ1. Following treatment, cells were lysed and luciferase levels were quantified and displayed relative to those in vehicle-

treated cells. The results marked a departure from the observations collected utilizing rGR. Regardless of the GRE employed, the monofunctional ligand RU-O3-N3 failed to appreciably elevate transcription of luciferase in both the absence and presence of (S)-JQ1-biotin. However, the bifunctional ligand produced a dose-dependent activation of transcription in all three systems, significantly raising transcriptional levels at concentrations as low as 0.1 μ M. While the magnitude of this activation ranged from 145% of the RU-O3-N3 produced level (Fig. 3.6C) to 165% (Fig. 3.6B), the absolute activation was less than 2-fold higher than that produced in response to DMSO, considerably lower than the responses observed utilizing rGR. Though these two receptors have been used relatively interchangeably in the literature, results like this highlight differences in activity that are imperative to note in future efforts.

In Fig. 3.5, we observed that mutation of the rat AF2 did not significantly alter RU-O3-JQ1's ability to act as an agonistic transcriptional modulator. In human pathology, numerous clinical cases of glucocorticoid resistance have presented in response to mutations in the AF2 of hGR. "Primary Generalized Familial or Sporadic Glucocorticoid Resistance," recently renamed as Chrousos syndrome⁵⁹, is typified by a broad range of symptoms, extending from mild hypertension to severe fatiguing and hypoglycemia. In cases where the biochemical mechanism driving the development of this syndrome has been elucidated, the presence of a single point mutation at a variety of positions throughout AF2 results in lowered ligand affinity, increased nuclear translocation times, and/or aberrant interactions with coactivators. As the RU-O3-JQ1 molecule was designed to act as a protein-protein interaction surrogate, we sought to investigate if the bifunctional molecule could 'rescue' the activity of impaired GRs incapable of normal coactivator interactions. Three representative receptors were chosen from the literature, described in Table 3.1, and expression plasmids coding for each were constructed.

Table 3.1: Glucocorticoid Receptor Mutations Resulting in Crousos Syndrome⁵⁹

Point Mutation			
cDNA	Protein	Genotype	Characteristics
N/A	hGR α	homozygous	normal transactivation normal ligand Affinity nuclear translocation: 12 min normal coactivator interactions
A1922T ⁶⁰	D641V	homozygous	transactivation decreased lowered ligand affinity (3x) nuclear translocation: 22 min aberrant coactivator interactions
G2185A ⁶¹	V729I	homozygous	transactivation down lowered ligand affinity (2x) nuclear translocation: 120 min aberrant coactivator interactions
T2209C ⁶²	F737L	heterozygous	transactivation decreased lowered ligand affinity (1.5x) nuclear translocation: 180 min

U2OS cells were transfected with a luciferase reporter plasmid driven by a promoter containing a GRE, along with an expression plasmid for one of the indicated mutant hGR isoforms (Fig. 3.7). Cells were treated with RU-O3-N3 or RU-O3-JQ1, lysed, and luciferase levels were quantified and displayed as transcriptional activity relative to levels produced in vehicle-treated cells.

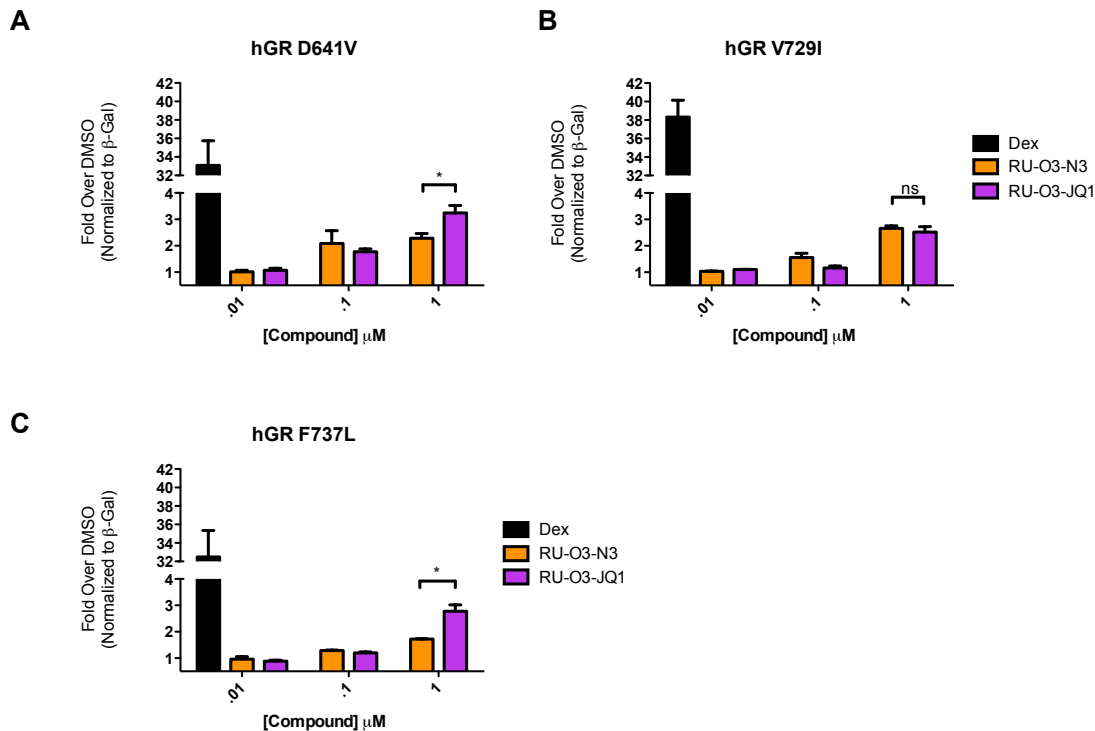


Figure 3.7 Mutational effects on hGR transcriptional activity. The transcriptional activity induced by the indicated compounds was measured in cells expressing hGR variants containing different point mutations that affect AF2 activity, including the point mutation D641V (A), V729I (B), and F737L (C). Statistical significance was determined using a one-tailed student's *t*-test.

While the introduction of each point mutation lowered the transactivation capability of dexamethasone (data not shown), the synthetic agonist was still capable of potently and effectively (30- to 40-fold activation, 0.01 μM dosing) induce GR activity; accordingly, it was unlikely that the weakly activating RU-O3-JQ1 would be able to rescue impaired GR activity.

Interestingly, the monofunctional ligand, in contrast to what was observed with the native receptor, weakly activated each of the mutant receptors. The bifunctional ligand also served to activate each of the mutants; however, individual mutations appeared to effect its ability to recruit BRD4 as it was only capable of activating the D641V and F737L point mutants to levels above that produced by RU-O3-N3 (140%, Fig. 3.7A; and 160%, Fig. 3.7C, respectively).

RU-O3-N3 and RU-O3-JQ1 produced similar responses in cells expressing the V729I mutant (Fig. 3.7B). The mutant-specific effects here may follow from altered LBD structures caused by the introduced mutations – the protein architecture may impair the ability of the RU-O3-JQ1 molecule to present the JQ1 moiety for effective BRD4 recruitment, or alternatively there may be hindrances introduced by the LBD. Further experimentation is necessary to define the roles that each mutation is playing in dictating whether the bifunctional ligand is capable of recruitment-mediated activation, though this knowledge may shed further light on the altered receptors themselves. Accordingly, though the antagonist-based ligands did not find utility as activity rescuers, they may find a use in further probing the functional effects of ligand binding by these therapeutically relevant receptor molecules.

Recruitment to endogenous genes

Previous work has demonstrated that luciferase reporter systems are more readily capable of amplifying transcriptional activity to detectable levels than experiments involving the quantification of endogenous transcripts. As observed in Fig. 3.6, the activation levels in a reporter system utilizing hGR were considerably lower than those produced by the agonist-based compounds described in Chapter 2. Surprisingly, we have not yet been able to discover a gene that is activated in a recruitment-based fashion by the bifunctional RU-O3-JQ1. However, these initial experiments have been limited to monitoring the production of three transcripts (*FKBP5*, *GILZ*, and *S100P*)^{55,63} in only two different cellular systems (A549 cells expressing endogenous levels of hGR, and U2OS cells transfected with a hGR expression plasmid), so it is possible that, as was seen with SDex-O3-JQ1, recruitment-facilitated transcriptional activity is gene-specific and we merely have not monitored the expression of an ideal candidate gene. Current efforts include transcriptomic profiling in response to RU-O3-N3 and RU-O3-JQ1 treatment to identify genes capable of harboring BRD4 recruitment.

Bifunctional-ligand induced transrepression

As has been discussed above and in Chapter 2, GR transrepression of NF- κ B activity is believed to be key signal in anti-inflammatory responses. RU486 has long been believed to be unable of downregulating the NF- κ B pathway; though there is evidence that RU486 continues to trigger an interaction between GR and NF- κ B^{64,65}, it does not arrange the two proteins in a transcriptionally defunct complex¹⁰. Recent work has shown that RU486 may even be capable of activating NF- κ B activity in certain contexts⁶⁶. Though the mechanism of this is unclear, it may arise through a recently discovered context-dependent synergism of activation observed between GR and NF- κ B⁶⁷.

If RU486 is indeed incapable of repressing NF- κ B signaling due to an inability to interfere with pTEF-b recruitment¹¹, BRD4 recruitment may provide a mechanism to disrupt the formation of a transcriptionally active complex. Alternatively, the presence of (S)-JQ1 in a bifunctional molecule, a moiety previously shown to inhibit NF- κ B signaling⁶⁸, may provide a means for NF- κ B transrepression. To this end we utilized the luciferase reporter assay described in Chapter 2 to monitor the effects of the antagonist-based molecules on NF- κ B activity.

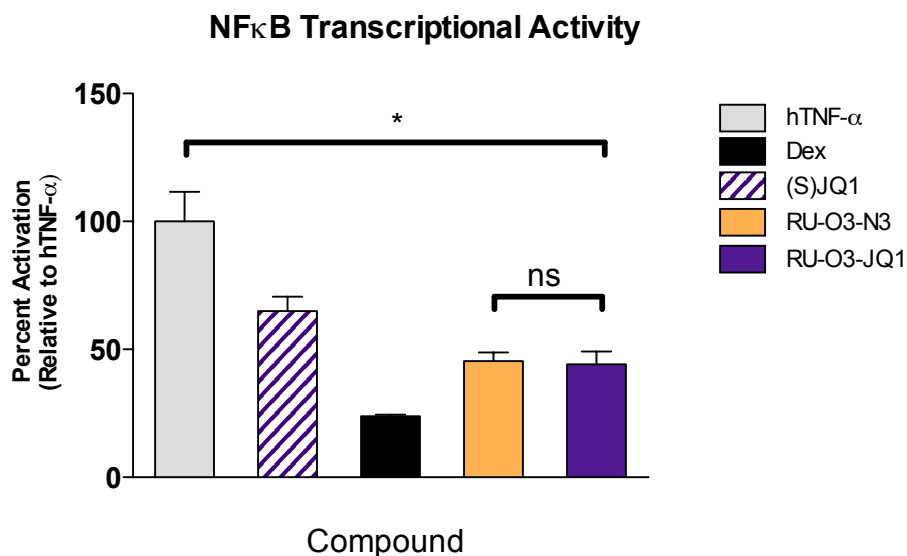


Figure 3.8 Transrepression of NF- κ B activity in a reporter assay. The effects of the indicated compounds on hTNF- α stimulated NF- κ B activity were measured in a transcriptional reporter system. Statistical significance was determined using a one-tailed student's *t*-test.

