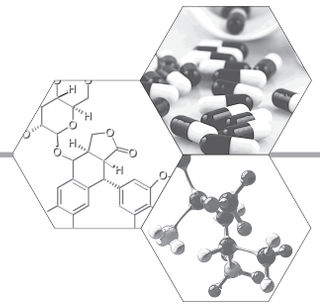

Rational ligand-based virtual screening and structure–activity relationship studies in the ligand-binding domain of the glucocorticoid receptor-α

The interest in developing synthetic glucocorticoids (GCs) arises from the utility of endogenous steroids as potent anti-inflammatory and immunosuppressant agents. The first GCs to be discovered, such as cortisol or dexamethasone, still represent the main treatment for conditions of the inflammatory process, despite the fact that they carry a significant risk of side effects. Hence, there is a continuing need to find drugs that preserve the immune effects of GCs without the side effects, such as those on metabolism (diabetes), bone tissue (osteoporosis), muscles (myopathy), eyes and skin. In this review, we focus on the recent use of ligand-based computational approaches in glucocorticoid receptor (GR) drug design efforts towards the determination of novel GR ligands. We examine a number of ligand-based (similarity searches, pharmacophore screens, quantitative structure–activity relationships) approaches that have been implemented in recent years. A recent virtual high throughput screening similarity search was successful in developing a novel series of nonsteroidal GR antagonists. Additionally, there has been considerable success in ligand-based structure–analysis relationship generation and lead optimization studies for the GR. Future trends towards integrated GR ligand design incorporating ligand- and structure-based methodologies are inevitable.

Glucocorticoids (GCs) are widely used to treat a variety of inflammatory and immune diseases, such as asthma and allergy conditions. The current pharmaceutical challenge is to find compounds that preserve the immune effects of GCs without the concurrent detrimental side effects.

Today's medicinal chemist can apply molecular modeling and computational techniques to drug-design problems to reduce the time and expense of the drug-discovery and -development process. In this review, we focus on the use of ligand-based computational approaches in drug-design efforts towards the determination of novel **GC receptor** (GR) ligands. These studies utilize the available information on known GR active ligands.

The GR belongs to the superfamily of ligand-regulated **nuclear receptors** (NRs) that includes the estrogen receptor (ER), progesterone receptor (PR), mineralcorticoid receptor (MR) androgen receptor (AR), peroxisome proliferator receptor, vitamin D receptor and the thyroid hormone receptor. The human GR ligand-binding domain (LBD) contains nuclear localization motifs as well as the ligand-dependent activation function domain 2 (AF-2) [1,2]. The GR LBD is the focus of all rational drug-design approaches towards the discovery and development of novel GCs and it was first crystallized by Bledsoe *et al.* in 2002 [3,101]. The structure consists of 12 α-helices and four small β-strands that fold into a three-layer helical domain arranged in a 'sandwich fashion' [4,5]. One side of the receptor is formed by helices 1 and 3, while the other side is formed by helices 7 and 10. Helices 4, 5, 8 and 9 are present in the top half of the protein and create a cavity where the ligand can bind.

The GR is a ligand-activated transcription factor that binds with high affinity to cortisol (COR) and other GCs **(Figure 1)** [6]. COR regulates or supports a variety of important cardiovascular, metabolic, immunologic and homeostatic functions. The anti-inflammatory effects of endogenous steroids on GR have stimulated the development of GC derivatives, such as **dexamethasone** (DEX) and prednisolone, which are widely used in the treatment of various inflammatory, immune and allergic disorders including rheumatoid arthritis [7], chronic obstructive pulmonary disease [8], Crohn's disease, systemic lupus erythematosus [9] and osteoarthritis. The synthetic GC DEX is a potent and long-acting GC in terms of anti-inflammatory activity without substantial sodium-retaining effects. This is in contrast to COR, which has approximately a 25-fold lower anti-inflammatory potency and presents some sodium-retaining effects [10]. Deacylcortivazol (DAC), which contains a

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Nuclear Receptor
Nuclear receptors are a class of proteins that are responsible for sensing the presence of hormones and other molecules

