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Review

Selective Glucocorticoid Receptor modulators☆

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ABSTRACT

The ancient two-faced Roman god Janus is often used as a metaphor to describe the characteristics of the Glucocorticoid Receptor (NR3C1), which exhibits both a beneficial side, that serves to halt inflammation, and a detrimental side responsible for undesirable effects. However, recent developments suggest that the Glucocorticoid Receptor has many more faces with the potential to express a range of different functionalities, depending on factors that include the tissue type, ligand type, receptor variants, cofactor surroundings and target gene promoters. This behavior of the receptor has made the development of safer ligands, that trigger the expression program of only a desirable subset of genes, a real challenge. Thus more knowledge-based fundamental research is needed to ensure the design and development of selective Glucocorticoid Receptor modulators capable of reaching the clinic. Recent advances in the characterization of novel selective Glucocorticoid Receptor modulators, specifically in the context of anti-inflammatory strategies, will be described in this review.

Contents

Glucocorticoids (GCs) are steroidal hormones, synthesized from cholesterol with the cyclo pentano perhydrophenanthrene ring structure as a scaffold. They enable the organism to adequately respond to physical or emotional stress. The name glucocorticoid (glucose + cortex + steroid) derives from their role in the regulation of the metabolism of glucose, their synthesis in the adrenal cortex, and their steroidal structure. Indeed, one of the roles of GCs is to stimulate gluconeogenesis. The synthesis of GCs by the adrenal gland is under the tight control of the hypothalamus–pituitary–adrenal (HPA) axis [1].

Glucocorticoids antagonize some of the effects to insulin, and are therefore also categorized as 'catabolic' hormones. In addition to the stimulation of gluconeogenesis they induce a decrease in glucose utilization, giving rise to increased glucose levels in the blood. They also induce the breakdown of proteins into amino acids and stimulate the mobilization of fatty acids. GCs also have an immunemodulatory function, which is the main reason for their use in the clinic. However the supra-physiological doses of exogenous GCs that are sometimes necessary to control chronic inflammation in patients can give rise to deleterious side effects. Among these

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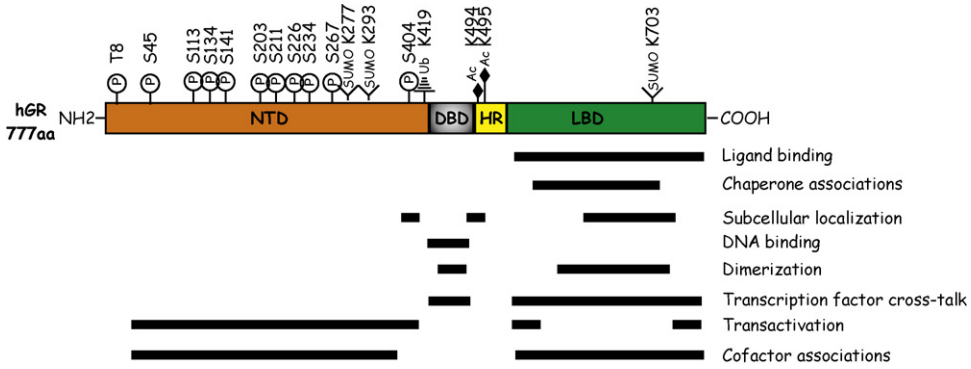

Fig. 1. The various described modifications as well as the modular structure/function relationship between the different domains of the Glucocorticoid Receptor is depicted. *Abbreviations*: NTD, N-terminal domain; DBD, DNA-binding domain; HR, hinge region; LBD, ligand-binding domain; AF, activation function; P, SUMO and Ub stand for phosphorylated, sumoylated and ubiquitinylated residues, respectively. The letter preceding the modification corresponds to the identity of the amino acid residue that is modified, the number corresponds to the position in human GR. For an extensive review on the role of GR modifications, please see [7].

are diabetes, osteoporosis, muscle wasting, growth retardation in children, disturbed water balance, fat redistribution, mood disorders and suppression of the HPA axis resulting in disturbed production and regulation of endogenous cortisol. In the clinic, GCs are administered either systemically to patients suffering from chronic inflammatory disorders such as rheumatoid arthritis, inflammatory bowel diseases, psoriasis, multiple sclerosis, or locally to patients suffering from asthma, allergic rhinitis and skin allergies [2,3]. Thus far, there is an unmet high need for drugs as effective as classic GCs, but with a reduced side effect profile.

