Review Article

Strategic targeting of the glucocorticoid receptor for anti-inflammation

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Glucocorticoids are produced in the adrenal cortex under the strict control of the hypothalamus-pituitary-adrenal axis and exert a variety of biological actions including the regulation of glucose and lipid metabolism, electrolyte balance, and modulation of the immune, cardiovascular, and central nervous system. Pharmacologically, glucocorticoids are estimated to be used long-term by 0.5-1% of the general population and up to 2.5% of older adults[1,2]. Despite the established role of glucocorticoids in controlling short-term inflammation, and despite emerging evidence supporting a disease-modifying role in various autoimmune disorders, concern for adverse events associated with glucocorticoids often limits their use. The glucocorticoid compounds bind the glucocorticoid receptor (GR), which is a member of the nuclear receptor superfamily, and elicit their pharmacological actions. Recent progress in molecular biology of the GR has extended our understanding of their mechanism of action, however, the molecular basis for the side effects have not been fully clarified. Indeed, dissociation of their therapeutic effects and adverse reactions is still one of the most challenging clinical issues to be solved.

In this lecture, I will focus on the recent understanding of the molecular mechanism of glucocorticoid action and our recent work with ursodeoxycholic acid and cortivazol and discuss rationale to develop novel glucocorticoid-like compounds.

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Current Glucocorticoid Therapy

Since the introduction of glucocorticoids in the treatment of rheumatoid arthritis in 1949[3], scientists and pharmaceutical companies made intensive efforts to maximize the beneficial and to minimize the side effects of the drug. Many synthetic compounds with glucocorticoid activity were produced and the pharmaco-

Table 1 Classification of the nuclear receptor

	Class I	Class II	Class III
	Steroid hormone receptors	Adopted orphan receptors	Orphan receptors
Representative receptors	Receptors for glucocorticoid (GR), estrogen (ER), progesterone (PR), androgen (AR), mineralocorticoid (MR)	Receptors for thyroid hormone (TR), vitamin D (VDR), all-trans retinoic acid (RAR) and the peroxisome proliferator-activated receptor (PPAR)	SF-1, DAX-1, ERRs, Nurs, CoupTFs, SHP, LRH-1, RORs
DNA binding form	Homodimer	Heterodimer with 9-cis retinoic acid retinoid X receptor (RXR)	Heterodimer with RXR and/or monomer
Response element	Palindrome	Direct repeat	Direct repeat (half site)

logic differences among these chemicals result from structural alterations of their basic backbone and its side groups. These alterations variably affect the bioavailability of these compounds (i.e., gastrointestinal and/or parenteral absorption, plasma half-life, and metabolism, and interaction with the GR). At first, such modification in the structure succeeded in abolishing mineralocorticoid activity in electrolyte handling. On the other hand, modification of physicochemical characteristics (i.e., water solubility/lipophilicity) is considered for parenteral administration or enhancement of topical potency. Most synthetic glucocorticoids (e.g., prednisolone and dexamethasone) are minimally bound to cortisol-binding globulin and circulate mostly bound to albumin, or in the free form.

The side effects occur only with supraphysiologic doses of glucocorticoids and not with proper replacement, which is equivalent to 12 to 15 mg of hydrocortisone/ m^2 body surface area per day[4]. The side effects of glucocorticoids have been shown to be strictly dose-dependent. Thus, as the dosage is escalated to improve efficacy, the side effects also increase. In addition, some side effects are known to be age- and sex-dependent. Major complications are unlikely with short-term treatment (e.g., less than 2 weeks) with high doses of glucocorticoids, although sleep disturbances and gastric irritation are common complaints, and depression, mania, or psychosis, may be infrequently encountered. On the other hand, many side effects are associated with chronic administration of pharmacologic amounts of glucocorticoids. These side effects include the development of varying degrees of the manifestations of Cushing's syndrome[4-6].