U2OS cells were transfected with an expression plasmid for hGR α and a reporter plasmid driven by a promoter containing five NF- κ B binding sites. Following transfection recovery, NF- κ B activity was stimulated by its ligand hTNF- α , and cells were dosed with vehicle or the indicated compounds at a final concentration of 1 μ M. Transcriptional activity is displayed as a measure of quantified luciferase relative to the levels in cells treated with vehicle and hTNF- α alone (Fig. 3.8). Expectedly, dexamethasone served to efficaciously repress NF- κ B activity to 24% of that observed in vehicle-treated cells, while (S)-JQ1 mildly repressed activity to 65% of vehicle. Unexpectedly, the monofunctional RU-O3-N3 acted to repress activity to a level 45% of that observed in vehicle-treated cells. The bifunctional molecule also acted in a near identical fashion, repressing activity to similar levels as the linker-containing antagonist.

While it was unexpected to see the level of transrepression produced by RU-O3-N3, it was unclear if this activity would similarly occur in the NF- κ B-driven expression of endogenous genes. The *IL-8* gene codes for the interleukin-8

protein, a key cytokine in the inflammation response⁶⁹. *IL-8* expression is extremely sensitive to both TNF- α induction (through the activation of NF- κ B) and to glucocorticoid agonist-induced transrepression, establishing it as a model gene for analyzing the effects of glucocorticoid ligands on transrepressive activities.

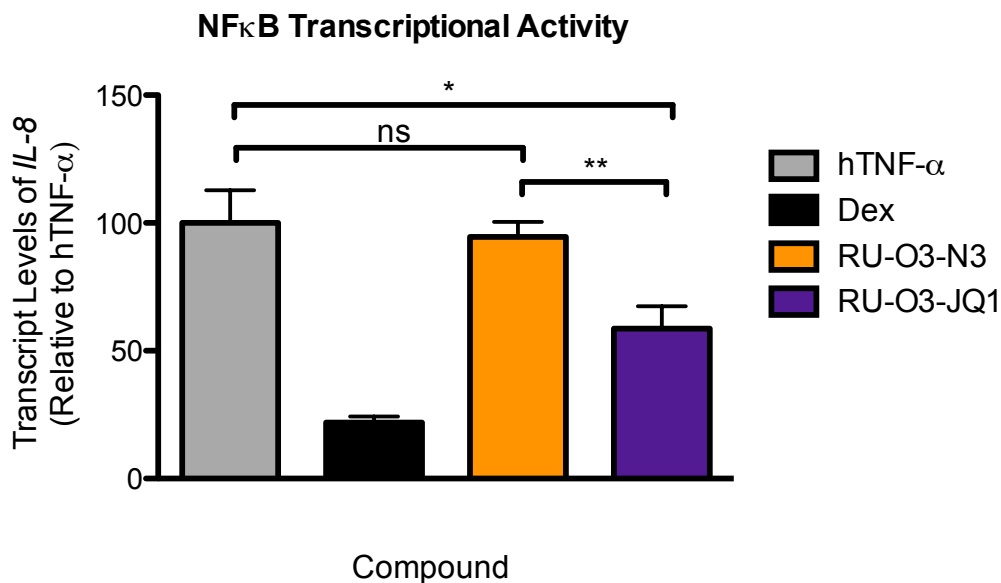


Figure 3.9 Transrepression of a NF- κ B-regulated gene. The effects of the indicated compounds on the modulation of a NF- κ B-regulated gene, *IL-8*, in *hTNF- α* stimulated cells were monitored. *IL-8* levels were normalized between samples through comparison to the housekeeping gene *RPL19* and effects of each compound on *IL-8* production were determined relative to DMSO-treatment via the $\Delta\Delta$ CT method.

The human adenocarcinoma lung epithelial cell line A549 has been well established as a model cell line for investigating GR-mediated transrepressive transcriptional activities³⁷. A549 cells were stimulated with the NF- κ B ligand *hTNF- α* and incubated with vehicle or the indicated compounds at a final concentration of 1 μ M (Fig. 3.9). Following treatment, transcript levels of *IL-8* were quantified and expressed relative to the levels produced in A549 cells stimulated with *hTNF- α* and treated with vehicle. As was seen in reporter experiments, dexamethasone acted as an effective transrepressor ligand. In

contrast to what was observed in Fig. 3.8, and seemingly mimicking what has been established in the literature, RU-O3-N3 did not act as a transcriptional repressor. However, the bifunctional molecule capably repressed the levels of *IL-8* to levels approximately 50% of those in vehicle-treated cells. Further experiments are necessary to discern if this mechanism is through ligand-facilitated recruitment of BRD4 to sterically hinder complex formation, though it is likely that this is simply occurring as a result of the (S)-JQ1-mediated inhibition of BRD4-NF- κ B interaction.

3.5 Recruitment to the androgen receptor

While a therapeutic limitation, a benefit to utilizing RU486 as a building block for creating bifunctional tool compounds is its promiscuity for NRs. One potential alternative target is the androgen receptor (AR)⁵¹. This aberrant activity of this member of the NR family is central to prostate cancer pathology, marking it as an important therapeutic target⁷⁰. As with GR, traditional approaches to modulate AR activities have centered on developing novel small molecules that sculpt the AR LBD surface in fashions that disfavor the expression of AR-controlled oncogenes⁷¹. This is confounded by observations that tumorigenic AR is prone to developing mutations in its LBD and coregulator binding surface, contributing to the development of resistance to therapeutic antiandrogens^{72,73}.

Acknowledging the limitations of the traditional approach described above, a recent focus shift has centered on the production of alternatively acting steroid-conjugates to combat this malady. Downregulating AR activity through the targeted recruitment of coregulators is an innovative strategy to accomplish this goal. In initial reporter experiments, we have observed that RU-O3-N3 acts as a mild (EC_{50} : 190 nM) partial agonist of AR transactivation while RU-O3-JQ1 is incapable of activating transcription, though currently, much further characterization is necessary.

3.6 Conclusions and discussion

Synthetic linkage of the glucocorticoid antagonist RU486 to the bromodomain inhibitor (S)-JQ1 provides a method for the recruitment of bromodomain-containing BRD4 to DNA-bound GR. In a three-hybrid experiment utilizing a Gal4 DBD and GR LBD chimera, this resulted in a potent, recruitment-dependent activation of transcription. However, in a reporter system absent of a Gal4 DBD to anchor GR to DNA, and instead resting on the inherent ability of full length GR to recognize and bind GREs, this effect is severely dampened.

The antagonist-based bifunctional ligand, RU-O3-JQ1, appeared to display both a species- and GRE-dependent ability to effect GR transactivation. The dampened transactivation induction of rGR by RU-O3-JQ1 was further magnified when characterizing the activation response by hGR, as RU-O3-JQ1 only weakly induced hGR transactivation. Further differences were observed regarding the GRE-dependent response to RU-O3-JQ1; whereas a stark difference in rGR activity was observed when utilizing alternative reporter plasmids bearing different GREs, swapping GREs did not appreciably perturb the responses in cells expressing hGR. While hGR and rGR have been presumptively interchanged in characterization studies, with relatively few comparative works described, these results seem to magnify the differences between the two, shedding light on the potential folly of assuming equivalence.

Despite the activity differences of rGR and hGR in reporter experiments, each isoform was capable of utilizing RU-O3-JQ1 agonistically while bearing mutation(s) in the respective AF2. By disrupting the rGR AF2, RU-O3-JQ1 was capable of activating mut-AF2 in both GRE-contexts investigated. In an alternative line of experiments, disruption of hGR AF2 resulted in a set of heterogeneous responses, where the ability of RU-O3-JQ1 to continue acting as an activator through recruitment was dependent on the location of the perturbation. The knowledge base regarding GR-coregulator interactions, and

the role that DNA sequence plays in determining these interactions, is rapidly expanding^{4,5} and molecular tools are necessary to further dissect the effects of altering AF2 topology, a therapeutically relevant occurrence⁵⁹. As such, the utility for compounds such as RU-O3-JQ1, capable of inducing novel GR-coregulator interactions, will similarly expand.

As discussed above, pharmaceutical efforts to develop anti-inflammatories has been driven by the paradigm of separating transactivation from transrepression, a viewpoint that may be myopic given recent indications of the role GR transactivation plays in the anti-inflammation response. Acknowledging this limitation, the observation that RU-O3-JQ1 is capable of repressing NF- κ B signaling while inducing a transactivation profile distinct from traditional GR agonists is a significant development. However, coordinating the inflammation response involves the up- and down-regulation of numerous pathways, notably the similarly GR-repressed AP-1 network. As such, these initial results provide encouragement to continue to determine the effects of RU-O3-JQ1 treatment on the expression of other genes in the inflammation network.

The developments described in Chapter 3 further validate bifunctional GR ligands as viable probes for studying this important receptor and as tools for producing novel transcriptional outputs. Notably, this strategy was applied to execute a change in a GR ligand's fundamental, intrinsic nature, employing extrinsic recruitment to convert an antagonist into a functional agonist. The transcriptional responses elicited by the bifunctional ligand display a selectivity that has not been observed in other steroidal modulators, providing excitement for additional characterization and, eventually, expanding this class of ligands to produce even further refined transcriptional responses.

3.7 Materials and Methods

Cell culture and transfections

U2OS (HTB-96) and A549 (CCL-185) cells were purchased from ATCC. Cells were cultured in DMEM with 10% FBS at 37°C under 5% CO₂. Transient transfections were done using Lipofectamine or Lipofectamine 2000 (Invitrogen) with conditions optimized using GFP expression plasmid and fluorescent analysis or by using a β -gal expression plasmid and activity assay.

Plasmids

All oligonucleotides for construction of plasmids were purchased from Integrated DNA Technologies.

pCMV- β -gal is a β -galactosidase coding plasmid driven by a CMV promoter and was generously provided by J. Iñiguez-Lluhí.

pCDNA3 hGR (human GR coding plasmid) and p6R GR (rat GR coding plasmid) were gifts from J. Iñiguez-Lluhí.

Coding plasmids for rat GR containing AF1 and AF2 mutations were constructed by A. Van Dyke.

pConsensus GRE-luc, pFKBP5(GRE)-luc, and pGILZ(GRE)-luc were generously provided by K. Yamamoto (UCSF).

Mutant hGR coding plasmids were constructed using the Stratagene Quikchange method. Relevant primers for each mutation were constructed and amplified using pCDNA3 hGR as a template. Following PCR amplification, synthetic plasmids were treated with DpnI restriction enzyme to remove template and transformed into *E. coli*. The primers for each mutation are as listed:

D641V F-Pr: 5'- ACTCTACCCTGCATGTACGTCCAATGTAAACACATGCTG -3'

D641V R-Pr: 5'- CAGCATGTGTTTACATTGGACGTACATGCAGGGTAGAGT -3'

V729I F-Pr: 5'- GATTCTATGCATGAAGTGATTGAAAATCTCCTTAACT -3'

V729I R-Pr: 5'- AGTTAAGGAGATTTTCAATCACTTCATGCATAGAATC -3'

pNF- κ B-luc was generously provided by G. Nuñez (Univ. of Michigan).

Transcriptional reporter assay (Figs. 3.4, 3.5, 3.6, 3.7)

U2OS cells were seeded at a density of 250,000 cells per well in a 6-well plate with 2 mL DMEM + 10% FBS. The following day, cells were transfected with 1 μ g of indicated pGRE-luc, 0.5 μ g of pCMV- β gal, 5 μ L of Lipofectamine 2000, and 0.5 μ g of the indicated GR coding plasmid. After 6 hours, the transfection mix was replaced with DMEM + 5% charcoal stripped (CS) FBS and allowed to recover overnight. The following day, cells were removed by trypsinization and replated at a density of 10,000 cells per well in a 96-well dish. Individual wells were treated with the indicated compounds to a final DMSO concentration of 0.01%. After 16 hours, media was removed and cells were lysed using a passive-lysis buffer. β -gal activity was assayed as described previously using a Molecular Devices Spectramax plate reader. Luciferase activity was determined as described previously using a Molecular Devices Spectramax L luminometer. Standard error from triplicate samples is represented by error bars on graph.

