2-2014

Bifunctional Ligands Allow Deliberate Extrinsic Reprogramming of the Glucocorticoid Receptor

Jonas W. Højfeldt

Osvaldo Cruz

Yasuhiro Imaeda

Aaron R. Van Dyke
Fairfield University, avandyke@fairfield.edu

James P. Carolan

Published Citation

Højfeldt, Jonas W; Cruz, Osvaldo; Imaeda, Yasuhiro; Van Dyke, Aaron R.; Carolan, James P.; Mapp, Anna K.; Iñiguez–Lluhí, Jorge. "Bifunctional Ligands Allow Deliberate Extrinsic Reprogramming of the Glucocorticoid Receptor," Molecular Endocrinology February 2014, 28(2), 249–259.

Bifunctional Ligands Allow Deliberate Extrinsic Reprogramming of the Glucocorticoid Receptor

Jonas W. Højfeldt,* Osvaldo Cruz-Rodríguez,* Yasuhiro Imaeda, Aaron R. Van Dyke, James P. Carolan, Anna K. Mapp, and Jorge A. Iñiguez-Lluhí

Department of Chemistry (J.W.H.,Y.I., J.P.C., A.K.M.), University of Michigan, and Department of Pharmacology (O.C.-R., J.A.I.-L.), University of Michigan Medical School, Ann Arbor, Michigan 48109; and Department of Chemistry and Biochemistry (A.R.V.D.), Fairfield University, Fairfield, Connecticut 06824

Therapies based on conventional nuclear receptor ligands are extremely powerful, yet their broad and long-term use is often hindered by undesired side effects that are often part of the receptor's biological function. Selective control of nuclear receptors such as the glucocorticoid receptor (GR) using conventional ligands has proven particularly challenging. Because they act solely in an allosteric manner, conventional ligands are constrained to act via cofactors that can intrinsically partner with the receptor. Furthermore, effective means to rationally encode a bias for specific coregulators are generally lacking. Using the (GR) as a framework, we demonstrate here a versatile approach, based on bifunctional ligands, that extends the regulatory repertoire of GR in a deliberate and controlled manner. By linking the macrolide FK506 to a conventional agonist (dexamethasone) or antagonist (RU-486), we demonstrate that it is possible to bridge the intact receptor to either positively or negatively acting coregulatory proteins bearing an FK506 binding protein domain. Using this strategy, we show that extrinsic recruitment of a strong activation function can enhance the efficacy of the full agonist dexamethasone and reverse the antagonist character of RU-486 at an endogenous locus. Notably, the extrinsic recruitment of histone deacetylase-1 reduces the ability of GR to activate transcription from a canonical GR response element while preserving ligand-mediated repression of nuclear factor-κB. By providing novel ways for the receptor to engage specific coregulators, this unique ligand design approach has the potential to yield both novel tools for GR study and more selective therapeutics. (***Molecular Endocrinology*** **28: 249–259, 2014)**

Synthetic glucocorticoids are one of the most widely used pharmacological agents, mainly because of their potent antiinflammatory and immunosuppressive effects. Given that maladaptive inflammation or inappropriate immune responses are a central part of many chronic diseases, glucocorticoids are an invaluable therapeutic tool in a wide range of conditions including arthritis, asthma, lupus, and allergy and are an important element of immunosuppressive regimens for organ transplantation (1). Despite these well-established and sometimes life-saving therapeutic applications, conventional glucocorticoid therapy is severely limited due to undesirable side effects. These are mainly due to the profound metabolic changes in energy and protein metabolism that endogenous glucocorticoids set in motion as an adaptive response to transient stress. Consequently, pharmacological glucocorticoid excess leads to hyperglycemia, visceral adiposity, and insulin resistance as well as muscle wasting and osteoporosis. Pharmacological approaches that mitigate the metabolic effects of glucocorticoids while preserving their immunomodulatory activity would be a major therapeutic advance. This, however, has proven elusive despite intense efforts (2–4).

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

Received October 26, 2013. Accepted December 10, 2013.
First Published Online January 6, 2014

* J.W.H. and O.C.-R. contributed equally to this work.

Abbreviations: AF-2, activation function-2; Dex, dexamethasone; HDAC, histone deacetylase; HEK, human embryonic kidney; FKBP, FK506 binding protein; GR, glucocorticoid receptor; LBD; ligand binding domain; NF-κB, nuclear factor-κB; NLS, nuclear localization signal; SDex, derivative of Dex that allows facile conjugation with other molecules; VP16, virion protein 16.

The effects of glucocorticoids are mediated by the glucocorticoid receptor (GR), a prototypic member of the nuclear receptor superfamily of sequence-specific, ligand-regulated transcription factors. Upon binding of an agonist to the C-terminal ligand binding domain (LBD), the GR translocates to the nucleus and localizes to specific loci through a central zinc finger region capable of direct recognition of specific sequences or through tethering to other transcription factors. From these sites, the GR influences the transcription of target genes by nucleating the assembly of specific coregulatory complexes through protein-protein interactions (5). The receptor orchestrates this process by integrating multiple signals (6), including variations in the target DNA sequence (7), intracellular signaling cascades, posttranslational modifications (8, 9), and uniquely, small cell-permeable ligands that bind to the LBD (Figure 1A).

The canonical mode of action of endogenous ligands involves their binding to the LBD and consequent reorientation of helix 12, leading to the engagement of the C-terminal activation domain [activation function-2 (AF-2)]. In concert with additional activation functions in the N-terminal region, these conformational changes alter the interaction surfaces for transcriptional coactivators and corepressors that are responsible for controlling chromatin remodeling as well as transcriptional initiation and elongation (10).

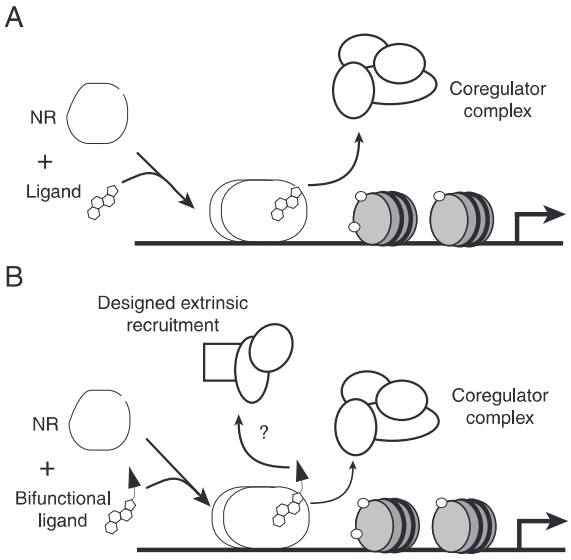

Figure 1. Extrinsic control of nuclear receptors. A, Transcriptional regulation by conventional ligands involves binding to the nuclear receptor (NR) and subsequent nucleation of coregulator complexes. The spectrum of targeted complexes is dictated mainly by the intrinsic conformation of the LBD induced by the ligand. B, Bifunctional ligands with unique targeting functionalities may allow the selective recruitment of coregulator complexes not accessible to conventional ligands.

Glucocorticoid responses are the integration of complex patterns of tissue-specific gene expression and involve both activation and repression of target genes (11, 12). Limiting metabolic side effects while preserving immunomodulatory actions therefore would require a clear identification of the on-pathway desirable responses as well as those involved in undesirable side effects and, importantly, an effective means to elicit one without the other. The ability of glucocorticoids to repress expression of multiple proinflammatory cytokines is a central component of its antiinflammatory effects, whereas the induction of metabolic enzymes such as phosphoenolpyruvate carboxykinase is an important component of the metabolic response. Initial efforts have therefore focused toward dissociated agonists that can support repression while minimizing transactivation but have met with limited success (2–4).