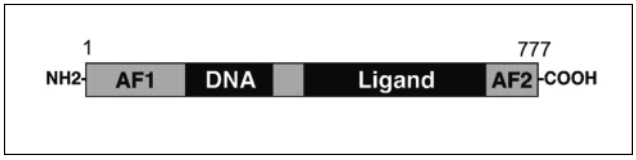

Fig.1 Primary structure of the human glucocorticoid receptor

GR is A Nuclear Receptor (NR)

NR is a large family of ligand-dependent transcription factors and regulates essential physiological processes including development, reproduction, metabolism, and homeostasis. NRs bind their cognate DNA sequences and modulate gene expression within target tissues. NRs can tentatively be subdivided into three major classes (Table 1). All members of the NR super family share a modular domain structure, consisting of an amino-terminal transcriptional activation function domain (AF1), a conserved zinc-finger DNA binding domain (DBD), a hinge region and a carboxyl-terminal ligand binding domain (LBD) that overlaps with a second transcriptional AF2 domain (Fig.1). Whereas the AF1 activity is constitutive in most cell types or under tissue-specific regulation, the AF2 activity is strictly ligand-dependent[7].

The GR LBD, similar to other NR LBDs, is composed of a-helices and β-strands folded into a three-layer helical sandwich. The ligand binding pocket is composed of residues from helices 3, 4, 5, 6, 7, 10, and the AF-2 helix as well as residues from β-strands between helices 5 and 6. Following AF-2 helix is an extended strand that forms a conserved β-sheet with a β-strand

between helices 8 and 9. This C-terminal β-strand also appears to play an important role in receptor activation by stabilizing AF-2 helix in an active conformation. Many AF-2 coactivators for the GR have been identified to date, including steroid receptor coactivator-1 (SRC-1), transcriptional intermediary factor (TIF) 2/GR-interacting protein-1 and cAMP response element binding protein-binding protein (CBP)/p300. These coactivators directly associate with the GR LBD via their LXXLL motif. For example, the LLRYLL sequence in the TIF2 forms a two-turn helix that orients the hydrophobic leucine side chains into a groove formed in part by the AF-2 helix and residues from helices 3, 3', 4, and 5. The N- and C-terminal ends of the coactivator helix are clamped by Glu-755 from the AF-2 helix and Lys-579 in helix 3, respectively. Mutations that disrupt either the first (Glu-755) or the second (Arg-585 and Asp-590) charge clamp dramatically reduce activation mediated by the GR LBD, demonstrating that they are critical for transactivation function of the GR. On the other hand, GR AF-1 coactivators have only recently been described. For example, basal transcription factors including TBP and TFIID have been shown to associate with the AF-1 of GR. TSG101 and DRIP150 have also been reported to interact with GR AF-1 and regulate GR function in a reciprocal manner; GR transcriptional activities are repressed by TSG101 but enhanced by DRIP150. These cofactors are shown to interact with distinct regions of AF-1. Although we now have at hand a large number of regulatory proteins that interact directly or indirectly with the various modular domains of NRs, how ligands differentially regulate the functional interplay between them remains poorly understood[8-10].

Unlike the GR, most nonsteroidal nuclear receptors like PPAR and RAR can interact with corepressors and repress transcription in the absence of ligand or in the presence of antagonists. These corepressors in turn have histone deacetylase activity that trims acetyl groups of nucleosomes, compacting and silencing the promoter to which unliganded nuclear receptor is bound[7].

GR-Mediated Anti-inflammation

In the absence of ligand, the GR is retained in the cytoplasm in association with chaperone proteins such as heat shock protein 90 (hsp90). Hormone binding initiates the release of the chaperone proteins and translocation of the receptor into the nucleus where GR binds to DNA promoter elements termed glucocorticoid response element (GRE) from which it can usually activate transcription of the target promoter. A dozens of the target genes of the GR have been identified, most of which transmit metabolic effects of glucocorticoids. A few genes are shown to be related anti-inflammatory properties of glucocorticoids. On the other hand, transcriptional repression activity is central to the glucocorticoid-mediated anti-inflammation and antiproliferative effects, and a number of transcriptional repression mechanisms mediated by GR have been described (Fig.2). Of note, in contrast to other classes of NR, repression by steroid receptors occurs only in the presence of ligand. The mechanism of repression varies significantly and ranges from effects at the level of DNA to effects on RNA polymerase and transcriptional elongation, and transcription factors directly. These include mechanisms dependent on either GR-DNA or GR-protein interactions. The variety of potential interactions suggests exquisite control over repression that is highly context-dependent. Initial studies emphasized that repression of proinflammatory transcription factors including AP-1 and NF-kB may be central in GR-mediated anti-inflammation[8,9].