Transrepression reporter assay (Fig. 3.8)

U2OS cells were seeded at a density of 250,000 cells per well in a 6-well plate with 2 mL DMEM + 10% FBS. The following day, cells were transfected with 1 μ g of indicated pNF- κ B-luc, 0.5 μ g of pCDNA3 hGR α , and 0.5 μ g of pCMV- β gal. After 6 hours, the transfection mix was replaced with DMEM + 5% charcoal stripped FBS and allowed to recover overnight. The following day, cells were removed by trypsinization and replated at a density of 10,000 cells per well in a 96-well dish. Individual wells were treated with the indicated compounds to a final DMSO concentration of 0.01%, along with hTNF- α (Roche) at a final concentration of 5 ng/mL. After 16 hours, media was removed and cells were lysed using a passive-lysis buffer. β -gal activity was assayed as described

previously using a Molecular Devices Spectramax plate reader. Luciferase activity was determined as described previously using a Molecular Devices Spectramax L luminometer. Standard error from triplicate samples is represented by error bars on graph.

RT-qPCR (Figs. 3.9)

50,000 transfected U2OS, A549, or MM1.S cells were plated in 24-well plate with 0.5 mL DMEM + 5% CS FBS. Next day, cells were treated with compounds or DMSO (0.2% final DMSO for all samples), along with hTNF- α (Roche) at a final concentration of 5 ng/mL. Total RNA was isolated with Qiagen RNAeasy Plus mini kit. cDNA synthesis was done with iScript RT Mastermix (Biorad). 20 μ L qPCR reaction mix: 2 μ L of produced cDNA, 0.2 μ L CXR, 0.4 μ L forward primer (200 nM final), 0.4 μ L reverse primer, 7 μ L H₂O, 10 μ L GoTaq qPCR master mix (Promega). qPCR was done on ABI StepOne Plus. Products were analyzed with melt curve for quality control. Transcript levels were normalized to the levels of a housekeeping gene, *RPL19*. Activation was displayed relative to transcript levels in DMSO-treated cells and determined using the $\Delta\Delta C_T$ method. Primer pairs used:

IL-8: F-Pr 5'- ATGACTTCCAAGCTGGCCGTGGCT -3'

IL-8: R-Pr 5'- TCTCAGCCCTCTTCAAAAATTCT -3'

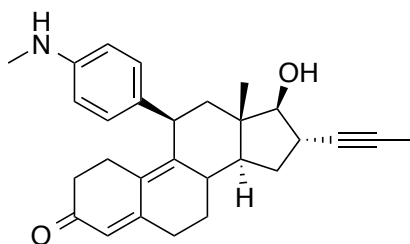
RPL19: F-Pr 5'- ATGTATCACAGCCTGTACCTG -3'

RPL19: R-Pr 5'- TTCTTGGTCTCTTCCTCCTTG -3'

Chemical synthesis

RU486 (Mifepristone) was purchased from Sigma-Aldrich. Commercially available reagents and solvents were used as received. Chromatographic separations were carried out on silica gel 60 (230-400 mesh, E. Merck) or by reverse phase HPLC on C18 column using the indicated eluents. Yields are unoptimized. ESI-MS spectra were obtained on Micromass LCT TOF mass spectrometer. High-resolution mass spectra (HRMS) were obtained on

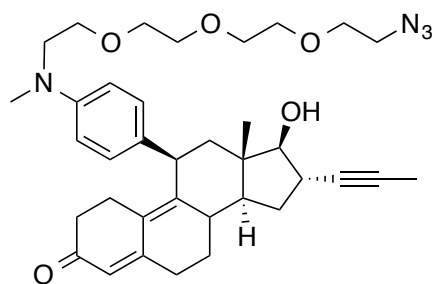
Micromass AutoSpec Ultima Magnetic sector mass spectrometer. $^1\text{H-NMR}$ spectra were obtained at 400MHz on a Varian MR-400 spectrometer. Chemical shifts are given in $\delta(\text{ppm})$ values.



RU486(-CH₃)

RU486(-CH₃) was synthesized by A. Van Dyke.

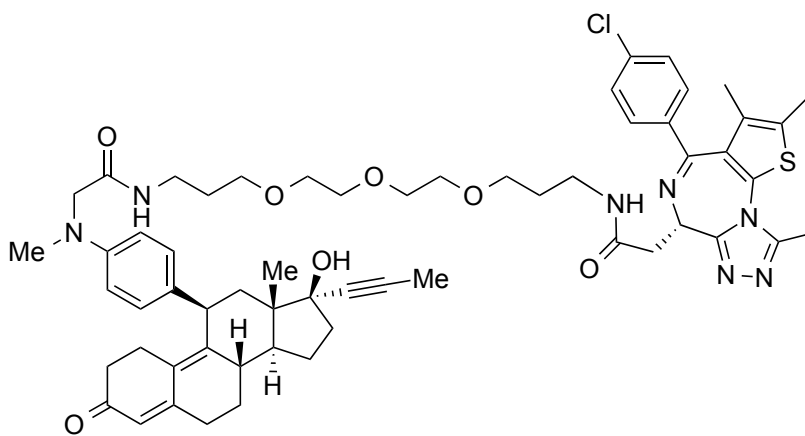
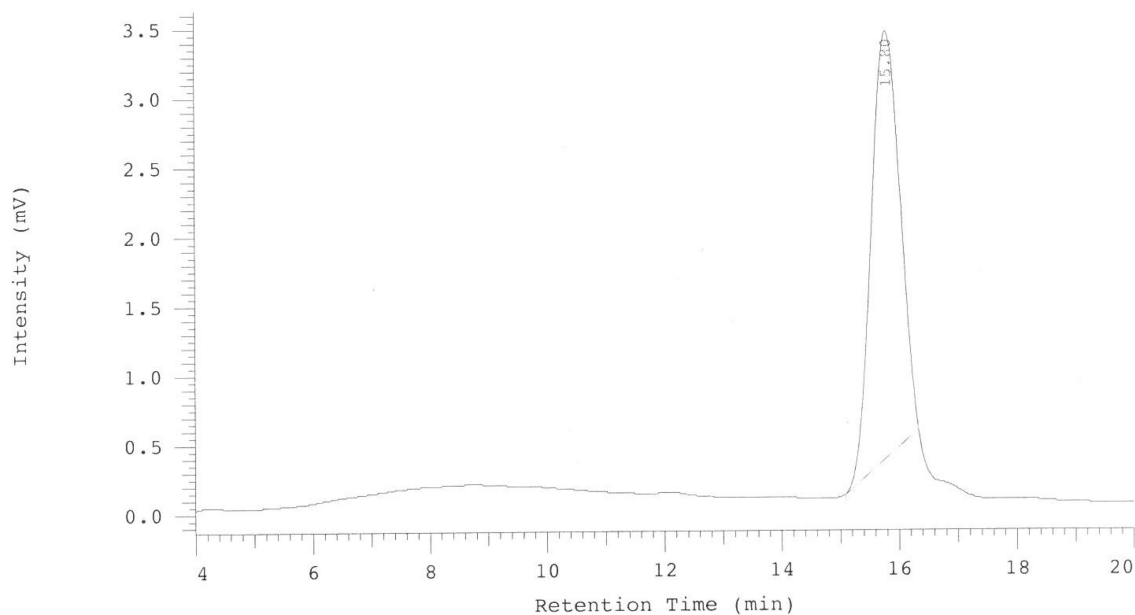
To a solution of RU486 (500 mg, 1.2 mmol) in dichloromethane (10 mL) was added NMO (0.61 g, 5.2 mmol) and tetrapropylammonium perruthenate (TPAP; 41 mg, 0.12 mmol). After stirring at room temperature for 22 h, additional TPAP (41 mg, 0.12 mmol) and NMO (0.61 g, 5.2 mmol) were added. After stirring at room temperature for 15 h, the reaction mixture was quenched with saturated aqueous NaHSO₃ solution. The dichloromethane was removed *in vacuo* and extracted with ethyl acetate. The extract was washed with brine, dried over Na₂SO₄ and concentrated *in vacuo*. The product was suspended in 1 M hydrochloric acid (10 mL) and methanol (10 mL) and heated at 55°C for 40 h. The mixture was neutralized with aqueous NaHCO₃ solution and extracted with dichloromethane. The extract was washed with brine, dried over Na₂SO₄, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (ethyl acetate/hexane = 4/1) to give compound RU486(-CH₃). ESI-MS calcd for [C₂₈H₃₃NO₂ + H⁺] = 415.3; found 416.2.



RU-O₃-N₃

RU-O₃-N₃ was synthesized by J. Carolan and J. Højfeldt.

To a mixture of RU486(-CH₃) (22 mg, 0.053 mmol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-amine (19 mg, 0.064 mmol) in acetonitrile (5 mL) in a sealed tube was added sodium iodide (10 mg, 0.064 mmol) and *N,N*-diisopropylethylamine (0.03 mL, 0.16 mmol). The reaction was heated to 100°C for 60 h. After concentration *in vacuo*, the residue was purified by silica gel chromatography (ethyl acetate/hexane = 4/6 to 6/4) to give compound RU486-O₃-N₃ (22 mg, 67%) as a yellow sticky oil. The product was further purified by HPLC (0.1% aqueous TFA/acetonitrile = 80/20 to 65/35) to give the trifluoroacetic acid salt of RU486-O₃-N₃ as a colorless amorphous powder, calculated to be 100% pure through peak integration. ¹H NMR (400MHz, CDCl₃): δ 0.53 (3H, s), 1.31-1.47 (2H, m), 1.65-1.78 (3H, m), 1.88 (3H, s), 1.89-2.03 (2H, m), 2.18-2.47 (7H, m), 2.55 (2H, m), 2.74-2.77 (1H, m), 2.93 (3H, s), 3.36-3.78 (2H, m), 3.46-3.48 (1H, m), 3.59-3.66 (12H, m), 4.32 (1H, d, *J* = 6.8 Hz), 5.74 (1H, s), 6.61 (2H, d, *J* = 8.8 Hz), 6.97 (2H, d, *J* = 8.4 Hz). HRMS (ESI) calcd for [C₃₆H₄₈N₄O₅ + H]⁺: 617.3697, found 617.3699. HPLC chromatogram of purified product is displayed below:



RU-O₃-JQ1

RU-O₃-JQ1 was synthesized by A. Van Dyke and J. Qi.

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Chapter 4

Introduction of a Guided-Inquiry Experimental Module into an Undergraduate Biochemistry Syllabus*

4.1 Abstract

The past two decades have seen the science education community define and develop several instructional methods that aim to increase cognition and long-term understanding in undergraduate science courses, such as a problem-based learning and peer-led teaching. Many of these methods have identified inquiry as a key component to transforming educative activities from low-level to high-level by Bloom's taxonomy of learning. Here, we describe efforts to incorporate guided-inquiry into the undergraduate biochemistry course syllabus at the University of Michigan through the development of a novel experimental module. Based on student feedback and performance, this module was an effective tool for raising student engagement and understanding, and it is easily modifiable for incorporation into curricula at other institutions.

4.2 The role of inquiry in undergraduate laboratory courses

Laboratory instruction allows young scientists-in-training to engage in practices reflective of trained specialists in a given discipline. Deriving from classical apprenticeships and constructivist theory and supported by Lave's work in

* The experimental module described here was designed by J. Carolan and K. Nolta. Assessment activities were designed by J. Carolan and influenced through discussions with B. Coppola and A. Mapp. Implementation and assessment activities were administered by K. Nolta and graduate student instructors associated with CHEM352.

situated learning¹, laboratory courses have historically been constructed to prepare students for future work through the repetition of established procedures. The limitations to this style of instruction have been noted frequently^{2,3}, but widespread attempts to address this have been often met without requisite enthusiasm. Despite good intentions, innovatively designed curricula were often simply spliced with existing methodology and practices, limiting or abrogating any potential efficacy⁴.