Future science part of fsg

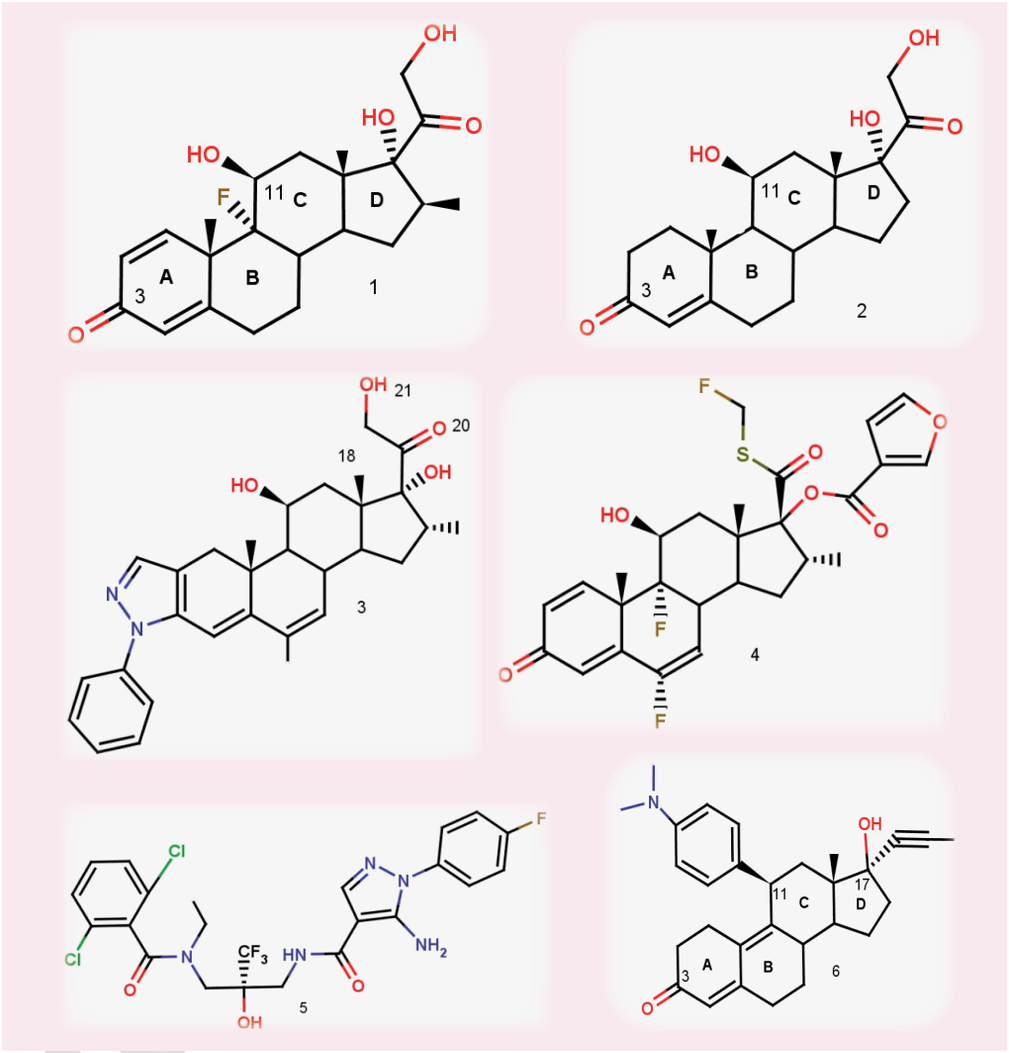


Figure 1. GR agonists (1) dexamethasone (DEX), (2) cortisol (COR), (3) deacylcortivazol (DAC), (4) fluticasone furoate (FF), (5) amino-pyrazole 2,6-dichloro-*N*-ethyl benzamide (GSK866) and (6) mifepristone (RU-486), a GR antagonist.

DEXAMETHASONE (DEX)

Dexamethasone is a potent synthetic glucocorticoid. It acts as an anti-inflammatory and immunosuppressant

MIFEPRISTONE (RU-486)

Mifepristone is a synthetic steroid that is used as an abortifacient in the first two months of pregnancy and in smaller doses as an emergency contraceptive

phenylpyrazole replacement at the conserved 3-ketone position of the steroid hormones, is also an extremely potent GC binder [11]. Additionally, fluticasone furoate (FF) is a novel enhanced-affinity GC that has been developed as a topical therapy for allergic rhinitis [12]. The interest in developing synthetic GCs arises from the ability of endogenous steroids to suppress a number of inflammatory diseases. However, in addition to their immunological suppressor activity, GCs are also able to invoke a broad range of metabolic effects, such as the stimulation of gluconeogenesis in the liver [13].

Finally, the antihormone mifepristone (RU-486) is an nonselective yet effective anti-progestin and anti-GC that has shown clinical efficacy in both functions [14]. Additionally, clinical studies have reported the efficacy of anti-GCs in the treatment of Cushing's syndrome [15,16], GC-dependent hypertension [17], depression [18–20] and glaucoma [21].

In recent years, considerable effort has been directed towards finding novel GR ligands that maintain the desired anti-inflammatory properties while limiting negative side effects. Much insight has been gained from the available GR crystal structures that provide a deeper understanding of ligands' binding modes and on the ability of accommodating different scaffolds due to the binding site flexibility.

In the first GR structure from Bledsoe *et al.* (Protein Data Bank [PDB]: 1M2Z), the agonist DEX [3] is held in place by the hydrogen bond (HB) interactions between the 3-carbonyl