The difficulties associated with conventional nuclear receptor ligands are likely to be due in large part to the fact that they act solely through the allosteric modulation of receptor conformation and are therefore constrained to recruit from a closed set of cofactors that are within the intrinsic interaction envelope of the receptor. This puts a limit to the range of achievable functional effects. Furthermore, despite the incorporation of novel protein dynamics criteria (13), it is not yet possible to rationally design into a conventional ligand the ability to recruit specific coregulator complexes or to generate a particular pattern of gene expression. A further challenge is that coactivators involved in transactivation such as GR-interacting protein-1 also participate in transcriptional repression mechanisms (14, 15). Furthermore, the development of clinical resistance is a significant problem, particularly in cancer therapy (16).

In a significant departure, we describe here a novel strategy that circumvents the limitations of conventional allosteric ligands and frees the receptor to engage in novel extrinsic functional interactions that are open to rational design. The approach leverages the versatility of GR bifunctional ligands, which, although have proven useful in three hybrid assays (17), have never been exploited to regulate the intact GR in its native context. By linking prototypic agonist and antagonist GR ligands to the small molecule FK506, we have thus generated bifunctional ligands that can bind the intact receptor and bridge it to designed transcriptional activators or corepressors containing the FK506 binding protein (FKBP) domain (Figure 1B). Using this strategy, we demonstrate for the first time that the regulatory repertoire of the native receptor at endogenous genomic loci can be expanded by directing the deliberate recruitment of extrinsic coregulators. This strategy allows the designed reprogram-

ming of the intact receptor and can dramatically enhance or completely reverse the efficacy of conventional ligands. By eliciting unique responses, this new class of GR ligands can thus serve as mechanistic probes to discern which are the relevant on-pathway transcriptional responses required for specific therapeutic effects and which are not required or causative of undesired side effects (18). Notably, this work demonstrates the viability of exploiting druggable small molecule binding sites in coregulator complexes for extrinsic recruitment to the receptor and opens the way for the purposeful recruitment of endogenous transcriptional coactivators and repressors to identify and drive therapeutically desirable responses.

Materials and Methods

Synthesis and binding affinity of GR bifunctional ligands

The macrolide FK506 was installed via a polyethylene glycol linker to a dexamethasone (Dex) derivative in which the 21-hydroxyl group is replaced by a thioether linkage (19). For the RU486 derivatives, a similar linker strategy was used using the aniline group of RU486 as the attachment point. Parallel compounds lacking the FK506 moiety were prepared as controls. Detailed information on compound synthesis is included as Supplemental Data, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. Binding affinities for Dex, RU486, and their derivatives were determined experimentally by radiolabeled competition binding assays using an extract from Hi5 insect cells expressing an N-terminally His- and green fluorescent protein-tagged rat GR. For extract generation, Hi5 cells were infected with H10 eGFP TEV GR baculovirus at a multiplicity of infection of 4 and lysed 48 hours after infection with a Dounce homogenizer in 20 mM HEPES (pH 7.4), 1 mM EDTA, 5% glycerol, 20 mM sodium molybdate, 5 mM dithiothreitol, and Complete (Roche) protease inhibitor cocktail (one tablet per 10 mL). The homogenate was centrifuged at 60 000 rpm at 4°C for 30 minutes in a TI-35 rotor (Beckman). The clear supernatant was aliquoted, flash frozen, and stored at 280°C until use. Binding assays were carried out in 10 mM HEPES (pH 7.4), 1 mM EDTA, and 20 mM sodium molybdate buffer in a 96-well plate format. The receptor extract (30 μg/well) was incubated with a mixture consisting of 10 nM 1,2,4,6,7-[^{3}H]dexamethasone (PerkinElmer) and increasing concentrations of test ligand at 4°C in a final volume of 50 μL. After a 2-hours incubation, 100 μL of a dextran coated charcoal solution (1% charcoal; 0.2% dextran in 10 mM HEPES, pH 7.4; 1 mM EDTA) was added to each well, followed by centrifugation for 2 minutes at 1000 3 g. Aliquots (80 μL) of the resulting supernatants were transferred to Optiplate 96-well plates (PerkinElmer), supplemented with 120 μL of MicroScint 40 (PerkinElmer) and read on a TopCount NXT microplate scintillation counter. Data were fit to a single binding site competition model using GraphPad Prism version 5.0 (GraphPad Software).

Plasmids, cell culture, and transfections

Plasmids p6R GR (20) and pRSV β-gal (21) are Rous sarcoma virus promoter-driven expression vectors for wild-type rat GR and β-galactosidase, respectively. pGBR 6.1 is a luciferase-based reporter harboring a 500-bp intronic region of the human FKBP5 gene and has been described previously (11, 22). The 53 nuclear factor-κB (NFκB) luciferase reporter (23) was a kind gift of Dr Gabriel Nuñez (University of Michigan). Plasmids pLIC FKBP and pNLS-FKBP are cytomegalovirus promoter-driven expression vectors for a protein bearing the Simian virus-40 nuclear localization signal (NLS) followed by human FKBP1A. The VP16 activation domain sequence (residues 411–456) was inserted between the NLS and FKBP1A sequences of pNLS-FKBP to generate pNLS-VP16-FKBP. A similar strategy was used to insert the mouse histone deacetylase (HDAC)-1 sequence at the same position of pLIC FKBP. For all constructed plasmids, relevant regions were sequenced and are available upon request. Human embryonic kidney (HEK) 293T cells were cultured in DMEM containing 10% fetal bovine serum (Life Technologies).

For functional assays, 3 3 10^6 cells seeded in 100-mm plates were transfected 24 hours later with 50 ng of p6R GR, 400 ng pGBR 6.1, 200 ng pCMV β-galactosidase, and 200 ng of pNLS-FKBP, pNLS-VP16-FKBP, or pLIC HDAC1-FKBP using Lipofectamine (Life Technologies) according to the manufacturer's instructions. Cells were trypsinized 16 hours after transfection and resuspended in DMEM supplemented with 10% heat-inactivated, charcoal-stripped fetal bovine serum and seeded onto 96-well plates at a density of 2 3 10^4 cells/well. After an additional 8 hours, cells were treated with either vehicle or the indicated ligands and harvested 16 hours later. Cells transfected with the 53 NF-κB luciferase reporter plasmid were treated with 10 ng/mL of human TNFα (Sigma) in addition to the indicated ligands. Luciferase and β-galactosidase activities were determined as described previously (20). For endogenous gene expression analysis, cells (1.5 3 10^4/well) were seeded onto 24-well plates and transfected 16 hours later with 50 ng of p6RGR and 25 ng of pLIC FKBP, pNLS VP16 FKBP, or pLIC HDAC1-FKBP using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Sixteen hours after transfection, cells were treated for an additional 6 hours with either vehicle or the indicated compounds. Total RNA was isolated using RNeasy RNA isolation kits (QIAGEN), and 500 ng of each RNA sample was used to synthesize cDNA using iScript cDNA synthesis kits (Bio-Rad Laboratories).