1. The Glucocorticoid Receptor

Glucocorticoids principally exert their main actions via an intracellular receptor, the Glucocorticoid Receptor α (GRα, further referred to as GR), which is a ligand-dependent transcription factor [4,5]. Full-length human GR protein comprises 777 amino acids (see Fig. 1). The GR protein comprises an N-terminal domain (NTD), deemed important for activation and basal transcription factor interaction as well as cofactor interaction, a conserved DNA-binding domain (DBD), involved in DNA binding, interaction with other transcription factors, dimerization and nuclear localization functions (nuclear import as well as nuclear export) and a C-terminally localized ligand-binding domain (LBD), necessary for ligand binding, transactivation functions, nuclear localization functions, chaperone interaction, cofactor interactions as well as the regulation of dimerization (reviewed in [6–8]). GR resides predominantly, but not exclusively, in the cytoplasm in the absence of ligand. The receptor has actually been demonstrated to shuttle back and forth between nucleus and cytoplasm [9,10]. A high-affinity hormone-binding competent complex is formed via association of the LBD with molecular chaperones [11,12]. Ligand binding results in a conformational change of the receptor [9], thus favoring nuclear localization over nuclear export and enabling GR to perform its role as a true transcription factor.

2. Transcriptional activities of GR

2.1. Transactivation

GR can support transactivation via direct binding to glucocorticoid responsive elements (GREs), which are typically organized as inverted repeats separated by three intervening nucleotides (imperfect palindromes), allowing the binding of GR as a dimer (Fig. 2). Classic examples are the mouse mammary tumour virus (MMTV) promoter, the tyrosine aminotransferase (TAT) promoter, the glucose-6-phosphatase promoter and the glucocorticoid-induced leucine zipper (GILZ) promoter [2,13]. Some promoters however contain atypical (intronic) GREs, or concerted half-site GREs. Examples of the latter include the IκB-α and phenyl-N-methyl-transferase (PNMT) promoters [14,15]. Within the nucleus a dynamic equilibrium exists between liganded receptor bound to promoter DNA and the DNA-free liganded receptor. The liganded receptor rapidly moves from a DNA-bound to a DNA-free situation, the mobility of which provides clues about its transcriptional activity. GR bound to ligands with a low transcriptional activity were found to be more mobile than GR bound to ligands with a high transcriptional activity [16,17]. GR mutants with a lower transcriptional activity also displayed a higher motility [18]. Ligand binding itself is also a highly dynamic process, although receptor clearance from DNA seems to occur independent of ligand dissociation [19]. The dynamics of the GRE/GR interaction may influence the design of novel ligands, and represents an important characteristic that requires monitoring in any ligand optimization program. For a long time, GRE binding sites were considered as mere docking sites. Using a combination of structural, biochemical, and cell-based assays, the group of Yamamoto recently showed that different GR binding sequences (GBS), some with only one base pair difference, can differentially affect GR conformation and its subsequent regulatory activity [20]. These data support a role for the DNA as a sequence-specific allosteric ligand of GR, able to sculpt the activity of the receptor toward specific target genes. Extending this concept further might suggest that ligands that do not allow transactivation on one type of GRE may still support transactivation on another type of GRE.

2.2. Cross-talk of GR with other transcription factors

An alternative form of gene regulation occurs via cross-talk mechanisms (Fig. 2) whereby activated GR does not bind DNA but interferes with the activation and/or activity of other DNA-bound transcription factors (TFs). Molecular mechanisms include transrepression via both tethering (the DNA-bound TF remains bound onto the promoter DNA) and squelching (the DNA-bound TF is removed from the promoter DNA). TFs targeted for transrepression include NF-κB, AP-1, NF-AT, T-bet, GATA-3 and IRF3 [21–24]. Since these TFs drive the expression of a whole range of cytokines, adhesion molecules and inflammatory enzymes, the gene-inhibitory characteristics of GR underpins the widespread use of GCs in the clinic as effective anti-inflammatory agents. A mouse model carrying a GR variant, GRdim, which was defective in its dimerization and DNA-binding potential, provided further support for the contribution of transrepression by GR to inhibit inflammatory processes *in vivo*. These mice showed *in vivo* an