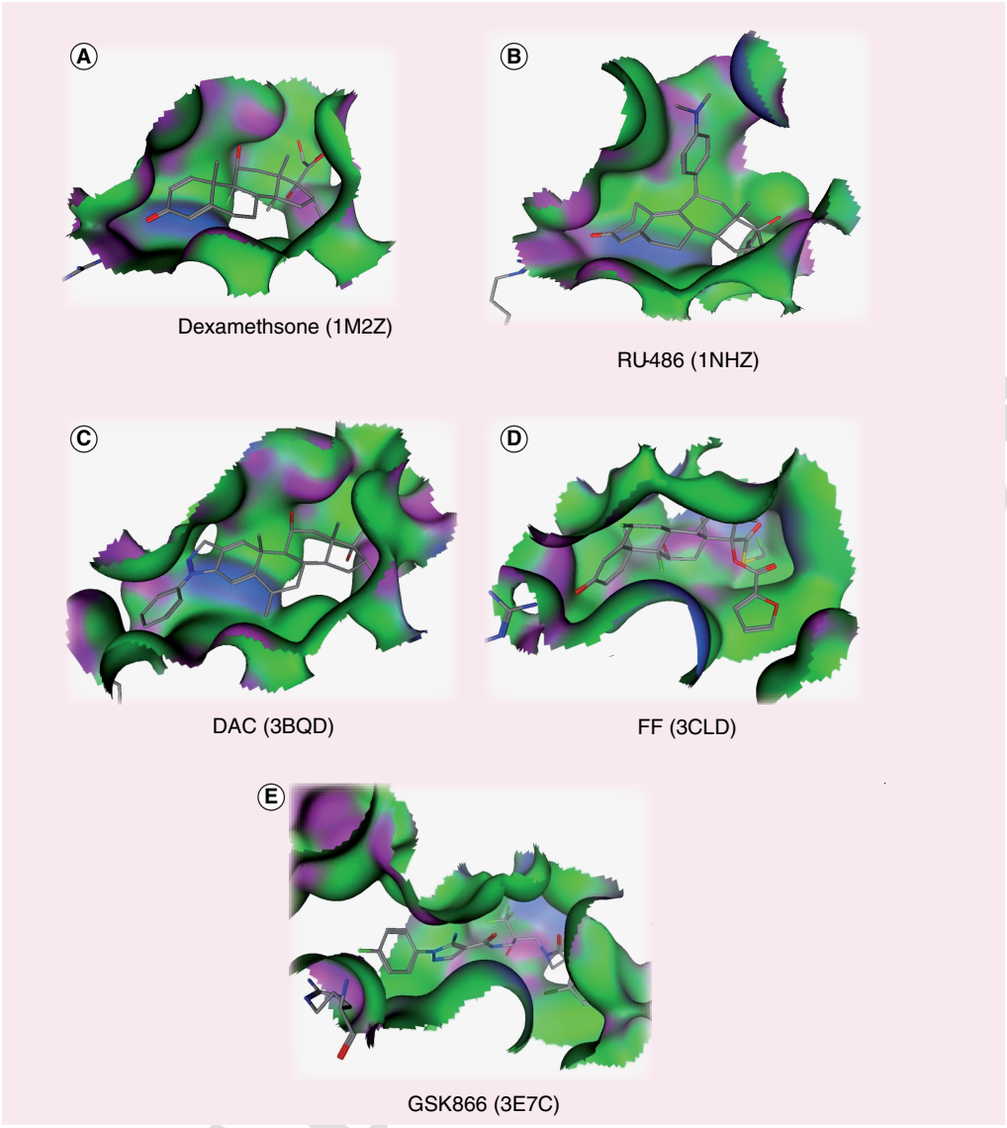


Figure 2. The GR-binding pocket co-crystallized with different ligands. The ligand-binding pocket surfaces were calculated on the protein residues within MOE [26]. **(A)** Dexamethasone in the 1M2Z crystal structure in the agonist-bound conformation. **(B)** RU-486 in the 1NHZ crystal structure. In this complex, the size of the LBD is increased due to the large conformational change adopted by helix 12. **(C)** DAC in the 3BQD crystal structure. This structure shows an expanded binding pocket in the A-ring region. **(D)** FF in the 3CLD crystal structure. Due to small movements of helices 3, 6, 7 and 10 and the loop preceding the AF2 helix, this structure shows an expanded 17α pocket occupied by the 17α furoate ester. **(E)** GSK866 in the 3E7C crystal structure. The di-chloro-phenyl group is directed into the 17α pocket on the D-ring region and the fluoro-phenyl group determine the expansion of the LBD in two directions relative to 1M2Z.

oxygen in the A-ring with the protein residues Gln570 and Arg611 and the 17-hydroxyl group on the D-ring with Gln642. In this protein–ligand complex, helix 12 becomes part of the ligand-dependent AF-2 domain and the protein adopts the so-called 'agonist-bound' conformation. The analysis of the GR structure co-crystallized with the antagonist RU-486 [22] highlights that the steroid core engages the same general interactions as the agonist DEX. However, the dimethylaniline side chain on RU-486 physically prevents helix 12 from becoming an integral part of the AF-2 site, as observed in the agonist-bound conformation. In the PDB: 3BQD crystal structure solved by Suino-Powell *et al.* [11], the agonist DAC maintains the general binding mode of the steroid cores but a dramatic expansion of

Transrepression
Transrepression is a decreased rate of gene expression and results in beneficial therapeutic anti-inflammatory effects

Transactivation
Transactivation is an increased rate of gene expression triggered either by biological processes or by artificial means and causes undesired clinical side effects

Ligand-Based Virtual Screening
In ligand-based virtual screening, the molecular and pharmacological properties and features of known active ligands for a particular target are exploited to identify new ligands with similar or improved properties

the LBD is observed due to the phenylpyrazole group replacing the 3-carbonyl oxygen on the A-ring. Similarly, an expanded LBD relative to the 1M2Z structure was observed in the GR crystal structure co-crystallized with FF (PDB: 3CLD) [23]. In this structure, the presence of the 17α ester on the FF scaffold is responsible for the enhanced GR-binding site due to small movements of the helices forming the 17α pocket. Finally, the GR structure co-crystallized with the amino-pyrazole 2,6-dichlore-*N*-ethyl benzamide (GSK866, **Figure 1**) (PDB: 3E7C) [24] shows a LBD expanded in both the A-ring region and the 17α pocket due to the presence of the fluoro-phenyl group and the dichloro-phenyl group, respectively.

Together, these results highlight the ability of the GR LBD to adapt to different scaffolds, opening the possibility of exploring extended regions of chemical space not limited to the steroid core. An overview of the co-crystallized binding pockets is presented in **Figure 2**, while a more detailed discussion of the crystal structures has been addressed elsewhere [25].