Within the larger educational community, recent outlooks on reform have focused on decreasing instructional-centered models of teaching in favor of focusing on enhancing the learning process of individual students⁵. This shift in mindset was reflected in the standards and recommendations recently released by the National Research Council⁶, highlighting the importance of incorporating inquiry into student practices as a means of increasing scientific literacy and understanding of scientific processing. This is amplified in laboratory settings, where it is important to convey to students the differences between learning science and doing science, and the goals associated with each process. As a result, in laboratory courses, this has manifested in multiple described efforts to incorporate student-driven inquiry into experiments. These examples belong in a continuum of loosely distinct styles of instruction that share in their goal of increasing active learning and vary in their level of inquiry incorporation^{2,7,8}, best described by Domin² for chemistry laboratories but applicable for other fields.

Expository learning

Recent meta-analyses of undergraduate laboratory experiments have uncovered that a vast majority in scientific disciplines are expository in nature, despite this style being the most heavily criticized^{7,8}. Expository laboratories define problems and procedures for students, who subsequently investigate an issue in order to define a known result. These experiments do allow for rote memorization and low level problem solving⁹, but they do so at the expense of higher order planning

and processing. However, their widespread use derives from ease of use, reproducibility, and lowered material costs, and in departments without financial flexibility or resources, this style is commonly the only available option.

Guided inquiry learning

Guided inquiry laboratories expand on the framework of expository experimentation by enlarging the responsibility thrust upon the students. While students are presented a problem and protocols to navigate towards a solution, it is largely up to the student to develop an overall method towards this destination and to determine an optimal method of communicating conclusions. Transitioning towards a more inductive method of learning, this style of instruction situates the student as the driver in his own learning, aiding deeper understanding^{10,11}. Unfortunately, even this limited incorporation of student-driven activity necessitates a significantly higher level of teacher effort, limiting its implementation.

Problem-based learning

Trained researchers identify and define problems, often with guidance from peers and advisors, and then approach those problems through self-constructed methodology. Problem-based learning (PBL) has emerged as an attractive style of instruction, across many disciplines, that emulates this style of inquisition. Here, instructors provide a concrete background of knowledge surrounding a concept alongside a loose framework for potential projects, but it is the students who are tasked with constructing a problem and developing the methods to solve it. Again, this approach serves to imitate that of a trained scientist, but in this scenario it is the methods of problem-solving that are emphasized over the solving of the problem itself. Accordingly, this approach is demonstrated to be more effective at developing many of the higher cognitive skills described in Bloom's taxonomy^{2,9,12}. The benefits described are paired with difficulties in

implementation – PBL requires substantively more effort on the part of both instructor and class, and the openness afforded to students yields significantly higher material costs.

4.3 Rationale for this work

Undergraduate biochemistry laboratory course have long provided an opportunity for students to connect the nebulous techniques learned in lecture to actual, “wet” application. As we evaluated the syllabus for the University of Michigan’s undergraduate laboratory course, CHEM352, we identified a set of key experiments that relied heavily on expository methods and, as such, were an ideal target for improvement.

Protein production, purification, and characterization form the nucleus of countless efforts in the biochemical laboratory. Navigating these techniques often requires a keen understanding of concepts like chromatography, protein-antibody recognition, or enzymatic catalysis, validating their importance in any biochemistry syllabus. Accordingly, recombinant protein characterization has been the subject of several academic efforts to produce more effective coursework¹³⁻¹⁶. However, these efforts can be difficult to adapt because they often require a full semester of instruction^{12,17-21} or expensive instrumentation²²⁻²⁵. Above, we described the benefits of utilizing guided-inquiry as a means of enhancing laboratory instruction – keeping true with a constructivist view of chemical education wherein student scientists must construct learning via their own frameworks, guided-inquiry allows students to benefit from their own built inquiries, developing their abilities to plan, infer, and critically analyze. We aimed to address the above limitations by developing an improved laboratory experiment that is brief and can be completed by large amounts of students in a financially reasonable fashion. Here, we describe a two-week experimental module centering on protein separation and identification that aims to improve on student engagement and learning through guided inquiry.

4.4 Incorporating inquiry into the biochemistry laboratory

The goals for this experiment center on introducing students to 1) the fundamentals and difficulties associated with separating proteins and 2) the necessity for different methods in identifying separated proteins, while gaining the benefits of a guided inquiry format^{26,27}. In this exercise, students are provided a designed mixture of proteins and are asked to determine a way to isolate and ascertain the molecular weight of each component. To represent different protein classes and features, each component is distinguishable either visually, by enzymatic assay, or by immunoassay. Following size-exclusion chromatographic separation, students determine which of their collected fractions contains each protein using these techniques. Each assay is tailored to provide data rapidly for student analysis. Students utilize SDS-PAGE and convoluted mass spectra of each protein component as alternative methods for molecular weight determination and must rationalize any conflicts in their collected data.

This two week experimental module was easily incorporated into the University of Michigan's biochemistry laboratory course, composed of students majoring in a range of disciplines (including biochemistry and chemistry, but also several majors associated with health professions) who typically had previously or were concurrently taking a biochemistry lecture course, though several students had no supplementary knowledge provided through this source. This module was optimized for a large (~ 90-110) group of students per implementation, and requires no uncommon instrumentation. It is easily incorporated into introductory biochemistry laboratory course curricula and serves as a valuable method for introducing students to protein separations and identifications.

4.5 Experimental details

Students work in groups of two to four depending on material limitations. This experiment was performed over two, 4 h periods following a one-hour lecture occurring during the week prior. During the lecture period, students are introduced to the concepts governing each potential portion of the experiment (chromatographic separations, protein-protein interactions, etc.), along with a generic background on each of the proteins they will be separating. Prior to beginning the experiment, students are provided with protocols for conducting each individual technique (Figure 4.8). The experiment is divided into two parts, allowing for a break to occur between sessions after completing Part I, and is summarized in Table 4.1.

Table 4.1: Experimental workflow

	Lab Activity
Part I (Week 1)	<ul style="list-style-type: none">• Students are provided a protein mixture• Separation by column chromatography• Visual analysis identifies fractions containing cytochrome c• Analysis by ELISA immunoassay indicates fractions containing BSA• Colorimetric assay identifies the presence of β-gal in fractions
Part II (Likely Week 2)	<ul style="list-style-type: none">• Students perform SDS-PAGE to aid in protein size determination• Using collected data and provided MS spectra, students determine size of protein components• Students describe, justify their methods, process, and conclusions

Part I: Separation and Identification of Proteins

Students are provided a mixture of proteins including cytochrome c, bovine serum albumin (BSA), and *E. coli* β -galactosidase (β -gal), along with a mixture of yellow riboflavin and blue dextran for determining maximum and minimum column elution volumes. Using a size-exclusion column, students collect fractions

of eluent using the dyed molecules as beginning and end point indicators (Fig. 4.1).

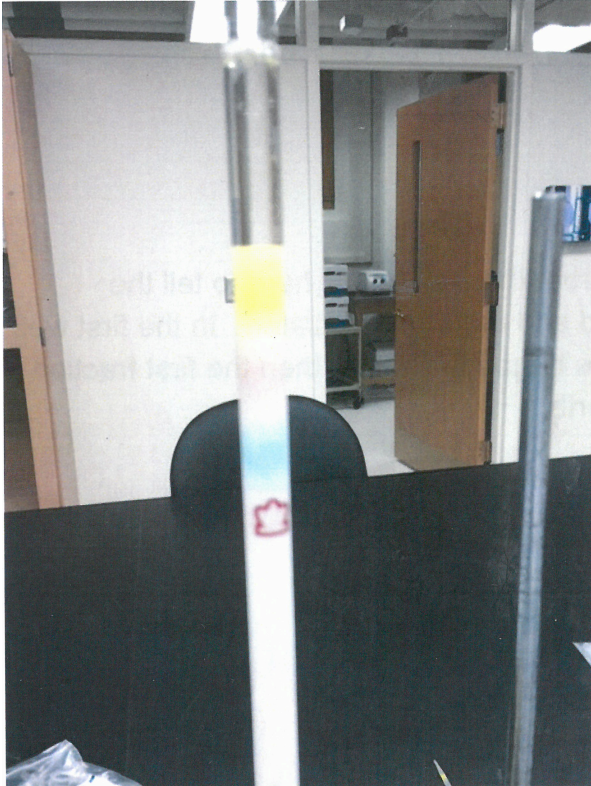
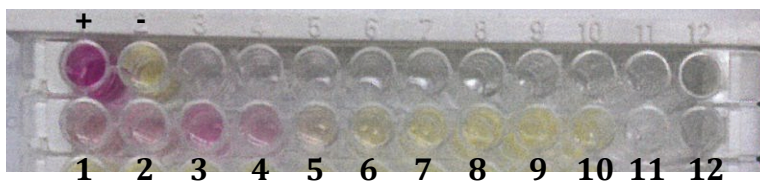


Figure 4.1 Students separate a mixture of proteins using column chromatography. Cytochrome c (red band) migration through the column is monitored visually, while riboflavin (yellow) and blue dextran (blue) serve as visual indicators for column total volume and void volume (respectively).

At this point, students attempt to determine which fractions contain each individual protein. Fractions containing cytochrome c are readily identifiable by a visible red tint. To identify fractions containing β -gal, students utilize a colorimetric assay fitted to β -gal detection (Figure 4.2A). Chlorophenol red- β -D-galactoside (CPRG) is a substrate for β -gal that provides a quantitative and readily visible color change in buffer medium and is frequently utilized to detect the presence of β -gal. Additionally, we have tailored an enzyme-linked immunosorbent assay (ELISA) to compatibility with a BSA-recognizing antibody

to produce an immunoassay for BSA detection (Fig. 4.2B). Students utilize these three methods to determine which fractions contain each component of their mixture. Students typically collect more fractions than there are wells available in a provided polyacrylamide gel for SDS-PAGE. As such, prior to continuing to Part II, students must process their data in order to determine which fractions to assess via SDS-PAGE.

A



B

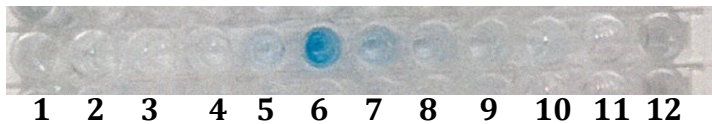


Figure 4.2 Visualization of each utilized assay. In the top panel (A), results of a β -galactosidase assay are depicted. +, -, and each number represent positive control, negative control, and reactions with samples from each indicated fraction, respectively. Red color change signifies the presence of β -gal. In the bottom panel (B), results of an ELISA assay are depicted. Development of blue color (as seen with a sample from fraction 6) indicates the presence of BSA in the collected fraction.

Part II: Size Determination of Each Protein

Students prepare samples for loading on to a polyacrylamide gel by mixing with a provided loading dye and denaturing with high heat. After sample preparation, students load and run gels. Following the SDS-PAGE separation, students stain their gels in Coumassie solution and proceed to destain the gels for visualization.

Students determine molecular weights by measuring migration distances of marker proteins, constructing a standard line, and comparing migration distances of their sample proteins (Figure 4.3).