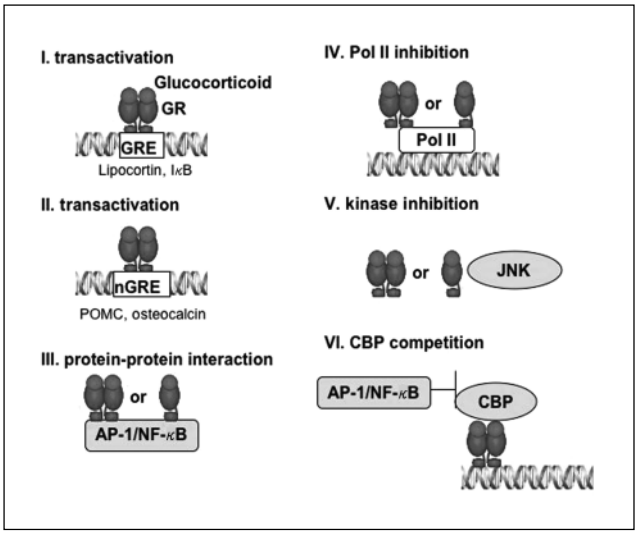

Fig.2 Multiple mechanisms for glucocorticoid receptor (GR)-mediated antiinflammation.

Development of Novel Glucocorticiod-like Compounds

Based on the development of molecular biology of the NRs, steroid pharmacology is increasingly focused in the development of novel ligands with selective modulatory activities[11]. Since dissociation of the side effects of glucocorticoids would definitely contribute to medical fields, GR could be one of the rational molecular targets for such purpose. It is obvious that a novel agent must have the same efficacy in such diseases for which glucocorticoids are currently-indicated, but with reduced side effects. Identification of novel GR ligands have resulted in a

number of divergent terminologies, and Rosen and Miner recently provided putative definitions of some of the key terms[12]:

- SGRM (selective GR modulator) and SeGRA (selective GR agonist). Both SGRM and SeGRA are general class descriptors used to describe compounds with an improved therapeutic index *in vivo* by whatever mechanism.
- Gene-selective compound. This term refers to compounds that act on the receptor to alter gene expression in a gene- or promoter-specific fashion. In other words, some genes might be activated, some might be repressed, but the resulting profile differs from that of currently used glucocorticoids.
- Dissociated compound. This term is usually used to refer to a compound that "dissociates" activation from repression. Compounds in this class fail to globally activate gene expression, but still significantly repress transcription.
- Soft steroids/glucocorticoids (also known as "antedrugs"). This describes corticosteroids that act at or near the site of administration but are inactivated by enzymes, thereby reducing systemic exposure and activity. These are often described for topical and inhaled therapies that act locally but are rapidly metabolized once they enter systemic circulation.

G. Schütz and his colleagues, using dimerization-deficient mutant GR that prevent gene activation by GR but do not affect repression, showed that the anti-inflammatory activity of steroids was maintained and suggested that repression may be sufficient for anti-inflammatory activity[13]. Although a variety of compounds have been tested in terms of dissociation of activation and repression, at this moment a complete dissociated compound is not available. Moreover, such compounds which were once shown to be a dissociated one *in vitro*, often failed to decrease glucocorticoid's metabolic side effects with keeping its anti-inflammatory activities *in vivo*. The other compounds fail to keep their dissociated characteristics at higher, or therapeutic concentrations. Given the battery of genes and the multitude of potential regulatory mechanisms, finding a compound that actually separates all activated genes from all repressed genes seems highly unlikely. It is also unclear whether such a compound would be truly desirable because activation of anti-inflammatory genes may also play a role in the treatment of inflammatory diseases. Of the proposed dissociated compounds that have been published, all have been shown to differentially regulate one or sometimes two genes. This is not the same as demonstrating that the compound is dissociated on all glucocorticoid target genes. It, however, should be noted that the activation/repression hypothesis has provided a very useful framework to find novel compounds with potential utility, and some success has already been achieved at least preclinically[12].