GR ligand-dependent activation

At the cellular level, GR resides in the cytoplasm as a multiprotein complex in association with multiple heat-shock proteins. Once within the cell, hormone binding causes dissociation of heat-shock proteins from GR [27] and the resulting ligand-activated complex translocates from the cytosol into the nucleus, where it regulates gene expression through different mechanisms in either a monomeric or dimeric form [28]. Although the mechanism is not fully understood, it is believed that **transrepression** (TR) action relies on the fact that GR prevents the transcription factors from binding to their consensus sites, thus limiting gene activation. The second mechanism by which GRs effect transcription is when they bind as a homodimer to consensus sequences known as GC response elements (GRE), in the promoter region of GC-sensitive genes, leading to an increase in **transactivation** (TA).

This TA is believed to be the basis of the majority of the side effects associated with clinical GCs, including edema, weight gain, muscle weakness, diabetes and osteoporosis [29]. In spite of much effort to improve treatment with GCs, the utility of these drugs still carries significant risks [27]. Long-term treatment with GCs can lead to a wide range of unwanted reactions, resulting in effects on metabolism (diabetes), bone tissue (osteoporosis), muscles (myopathy), ulcers, hypertension, hyperglycemia, neuroses and psychoses [13,30]. As a consequence of these side effects, there have been increased pharmaceutical efforts towards developing selective GR modulators with improved risk–benefit profiles. The aim is to separate the beneficial anti-inflammatory effects, derived from TR pathways, from the side effects, derived from TA pathways [30,31], leading to a new generation of compounds known as dissociated GR agonists or GR modulators.

Thorough reviews of current GR ligand patents can be found in the work of Mohler *et al.* [32] for agonists and antagonists. Also, an examination of medicinal chemistry approaches [1], novel ligands [33] and structure-based (SB) virtual screening (VS) techniques applied to the GR are discussed elsewhere [25]. This review will have a particular focus on the application of ligand-based computational studies involving the GR LBD and structure–activity relationship (SAR) studies towards lead optimization. We will discuss the evident merits and failures of these computational techniques that have been brought to bear on the challenging search for novel GR modulators.

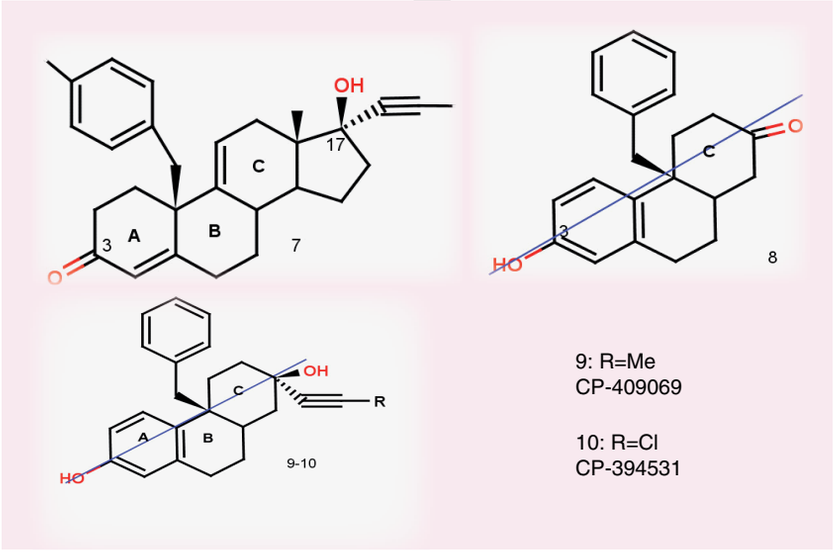


Figure 3. (7) RU43044. **(8)** a tricyclic compound with the pseudosymmetry axis indicated. **(9)** CP-406069 and **(10)** CP-394531 were identified by Morgan *et al.* [52].

Molecular modeling techniques

In parallel, with the increased performance and functionality of computer hardware and software, advances in protein crystallography have laid the foundation for the development of

computer-aided drug design [34–36]. Molecular modeling is now a key component in contemporary drug discovery with many tools available, among which VS is well known [37]. VS approaches are implemented for examining large compound databases *in silico* and to identify a selected number of molecules for *in vitro* testing. Such rational drug design can be divided into two generic categories: structure-based (direct methods) or ligand-based (indirect methods) VS (LBVS) [38].

In LBVS, the molecular and pharmacological properties and features of known active ligands for a particular target are exploited to identify new ligands with similar or improved properties. Based on the assumption that active molecules should have similar physical and chemical characteristics, these properties are used as a rational filter or guide towards the identification of compounds with similar activities. LBVS can be further divided into subclasses depending on the type of properties calculated and employed. LBVS can employ 1D (molecular weight filtering) [39,40], 2D (substructure searching, identification of 2D-maximum common substructure) [41–43] or 3D properties (pharmacophore, 3D similarity, 3D fingerprints and 3D-maximum common substructures) [35,36,44–46]. The prevailing paradigm that pervades these methodologies is that the 3D coordinates or structure of the biological target protein is not mandatory for progression of the screen, but rather a set of known active molecules that engage the target is required, from which specific properties to direct the screen are derived.

The understanding that TA and TR are distinct mechanisms activated by the ligand–protein complex focused scientific research towards the identification of 'modulators' of the GR – aiming to separate therapeutic from side effects. Intense research has delivered a wide set of GR binders with a large variety of biological profiles that can be well exploited in LBVS [1]. For example, with pharmacophore-based approaches, the most relevant structural features of active compounds are collected to represent the positive or beneficial molecular features to be included in the screen and, with quantitative structure–activity relationships (QSAR) approaches, SAR data can be taken into account. Ligand-based computational work towards VS and SAR studies in relation to GR shall now be explored.