Quantitative real-time PCRs were carried out in duplicate in a Roche 480 LightCycler using QuantiTect SybrGreen reagents (QIAGEN) and primers for human RPL19 (forward, 59-ATGTATCACAGCCTGTACCTG-39; reverse 59-TTCTTGGTCTCTTCCTCCTTG-39) and S100P (forward, 59-CGGAACTAGAGACAGCCATGGGCAT-39; reverse 59-AGACGTGATTGCAGCCACGAACAC-39) genes. LinRegPCR (version 11.0) (24) software was used to estimate S100P mRNA levels relative to the reference RPL19 transcript. For protein level analysis, parallel cultures were harvested directly in SDS-PAGE sample buffer. After brief sonication, lysates were centrifuged for 2 minutes at 16 000 3 g, and supernatants were resolved by SDS-PAGE and transferred to polyvinyl difluoride membranes (Millipore). FKBP12 (Abcam ab58072) and BuGR2 antibodies were used at 1:2000 dilution. Images were captured in a Li-Cor Odyssey Fc reader using stabilized goat antimouse horseradish per-

oxidase-conjugated antibodies (Pierce) and Super Signal West Femto chemiluminescence reagents (Pierce).

Results

Design of bifunctional ligands

Using the GR as a paradigm and building on the significant experience in structure-activity relationship for GR ligand conjugates (17, 19, 25–28), we created bifunctional molecules based on the agonist Dex and the antagonist RU486. The least perturbing derivative of Dex that allows facile conjugation with other molecules has been termed SDex, in which the 21-hydroxyl group is replaced by thioether linkages (19) (Figure 2). RU486 has been linked to bile acids through its aniline group and retained antagonistic activity (28). Using a polyethylene-glycol linker as a spacer, we have thus conjugated these ligand derivatives to the natural product FK506 (Figure 2). Our choice was based on the extensive experience using FK506 conjugates for ligand-induced protein complex assembly (29, 30). Compounds bearing a linker only were synthesized as controls (Figure 2).

The binding affinity of the conjugates for full-length GR was determined in a radiolabeled Dex competition binding assay. As can be seen in Figure 3A, the affinities of SDex-O_3-OMe and SDex-O_2-FK506 are approximately 50 and 100 nM, respectively. Although this is 10- and 20-fold lower than the affinity of Dex (5 nM), these values are in a range comparable with the affinity of endogenous steroids such as cortisol (20 nM). Although the conjugation of FK506 to SDex lowers the binding affinity a modest 2-fold, this difference is not due to effects of binding cellular FK506-binding proteins because binding studies done in the presence of free FK506 yielded similar results (data not shown). In the case of the RU486 derivatives, both compounds displayed comparable affinities (; 90 nM), which are less than an order of magnitude (; 6-fold) lower than that of unmodified RU486 (Figure 3B). From this analysis, it is clear that the synthetic strategy yielded bifunctional ligands that retain a significantly high affinity for GR.

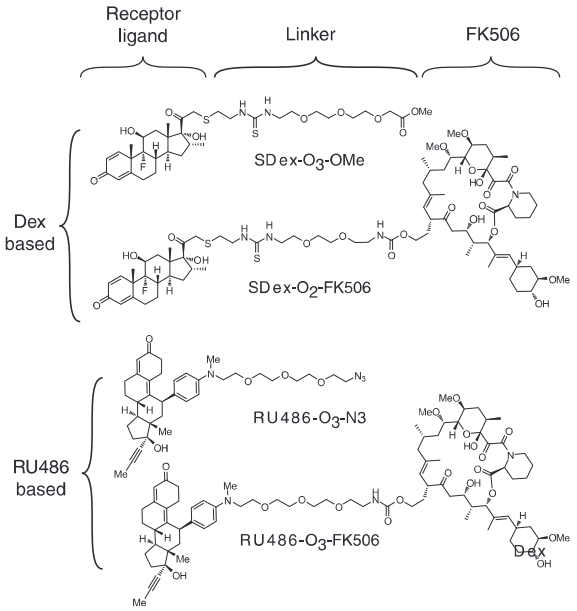

Figure 2. GR bifunctional ligands. Structures of GR bifunctional ligands based on the agonist dexamethasone (top panel) or the antagonist RU486 (bottom panel). Receptor binding, linker, and FK506 moieties are indicated.

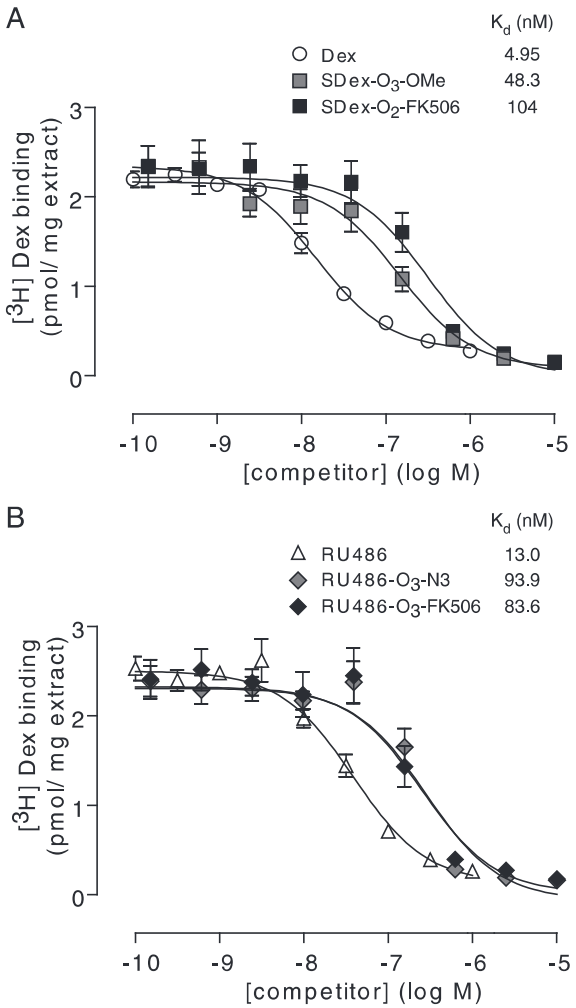

Figure 3. Bifunctional ligands bind to the GR. Competition binding assays based on displacement of ^{3}Isqb]H]Dex (10 nM) from GR were carried out as described in Materials and Methods. All curves are fits to a competitive single binding site model. Values for the calculated dissociation constant (K_d) are indicated in the inset. A, Competition using Dex- or SDex-derived ligands. B, Data for RU486-based ligands.

Extrinsic recruitment of a designed coactivator enhances GR ligand efficacy

FK506-binding proteins such as human FKBP1A bind with subnanomolar affinity to FK506, and the fact that this high-affinity interaction is retained, even when the macrolide is conjugated to other molecules, has made this pairing the basis for multiple successful small molecule-mediated protein recruitment strategies (31). As a first approach, we designed and constructed a coactivator fusion protein consisting of a nuclear localization signal, the strong transcriptional activation domain of the herpes simplex virion protein 16 (VP16), and FKBP1A and examined its ability to modulate GR activity by monitoring the transcriptional output of a GR-stimulated reporter driven by a natural GR enhancer sequence. As can be seen in Figure 4A, in the absence of any fusion protein, the Dex-derived bifunctional molecules (SDex-O_2-FK506 and SDex-O_3-OMe) led to a dose-dependent enhancement of activity with both compounds achieving maximal responses comparable with that of the parent agonist Dex. As expected from their somewhat reduced binding affinity, these compounds activated with lower potency relative to Dex. Interestingly, even though SDex-O_2-FK506 has discernibly lower affinity than SDex-O_3-OMe, both compounds activated with comparable potencies, suggesting an advantage for the FK506 conjugate. From these data, it is apparent that despite the presence of the linker and FK506 moiety, the bifunctional ligands remain cell permeable and retain full efficacy, revealing a significant degree of steric tolerance by the native receptor.