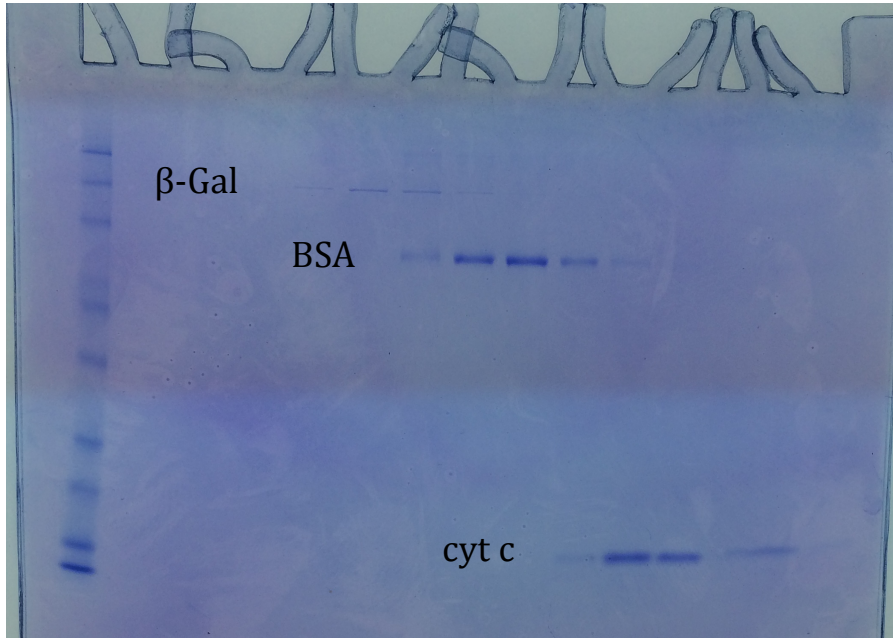


Figure 4.3 SDS-PAGE results displaying electrophoresed collected fractions. Following gel stain with coumassie blue, proteins were visualized. The annotation by each set of bands indicates the protein identity.

Prior to the beginning of the lab, mass spectra for each protein were collected (or, when unavailable, a representative spectrum was constructed). At this point, students are provided these, with each spectrum only labeled with the position in which the protein eluted. Students are tasked with utilizing the differing (and potentially conflicting) data collected from assaying, electrophoresis, and mass spectrometry in order to determine protein elution order and molecular weight.

After completing the experimental portion of this module, students report the determined size of each protein in their mixture. This report includes a detailed explanation of how they came to each determination, how they reconciled and prioritized any potential conflicting data, and a discussion of the benefits and limitations of each technique that contributed to their final results.

4.6 Results

A majority of students (69 of 87) attempting this experiment were able to successfully design a path to complete the module and provide an acceptable rationale for their determined protein molecular weights. One common error arose from the β -gal protein existing as a homotetramer, so molecular weight information obtained from SDS-PAGE yields a deceptively small mass; a majority were able to identify this and provide an attempted explanation, but a significant minority were unable to reconcile the mass differences. One common technical mistake performed was protein mixture dilution prior to column entry; this often resulted in difficulty monitoring the visible components of the mixture during separation and an excessive number of collected fractions. Second, many students reported having difficulty interpreting ELISA results; color development is time sensitive and it is likely that slow reagent addition played a role. However, the β -gal activity assay used proved extremely robust and, with access to a plate reader, a quantitative component could be readily incorporated into this module.

4.7 Discussion

Following an informal implementation, a formal implementation was conducted where three sections of 28-32 students completed this experiment. Chromatographic separations, ELISAs, and protein mass spectrometry were typically introduced in separate experiments prior to this and students often had trouble connecting the utility of each technique to solving larger problems. Student response to this laboratory module was highly favorable. To assess the impact of incorporating guided inquiry, students engaged in a group retrospective analysis^{28,29} to rate and rank this experimental module in addition to others in the course (questions are provided in 4.9), including experiments that focused on: pipetting; titrating an amino acid; restriction mapping; determining protein concentrations; characterizing forms of hemoglobin using spectrophotometry;

enzyme kinetics; and protein isolation and assaying. Ratings were collected as numerical inputs to signify how well a group of students believed each experiment was able to accomplish an educational goal, with a rating of '7' signifying that this was significantly accomplished and a rating of '1' signifying that this was not accomplished at all. Rankings were utilized to validate the data collected from rating questions, and the inverted numerical scale, purposefully inserted, prevent students from simply transcribing answers from the paired question. The results of the ratings, including standard error of the mean and a statistical p value, are displayed in Table 4.2. P values were determined utilizing a two-tailed student's t -test assuming equal variance that compared collected rankings against a hypothetical, purely neutral rating.

Table 4.2: Average Ratings Score of Each Experiment as it Accomplishes an Educational Goal

Experiment	Educational Goal		
	Utility/Relevance	Mimics Biochemical Research	Critically Evaluate Data
Pipetting/Buffer Preparation	5.83 (0.26), 0.00000	5.93 (0.44), 0.00015	2.80 (0.42), 0.00750
Amino Acid Titration	4.47 (0.42), 0.28031	4.27 (0.38), 0.49290	4.53 (0.39), 0.18096
Analysis of DNA/Restriction Mapping	5.80 (0.33), 0.00001	5.93 (0.32), 0.00000	6.07 (0.33), 0.00000
Determining Protein Concentrations	5.87 (0.27), 0.00000	6.27 (0.21), 0.00000	6.20 (0.28), 0.00000
Characterizing Forms of Hemoglobin	5.00 (0.34), 0.006232	5.07 (0.36), 0.00592	5.33 (0.27), 0.00003
Enzyme Kinetics	4.00 (0.40), 1.00000	4.33 (0.41), 0.42328	5.13 (0.58), 0.06262
Protein Isolation/Assaying	6.03 (0.29), 0.00000	6.60 (0.16), 0.00000	5.67 (0.41), 0.00035

Several interesting observations are revealed in Table 4.2, but of most significant importance is that student groups indicated that this new experimental module had high utility and relevance and was able to significantly prepare them for work in a research environment. Additionally, this module was able to accomplish these more capably than other experiments in the course. However, this module falls short when evaluating our desire for students to feel as if they are generating and critically evaluating data. This may have been a linguistic design flaw in the construction of our post-laboratory solicitation of feedback – it was later identified that many students disconnected the colorimetric results from the assays they performed as data, whereas this was not the case in experiments

that dealt with collected numerical outputs (such as in determining the concentration of a protein spectrophotometrically).

As a means of assessing long-term cognitive impact of this experimental module and the ability of students completing it to reappropriate their knowledge in new settings, an end of semester assessment was conducted. Students were tasked with providing written feedback and responses to challenges built off of techniques and concepts learned throughout the course, including an assessment module built off of concepts learned in this described experiment (Figure 4.10). This assessment was constructed not as an attempt to elicit 'correct' responses from students, but rather to determine if students are able to re-conceptualize concepts and apply them to new problems (as they would in a research setting); as such, detailed analysis of the assessment results is difficult. However, an independent member of the chemistry department rated and ranked student responses to this module, and we were encouraged to find that 82% of responding students (72 of 88) were able to provide adequate responses, and 40% (35 of 88) were able to provide exceptional responses.

From a student's perspective, there are many benefits from completing this module. Though not using cellular lysates, students are introduced to the difficulties and limitations of protein purification. Students also gain familiarity with several protein identification techniques and how protein characteristics necessitate different methods in different situations. This also illustrates disparate sensitivities and reliabilities from one method to the next (e.g. comparing an enzymatic assay to an immunoassay).

Finally, students benefit from situating this experiment in the context of a guided inquiry. Presented with different techniques and tasked with determining the best method to tackle their problem, students have described this module as feeling more like a research project than other experimental modules. Connecting data collected from different sources helps contextualize the techniques introduced in

this module to laboratory settings outside of the course, increasing student interest and engagement. The benefits of completing this laboratory were acknowledged as this experiment has been incorporated into the syllabus and, as of April 2015, has been implemented by instructors independent of those behind the design of the module. Based on the positive reception described herein, the implementation of this module and subsequent results are being prepared for communication with the larger chemical education audience through publication.

4.8 Student protocol

Chemistry 352 – Techniques in Biochemistry Experiment 9 Separation, Identification, and Analysis of Proteins

Protein purification is one of the most important facets of biochemical investigation. A successful purification procedure may require a number of different techniques. Chromatographic methods are some of the most common (and most successful) tools that a protein chemist can use. A wide variety of chromatographic techniques have been developed, and each utilizes a different means of discriminating between proteins (i.e. size, affinity for a particular ligand, pI, etc.). Gel filtration, which is used to separate proteins based on their native size, is among the most common, and you will be performing that procedure in the lab.

Identification of proteins following separation by chromatography is an integral step in purification. Most proteins are not visible in solution and indirect methods necessary for detecting the presence of a given specific protein. Common methods for protein identification include enzymatic assays and immunogenic assays, two indirect methods of determining the presence of a protein. During this experiment, you will utilize both types of assays to identify the presence of two distinct proteins in your column fractions.

Electrophoretic techniques are also quite useful when isolating and characterizing proteins. It can also be an attractive method to identify proteins when no identification assay is readily available. Like gel filtration, it can separate proteins based on size (or relative molecular weight, M_r), but it is often done under denaturing conditions. You will analyze the size, composition, and purity of various proteins separated from a mixture using SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), which is one of the most commonly used electrophoretic techniques for analyzing proteins.

Background

Suggested reading: Lehninger, 3.3; Voet & Voet, 6-3B, 6-4B,C

Gel filtration chromatography (gel permeation or size exclusion chromatography) uses a matrix (gel) of semi-porous microscopic beads to effect separation of molecules on the basis of size (and also shape). Such beads are typically made from hydrated polymers of dextran, acrylamide, or agarose, and they can be used to separate molecules ranging in size from molecular weight (M_r) ~ 100 to M_r ~ 10,000,000, depending on the size of the beads. Specific beads will have specific ranges of molecular weight that can be separated; solvent and molecules in this range will freely enter the beads while molecules outside the range are

eluted from the beads entirely. Molecules of sizes in a bead's range spend part of the time within the bead and part of the time excluded. The elution volume of molecules from the column therefore depends on how much time a molecule spends within the pores of the bead—larger molecules spend less time in the pores and thus elute faster.

Remember that gel filtration chromatography is usually run under native conditions: proteins remain folded in their native conformations and are able to interact with each other. Proteins that consist of more than one polypeptide chain (complexes) remain intact and run at the size of the complex, not the size of the individual components. Polyacrylamide gel electrophoresis (PAGE) uses a gel-based matrix to size proteins, but the proteins move through the gel based on their charge as well as effective size. Denaturing SDS-PAGE, in contrast, uses the detergent SDS (and often reducing agents) to denature proteins completely, breaking complexes apart. Each individual subunit will be coated with the detergent, giving each polypeptide a negative charge. Proteins will move through the gel due to this charge, but become hung up on the gel matrix based on their individual sizes. The relative distance that a protein migrates in the gel is inversely proportional to its molecular weight. By comparing the distance migrated by an unknown protein to a set of protein standards of known molecular weight (the standard “ladder”), the molecular weight of the denatured protein can be estimated.

ELISA (enzyme-linked immunosorbent assay) is a powerful tool for detecting the presence of very specific target molecules, often being able to detect incredibly small amounts of molecule. This method traditionally relies on the use of antibody-protein binding interactions, but it can and has been used in a much wider variety of general ligand binding tests. The ELISA method has been used for years as a diagnostic tool in medicine and it is widely used in industry as a means of checking quality control. It is such a practical tool that many consumer-oriented products have been developed from it (i.e. pregnancy tests). This method (with other immunoassay techniques like radioimmunoassay) is one of the most powerful biotechnological tools to have come out of the biochemical sciences.

Enzymatic assays provide another method to study and characterize specific proteins. Though limited in that a specific assay can only be used for a specific protein, an appropriate enzymatic assay can be a very powerful method to study that enzyme. Oftentimes, a reagent that mimics an enzyme's natural substrate can be used as a tool for study. One commonly used enzymatic assay is the β -galactosidase assay. Chlorophenyl red- β -D-galactopyranoside (CPRG) is a modified galactoside sugar that is chemically linked to a red dye. In the presence

of β -galactosidase, the enzyme cleaves the dye from the sugar and a red color is produced in solution. This assay is frequently used to determine the presence and concentration of β -galactosidase and is representative of similar colorimetric enzymatic assays.

Experiment 9 Goal:

You will be given a mixture of three proteins and are tasked with separating, identifying, and determining the molecular weight of each protein using the techniques described above.