Conformation generation analysis

Carta *et al.* performed a study to determine how preprocessing of strings input (SMILES) affects the generation of conformers in the context of VS [47]. To examine this concept, the work explores the effect that permutation of SMILES strings has on the conformers generated using data from a set of 17 crystal structures, including the 1P93 (GR co-crystallized with DEX). The aim was to quantitatively show the influence that each permutation of a SMILES string, generated by several software programs (Corina [48], Rubicon [49], Catalyst [201] and Omega [50]), has on the production of conformers. Although significant differences were observed for many crystal structures and with different software, in the case of 1P93, the permutations did not affect the conformer generation, probably due to the relative rigidity of DEX.

Pharmacophore design

The term 'pharmacophore' was first defined by Paul Ehrlich in 1909 as 'a molecular framework that carries (phoros) the essential features responsible for a drug's (pharmacon's) biological activity'. A later definition is 'a set of structural features in a molecule that is recognized at a receptor site and is responsible for that molecule's biological activity' [51]. In computer-aided drug design terms, a pharmacophore is a specific, 3D map of biological properties common to all active conformations of a set of ligands that exhibit a particular activity.

Morgan *et al.* applied pharmacophore design to GCs to assist the drug-discovery process [52]. The concept was to exploit the similarity between the ER and GR to derive GR antagonists. In fact, the presence of a C-2 or a pseudo-C-2 **(Figure 3)** symmetry in ligand binding to the ER is believed to be important for ligand orientation within its LBD [53]. The authors embodied this symmetry in their

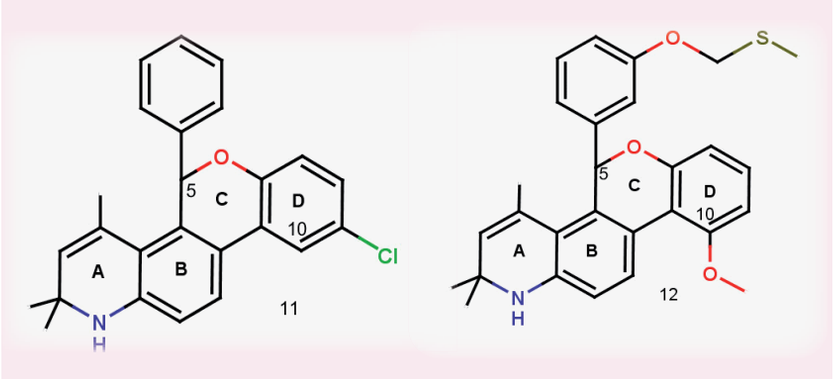


Figure 4. Compounds 11 and 12 from the study by Coghlan *et al.* [1,54].

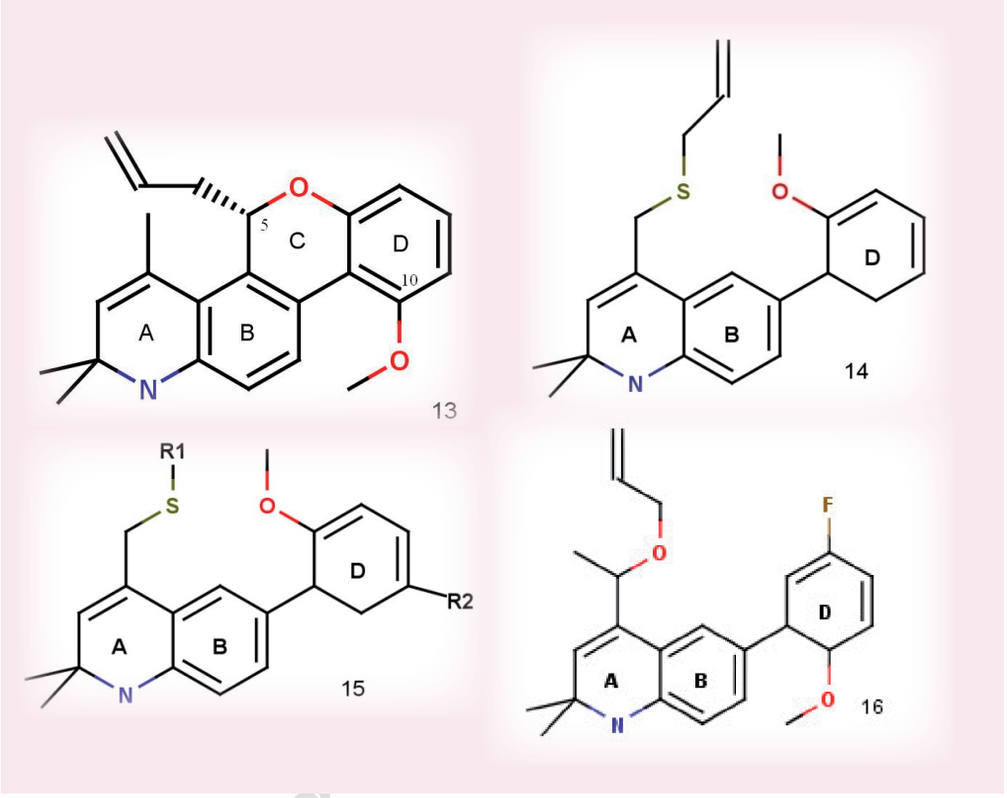


Figure 5. Compound 13 (AL-438) and compounds 14–16 from Takahashi *et al.* [55].

pharmacophore model built on the nonselective RU-486 (which displays a very high affinity for PRs and GRs) and the GR-selective steroidal antagonist RU-43044 **(Figure 3)**.