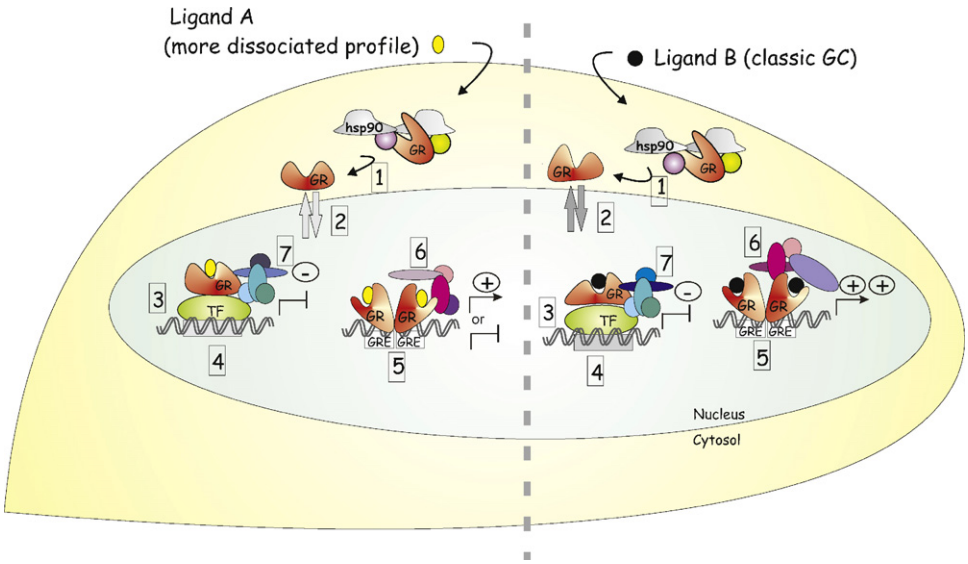

Fig. 2. The figure depicts different subtle mechanistic and differential transcriptional consequences that may arise from the usage of different modulators for GR, for example using a ligand with a more dissociated profile (Ligand A) versus a classic ligand (Ligand B). Different GR modulators induce slightly different structures of GR, which may already have an impact in the cytosol on different kinase pathways (marked by 1). Different GR modulators may further influence the shuttling characteristics of the receptor/nuclear retention time (marked by 2). In the transrepression model, differential GR modulation may result in a different transcription factor selectivity and/or potency (marked by 3). Slight variations on the responsive element, for a given transcription factor, may also influence the transcriptional outcome (marked by 4). Similarly, different GRE's also influence the transcriptional behavior of ligand-bound and dimerized GR (marked by 5). Further, it is expected that different modulators of the receptor, changing its structure, will attract a differential cofactor surrounding (marked by 6 for transactivation and marked by 7 for transrepression). Cofactors functional for the transactivation mode can be recycled for the transrepression mode, or even change their functionalities, dependent on the context, e.g. the presence of other cofactors, chromatin modifying/remodeling factors or basal transcription factors.

impaired ability for GR to transactivate GRE-driven genes including TAT, Hsp27 and Glutathione-3-peroxidase genes, whilst retaining AP-1-targeted transrepression [25,26]. No difference in the DEX-mediated anti-inflammatory activity in the TPA-induced ear edema skin inflammation model was seen when comparing GRdim mice with wild-type animals [27]. This study, in the late nineties, spurred the quest for ligands that only induce GR monomer formation (see further Section 3). Some years ago, the group of Pearce found however that the GRdim variant was still able to transactivate a so-called 'concerted' GRE-bearing gene promoter of the PNMT gene [14]. Furthermore, when screening a large number of primary GR targets, a substantial number of genes were found that were still transactivated by the GR dimerization mutant. Most probably, the AF-1 and AF-2 domains of the receptor contribute to the support of activation mechanisms [28]. Recent findings using the GRdim mouse model demonstrated that the animals still exhibit a reduced bone formation comparable to the GRwt mice, suggesting that a strategy favoring the formation of monomeric GR may perhaps not have the benefit of avoiding GC-induced osteoporosis (Rauch and Tuckermann, communication at the EMBO nuclear receptor meeting, 2009).

Protein–protein interferences are believed to form the basis of the GC-mediated negative feedback on the HPA axis. Here the interaction between activated GR and NGF-IB results in repression of POMC gene transcription and requires recruitment of the histone deacetylase HDAC2 to the promoter [29], which is in accord with the general gene-repressive state following histone deacetylation [30]. In the case of the transrepression mechanism it is conceivable that the physical interaction between activated GR and other transcription factors will also be subject to similar dynamic regulatory processes although presently experimental evidence is lacking.

Although transrepression is the most frequently described cross-talk mechanism, expression profiling and ChIP sequencing revealed that GC-mediated gene upregulation occurs in the virtual absence of GREs in a substantial proportion of regulated target genes, arguing for additional tethering mechanisms [15]. One example of this is the interaction between activated GR and DNA-bound STAT5 [31], which results in stimulation of the β-casein promoter. Prolactin- and GC-mediated induction of β-casein gene expression has recently been shown to involve not only a proximal promoter but also a distal enhancer, which communicate with each other through direct physical interactions via the recruitment of a specific TF and p300 that induces the formation of a chromatin loop [32].

A final mode of regulation, albeit rare, is exemplified by direct binding of GR to the osteocalcin promoter, via a so-called negative GRE (nGRE), whereby osteocalcin gene transcription is inhibited.