Experimental Design

You are provided with the following:

- A column of sephacryl S300 filtration material, bed volume ~10 mL, equilibrated in PBS buffer – this column matrix can separate molecules between 10 and 1500 kDa
- A concentrated solution of the following mix of proteins:
 - Cytochrome C, a pink/red heme-containing protein involved in electron transfer
 - A galactosidase, a colorless enzyme that hydrolyzes galactosides into monosaccharides
 - A serum albumin, a colorless protein that binds lipids in blood
- A solution of blue dextran (a large colored sugar with a Mr ~ 2,000 kDa) and riboflavin (a brightly colored small molecule that will spend a maximal amount of time on the column)

Running the column

To get the best separation, the sample volume (the “load”) should be no more than 1% of the column volume; loading the sample properly is the key to attaining good chromatographic separation. To help keep your protein sample concentrated, you may add your blue dextran/riboflavin mixture to the column prior to adding your protein sample. To do this, it’s important to allow the buffer above your column to run to just above the column matrix. At this point, add the mixture, allow the mixture to run into the column, and add a small amount of PBS to further allow the column. When the mixture has fully entered the column, repeat this process with the protein sample; the aim is to have sample enter as a tight band with minimal dilution. When the protein sample has fully entered the column, top up the column with PBS, taking care to not disturb the column matrix. It is important to maintain a level of PBS above the column, never allowing it to dry.

Collect PBS in a graduated cylinder until you are ready to begin collecting fractions (hint: when will this be? what should be coming off the column first?). It

is up to you to determine fraction size - more fractions will result in better separation of proteins, but there are limits to how many fractions you can assay.

It is likely that you will have too many fractions to analyze on a single SDS-PAGE gel. To help narrow down which fractions to run on your gel, you have the option of performing a galactosidase assay and an ELISA assay designed to detect the serum albumin protein.

Identification by enzymatic activity – running the galactosidase assay

In order to determine which fractions contain your galactosidase protein, you will be utilizing an enzymatic assay that specifically detects the presence of this protein.

- Add 50 μL of positive control, negative control, or of your fraction to your microplate strip
- Add 150 μL of assay buffer to each well
- Monitor for color change and record which wells/fractions tested positive

Using this will allow you to determine which fractions contain your protein and can be used, along with the SDS-PAGE gel results, to determine the size of galactosidase. Note – this is a time sensitive assay, so be sure to add assay buffer quickly.

Identification by an immunogenic assay – running the ELISA

ELISA assays can be tailored to detect different proteins using different primary antibodies. You have an antibody that recognizes this serum albumin protein and can utilize it in an ELISA assay to determine which fractions contain this protein.

- Add 50 μL of positive control, negative control, or of your fraction to a well in your microplate strip
- After 5 minutes, dump out your wells by inverting them onto a stack of paper towels and gently tapping them. Add 150 μL of wash buffer to each of your wells. Discard your wash buffer. Repeat, but allow your wash buffer to sit in the wells for 5 minutes. The wash buffer will wash away any unbound proteins and block unoccupied binding sites.
- Add 50 μL of primary antibody to your wells. Allow it to bind for 5 minutes, after which time, dump out the antibody solution and wash 2x with wash buffer. You no longer need to incubate the wells with wash buffer.
- Add 50 μL of HRP-conjugated secondary antibody. Allow it to bind for 5 minutes, after which time, dump out the antibody solution and wash 2x with wash buffer.
- Add 100 μL of enzyme substrate to the wells.
- Monitor for color change and record which wells/fractions tested positive.

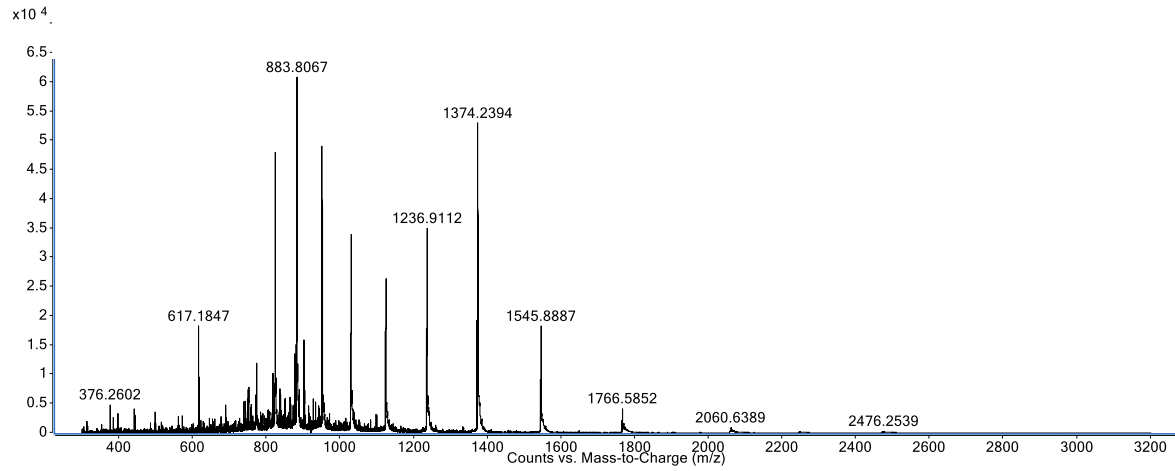
Analysis by SDS-PAGE - right after the column or in the following week

Your GSI will help you load and run your gel. If you are unfamiliar with running SDS-PAGE gels, get help before you start.

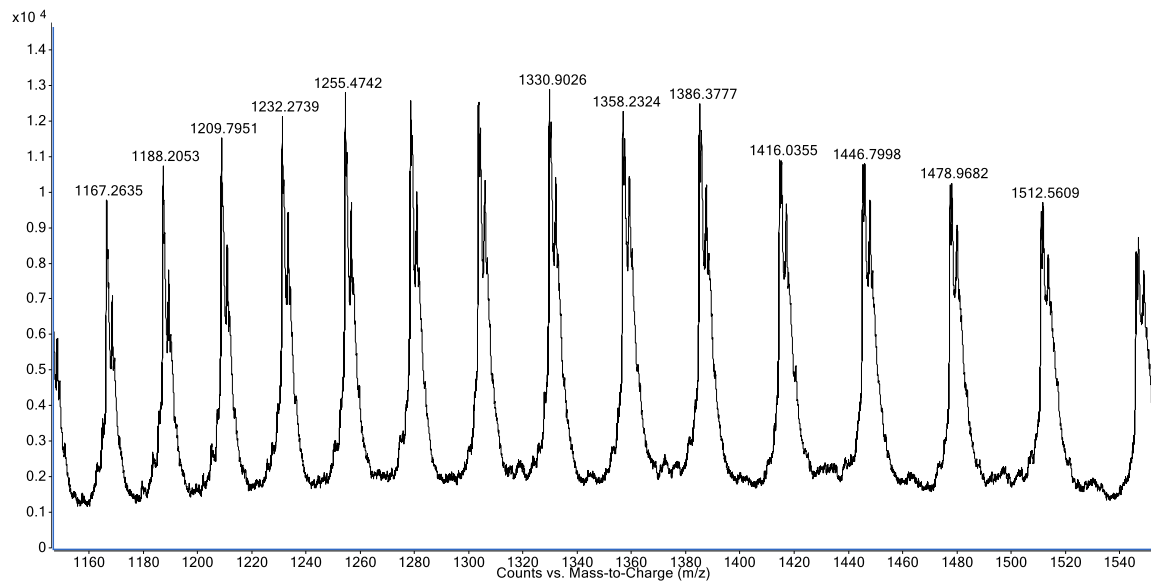
- Pipet 10 μL from each fraction into a separate eppendorf tube and add 10 μL of 2X gel loading buffer.
- Mix the sample, cap the tube and heat at 95 $^{\circ}\text{C}$ in a heating block for 5 min. (Question: what does heating in loading buffer do to the proteins?).
- During this time, set up your gel apparatus. Make sure you position the gel carefully to avoid leaking buffer. And don't forget – **remove the tape** from the bottom of the gel before setting up the apparatus.
- To run the gel, carefully load $\sim 15 \mu\text{L}$ (this will be based on the well capacity) of each sample into one of the sample wells on the top edge of the gel—use a pipetman to do this and make sure the wells are completely submerged with buffer before loading. Include a “ladder” lane of standards (**10 μL** of ladder should be fine).
- Run the gel at $\sim 180\text{V}$ until the blue marker dye is about 5 mm from the bottom edge of the gel.
- Carefully remove the gel cassette and pry apart the plates.
- Remove the gel—it can rip quite easily so be gentle—and transfer it to a container. Submerge the gel in enough Coomassie solution so that it completely covers the gel.
- Once the Coomassie dye has penetrated the gel, ($\sim 10\text{-}30$ min or until the entire gel is a deep deep blue), pour the Coomassie dye back into the bottle it came from and rinse the gel once with dH_2O .
- Pour the water in the appropriate waste and pour in enough destaining solution to completely submerge the gel. You can add a couple of balled-up Kimwipes in with your gel as the Kimwipes have an affinity for Coomassie and will help with the destain process.
- As unbound stain diffuses out of the gel, protein bands should become visible. If necessary, your gel may be left to destain until the next lab session, when you can photograph your gels to include as part of your results. Label your gel if you want to leave it over the week.
- When you are ready to analyze band migration, place your gel on a lightbox and measure the distance traveled by each band, including each band in your ladder

The following mass spectra were obtained to aid in precise protein size determination:

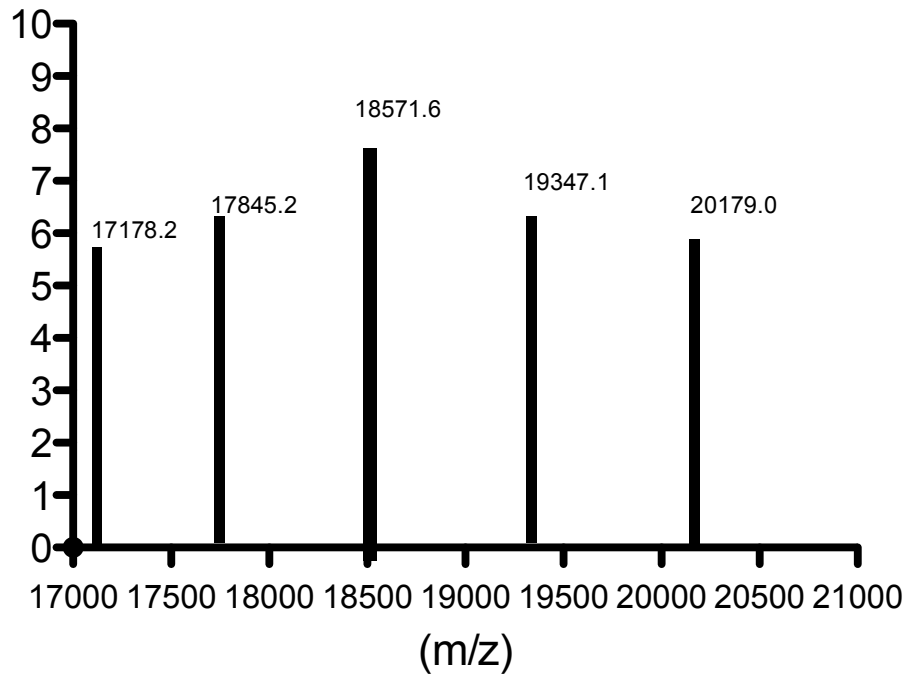
The last protein to elute from the column:



The second protein to elute from the column:



The first protein to elute from the column:



Write-up

In your post-lab write up, include the following:

- a digitized version of your gel including:
 - distances migrated for each band (including each band in your protein ladder)
 - a standard curve constructed using your protein ladder band migrations
- the determined molecular weights for cytochrome C, BSA, and β -galactosidase
- a detailed description of how you determined the molecular weight for each protein, including:
 - which data you utilized in your determination, noting any inconsistencies in information collected from different sources
 - how you prioritized data collected from different sources, and, more importantly, why
 - include an explanation of why you disregarded, if applicable, certain data sets
- a brief discussion of the benefits and limitations of each utilized technique (MS, size-exclusion chromatography, and each assay) – this will likely tie into your discussion on data prioritization

4.9 Student feedback handout

Experimental Evaluations

We are constantly striving to improve the designs of each experimental project in CHEM352, aiming to enhance both the scientific design and the educational utility of each lab. In order to aid in this process, we are asking that you help provide feedback so that future versions of this course can benefit.