The model includes a feature for the A- and B-rings of steroid molecules, deemed essential for potency and specificity, a second feature for the benzyl side of RU-43044 chain, believed to play a key role in the antagonistic activity, and a third pharmacophore feature accounting for the alkyne attached to the D-ring, believed to be essential for tight binding compounds. Tricyclic compounds matching the first two pharmacophore points (compound **8**) and containing a pseudo-symmetric axis structurally distinct from the steroid core, were designed from the model aiming to be specific and selective with antagonist activity for GR. Both enantiomers (benzyl group stereocenter of compound **8**) were synthesized so that the *R*-enantiomer could be used as a control when evaluating the symmetric position of the features in the *S*-enantiomer, which was believed to be the active form. In contrast to what the authors expected, the *R*-enantiomer exhibited a binding affinity in the nanomolar range, which was not exhibited by the *S*-enantiomer. Finally, the third pharmacophore feature was 'manually' introduced, leading to the design of compounds **9–10**, CP-409069 and CP-394531. The molecular modeling studies were validated by the ameliorated binding affinity of these compounds, which carry both an axial propyne moiety and an equatorial hydroxyl on the saturated C-ring. That is, different substituents on the C-ring have proved to be extremely relevant for the binding potency of the tricyclic compounds, likely due to the ability to form van der Waals interactions between the saturated C-ring and key active site residues. Selectivity studies for the GR versus the MR and PR were performed in transiently transfected T47D cells. The model successfully led to the discovery of potent and selective antagonists for the GR over the MR and PR.

Structure–activity relationships

Coghlan *et al.* examined multiple regions of substitution around a novel tetracyclic quinoline scaffold [54]. Their initial hit **(Figure 4, compound 11)**, was originally designed to target PR but, on assay, displayed GR cross-reactivity. It is important to stress that the identification of a compound active within reasonable thresholds for a particular target is the first critical step in

the drug-discovery process. Moreover, as hit-identification for some proteins can be more difficult compared with other targets, the value of a good hit cannot be stated *a priori* but should be based on an overall consideration as with the target complexity. In particular, developing novel chemical scaffolds for GR has proved to be a tough challenge. Therefore, although compound **11** displayed poor GR activity compared with classical steroids and little selectivity for GR, it was selected as a scaffold starting point upon which to develop selective ligands with better biological profiles. The initial goal was to decrease PR binding and a substitution study around the D-ring revealed that the removal of the C-9 chlorine and the introduction of a methoxy group at the C-10 position yielded a selective analogue with poor affinity for PR. As ligands with favorable binding profiles were synthesized, Coghlan's team worked on improving the functional behavior of the molecules. By focusing on substitutions around the C-5 phenyl group, a significant number of active compounds, such as **12 (Figure 4)**, with functional profiles in GR TA and TR assays comparable to prednisolone, were discovered [54].

Based on the observation that RU-486 and the nonsteroidal dissociated GR ligand AL-438 **(Figure 5, compound 13)** share common chemical features in different scaffolds, a SAR study was performed to develop a novel class of dihydroquinoline derivatives with dissociated GC properties [55]. The selected initial core that was structurally related to AL-438 **(Figure 5)** presented an open C-ring and a C-4 allyl group containing a sulfur linker **(Figure 5, compound 14)**. An additional important issue with this lead molecule was the metabolic lability of the thioether group. The α-position on the C-4 allyl substituent plays a key role in the binding affinity and GR selectivity over PR with a methyl substituent displaying the optimal profile. The introduction of a broad series of substituents, such as benzyl, acetyl derivatives or polar groups, in place of the allyl group resulted in a two-to-ten-fold reduction in affinity for GR. A SAR study based on thioether and ether analogues of compound **15** was developed with respect to substitutions at the phenyl ring at the C-6-position of the dihydroquinoline core and adjacent to the ether. An improvement in binding affinity and selectivity was obtained by introducing a fluorine atom at the 5 positions of the phenyl ring. Compound **16** in **Figure 5** was found to display the best affinity and selectivity profile. However, compound **16** failed to significantly exhibit agonist activity in an IL-6 TR assay, while it displayed nanomolar activity as a selective GR antagonist in a MMTV reporter gene assay.