In notable contrast, in the presence of the VP16-FKBP fusion (Figure 4B), the FK506-conjugated ligand (SDex-O_2-FK506) displayed a nearly 2-fold increase in maximal activity relative to Dex, and the corresponding EC_{50} of 0.76 nM reflects an approximately 7-fold increase in potency relative to the linker-only ligand (SDex-O_3-OMe). Notably, these effects were specific to the FK506-conjugated ligand because the presence of the fusion did not appreciably alter the response to Dex or SDex-O_3-OMe. Thus, the enhancement depends on both the fusion and the FK506 moiety of the ligand. These data argue that the enhanced activation in the presence of the fusion is due to extrinsic recruitment by the bifunctional ligand. If this is indeed the case, it can be anticipated that the effect should be disrupted by excess unconjugated FK506 (32). In support of this prediction, increasing concentrations of free FK506 reduced the activity of the bifunctional ligand (Figure 4C) until it was essentially indistinguishable from the behavior of the ligand lacking FK506 (except for a small increase in basal activity at the highest concentration of FK506). As expected, the dampening effect of free FK506 depended on the presence of the fusion and was

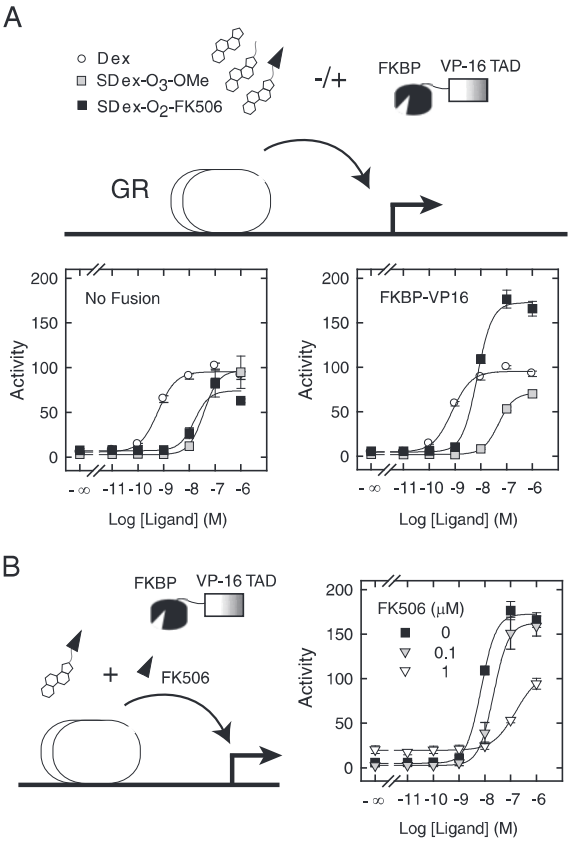

Figure 4. Directed enhancement of efficacy using bifunctional GR ligands. Dose-response curves for transcriptional activation by Dex, SDex-O_3-Ome, and SDex-O_2-FK506 in HEK 293T cells expressing GR alone (A), or coexpressing GR and the fusion protein FKBP-VP16 (B). Note the bifunctional ligand and fusion-dependent enhancement in efficacy. C, Dose-response curves for SDex-O_2-FK506 in the absence (filled squares) or presence of 0.1 (gray triangles) or 1 μM (open triangles) of free FK506 in cells coexpressing GR and the fusion protein FKBP-VP16. Note that at the highest concentration of FK506, the response reverts to that seen in the absence of extrinsic recruitment. Data represent the average 6 SEM of at least three experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex.

not observed for SDex-O_3-OMe (data not shown). Taken together, these results clearly indicate that by using the extrinsic recruitment strategy, bifunctional ligands endow GR with the ability to activate transcription well beyond what can be achieved with one of the most efficacious conventional ligands. This strategy opens the way for uniquely high-efficacy ligands that could serve to overcome clinical resistance or to restore function due to inborn deficits in GR transactivation.

Extrinsic recruitment of HDAC1 selectively reduces agonist efficacy in activation but not repression contexts

Therapeutically, GR ligands that have reduced transactivation efficacy but retain full agonism in repression

contexts have been sought after because they may display more favorable efficacy vs side effect profiles. Because these properties have proven to be difficult to obtain using conventional ligands, we sought to build on our initial results and use the extrinsic control approach to explicitly design and implement this desired regulatory outcome. Because recruitment of corepressor complexes is a common strategy used by transcription factors to negatively regulate transcription, we constructed a designed coregulator in which FKBP1A is fused to the histone deacetylase HDAC1, an enzyme that is an integral component of multiple corepressor complexes (33, 34). In the presence of this coregulator, we then examined the ability of SDex-based bifunctional ligands to mediate GR activity in both a canonical activation context (as examined in the experiments above, Figure 5A, left panel) as well as in a repression context (Figure 5A, right panel) in which GR inhibits TNFα-stimulated NF-κB activity. As in the case of proinflammatory cytokine genes, GR is recruited to DNA indirectly by tethering to NF-κB and inhibits transcription in an agonist-dependent manner, likely at a step downstream of RNA polymerase II recruitment (35, 36). As can be seen in Figure 5B, left panel, in the presence of the FKBP-HDAC1 coregulator, the maximal response elicited at the activation context by SDex-O_2-FK506 was significantly blunted, reaching only approximately 30% of the response induced by Dex. In contrast, the fusion did not alter the activity elicited by either Dex itself or SDex-O_3-OMe (compare with Figure 4). These results indicate that the extrinsic recruitment of HDAC1 can successfully oppose the intrinsic efficacy of a conventional ligand in an activation context. In notable contrast, analysis of the tethering repression context (Figure 5B, right panel) revealed that both the control and FK506-conjugated SDex bifunctional ligands supported GR-mediated inhibition of NF-κB to the same extent as Dex (Figure 5B). Because this same repression activity profile was observed in the absence of any fusion protein (data not shown), this indicates that the FKBP-HDAC1 coregulator did not interfere with the ability of the ligands to repress in this context. As expected, given their lower intrinsic affinity, the bifunctional ligands were less potent than Dex in both the activation and repression contexts. Taken together, the results indicate the successful establishment of the desired outcome and underscore the deliberate design potential of the extrinsic recruitment approach.

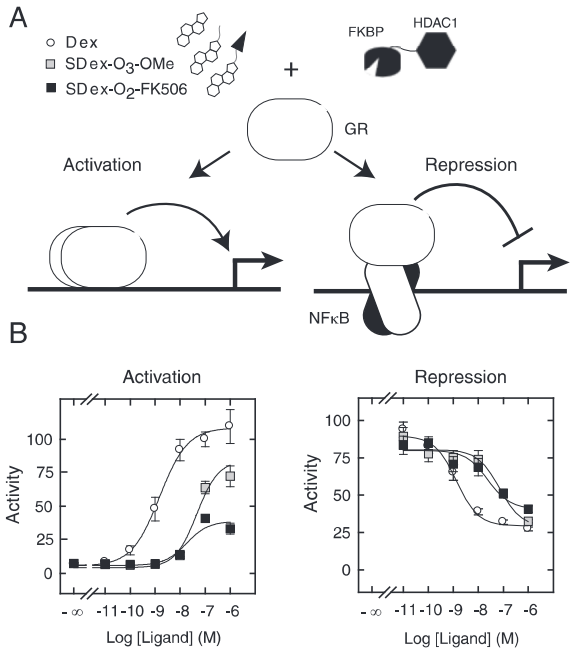

Figure 5. Suppressed transactivation with preserved transrepression using extrinsic recruitment. A, Diagram depicting the GR-mediated activation (left panel) and repression (right panel) contexts for functional assays. The bifunctional ligands with associated legends are also shown. B, Dose-response curves for transcriptional activation by Dex, SDex-O_3-Ome, and SDex-O_2-FK506 in HEK 293T cells coexpressing GR and the fusion protein HDAC1-FKBP are shown on the left (see Figure 4 for comparison). Transcriptional repression of TNFα stimulated NF-κB activity by Dex and the bifunctional ligands in the presence of the fusion protein HDAC1-FKBP is shown on the right. Data represent the average ± SEM of at least three experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex (left panel) or 10 ng/mL TNFα alone (right panel). The basal activity in the absence of TNFα was less than 1%.