The following questions ask you about your experience with each laboratory in CHEM352 and how you felt they helped accomplish course educational goals.

In place of individual survey, you will be working in groups to provide these assessments. It is imperative that each group member is vocal and included; collective responses about past experiments will help provide much more useful feedback. With the other lab members at your bench, work together to provide consensus responses, using reasoned arguments to describe your answers. In the event of a disagreement, work together to provide the best possible consensus response.

Question 1: An important part of any undergraduate laboratory course is introducing students to techniques that are relevant and useful (i.e. potentially used in a research setting). To what degree do you feel that each experiment accomplished this?

	Not at all			Neutral		Significantly	
	1	2	3	4	5	6	7
Pipetting/buffers:	1	2	3	4	5	6	7
Amino acid titration:	1	2	3	4	5	6	7
Analysis of DNA/GMOs:	1	2	3	4	5	6	7
Protein concentrations/spectrophotometry:	1	2	3	4	5	6	7
Hemoglobin separation:	1	2	3	4	5	6	7
Enzyme kinetics:	1	2	3	4	5	6	7
Protein isolation/assaying:	1	2	3	4	5	6	7

Question 2: Rank each experiment in terms of how each accomplished this goal; 1 = most significantly accomplished, 7 = least significantly accomplished

Pipetting/buffers:	1	2	3	4	5	6	7
Amino acid titration:	1	2	3	4	5	6	7
Analysis of DNA/GMOs:	1	2	3	4	5	6	7
Protein concentrations/spectrophotometry:	1	2	3	4	5	6	7
Hemoglobin separation:	1	2	3	4	5	6	7
Enzyme kinetics:	1	2	3	4	5	6	7
Protein isolation/assaying:	1	2	3	4	5	6	7

Include any comments/reasoning below:

Question 3: Unlike lecture courses, laboratory courses provide an opportunity for “situated learning,” allowing students to learn through doing by mimicking research activities. Though it’s unlikely any person can gain mastery of a skill/technique after one use, it is a goal of this course to introduce students to techniques in a setting that they would then be able to envision applying those techniques to alternative settings (such as to solving problems in a research lab). To what degree do you feel each experiment provided opportunities that would better prepare you for working in a research environment?

	Not at all			Neutral		Significantly	
Pipetting/buffers:	1	2	3	4	5	6	7
Amino acid titration:	1	2	3	4	5	6	7
Analysis of DNA/GMOs:	1	2	3	4	5	6	7
Protein concentrations/spectrophotometry:	1	2	3	4	5	6	7
Hemoglobin separation:	1	2	3	4	5	6	7
Enzyme kinetics:	1	2	3	4	5	6	7
Protein isolation/assaying:	1	2	3	4	5	6	7

Question 4: rank each experiment in terms of how each accomplished this goal; 1 = most significantly accomplished, 7 = least significantly accomplished

Pipetting/buffers:	1	2	3	4	5	6	7
Amino acid titration:	1	2	3	4	5	6	7
Analysis of DNA/GMOs:	1	2	3	4	5	6	7
Protein concentrations/spectrophotometry:	1	2	3	4	5	6	7
Hemoglobin separation:	1	2	3	4	5	6	7
Enzyme kinetics:	1	2	3	4	5	6	7
Protein isolation/assaying:	1	2	3	4	5	6	7

Include any comments/reasoning below:

Question 5: Laboratory courses provide an opportunity to produce real data. A goal of this course is to provide an opportunity to critique and evaluate data. Occasionally, it may even be necessary to assess and reconcile conflicting data in order to come to an experimental conclusion. To what degree do you feel did each experiment aid in your ability to critically evaluate data?

	Not at all			Neutral		Significantly	
Pipetting/buffers:	1	2	3	4	5	6	7
Amino acid titration:	1	2	3	4	5	6	7
Analysis of DNA/GMOs:	1	2	3	4	5	6	7
Protein concentrations/spectrophotometry:	1	2	3	4	5	6	7
Hemoglobin separation:	1	2	3	4	5	6	7
Enzyme kinetics:	1	2	3	4	5	6	7
Protein isolation/assaying:	1	2	3	4	5	6	7

Question 6: rank each experiment in terms of how each accomplished this goal; 1 = most significantly accomplished, 7 = least significantly accomplished

Pipetting/buffers:	1	2	3	4	5	6	7
Amino acid titration:	1	2	3	4	5	6	7
Analysis of DNA/GMOs:	1	2	3	4	5	6	7
Protein concentrations/spectrophotometry:	1	2	3	4	5	6	7
Hemoglobin separation:	1	2	3	4	5	6	7
Enzyme kinetics:	1	2	3	4	5	6	7
Protein isolation/assaying:	1	2	3	4	5	6	7

Include any comments/reasoning below:

Question 7: Are there other learning goals that you believe CHEM352 should be aiming to achieve? Please list any below.

Question 8: If you have any other critiques that you wish to include, please list them below.

4.10 End of semester assessment

Module 2: Protein Isolation

Scenario:

In your research lab, you have been attempting to purify a specific NADH-oxidizing enzyme to study *in vitro*. You believe you have isolated this enzyme from cellular lysate, but when you run a SDS-PAGE gel analyzing your protein, you notice an extra band that indicates an impurity smaller than your desired protein. You have a few hypotheses as to why you may be seeing two bands instead of one; suggest a method for testing each hypothesis:

- the enzyme is a heterodimer

- your purification method isolated an extra NADH-oxidizing enzyme, giving you a mixture of two different dehydrogenases

- a truncated version of the protein was also produced, missing a part of the C-terminus; according to the literature, the C-terminus is where the catalytic site of the enzyme is

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Chapter 5

Conclusions and Future Directions

5.1 Design and characterization of bifunctional glucocorticoids

Conclusions

The glucocorticoid receptor (GR) has been a drug target for the better part of a century, with early drugs even predating the first expression of a nuclear receptor by several decades^{1,2}, but the strategies to target this class of transcription factors have largely been stagnant in their methodology. Obtaining a crystal structure of the GR ligand-binding domain (LBD)³ was a breakthrough that allowed researchers to break away from traditional structure-activity relationships to guide ligand design⁴, but these efforts have overwhelmingly subscribed to a paradigm of targeting the LBD alone^{5,6}, ignoring discoveries that indicate factors outside the LBD play key roles in determining GR activity⁷⁻⁹. At the outset of my dissertation research, I set out to validate an alternative method of modulating GR activity. Chemical inducers of dimerization have been established as a novel, straightforward method for selectively inducing protein-protein interactions (PPIs)^{10,11} and provided an attractive method for guiding the construction of transcriptional complexes at GR-regulated genes.

Preliminary efforts focused on adapting the well established yeast three-hybrid assay¹² to a mammalian system utilizing human GR. In a standard three-hybrid assay, a Gal4 DNA-binding domain (DBD) is fused to a GR LBD, removing the need for a GR ligand to induce nuclear translocation (and effectively removing a

variable), while the removal of the GR N-terminal domain (AF1) removes potential PPIs from confounding an otherwise straightforward transcriptional output¹³. Despite these differences, we were able to develop a three-hybrid reporter system wherein transcriptional output was dependent on a bifunctional molecule bridging full-length GR and a fusion protein and dictated by the nature of the fusion. While the recruitment of a fusion-activator triggered sharp transcriptional activation, consistent with traditional three-hybrid experiments^{12,14}, it was especially promising to see that a corepressor fusion protein was able to inhibit transcription when recruited.

Extensive efforts were devoted to recreating recruitment-dependent transcriptional activity at GR-regulated genes. We were encouraged to find that the *S100P* gene appeared to be particularly sensitive to recruitment, but it was unclear why we were not able to reproduce similar activities at other genes. We were met with similar limitations when evaluating the activity of our BRD4-targeting bifunctional molecule. Recruitment-dependent effects on the transcription of certain endogenous genes appeared to be evident, but we were unable to make any certain conclusions about the susceptibility of a gene to transcriptional modulation by a recruited target. Predicting the activity of glucocorticoid-bound GR is difficult¹⁵⁻¹⁷, and our understanding of GR interactions within the larger environment of complex chromatin architecture is still in its infancy. These factors make it difficult to determine *a priori* when recruitment at endogenous genes is a possibility, but as sequencing technologies continue to develop and become more prevalent, our future ability to predict the transcriptional outcome of bifunctional molecule treatment will grow.

During the initial characterization of the functional effects of BRD4-recruitment to the GR LBD, it appeared that recruitment, in the absence of competing factors through other GR domains^{13,18,19}, could yield potent transactivation. This consequence did not translate to activity produced by full-length GR, but it nonetheless indicated that, under certain conditions, BRD4-recruitment might

result in transactivation. Following from this, we sought to determine if BRD4-recruitment to *antagonist*-bound GR, lacking appreciable activation capabilities, would result in GR transactivation. The rationale for this was two-fold: the production of novel GR transcriptional outputs, as would be a predicted result by this molecule, is desirable, and the functional conversion of an antagonistic molecule to an acting agonistic as a result of extrinsic recruitment would be a powerful display of the potential of this system. As was the case with our agonist-based bifunctional molecules, the activities stimulated by an antagonist-based bifunctional molecule were difficult to predict but highly encouraging. In a variety of systems, this molecule acted as a transcriptional activator relative to its parent antagonist. Investigating this molecule's interactions with abnormal GR isoforms, an interesting pattern of selectivity in activation induction emerged, hinting at the possibility for future mechanistic studies on recruitment. Most encouragingly, however, was the observation that this molecule is capable of potent transrepression. The exact mechanism of GR-mediated suppression of inflammation is still elusive, but it has become clear that an ideal transcriptional response involves a profile reflecting a mixture of transactivation and transrepression^{20,21}, hinting at the unique utility of this bifunctional molecule.

Future directions

Development of glucocorticoid-FK506 conjugates, preliminarily, was performed as a necessary step in confirming that full-length GR is a suitable partner in further three-hybrid studies, prior towards developing conjugates that target endogenous proteins. However, the success we achieved in recruitment of fusion proteins to GR, and subsequently affecting its activity, mark this as a potential method for monitoring the effects of recruiting alternative protein modulators to GR²². Advanced bifunctional ligands have been developed that recruit ubiquitin E3 ligases to targets, inducing the ubiquitylation of bound proteins²³; however, similar strategies to effect other post-translational modifications have largely been unexplored. The modularity of our system allows for the facile replacement

of recruited moieties, making the recruitment of kinase-, acetylase-, or sumoylating-fusion proteins, to name just a few potential partners, a possibility (Figure 5.1). Though modification of GR *in vitro* is a straightforward process, the ability to trigger modification through the introduction of an extracellular stimulus is intriguing and provides a new method for mechanistic studies of GR.