Recent structural analyses of the nuclear receptors establish a paradigm of receptor activation, in which agonist binding induces the LBD/AF-2 helix to form a charge clamp for coactivator recruitment[10]. However, these analyses have not sufficiently addressed the mechanisms for differential actions of various synthetic steroids in terms of fine tuning of multiple functions of whole receptor molecules. In this line, our studies with two distinct compounds, ursodeoxycholic acid (UDCA) and cortivazol (CVZ), provided the rationale for ligand-based selective modulation of the receptor activities.

Ursodeoxycholic Acid (UDCA)

UDCA is the current mainstay of treatment for various liver diseases including primary biliary cirrhosis, autoimmune hepatitis, and hepatitis C. UDCA has multiple functions, acting not only as a bile secretagogue, but also as a cytoprotective agent, immunomodulator, and inhibitor of cellular apoptosis[14-18]. Based on this cumulative evidence of the cytoprotective and immunomodulatory effects of UDCA, we tried to identify the target molecule and pathway of UDCA action. It was shown that UDCA specifically translocates the GR into the nucleus as a DNA binding species but does not elicit its transactivational function in a transient transfection assay (Fig.3). Moreover, the LBD of the GR is responsible for UDCA-dependent nuclear translocation of the GR. Indeed, we demonstrated that UDCA acts on the distinct region of the LBD when compared with the classical GR agonist dexamethasone, resulting in loss of coactivator recruitment and differential regulation of gene expression by the GR (Fig.4). Our data clearly indicated that UDCA, at least in part via activation of the GR, suppresses NF-κB-dependent transcription through the intervention of GR-p65 interaction[19,20]. Together with the established clinical safety of UDCA, we may propose that UDCA could be a prototypical compound for development of a novel and selective GR modifier. Recently, using a flourescently labeled UDCA molecule, we showed that UDCA shows similar distibution pattern with GR in hepatocytes and that GR is crucial for the nuclear translocation of UDCA for reducing apoptosis[21]. In fact, it is now wellknown that bile acids are ligands of a various NR including the farnesoid X-activated receptor (FXR) and VDR[22-25]. Our understanding the physiological role of bile acids, thus, is still expanding and NR-target drug development may provide a novel milieu for treatment of various gastrointestinal diseases.

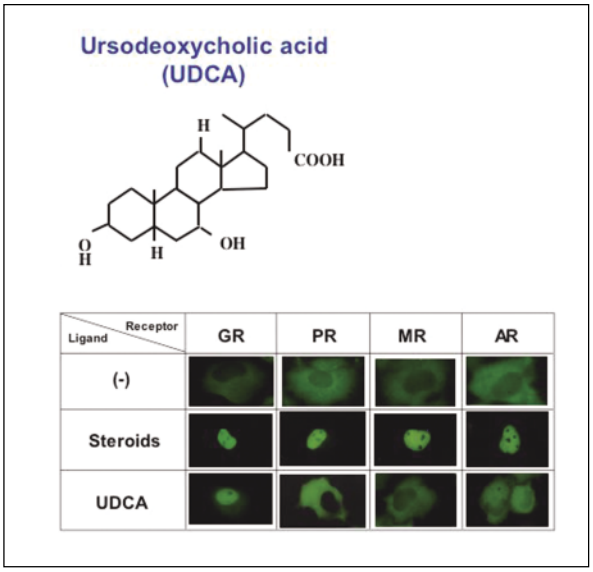

Fig.3 GR-selective activation by ursodeoxycholic acid (UDCA)

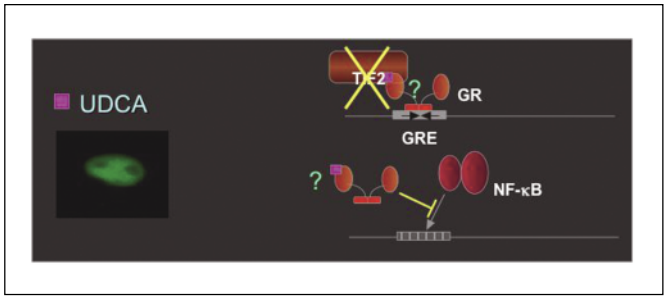

Fig.4 Proposed mechanism of UDCA in GR-mediated immunomodulation

Cortivazol (CVZ)