Efficacy switch from antagonist to agonist through extrinsic recruitment

The above experiments show that in an activation context, it is possible to positively or negatively modulate the efficacy of an agonist ligand by the judicious extrinsic recruitment of coregulators. To probe the design versatility and scope of this approach, we sought to determine to what extent the regulatory effects of extrinsically recruited factors can be dissociated from the efficacy intrinsic to the GR binding moiety of the bifunctional ligand. To this end, we examined the properties of RU486-based ligands in which the receptor binding moiety is an antagonist. As can be seen in the left panels of Figure 6, in the absence of any fusion, RU486 and its derivatives showed no detectable activation of the GR-responsive promoter (Figure 6A, left panel). The compounds, however, are active and cell permeable because they are able to antagonize the activity of 3 nM Dex in a dose-dependent manner (Figure 6B, left panel). Interestingly, even though both RU486-based ligands have indistinguishable affinities (Figure 3B), the FK506 conjugate antagonized Dex with an IC_{50} approximately 6-fold lower than the linker-only ligand. This makes the FK506 conjugate comparable with

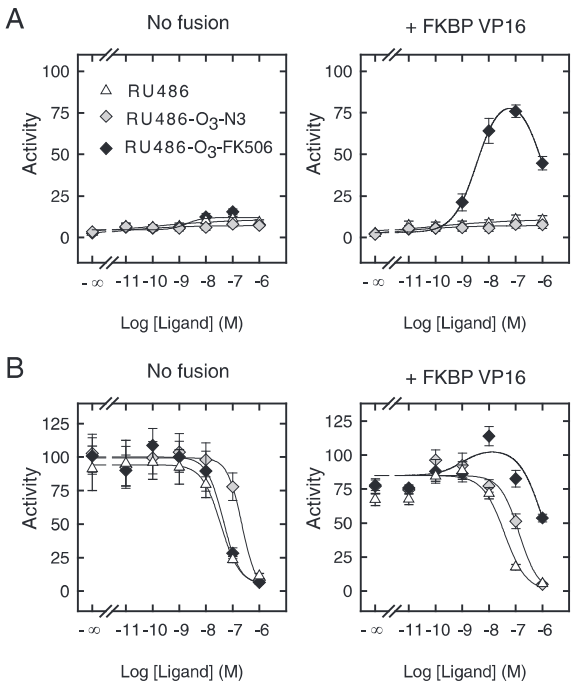

Figure 6. Antagonist to agonist conversion through extrinsic recruitment. A, Dose-response curves for transcriptional activation by RU486-based ligands in HEK 293T cells expressing GR alone (left panel) or coexpressing GR and the fusion protein FKBP-VP16 (right panel). B, Effect of the ligands in the presence of 3 nM Dex. Data represent the average 6 SEM of at least three experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex.

the parental RU486, despite its lower binding affinity. This indicates that the FK506 moiety imparts additional properties to the ligand, which could include effects on the cellular accumulation of the ligand.

In contrast to the above data, in the presence of the VP16-FKBP fusion, RU486-O_3-FK506 became an effective inducer of transcriptional activation, reaching a maximal activity nearly as strong as that of Dex (Figure 6A, right panel). Interestingly, the response is biphasic because activation is reduced at the highest concentration. This behavior, however, is consistent with the properties of bifunctional ligands because at sufficiently high concentrations, the formation of binary ligand-protein complexes is favored over the ternary complex (32, 37). It is also notable that the half-maximal activation by the FK506 conjugate occurred at concentrations (EC_{50} ; 3 nM) significantly lower than the intrinsic receptor affinity (; 90 nM). Importantly, and similar to the case of the Dex derivatives, the activity depends on both the fusion protein and the FK506 moiety and can be disrupted with free FK506 (Figure 6A and data not shown).

Consistent with a large gain in efficacy, the behavior of the ligands in the presence of 3 nM Dex (Figure 6B, right panel) indicated that RU486-O_3-FK506 behaved as an agonist and increased activity beyond 3 nM Dex. The fact that the peak activity is higher than that observed with RU486-O_3-FK506 alone also suggests that at these concentrations, in which mixed occupancy is likely, cooperation between intrinsic (Dex bound GR) and extrinsic (RU486-O_3-FK506 bound GR) mechanisms is occurring. As anticipated from competitive displacement, the behavior at the highest concentrations is comparable with that of RU486-O_3-FK506 alone. Furthermore, the presence of the VP16-FKBP fusion did not appreciably alter the antagonistic behavior of the parental RU486 or the conjugate lacking FK506, indicating that the observed effects require both the appropriate coregulator and the FK506 moiety in the bifunctional ligand. Taken together, these data clearly show that the regulatory effects elicited through extrinsic recruitment can be fully dissociated from the intrinsic properties of the GR binding moiety. The versatility of the approach is such that it allows for the predictable reprogramming of the transcriptional output of GR such that the behavior of a ligand can be completely reversed from an antagonist to an agonist.

Regulation in an intact chromatin environment through extrinsic recruitment

The implementation of the overall GR transcriptional program in vivo occurs in the context of a complex chromatin environment, and any successful ligand strategy must be able to operate under these circumstances. To demonstrate that GR-extrinsic transcriptional control can also be achieved at endogenous GR target genes in their native chromatin context, we focused on the S100P gene. The basal expression of this gene in HEK 293T cells is comparatively low and can be stimulated approximately 50- to 100-fold by Dex only upon GR expression. These properties indicate that the receptor is a major determinant of S100P transcription and make it a suitable target for analysis. In cells coexpressing FKBP alone, both of the SDex derivatives at 100 nM (which is comparable with their dissociation constant) were able to activate the S100P gene approximately 20-fold (Figure 7A, left panel). In contrast, in cells coexpressing the VP16-FKBP fusion, S100P expression was 3-fold higher in the presence of SDex-O_2-FK506 compared with the control ligand SDex-O_3-OMe, which lacks the FK506 moiety (Figure 7A, center panel). In fact, the activity elicited by the FK506 conjugate reached levels comparable with those obtained with the same concentration of Dex (which in comparison, corresponds to a 20 fold excess over its own dissociation constant). On the other hand, in cells ex-