3. Dissociated GCs

Insights into how transactivation mechanistically differs from transrepression are crucial for the development of so-called dissociated ligands for GR. As soon as it became clear that the transactivation mechanism could account for the expression of a number of the GC-influenced genes responsible for side effects (e.g. genes involved in gluconeogenesis) and that the transrepression mechanism could account for the greater part of the therapeutic benefits of GCs, novel ligands were developed aiming to separate or dissociate transactivation from transrepression (see Table 1). Although at present an increasing number of exceptions testify to the obvious limitations of the dissociative model, the widespread initial acceptance of this hypothesis has contributed to the generation of a growing number of improved GR ligands, currently still under preclinical testing.

A first generation of dissociated ligands has been described by Sanofi-Aventis, formerly known as Roussel Uclaf (France) [33]. Following the screening of a GC library, three molecules were identified, RU24782, RU24858, and RU40066 that exhibited dissociated characteristics. Some residual transactivation activity was retained (between 9% and 35% of that of DEX), in combination with a transrepressive activity on AP-1 (between 58% and 83% of that of the potent DEX), but with similar activity to prednisolone [33]. In the

Table 1
Dissociated GR modulators.

SGRM	Transrepression on	Test system *in vivo*	Side effect advantages (compared to classic GCs)	Ref.
Steroidal				
RU24782	AP-1-dependent reporter genes, IL1β, MMP-9, tPA	Cotton-pellet granuloma model, Croton oil-induced ear edema	n.d.	[33,37,83]
RU24858	AP-1-dependent reporter genes, IL1β, MMP-9, tPA	Cotton-pellet granuloma model, Croton oil-induced ear edema Sephadex-induced lung edema	Decrease in body weight gain Less thymus involution Less femur osteopenia	[33,35,37,83]
RU00466	AP-1-dependent reporter genes, IL1β	Not effective in AI models		[35]
Non-steroidal				
AL-438	IL-6, E-selectin	Rat asthma model Carrageenan-induced rat paw edema Freund's complete adjuvant-induced edema	Reduced hyperglycemia More favorable bone profile *in vivo*	[42]
ZK 216348	IL-8, TNF-α, IL12p70	Topical in a croton oil-induced rat ear inflammation model	Reduced hyperglycemia No adverse effect on osteoblasts *in vitro* Less reduction of skin thickness and skin-breaking strength Topical and i.p.: lower or no reduction in thymus and spleen weight at effective doses Less ACTH suppression	[49]
ZK 245186	Collagenase promoter activity, IFN-γ, IL-12p40	Irritant contact dermatitis in rodents (topical)	Less thymocyte apoptosis	[84]
LGD5552	IL-6, E-selectin, MCP-1, COX2	Adjuvant-induced arthritis model	No increase in % body fat Decreases in bone formation rate less Lowered increase in mean arterial blood pressure Decreased loss in adrenal weight	[50]
CpdA	IL-6, IL-8, E-selectin, TNFα, IL1β	Zymosan-induced paw swelling model CIA model EAE model EAN model	Absence of hyperglycemia Absence of hyperinsulinemia Absence of HPA axis suppression	[52–54,57,58]
(Aryl)pyrazoles (L5)	MCP-1, GM-CSF, IL-6, RANTES, IL-8, GRO-1	n.d.	n.d.	[66]
Octahydrophenanthrene-2,7-diol analogues (Compound 2)	IL-8, MMP-13	n.d.	n.d.	[65]
Compound 15	IL-6	Mouse LPS-induced TNFα assay	n.d.	[85]
Phenylpyrazole fused Wieland–Miescher ketone derivatives	NF-κB, AP-1 reporter genes	n.d.	n.d.	[41]
Benzoxazinone (Compound 36)	IL-6	n.d.	n.d.	[61]
Isoquinoline 49D1E2	NF-κB	n.d.	n.d.	[62]
Quinol-4-one	IL-6	*In vivo* LPS challenge assay (TNFα)	n.d.	[86]

Steroidal and non-steroidal selective GR modulators (all compounds display a markedly reduced transactivation potential on GRE target genes or reporter gene assays). n.d., not determined. AI, anti-inflammatory; CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; EAN, experimental autoimmune neuritis.

croton oil-induced ear edema model, which relies on phorbol ester-induced skin inflammation, RU24858 was 2-fold more active than prednisolone, whereas RU24782 was about half as active as prednisolone. RU40066, an analogue with no hydroxyl radical on C17 or a ketone radical on C11, a typical hallmark of many therapeutically active GCs [34], turned out to be inactive *in vivo* [33]. However, transrepressive activities of RU40066 could be observed *in vitro*. Obviously there is not necessarily a direct correlation between transrepression *in vitro* and transrepression *in vivo* for a number of reasons. The initial enthusiasm for dissociated GCs cooled when it was recognized that Roussel Uclaf's most promising compound RU24858 despite displaying clear transactivation properties in human eosinophils or rat mesanglial cells still evoked typical GC-associated side effects such as weight loss and a reduced bone mass [35–37]. Similarly, mouse abdominal skin inoculated with GRE-dependent reporter genes displayed a higher reporter gene activity for RU24858 than for prednisolone; a phenomenon that may be explained by metabolic conversion of the ligand *in vivo* [38]. Recent findings have pointed out that chemokine mRNA degradation is increased by both DEX and RU24858. Furthermore, inhibitors of transcription and protein synthesis attenuate DEX- and RU24858-dependent repression of IL-1β-induced steady-state mRNA levels for IL-8 and COX-2. Consequently, it was proposed that the gene repression induced by both glucocorticoids was at least partly dependent on GC-induced gene expression [39]. Despite the fact that RU24858 exhibits defective transactivation by the clas-