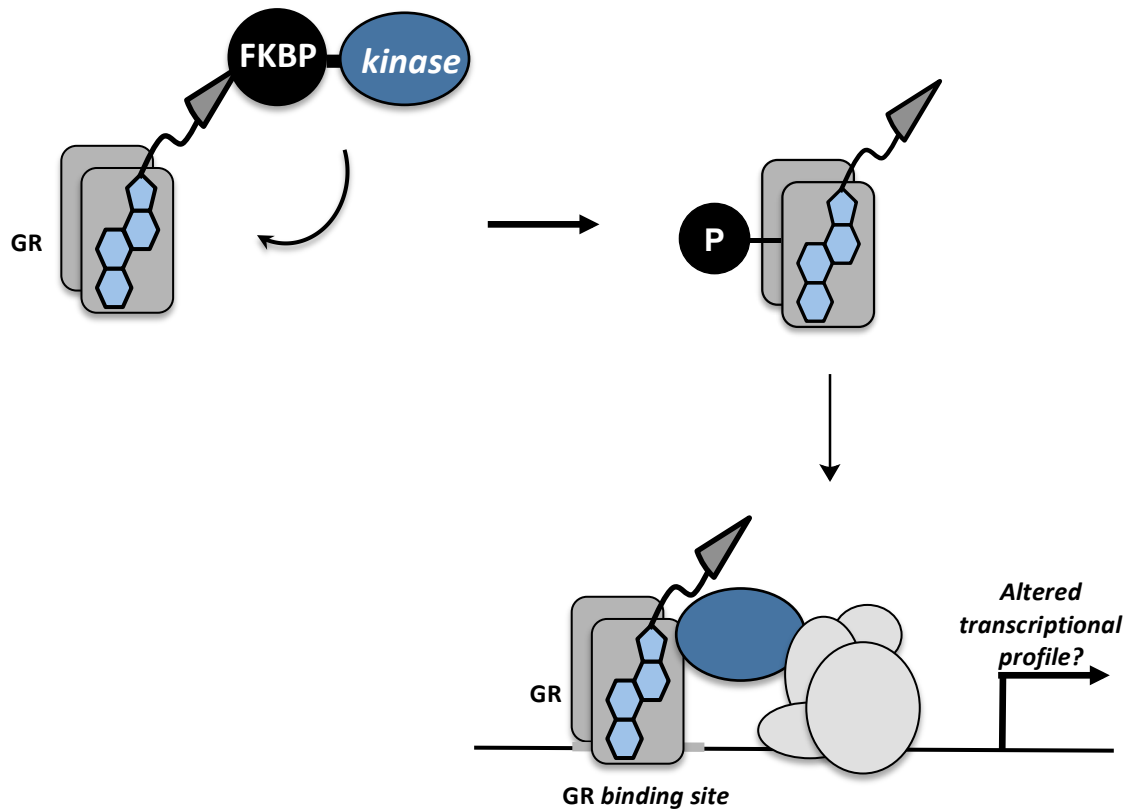


Figure 5.1 Inducible post-translational modification. The recruitment of specific fusion proteins to GR provides a potential method for triggering post-translational modifications, such as phosphorylation, in response to an extracellular stimulus by localizing relevant enzymatic proteins to the receptor.

One observation that bore out from our three-hybrid studies was that recruitment-dependent activity seemed to be dependent on cellular levels of GR. It is unclear if modulating levels of BRD4 will accordingly result in differential effects on GR activity following bifunctional molecule-treatment. In preliminary experiments, even slight overexpression of BRD4 lead to considerable cellular cytotoxicity, precluding an investigation into the role of BRD4 expression levels on

recruitment. If it bears out that recruitment and, subsequently, transcriptional suppression of GR is weakened in cells expressing lower levels of BRD4 (or alternatively, strengthened in highly expressing cells), this may provide a method for further selectivity in action. This concept, moreover, may provide additional layers of complexity regarding selectivity in action. Throughout the work described here, several different cell lines were utilized for varying reasons as indicated. Ligand-mediated recruitment is likely an event that is affected by competitive intrinsic interactions, and differences in coregulator expression levels among different cell lines and tissue types likely plays a role in the ability for and outcome of recruitment. Accordingly, it would be naïve to assume that our bifunctional ligands would effect similar outcomes across a variety of cell lines. As such, it will be important to expand our studies to include multiple cellular environments to cast light on the effects that this variable plays within our system.

In the construction of each bifunctional ligand, dexamethasone was chosen as a constituent member owing to its high affinity for GR and propensity for effective stimulation of nuclear translocation. As our investigations uncovered that BRD4 recruitment is a method for suppressing GR activity, however, it became evident that many of the initial qualities that made dexamethasone attractive could potentially be detrimental. Though likely not the sole cause, the strength of the dexamethasone-GR interaction relative to the JQ1-BRD4 interaction may provide an explanation for the persistent activation capabilities of our bifunctional ligand at low dosing concentrations. Prednisolone and methylprednisolone are less potent glucocorticoids that are established conjugation candidates²⁴ that could be used in the construction of ligands that provoke even further specialized transcriptional profiles, likely with low transactivation potential but maintained transrepression.

Though the antagonist-based bifunctional molecule was incapable of ‘rescuing’ the activity of defective receptors implicated in glucocorticoid resistance, this

molecule may provide a key towards targeting an alternative instigator of glucocorticoid resistance. The *NR3C1* gene is known to produce at least nine physiologically relevant GR isoforms²⁵, and though the α -isoform, described throughout this thesis, is believed to be the dominant receptor, a single alternative isoform, hGR β ²⁶, is believed to be expressed at significant levels in many, if not all, tissues. Produced by an alternative splicing event, hGR β has a truncated LBD containing fifteen unique residues relative to hGR α (Figure 5.2). Canonically, hGR β is incapable of ligand binding and serves simply as a dominant-negative receptor, serving to inhibit hGR α activity when overexpressed. However, recent reports have indicated that this isoform binds RU486 and undergoes nuclear translocation²⁷, though this observation is in some dispute²⁸. If hGR β does indeed bind RU486, the antagonist-based bifunctional molecule would provide a unique method of modulating this isoform's transcriptional activity, a highly desirable outcome given its believed role in the development of resistance in cancers such as multiple myeloma²⁹.

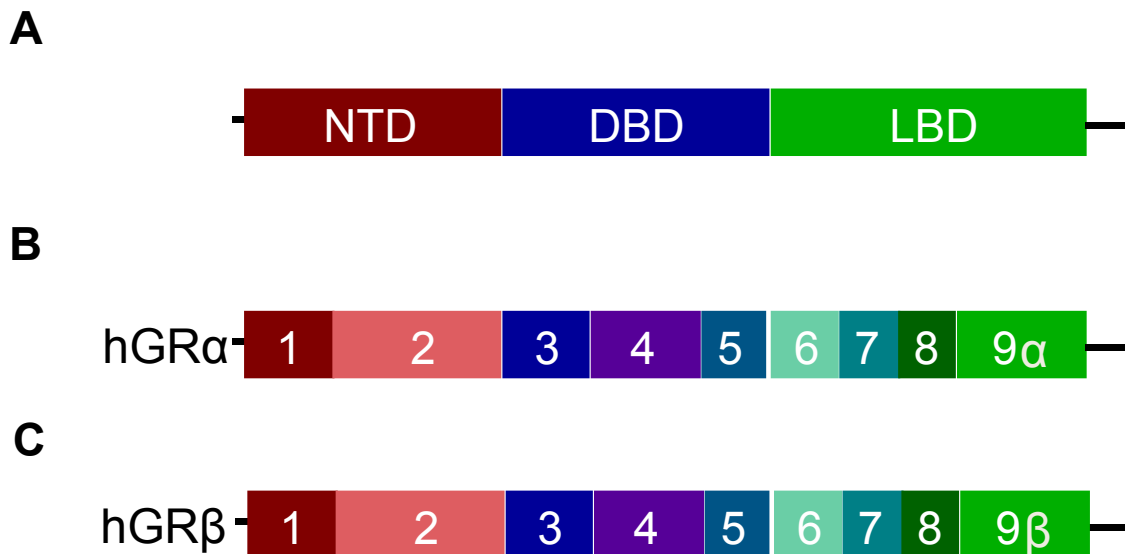


Figure 5.2 Glucocorticoid receptor isoforms. A: the glucocorticoid receptor is multidomain protein, consisting of a N-terminal domain (NTD), DNA-binding domain (DBD), and a ligand-binding domain (LBD). B and C illustrate the similarities between the dominantly expressed human GR isoforms, hGR α and hGR β . Numbers represent standard notation for exons of the *NR3C1* gene. The divergence of hGR α and hGR β arises from a splice variation, with each isoform incorporating a different ninth exon.

Many of these ideas touch on potential therapeutic applications for using our designed bifunctional steroids. However, the greatest power of this platform rests in the underlying modularity of the designed ligands and the proteins they target. Picking new targets and synthetically appending new ligands is relatively straightforward, and our ability to produce distinct transcriptional profiles accordingly is magnified exponentially relative to efforts that attempt to achieve this outcome through new monofunctional ligands. Currently, the prevailing limitation to this method is in our ability to predict the outcome of gene-specific regulation – it is highly beneficial that only certain genes are acutely sensitive to recruitment-dependent modulation, but we are currently unable to predict which factors contribute to this state. Despite this, we have demonstrated the much-needed development of molecules capable of producing complex, novel transcriptional profiles. As we expand our ability to predict genes sensitive to this effect, we will be able to better design bifunctional ligands that provoke desired activation and suppression states of sensitive targets across the genome. Expanding beyond our system, future efforts will focus on using this knowledge in the design of new ligands, expanding our matrix of utilized constituent ligands and exponentially increasing our ability to induce novel transcriptional profiles.

5.2 Effects of incorporating guided-inquiry into an educational laboratory

Conclusions

The course syllabus for CHEM352 at the University of Michigan, Biochemistry Techniques, is designed to provide students a broad immersion into techniques commonly performed in biochemical and molecular biological research laboratories. This design, coupled with rotating instructors for the course and limited resources for reform, precludes students from engaging in higher level cognitive activities³⁰ in a biochemical environment. Though a small step forward from the expository design, the guided-inquiry laboratory style has been

demonstrated to provide significantly expanded benefits for students, raising engagement, understanding, and long-term cognition in a variety of settings³¹⁻³⁶.

Identifying a three-week portion of a previous syllabus that had been identified as underwhelming by students and instructors, we aimed to reform the experiments therein by introducing students to the same techniques (protein chromatography, mass spectrometric analysis of proteins, protein electrophoresis, and immunoassays) in the form of a guided-inquiry investigation. Extensive optimization of the module yielded a reproducible experiment capable of being administered to large (> 75) groups of students reliably without necessitating the purchase of any prohibitively expensive reagents; indeed, the most expensive reagent, a primary antibody recognizing bovine serum albumin, was purchased inexpensively (\$135) at a quantity that is estimated to be adequate for six hundred students.

Informal student response to the module was overwhelmingly positive, with students commonly remarking that it was enjoyable to engage in an experiment where there was a perception of problem solving. Formal elicited feedback confirmed this, as students identified the laboratory as engaging and containing a higher level of utility (i.e. transferability of skills to a non-educational setting). In an end of term assessment, a majority of students were capable of transferring learned skills in the module to the design of methods for investigating related 'research' questions. As a result of this response, the experimental module has been formally incorporated into the course syllabus and is currently (April, 2015) being implemented by an instructor not involved with the design of the module.

The reproducibility of this module, paired with the relative low cost of performing it by large groups of students, marks it as especially attractive for incorporation by educators at institutions similar to the University of Michigan, where efforts for substantive renovation can be difficult.

Future directions

The construction of this laboratory module marked an important step forward in progressing towards a more significant overhaul of the CHEM352 syllabus, but there are still opportunities to continue to modify this experiment, raising its utility. For example, the experiment was intentionally designed to provide for a level of ambiguity to results obtained from the immunoassay portion of the module, believing this to be an appropriate method for students to have to interpret and reconcile unclear data. However, a significant portion of students met this with result by simply shutting down, believing the experiment itself to be flawed and without utility. This shed light on an important observation – many students observe experimental protocols as sacrosanct and do not have to go through the rigor of repeating and refining techniques, providing an inadequate impression of actual research. Though difficult to implement, allowing students to engage in method refinement would be an important way to further develop this module, and I believe the subsequent cognitive benefits would make the work well worth the effort.

5.3 References

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