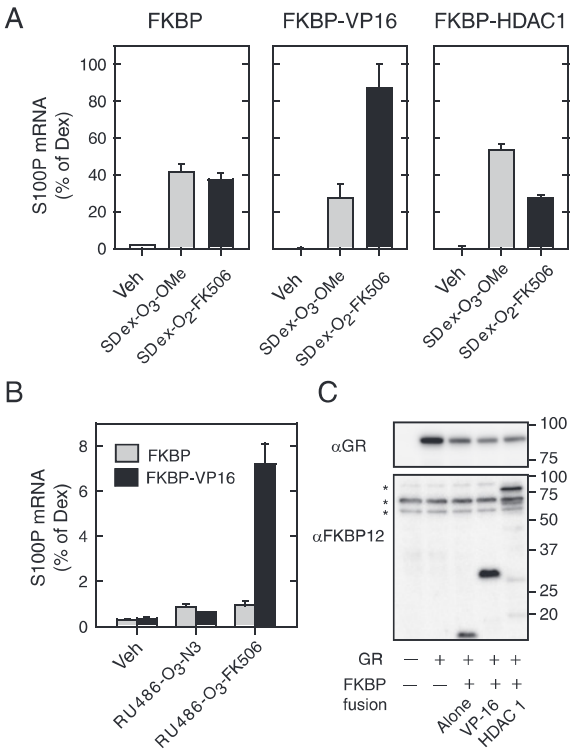

Figure 7. Directed regulation of the endogenous S100P gene by bifunctional ligands. A, mRNA levels of the S100P gene in response to SDex-O_3-OMe and SDex-O_2-FK506 in HEK 293T cells coexpressing GR and FKBP alone (left panel),VP16-FKBP (center panel), or HDAC1-FKBP (right panel). B, Response to RU486-based ligands in cells coexpressing GR and either FKBP alone (gray bars) or the VP16-FKBP fusion (black bars). Data are the averages 6 SEM of at least four independent experiments performed in duplicate and are expressed as a percentage of the levels observed in response to 100 nM Dex. Except for the control FKBP-alone data, all comparisons between linker only and FK506 conjugates were statistically significant (two tailed Student's t test, $P \leq .01$). C, Western blot analysis of parallel cultures using anti GR (top panel) or FKBP12 (lower panel). Molecular masses of standards (in kilodaltons) are indicated on the right of each panel, and nonspecific species are indicated by asterisks. Predicted molecular masses for the FKBP, VP16-FKBP, and HDAC1-FKBP fusion constructs are 15.8, 20.3, and 71.1 kDa, respectively. The anomalous migration conferred by the acidic VP16 activation domain has been well described (58).

pressing the HDAC1-FKBP fusion, S100P expression in response to SDex-O_2-FK506 was reduced by half in comparison with SDex-O_3-OMe (Figure 7A, right panel). Thus, for both the positive and negative modulation, successful extrinsic control can be demonstrated in a manner that depends on both the appropriate coregulator partner and the FK506 moiety in the ligand.

A parallel analysis of the effects of the RU486-based ligands (Figure 7B) revealed that in the presence of FKBP alone, the ligands did not appreciably activate S100P expression. This is expected from the intrinsic antagonist nature of RU486. In the presence of the VP16-FKBP fusion, however, the FK506 conjugate successfully activated S100P expression more than 10-fold compared with the ligand lacking the FK506 moiety. Importantly, Western blot analysis demonstrated successful expression of the fusion proteins (Figure 7C, bottom panel) and comparable expression of the receptor (Figure 7C, top panel). Taken together, the data clearly indicate that the extrinsic recruitment approach can be readily used to reprogram the expression of genes in their natural context and thus is amenable to further development for potential therapeutic applications.

Discussion

The ligand design we have implemented demonstrates that it is possible to independently manipulate intrinsic as well as extrinsic pathways of GR control and that their combinatorial coupling via bifunctional ligands can yield a variety of regulatory outcomes in both activation and repression contexts (Table 1). The approach is instructive in multiple ways because it reveals mechanistic features of GR function and opens up numerous design opportunities for its directed manipulation. The ability of the intact GR LBD to accommodate both agonist- and antagonist-based bifunctional ligands is notable because the predicted exit trajectory of the linker from the LBD based on structural information is quite different. For the Dex derivatives, the linker attachment site is very close to the surface and projects outward between helix 3 and 11 on the opposite side of helix 12. The linker is likely accommodated with minor movements of nearby residues such as T739 and Ile 747. The ability of the bifunctional ligands on their own to mount maximal responses comparable with Dex argues that the linker is accommodated while preserving a functional AF-2. For the RU406 derivatives, the linker extends from the aniline moiety, which is responsible for preventing helix 12 from adopting an active conformation and is already solvent exposed. Despite opposite exit points from the LBD and a relatively short linker, both bifunctional ligands are proficient for the recruitment of designed coregulators, which indicates a significant degree of flexibility and steric tolerance. These properties are favorable to the further development of the extrinsic recruitment approach.

The ability of the HDAC1-FKBP coregulator to limit transactivation by GR is also revealing. On the one hand, the inhibitory effect does not appear to be due to steric hindrance because FKBP alone or fusions to other proteins of comparable size are inactive (data not shown). Furthermore, although our data are consistent with the established role of HDAC1 as a component of corepres-

Table 1. Outcomes of Combinatorial Intrinsic and Extrinsic Pathways

Pathway		Maximal Effect (Relative to Dex)	
Intrinsic (Ligand character)	**Extrinsic (Cofactor recruitment)**	**Activation**	**Repression**
Agonist (Dex)	None	100%	100%
	VP16	200%	n.t.
	HDAC-1	30%	100%
Antagonist (RU-486)	None	0%	n.t.
	VP16	75%	n.t.

Abbreviation: n.t., not tested.

sor complexes, recent data based on the functional effects of HDAC1 knockdown have been interpreted as HDAC1 playing a positive role in GR transactivation (38). Whether this reflects indirect effects of HDAC1, or more complex interactions as has been suggested recently (39), remains to be determined. It is also important to note that HDACs can play key scaffolding roles in corepressor complexes that do not depend on their HDAC activity (34, 40). Consistent with this view, initial data indicate that the catalytic activity of HDAC1 is not required for its ability to suppress GR transactivation in the extrinsic recruitment approach (data not shown). This would indicate that the catalytic site of HDAC1 could be targeted by bifunctional ligands to recruit functional corepressor complexes. Importantly, targeting HDAC1 allowed selective reduction of GR transactivation while preserving agonist-mediated repression. Such an outcome is difficult to obtain with conventional ligands because the same AF-2-directed coregulators, such as GR interacting protein-1, can participate in both contexts (15).

The ligand design we have implemented has unique properties that make it amenable to directed design strategies. The nearly independent manipulation of the properties of both the receptor binding moiety and the additional chemical functionality is a significant advantage. This modularity extends the receptor's own design in which the DNA and ligand specificity have divergently evolved to generate distinct receptors with unique properties and functions. The substantial level of flexibility afforded by this approach greatly increases the types of ligands that can be envisioned. Although we have demonstrated a controlled change in efficacy with prototypic agonist and antagonist receptor ligands, the strategy could be combined with compounds with some conventional dissociated properties (2) or the arylpyrazole nonsteroidal series (3) that display some cell- and gene-selective properties to leverage both intrinsic and extrinsic effects. The experiments described here demonstrate that the added functionality afforded by the bifunctional ligand can be used in explicit design efforts to direct a desired transcriptional output and override the intrinsic properties of the receptor binding moiety. This also means that ligands with very high affinity but weak efficacy could be used as scaffolds. Such a strategy could counteract the mild penalty in affinity incurred by the introduction of the linker.