sic GRE-dependent mechanism, both DEX and RU24858 induced the expression of anti-inflammatory genes and genes involved in metabolism, thus supporting a potential role for nontraditional GR-dependent gene expression. However on the positive side the GC-responsive gene coding for receptor-activator of NF-κB ligand (RANKL), involved in stimulating bone resorption, did not show activation by the RU24858 dissociated ligand in osteoblasts [40]. Taken together, these findings illustrate the context-dependent actions of different GR ligands. In this respect, Fig. 2 summarizes the different mechanistic and differential transcriptional consequences that may arise from the usage of different modulators of GR.

4. Non-steroidal GR modulators

The complex nature of GR-induced gene regulation has refocused pharmaceutical attention from modifying GCs with a steroidal scaffold, towards a broader evaluation of non-steroidal ligands, often using high-throughput screening methodologies. An example of such a screening strategy is described by Shah and Scanlan [41]. Traditionally, receptor binding assays and cellular *in vitro* reporter gene assays precede proof-of-principle tests using various inflammatory animal models. Hits identified with high affinity for the GR are screened for their potential to inhibit NF-κB- or AP-1-driven gene expression and their potential to mediate GRE-driven gene expression is being explored. Because of structural similarities between steroid receptor family members, candidate ligands are also screened for affinity to the mineralocorticoid receptor (MR), the progesterone receptor (PR), the androgen receptor (AR) and the estrogen receptor (ER) in order to minimize the potential for off-target effects. This is particularly the case for the MR that recognizes the endogenous ligands for GR, including cortisol, with comparable affinities. The enzyme 11-β-hydroxysteroid dehydrogenase, which converts cortisol into the inactive cortisone prevents this recognition by both receptors from being a problem in specific MR target tissues.

As such, a number of non-steroidal GR modulators have been described (see Table 1). Extensive modifications of a synthetic progestin resulted in the synthesis of AL-438, a benzopyrano[3,4-f]quinoline derivative, which displayed a preferential binding to GR over binding to MR, AR, PR and ER. AL-438 efficiently transrepresses the production of the inflammatory mediators IL-6 and E-selectin, but shows a lower transactivation potential as compared to classic GCs [42]. *In vivo*, AL-438 inhibits inflammation in a rat asthma model while showing a decreased potential to enhance blood glucose, a side effect directly associated with transactivation mechanisms. Differential ligand binding results in alternative conformations of helices from the LBD, and a subsequent differential receptor:cofactor binding profile. The principle of allosteric ligand control (and its effects on cofactor binding) was originally derived from crystallographic studies on holo-receptor complexes such as the RAR LBD or the RAR/RXR heterodimer when associated with agonists as compared to antagonists [43,44]. In line with this generally accepted viewpoint, AL-438-activated GR showed different preferences in its cofactor association profile than GC-activated GR. AL-438-activated GR did not associate with PGC-1, a coactivator implicated in hepatic glucose metabolism [45,46], but did show, similar to prednisolone-activated GR, an interaction with GRIP1, a cofactor that previously had been implicated in AP-1-targeting transrepression mechanisms [47]. It is unclear at this stage how stringent and predictive this particular differential cofactor profile may be or whether other non-steroidal GR modulators display a similar preferential binding profile, in connection to their dissociated potential. Apart from induction of diabetes, osteoporosis induced by GCs also represents a clinical burden. AL-438 was suggested to exhibit a greater separation of its anti-inflammatory activity from bone growth-related side effects, including chondrocyte proliferation and longitudinal bone growth, and to affect osteoblasts at a lesser extent than DEX or prednisolone [48]. Alternatively, as GCs influence both osteoblast differentiation and osteoclastogenesis, the functional interpretation also depends on which process and markers are studied. It is however conceivable that a complex mechanism like GC-induced osteoporosis, requiring different factors and many cellular players, will have both transrepression and transactivation mechanisms at play in the same system. It is clear that the final outcome on bone metabolism will be a composite with all GC-dependent processes involved. At any rate, these results already indicate that data obtained with the GRdim mouse model may not necessarily allow one to predict the behavior of particular transrepression-favoring ligands.