The phenylpyrazologlucocorticoid CVZ is a unique synthetic glucocorticoid agonist with complex binding properties and is more potent than DEX[6] (Fig.5). We demonstrated that CVZ selectively binds to the GR but not to the MR (Fig. 6) and, based on two criteria, we proposed that the functional interaction of CVZ with the GR LBD is different from that of DEX (Fig.7). Firstly, deletion of the last 12 amino acids of GR severely compromises DEX but not CVZ binding and secondly, the point mutant L753F, in which Leu-753 in AF-2 is substituted to Phe, can efficiently recruit TIF2 to the LBD when bound to CVZ but not when bound to DEX[27]. These results prompted us to propose that occupancy of the GR LBD by CVZ might lead to a more stable active conformation that can tolerate the disrupting effects of LBD mutations and may have unique effects on the structure and function of the whole GR molecule. Structural docking analysis revealed that although CVZ is more bulky than other agonists, it can be accommodated in the ligand binding pocket of the GR by reorientation of several amino acid side chains but without major alterations in the active conformation of the LBD. In this induced fit model, the phenylpyrazole A-ring of CVZ establishes additional contacts with helices 3 and 5 of the LBD that may contribute to a more stable LBD configuration. Structural and functional analysis revealed that CVZ is able to compensate for the deleterious effects of a C-terminal deletion of the LBD in a manner that mimics the stabilizing influence of the F602S point mutation. CVZ-mediated productive recruitment of TIF2 to the C-terminally deleted LBD requires the receptor's own DNA binding domain and is positively influenced by the N-terminal regions of GR or progesterone receptor. These results support a model where ligand-dependent conformational changes in the LBD play a role in GR-mediated gene regulation via modular interaction with the DBD and AF-1. Steroid pharmacology is increasingly focused in the development of ligands with selective modulatory activities. Because the mode of interdomain communication may be distinct for each receptor and may be modulated in a ligand-, tissue-, and promoter-context-dependent manner, ligands such as CVZ and other phenylpyrazole analogs that manipulate this regulatory avenue will not only provide a better understanding of the mechanisms of interdomain communication but also provide novel leads in the development of selective GR modulators[28]. Indeed, a further series of compounds based on an arylpyrazole structure have recently been published. These compounds specifically bind the GR with relatively high affinity. These compounds differ in their relative activity to inhibit cell-based assays of proliferation, adipocyte differentiation or osteoblast differentiation. Moreover, transcriptome profile analyses revealed that the different molecular structures have differential effects on individual target genes. It was striking that, as also indicated in the case of CVZ, subtle changes in structure of the ligand caused markedly distinct GR regulatory effects in more than one cell line. Chromatin immunoprecipitation assays suggested that the different compounds alter the relative affinity of the GR for specific DNA sequences. The authors concluded that the induced structure of the LBD of the GR appears to influence the interaction with DNA sequence and thereby specify a distinct profile of gene regulatory events[29]. This study overall supports the idea that pursuit of the perfect dissociating glucocorticoid ligand may well be complicated, but it is certainly possible that even an imperfectly dissociating compound may be more than sufficient to offer an improved therapeutic index and thereby

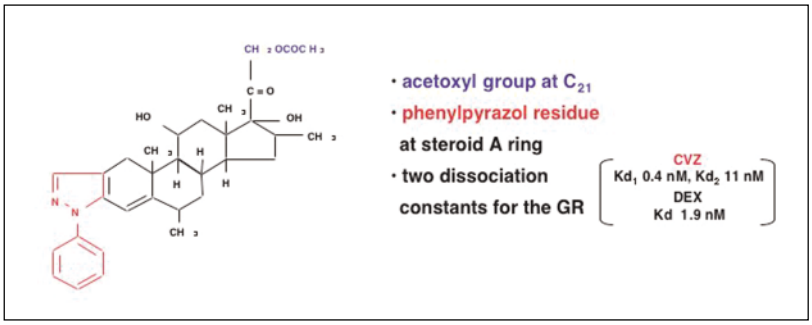

Fig.5 Characteristics of cortivazol (CVZ)