It is also important to note that in addition to affecting the pharmacodynamic properties of a receptor ligand, the linker as well as the additional chemical functionality in the bifunctional ligand can influence its pharmacokinetics and this can be advantageous. Thus, studies of an analogous Dex conjugate series indicate that the thiourea linker used here confers favorable properties both for cellular permeability (44) and transscleral transport in the context of ocular delivery (45). Our data also indicate that the FK506 moiety enhances the cellular potency of Dex (Figure 4) as well as RU486-based bifunctional ligands (Figure 6) relative to their intrinsic receptor affinities (Figure 3), an effect observed even in the absence of designed FKBP fusion proteins (Figure 6B, left panel). The enhanced potency may be a reflection of increased cellular uptake or retention provided by the FK506 group, particularly because FK506 can serve as a substrate and inhibitor of drug efflux transporters such as multidrug resistance protein 1 (41). Notably, the favorable pharmacokinetic properties provided by FK506 have been recently demonstrated for drug conjugates both in vitro and in vivo (42, 43). Similarly, conjugation of GR antagonists to bile acids has been explored as a means to target GR antagonism to the liver (28). The oral bioavailability, tissue distribution, and microsomal stability of such conjugates (28) indicate that GR bifunctional ligands can have pharmacokinetic properties suitable for further clinical development.

The experiments presented here depend on genetic manipulation of the designed coregulator. Although this may be incorporated as part of gene therapy strategies, transition to bifunctional ligands acting on purely endogenous proteins will obviously depend on appropriate functionalities that can target coregulator complexes. In this regard, the recent progress by our group (46–48) and others (49) in the development of small molecules that can

mimic activation domains offers some clear opportunities (50). Enhancing the agonist efficacy of glucocorticoid ligands in this manner could provide a means to overcome or alleviate steroid resistance, which is an important clinical problem in diseases such as asthma (51, 52), nephrotic syndrome (53), and malignancies such as acute lymphoblastic leukemia (54). Similarly, such ligands could restore function to carriers of mutations in GR that impair interaction with coactivators (55, 56). Indeed, the modular design of our bifunctional ligands argues for the successful incorporation of these chemical motifs to target endogenous regulatory complexes.

Bifunctional ligands made as FK506 conjugates as in this study have intriguing prospects in their own right as mechanistic tools. They can be used to directly recruit specific FKBP-coactivator or -corepressor fusions. This can provide a means to not only identify factors that can overcome gene-specific, rate-limiting barriers to activation but also to provide novel mechanisms of repression for specific GR target genes. For example, extrinsic recruitment of factors implicated in the GR-mediated repression at tethering sites such as negative elongation factor (14) could enhance the repressive effects of agonists in a gene-selective manner. By examining multiple cofactors in parallel, this approach could also be used to establish epistatic relationships between them and the gene subsets affected by them. It is precisely this type of knowledge that is required to identify the most desirable regulatory profile for a given therapeutic application. Furthermore, the approach could also be used to establish or monitor specific epigenetic marks at GR-targeted loci in a ligand-dependent manner as has been recently illustrated for octamer-binding transcription factor 4 (57). Clearly the strategy outlined here has significant potential and opens up the possibility of an instructive ligand design not only for GR but also for the entire nuclear receptor class.

Acknowledgments

We thank the members of the Mapp and Iñiguez laboratories for helpful suggestions as well as the laboratory of Dr Gabriel Nuñez for providing reagents.

Address all correspondence and requests for reprints to: Jorge A. Iñiguez-Lluhí, Department of Pharmacology, University of Michigan Medical School, 1150 West Medical Center Drive, 1301 MSRB III, Ann Arbor, MI 48109. E-mail: iniguez@umich.edu.

This work was supported by US Public Health Service Grant R56 DK061656 (to J.A.I.-L.); Department of Defense Congressionally Directed Medical Research Program Idea Award PC101214 (to A.K.M.); the American Cancer Society Michigan Cancer Research Fund award 120684-PF-11-085-01-TBE (to A.R.V.D.) and the Division of Medicinal Chemistry of the American Chemical Society (to J.W.H.). Core facility use was also provided through US Public Health Service Grant P60 DK20572 and DK089503 of the National Institutes of Health (to the University of Michigan).

Disclosure Summary: The authors have nothing to disclose.

References

1. Ramamoorthy S, Cidlowski JA. Exploring the molecular mechanisms of glucocorticoid receptor action from sensitivity to resistance. Endocr Dev. 2013;24:41–56.
2. Schacke H, Rehwinkel H, Asadullah K. Dissociated glucocorticoid receptor ligands: compounds with an improved therapeutic index. Curr Opin Investig Drugs. 2005;6:503–507.
3. Wang JC, Shah N, Pantoja C, et al. Novel arylpyrazole compounds selectively modulate glucocorticoid receptor regulatory activity. Genes Dev. 2006;20:689–699.
4. Xiao HY, Wu DR, Sheppeck JE 2nd, et al. Heterocyclic glucocorticoid receptor modulators with a 2,2-dimethyl-3-phenyl-N-(thiazol or thiadiazol-2-yl)propanamide core. Bioorg Med Chem Lett. 2013;23:5571–5574.
5. Johnson AB, O'Malley BW. Steroid receptor coactivators 1, 2, and 3: critical regulators of nuclear receptor activity and steroid receptor modulator (SRM)-based cancer therapy. Mol Cell Endocrinol. 2012;348:430–439.
6. Yamamoto KR, Darimont BD, Wagner RL, Iñiguez-Lluh□JA. Building transcriptional regulatory complexes: signals and surfaces. Cold Spring HarbSymp Quant Biol. 1998;63:587–598.
7. Thomas-Chollier M, Watson LC, Cooper SB, et al. A naturally occurring insertion of a single amino acid rewires transcriptional regulation by glucocorticoid receptor isoforms. Proc Natl Acad Sci USA. 2013;110(44):17826–17831.
8. Druker J, Liberman AC, Antunica-Noguerol M, et al. RSUME enhances glucocorticoid receptor SUMOylation and transcriptional activity. Mol Cell Biol. 2013;33:2116–2127.
9. Ismaili N, Garabedian MJ. Modulation of glucocorticoid receptor function via phosphorylation. Ann NY Acad Sci. 2004;1024:86–101.
10. Bain DL, Heneghan AF, Connaghan-Jones KD, Miura MT. Nuclear receptor structure: implications for function. Annu Rev Physiol. 2007;69:201–220.
11. So AY, Chaivorapol C, Bolton EC, Li H, Yamamoto KR. Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor. PLoS Genet. 2007;3:e94.
12. Uhlenhaut NH, Barish GD, Yu RT, et al. Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. Mol Cell. 2013;49:158–171.
13. Dai SY, Chalmers MJ, Bruning J, et al. Prediction of the tissue-specificity of selective estrogen receptor modulators by using a single biochemical method. Proc Natl Acad Sci USA. 2008;105:7171–7176.
14. Gupte R, Muse GW, Chinenov Y, Adelman K, Rogatsky I. Glucocorticoid receptor represses proinflammatory genes at distinct steps of the transcription cycle. Proc Natl Acad Sci USA. 2013;110:14616–14621.
15. Chinenov Y, Gupte R, Dobrovolna J, et al. Role of transcriptional coregulator GRIP1 in the anti-inflammatory actions of glucocorticoids. Proc Natl Acad Sci USA. 2012;109:11776–11781.
16. van Rossum EF, van den Akker EL. Glucocorticoid resistance. Endocr Dev. 2011;20:127–136.
17. Licitra EJ, Liu JO. A three-hybrid system for detecting small ligand-protein receptor interactions. Proc Natl Acad Sci USA. 1996;93:12817–12821.