Another non-steroidal ligand identified is ZK 216348, a pentanoic acid 4-methyl-1-oxo-1H-2,3-benzoxazinamide, synthesized by Bayer Schering Pharma [49]. *In vitro*, ZK 216348 inhibits IL-8 production in the absence of a strong tyrosine aminotransferase (TAT) induction, illustrating its dissociating properties. The dissociated profile of ZK 216348 is maintained *in vivo* after topical or subcutaneous administration, as evidenced by a reduction in the severity of croton oil-induced ear inflammation and by its failure to induce TAT and to enhance blood glucose levels. Similar to AL-438, ZK 216348 also has been reported to display no adverse effects on osteoblasts, at least *in vitro*.

LGD5552-((5Z)-5-[(2-Fluoro-3-methylphenyl)methylene]-2,5-dihydro-10-methoxy-2,2,4-trimethyl-1H-[1]benzopyrano[3,4-f]quinolin-9-ol) is a non-steroidal compound of molecular weight similar to prednisolone but differing in its ring saturation as well as in its substituents [50]. LGD5552 behaves as a weak GR-transactivator on MMTV-dependent reporter genes, but as a potent agonist of GR in terms of transrepression on IL-1β/TNFα-induced activation of IL-6 and E-selectin promoters. In contrast to DEX, LGD5552 is an antagonist of MR which may result *in vivo* in changes in water and ion balance, potentially causing a decreased blood pressure. Further support of its therapeutic potential was provided by data from the adjuvant-induced arthritis model that showed a strong repression of serum MCP-1 following LGD5552 treatment [50].

Not all dissociated non-steroidal ligands have been discovered via extensive scaffold modifications or compound library screenings. Compound A (CpdA), a stable analog of the hydroxyphenyl aziridine precursor found in the Namibian shrub Salsola tuberculatiformis Botschantzev was found in a rather serendipitous fashion, while looking for the mediator responsible for causing prolonged gestation in sheep when placed on a predominant Salsola-based diet during periods of drought [51]. When CpdA was submitted to GR-dependent whole cell ligand-binding assays and *in vitro* transrepression and transactivation assays, it turned out that this simple molecule could not only bind to GR, but surprisingly also behaved as a selective GR modulator [52]. CpdA was demonstrated to interfere with the transactivation capacity of NF-κB, similar as classic GCs [52] but failed to support GRE-dependent transactivation. The dissociated character was explained by the GR monomer-favoring potential of CpdA, which is in contrast to the dimer-favoring potential of DEX [53,54]. A detailed study of the NF-κB pathway as a target in primary synovial fibroblasts revealed that CpdA treatment resulted in a strikingly more efficient down-regulation of MAPK phosphorylation, and thus of MAPK activation, in comparison to DEX. It should be noted however that the effect of CpdA on MAPK activation displays striking differences depending on the cell type (De Bosscher K., unpublished observations). Furthermore in contrast to the classic steroid DEX, CpdA treatment leads to a clear cytoplasmic relocalization of NF-κB in RA primary synovial fibroblasts (FLS). A dual pathway, partially dependent and partially independent of GR, was proposed

to explain the gene-inhibitory effects of CpdA on NF-κB in RA FLS [55]. Recently, the CpdA-mediated decrease of corticosteroid-binding globulin (CBG), adrenocorticotropic hormone (ACTH) and luteinizing hormone (LH) levels in rats has been linked to direct transcriptional regulatory events at the promoters of these genes [54]. The *in vivo* anti-inflammatory potential of CpdA was confirmed in an acute zymosan-induced arthritis model and in the collagen-induced arthritis (CIA) model and these benefits were observed in the absence of hyperglycemia and hyperinsulinemia [52,53]. CpdA was further independently studied in three models of neuroinflammation. First, in a rat model of experimental autoimmune neuritis, CpdA was found to efficiently attenuate inflammation [56]. In lymph nodes, CpdA inhibited Th1 and Th17 cytokines but enhanced Th2 and regulatory T cell cytokines, thereby suppressing an autoimmune response in EAN and producing a beneficial outcome [56]. Administration of CpdA to mice markedly suppressed the clinical symptoms of myelin oligodendrocyte glycoprotein peptide (MOG35-55)-induced EAE, a mouse model known to serve as an excellent model for multiple sclerosis in man, both in early stages and at the peak of the disease [57]. Attenuation of the clinical symptoms of EAE by CpdA was accompanied by reduced leukocyte infiltration in the spinal cord, reduced expression of inflammatory cytokines and chemokines, and reduced neuronal damage and demyelination. In agreement with the previous animal model experiments, CpdA did not cause hyperglycemia or hyperinsulinemia in either EAN rats or EAE mice unlike classic steroid treatment [56,57]. Finally, in EAE mice the therapeutic effect of CpdA, in contrast to that of DEX, occurred in absence of a suppressive effect on the HPA axis, documenting an additional advantageous effect of CpdA over classic GCs *in vivo* [57]. The latter finding is intriguing, especially in the light of the general belief that GR-mediated transrepression contributes substantially to HPA axis feedback mechanisms. The third study by the group of Reichardt, also addressing EAE, largely confirmed the findings of van Loo et al. with respect to the dissociated character and the anti-inflammatory potential of CpdA [58]. Importantly however, their study provided additional insights on the narrow therapeutic range of CpdA [58], reported previously by Dewint et al. [53]. CpdA's inherent lability, that is dependent on the buffer system used to dissolve the compound, results in the potential to generate metabolites that have the ability to provoke GR-independent apoptosis, thereby compromising the use and anti-inflammatory potential of the original molecule [58]. Based on these findings, the group investigated and identified conditions that allowed a successful treatment of EAE induced in C57Bl/6 mice [58].