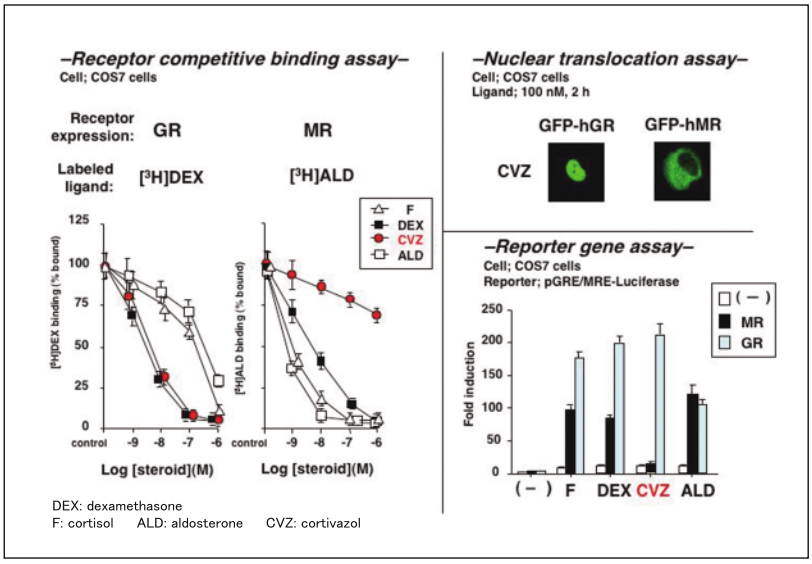

Fig.6 CVZ is a specific ligand for the GR

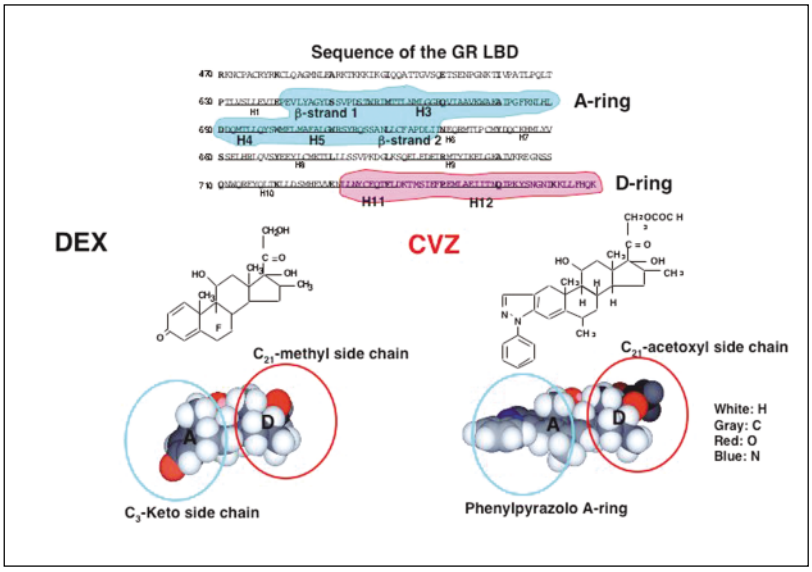

Fig.7 Distinct binding poperties of CVZ to the GR -- comparison with dexamethasone (DEX)

unleash the full anti-inflammatory potency of glucocorticoids without the side-effect profile.

Future Directions

A safer glucocorticoid should have full efficacy in anti-inflammatory activity, but reduced efficacy and potency in one or more side effects. The development of new safer anti-inflammatory

agents that target the GR is now gaining momentum after years of work on steroids and, more recently, nonsteroidal molecules. The molecular details behind the action of the newer compounds being described may point the way to more effective assays capable of detecting novel anti-inflammatory agents.

The detection of a tissue selective or a functionally selective ligand for the GR will be difficult, and there is no guarantee, once such a ligand is found, that it will have the necessary profile *in vivo*. However, recent reports of SGRMs with equal efficacy and improved side effect profiles compared with steroids together with molecular discoveries of the receptor mechanism of action provide fertile ground for additional efforts. Thus, despite the difficulties associated with developing a novel glucocorticoid, progress in this area would be a major benefit to the large number of patients suffering from the side effects of steroids, but needing the anti-inflammatory and anti-cancer activity to maintain their quality of life.

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