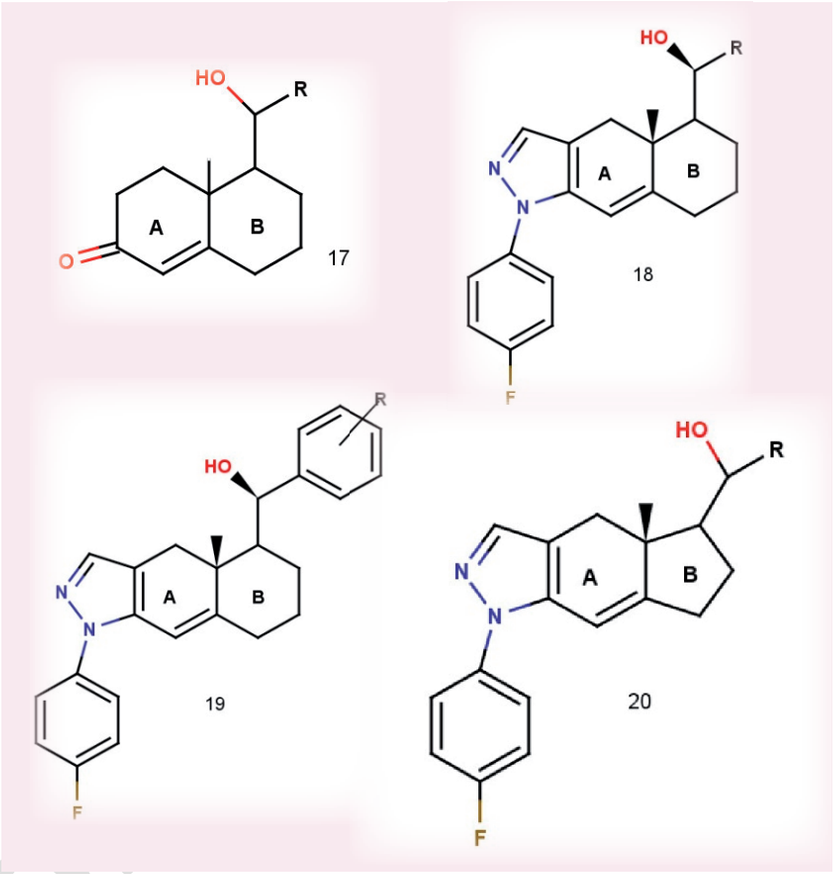


Figure 6. Compounds 17–20 developed around the A–B ring core.

A new scaffold **(Figure 6, compound 17)** binding to GR was studied by a group based in the University of California after observing that, in general, GR can accommodate a wide range of manipulations in the C- and D-ring of the steroid core while the A- and B-ring are subject to stricter constraints [36]. This core retains the A-B rings and the hydroxyl group (which, in the steroidal structure, would correspond to the 11α position), as it was thought to be important for GR selectivity [56]. Thus, compound **17** acted as a starting point for further development in the C–D region through a SAR study. The first substitution pattern confirmed that the equilibrium between TA and TR could be easily affected by substituent at the R position. In an attempt to enhance the binding affinity for GR, a p-fluorophenyl pyrazole moiety was appended to the A-ring and a series of analogues differing only at the substituent of the C-11 of the aryl-pyrazole nucleus was developed (e.g., compound

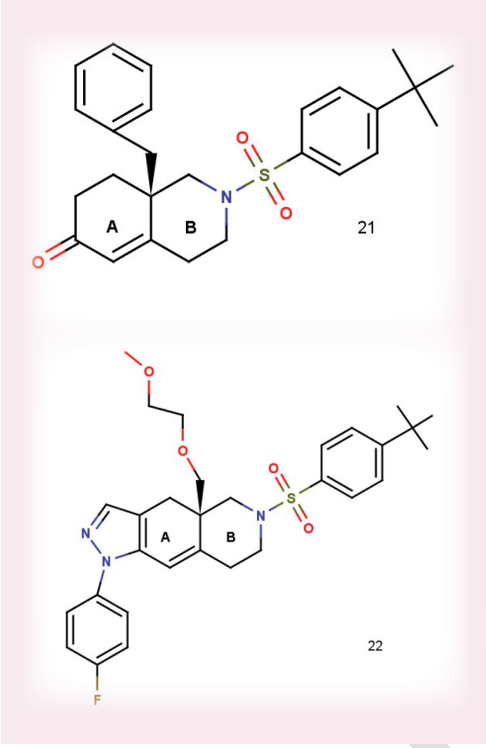


Figure 7. 2-azadecalin structures (compounds 21 and 22).

18). All but one of these compounds acted successfully as an agonist in the TA NF-κB and AP-1 reporter-gene assay.

Independent of the research described above, Merck Research Laboratories developed SAR around the same scaffold [57] following the observation that the C-11 hydroxyl group in the steroid core plays a role in GR selectivity and that the replacement of the A-ring with heterocyclic A-ring mimetics causes a significant improvement in the biological profile [58]. Moreover, the observation that dissociated GCs often rely on structural modifications of the C–D region with regards to the steroidal core offered the possibility of investigating the SAR of this region [59]. An 'ideal' scaffold **(Figure 6 compound 19)** is able to retain affinity and selectivity for the GR and, therefore, provides a starting point for investigation of the SAR of the C–D region. The substitution pattern on the phenyl ring produced high GR-affinity analogues with a preference for small hydrophobic groups at the ortho and para positions, while polar substituents, such as a hydroxyl group or amine, were not tolerated, causing a reduction in affinity and functional activity. Therefore, it was concluded that the insertion of substituents in the C and D region provided a large number of analogues capable of binding to GR and displaying a wide range of dissociation profiles between functional TA and TR activities.

In the case above, the A–B-ring system was a classical steroid 6,6-ring system. Based on a trend observed by Smith *et al.* [60] of improved TR for compounds containing a five-membered B-ring **(Figure 6, compound 20)**, an exploration of this core structure was undertaken with hydroxy-aromatic and heteroaromatic substituents on the B-ring [61]. The SAR of the 6,5-bicyclic series differed markedly from the previously reported 6,6-series. It was found that monocyclic aromatic or heteroaromatic B-ring substituents lacked potency in TR and TA assays, particularly in mouse cell lines. Bicyclic ring systems displayed strong activity in both mouse and human cell lines, although the SAR was much tighter than in the 6,6-ring series, with the point of attachment to the aryl or heteroaryl substituent of critical importance. Contrary to what was expected by the authors, after analyzing a wide range of mono-cyclic and bicyclic hydroxyl-aromatic and hetero-aromatic substituents, it was deemed that the 6,5-ring series SAR was subject to stricter constraints compared with the 6,6-ring series. In fact, all but a few molecules of the 6,5-ring series exhibited a reduced TR assay potency showing a poorer functional profile than the analogues in the 6,6-ring series.