Recently, another fascinating molecular aspect of GR biology surfaced that is relevant to the development of new, improved GR activators. Homologous down-regulation of GR is the process whereby a GR ligand is responsible for a down-regulation of its own receptor, thus serving as an appropriate physiological feedback mechanism. Although this process has been extensively discussed, a direct link with the anti-inflammatory potential of exogenous GCs has not been firmly established. Recent data indicate that primary synovial fibroblasts (FLS) isolated from the inflamed synovium of arthritis patients were found to be only initially responsive to the therapeutic effects of DEX following a short-term treatment protocol. Intriguingly, the DEX-induced homologous down-regulation of GR in FLS from RA patients dramatically suppressed the transrepression function of the receptor, whilst retaining most of its transactivation function [59]. This finding may have a significant therapeutic relevance for many chronic inflammatory diseases requiring long-term immunosuppressant therapies. Indeed, it suggests that a developing localized GC resistance resulting from defective transrepression, and the consequent suboptimal therapeutic benefit, may proceed unnoticed, when assessed by a read-out dependent on transactivation (e.g. blood glucose levels). However, not all GR modulators evoke homologous receptor down-regulation. CpdA, which induces nuclear translocation of the receptor in FLS, does not lead to a ligand-induced GR down-regulation in the ex vivo samples from patients, an observation which may be an encouraging finding for future drug discovery programs focusing on druggable dissociated GR ligands. Evidence of the clear interest in the field of non-steroidal dissociated GR ligands is provided by the considerable number of compounds reported (reviewed in [60]) that are able to bind GR with a more favorable profile as compared to standard classic GCs. These include Boehringer Ingelheim's BI 115 and Compound 36 [61], GSK's tetrahydronaphthaline-methylbenzoxazinones [62] and arylpyrazoles [63] or a 6,5-bicyclic core fused to a pyrazole ring (Merck) [64], Compound 15 (UCSF), Compound 25 (Merck), or the recently reported Compound 2 (Pfizer) [65]. Data on side effect parameters are still lacking for many of these compounds.

A very complex profile was observed when comparing structural variants of GR-binding arylpyrazole compounds for their potential to inhibit a panel of TNF-α-induced cytokines [66]. Some of the ligands inhibited the expression of most of the TNF-α-induced genes, whereas other ligands had little effect. Yet others inhibited only a subset of the monitored genes. For example, particular arylpyrazole ligands able to inhibit MCP-1, GM-CSF, and IL-6 were ineffective against IL-8, RANTES, and GRO1. These findings illustrate that modest changes in ligand chemistry can provoke dramatic differences in gene expression profiles, which is especially relevant in the context of natural response elements in their normal chromosomal settings [66].

Of note, the therapeutic advantages of non-steroidal GR agonists over classic GCs are being explored not only for anti-inflammatory diseases but also for the purpose of treating cancers, including multiple myeloma or prostate cancer [67,68].

5. Future directions

Insights from novel basic research on GR, using classic GCs, remain important for the dissociated GR modulator concept and are listed below. To date it is still unclear why so few dissociated GR modulators have reached the clinic. Obvious points of attention include a better understanding of the *in vitro–in vivo* discrepancies, solving narrow pharmacological windows of efficacy *in vivo* (e.g. for CpdA-like molecules), understanding mechanistic differences between the GRdim mouse model and ligand-induced GR monomerization, as well as understanding the tissue- and disease-dependent differential activities and mechanisms used by GR.