18. Huang P, Chandra V, Rastinejad F. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. Annu Rev Physiol. 2010;72:247–272.
19. Lopez S, Simons SS Jr. Dexamethasone 21-(β-isothiocyanatoethyl) thioether: a new affinity label for glucocorticoid receptors. J Med Chem. 1991;34:1762–1767.
20. Inïguez-Lluh□JA, Pearce D. A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. Mol Cell Biol. 2000;20:6040–6050.
21. Inïguez-Lluh□JA, Lou DY, Yamamoto KR. Three amino acid substitutions selectively disrupt the activation but not the repression function of the glucocorticoid receptor N terminus. J Biol Chem. 1997;272:4149–4156.
22. Bolton EC, So AY, Chaivorapol C, Haqq CM, Li H, Yamamoto KR. Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev. 2007;21:2005–2017.
23. Inohara N, Koseki T, Lin J, et al. An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem. 2000;275:27823–27831.
24. Ruijter JM, Ramakers C, Hoogaars WM, et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 2009;37:e45.
25. Manz B, Heubner A, Köhler I, Grill H-J, Pollow K. Synthesis of biotin-labelled dexamethasone derivatives. Eur J Biochem. 1983; 131:333–338.
26. Lin H, Abida WM, Sauer RT, Cornish VW. Dexamethasone-methotrexate: an efficient chemical inducer of protein dimerization in vivo. J Am Chem Soc. 2000;122:4247–4248.
27. Gallagher SS, Miller LW, Cornish VW. An orthogonal dexamethasone-trimethoprim yeast three-hybrid system. Anal Biochem. 2007;363:160–162.
28. Richards SJ, von Geldern TW, Jacobson P, et al. Synthesis and activity of novel bile-acid conjugated glucocorticoid receptor antagonists. Bioorg Med Chem Lett. 2006;16:6086–6090.
29. Ho SN, Biggar SR, Spencer DM, Schreiber SL, Crabtree GR. Dimeric ligands define a role for transcriptional activation domains in reinitiation. Nature. 1996;382:822–826.
30. Spencer DM, Wandless TJ, Schreiber SL, Crabtree GR. Controlling signal transduction with synthetic ligands. Science. 1993;262: 1019–1924.
31. Gestwicki JE, Marinec PS. Chemical control over protein-protein interactions: beyond inhibitors. Comb Chem High Throughput Screen. 2007;10:667–675.
32. Fegan A, White B, Carlson JC, Wagner CR. Chemically controlled protein assembly: techniques and applications. Chem Rev. 2010; 110:3315–3336.
33. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet. 2009;10:32–42.
34. Fischle W, Dequiedt F, Hendzel MJ, et al. Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol Cell. 2002;9:45–57.
35. Nissen RM, Yamamoto KR. The glucocorticoid receptor inhibits NFκB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev. 2000;14: 2314–2329.
36. Luecke HF, Yamamoto KR. The glucocorticoid receptor blocks P-TEFb recruitment by NFκB to effect promoter-specific transcriptional repression. Genes Dev. 2005;19:1116–1127.
37. Mack ET, Perez-Castillejos R, Suo Z, Whitesides GM. Exact analysis of ligand-induced dimerization of monomeric receptors. Anal Chem. 2008;80:5550–5555.
38. Kadiyala V, Patrick NM, Mathieu W, et al. Class I lysine deacetylases facilitate glucocorticoid-induced transcription. J Biol Chem. 2013;288(40):28900–28912.
39. Qiu Y, Stavreva DA, Luo Y, Indrawan A, Chang M, Hager GL. Dynamic interaction of HDAC1 with a glucocorticoid receptor-regulated gene is modulated by the activity state of the promoter. J Biol Chem. 2011;286:7641–7647.
40. Martin M, Kettmann R, Dequiedt F. Class IIa histone deacetylases: conducting development and differentiation. Int J Dev Biol. 2009; 53:291–301.
41. Li Y, Hu X, Cai B, et al. Meta-analysis of the effect of MDR1 C3435 polymorphism on tacrolimus pharmacokinetics in renal transplant recipients. Transpl Immunol. 2012;27:12–18.
42. Marinec PS, Lancia JK, Gestwicki JE. Bifunctional molecules evade cytochrome P(450) metabolism by forming protective complexes with FK506-binding protein. Mol Biosyst. 2008;4:571–578.
43. Marinec PS, Chen L, Barr KJ, Mutz MW, Crabtree GR, Gestwicki JE. FK506-binding protein (FKBP) partitions a modified HIV protease inhibitor into blood cells and prolongs its lifetime in vivo. Proc Natl Acad Sci USA. 2009;106:1336–1341.
44. Liu B, Kodadek T. Investigation of the relative cellular permeability of DNA-binding pyrrole-imidazole polyamides. J Med Chem. 2009;52:4604–4612.
45. Cruysberg LP, Nuijts RM, Geroski DH, Koole LH, Hendrikse F, Edelhauser HF. In vitro human scleral permeability of fluorescein, dexamethasone-fluorescein, methotrexate-fluorescein and rhodamine 6G and the use of a coated coil as a new drug delivery system. J Ocul Pharmacol Ther. 2002;18:559–569.
46. Buhrlage SJ, Bates CA, Rowe SP, et al. Amphipathic small molecules mimic the binding mode and function of endogenous transcription factors. ACS Chem Biol. 2009;4:335–344.
47. Rowe SP, Casey RJ, Brennan BB, Buhrlage SJ, Mapp AK. Transcriptional up-regulation in cells mediated by a small molecule. J Am Chem Soc. 2007;129:10654–10655.
48. Hojfeldt JW, Van Dyke AR, Mapp AK. Transforming ligands into transcriptional regulators: building blocks for bifunctional molecules. Chem Soc Rev. 2011;40:4286–4294.
49. Jung D, Shimogawa H, Kwon Y, et al. Wrenchnolol derivative optimized for gene activation in cells. J Am Chem Soc. 2009;131: 4774–4782.
50. Lee LW, Mapp AK. Transcriptional switches: chemical approaches to gene regulation. J Biol Chem. 2010;285:11033–11038.
51. Barnes PJ. Biochemical basis of asthma therapy. J Biol Chem. 2011; 286:32899–32905.
52. Durham A, Adcock IM, Tliba O. Steroid resistance in severe asthma: current mechanisms and future treatment. Curr Pharm Des. 2011;17:674–684.
53. Gipson DS, Massengill SF, Yao L, et al. Management of childhood onset nephrotic syndrome. Pediatrics. 2009;124:747–757.
54. Bhadri VA, Trahair TN, Lock RB. Glucocorticoid resistance in paediatric acute lymphoblastic leukaemia. J Paediatr Child Health. 2012;48:634–640.
55. Charmandari E, Raji A, Kino T, et al. A novel point mutation in the ligand-binding domain (LBD) of the human glucocorticoid receptor (hGR) causing generalized glucocorticoid resistance: the importance of the C terminus of hGR LBD in conferring transactivational activity. J Clin Endocrinol Metab. 2005;90:3696–3705.
56. Charmandari E, Kino T, Ichijo T, et al. A novel point mutation in helix 11 of the ligand-binding domain of the human glucocorticoid receptor gene causing generalized glucocorticoid resistance. J Clin Endocrinol Metab. 2007;92:3986–3990.
57. Hathaway Nathaniel A, Bell O, Hodges C, Miller Erik L, Neel Dana S, Crabtree Gerald R. Dynamics and memory of heterochromatin in living cells. Cell. 2012;149:1447–1460.
58. O'Reilly D, Hanscombe O, O'Hare P. A single serine residue at position 375 of VP16 is critical for complex assembly with Oct-1 and HCF and is a target of phosphorylation by casein kinase II. EMBO J. 1997;16:2420–2430.