5.1. Impact of GRα variants?

In recent years it has become clear that hGRα mRNA can be translated from at least eight alternative initiation sites into multiple GRα isoforms, termed GRα-A to D (A, B, C1, C2, C3, D1, D2 and D3), and which display different expression patterns depending on the tissue type (reviewed in [69,61,62]). Therefore, not only the different ligands but also the pre-existence in a particular tissue type of different receptor isoforms will co-determine the biological response. For example, the fact that the GR-D isoform reduced U2OS osteosarcoma cell-killing capability whilst maintaining the NF-κB-repressing activity [70] may provide a basis for the development of improved glucocorticoid regimens with reduced bone cell-killing side effects. Towards this goal, deciphering the exact coregulator profile associated with GR-D will be instructive. Nevertheless, whether the conclusions for GR isoform dependent apoptotic activity drawn from U2OS sarcoma cells can be generalized requires the investigation of primary cells lacking the endogenous GR.

5.2. Insights on mechanisms contributing to side effects

Recently, a comparison was made between the effects of the arylpyrazole GR modulator L5 and prednisolone on various side effect pathways. This employed a heavy water labeling strategy that allowed a concurrent measurement of fluxes through multiple target metabolic pathways that included the dynamics of lipids, proteins and cells, in the whole organism. This approach is sufficiently powerful to enable interrogation of known side effects pathways [71].

It remains important to consider not only the classic mechanisms but also the impact of novel findings on these side effect pathways. Functional glucocorticoid response elements (GREs) have recently been identified on multiple clock genes, including the core clock gene Per2. Mice with a genomic deletion of this GRE expressed elevated leptin levels and were concomitantly protected from glucose intolerance and insulin resistance following glucocorticoid treatment. These results suggest that the GC regulation of the circadian rhythm cross-talks with the regulation of glucose homeostasis, a relationship which will have implications for GR-associated metabolic diseases in general and may reveal potential therapeutic targets from future insights [72]. Therefore, it would be of interest to study the effect of various dissociated GCs or non-steroidal GR modulators on circadian rhythm. GCs have recently been described to signal via membrane-bound GPCRs [73]; however, the influence of various non-steroidal GR ligands on this GR receptor-independent signaling pathway remains largely unexplored. Another recent development in the field is the GC-mediated induction of miRNA with implications for its role in therapy resistance [74]. Investigation of the effect of various dissociated and/or non-steroidal ligands on miRNA induction will hopefully shed more light on the question of whether novel ligands may be more able to circumvent resistance to therapy.

5.3. Advantage of combinatorial approaches?

It has been postulated that the combined administration of two therapeutics may have an additive or synergistic influence via different signaling pathways. This is believed to lead to an increased benefit to risk ratio of therapy by allowing for lower dosages while maintaining the therapeutic efficacy. Consequently, risks of toxicity and possible drug resistance issues would to a certain degree be avoidable [75]. A recent example is the multi-target mechanism achieved by combining low-dose prednisolone with the antithrombotic drug dipyridamole or a tricyclic antidepressant, both of which can generate a dissociated activity profile with an increased therapeutic window, through a selective amplification of glucocorticoid-mediated anti-inflammatory signaling [76,77]. This approach is further supported by studies of the combination of GCs with PPARα agonists, which showed an additive anti-inflammatory potential targeting NF-κB. Quite surprisingly, PPARα agonists were also able to counteract the GC-aggravated insulin resistance phenotype in a high fat diet-fed animal model [78,79]. GR activation may also be combined with small molecule inhibitors of the NF-κB activating or activity-modulating pathway. *In vitro*, the combined treatment of TNF-induced cells with GR ligands, including DEX and CpdA, and MSK1 (a key kinase able to phosphorylate the p65 subunit of NF-κB) inhibitors or MAPK inhibitors was found to give rise to additional repression of inflammatory gene expression. GCs are already widely used in the clinic, but novel and more specific MSK1 and MAPK inhibitors should be developed to prevent off-target effects and to warrant the safety of the combination [80]. Another combination with potential for the clinic is the combination of GCs with hop bitter acids, the latter being natural compounds which have been shown to inhibit NF-κB activation [81].

5.4. Impact of protein surface modulators?

A completely novel research area, at least with respect to nuclear receptors, is the exciting field of protein surface modulators [69,70]. Here the goal is not to focus on ligand interactions with the classic ligand-binding pocket only, but to investigate how the surface of the protein can be functionally modulated. It will be informative to explore in detail what other receptor sites may be of importance to support only a subset of GR functions. Therapeutic opportunities, away from the classic ligand-binding pocket, are being explored. Indeed, X-ray crystal structure determination of an indazole amide variant functioning as a non-steroidal GR modulator, confirmed the existence and occupation of a predicted "meta" channel in the GR LBD [82]. This offers additional possibilities for novel GR ligand design.

5.5. Towards a customized treatment?

Finally, taking into account large variations in patient responsiveness, future directions for the clinic are geared toward support of a customized treatment to meet each individual patient's needs. This will extend the strategy that is already being pursued to some extent, namely that different GCs are being prescribed for different diseases, according to the corresponding GC characteristic that fits the patients' need the most (topical or oral, or desired length of action).

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