Molecular modeling studies were employed to understand the conformational differences between the two series of compounds, which may explain their divergent activity and shed light on the correlation between the B-ring size and functional activity [60,61]. Conformational analysis was performed on a small subset of compounds, selected for their activity profiles, using an implementation of a distance geometry algorithm [62] and, subsequently, minimized with MMFF. Finally, compounds were aligned with regards to the common pyrazole ring. From the analysis of the superposition of the lowest energy conformer, it became evident that the combination of the orientation of the pendant aromatic and the chirality of the hydroxyl group contribute the most to the dissociative profile.

Recently, Clark *et al.* pursued a SAR study around a 2-azadecalin GR antagonist **(Figure 7, compound 21)** that is structurally related to the antagonist RU-43044 **(Figure 3, compound 7)** [63]. It is possible that the antagonist behavior of compound **21** is due to the presence of the angular benzyl substituent between the

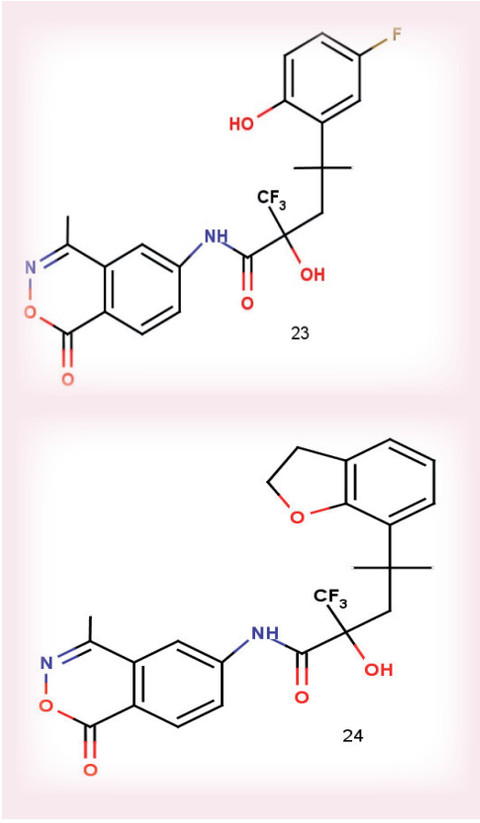


Figure 8. Compounds 23 and 24 (ZK 2163482).

A- and B-rings, which physically prevents the helix 12 from adopting the agonist conformation. The addition of a 4-fluorophenylpyrazole group **(Figure 7, compound 22)** to the 2-azadecalin resulted in a 40-fold enhancement in functional activity compared with 2-azadecalinone. SAR of the bridgehead substituent indicated that, while small groups such as methyl afforded high GR binding, GR functional activity was enhanced by larger groups such as benzyl, substituted ethers and amino alkyl derivatives. The bridgehead benzyl group was found to not be essential for binding but did impact upon the antagonist effect of the ligands. GR antagonists with binding and functional activity comparable to RU486 were discovered (e.g., compound **22** with GR binding of K_i 0.7 nM and functional K_i 0.6 nM; RU486 is 0.4 and 1.2 nM, respectively) and found to be highly selective over other steroid receptors.

Several noncomputational 'pharmacophore models' based on the dissociated GC compound **23** [102] and ZK 2163482 **(Figure 8, compound 24)** were developed in Boehringer Ingelheim Pharmaceuticals, leading to the design of a new core active on GR. A first analysis aimed to investigate the effect of replacing the benzoxaninone ring with an A-ring mimetic and then exploring the impact on the anti-inflammatory activity **(Figure 8)**. A second model focused on the role played by the central hydroxyl and trifluoromethyl group [64]. Finally, the last study centered on substituting the benzoxazinone amide with a phenyl ring and exploring the effect of different length alkyl chains.

The first model was a pharmacophore based on the GR agonists compounds **23** [103] and **24**, aimed at developing dissociated, nonsteroidal, anti-inflammatory agents [65]. From those starting molecules, the model retains two features placed on the central hydroxyl and trifluoromethyl group with the purpose of preserving the scaffold and thereby the agonist activity. The addition of a HB-acceptor feature derived from the C-3 carbonyl group of the A-ring of steroidal structures gave the opportunity to analyze the effect that replacing the benzoxazinone group with several A-ring mimetics had on the binding potency and TR activity [101]. Lipophilic groups fused with an aromatic ring produced an ameliorated binding affinity combined with a dramatic loss of TR activity confirming that 'often there is a lack of correlation between GR binding affinity and trans-repression activity' as observed for other series of compounds [1,60,65–67] The inclusion of nitrogen atoms in the A-ring mimetic, such as quinazolinone and naphthyridin-4-one **(Table 1, compounds 25 & 26)**, while yielding a drop in binding affinity when compared with a non-aza analogue, is however able to re-establish the TR activity in a IL-6 assay. Molecular modeling studies further guided modifications of the phenyl ring, which was suggested to lie within the steroid D-ring domain. The analogue with a hydroxyl substituent at C-2 on the phenyl ring (compound **27**) displayed a clear improvement in GR affinity and TR activity. However, it is devoid of selectivity over PR and MR. It was concluded that the degree of dissociation activity

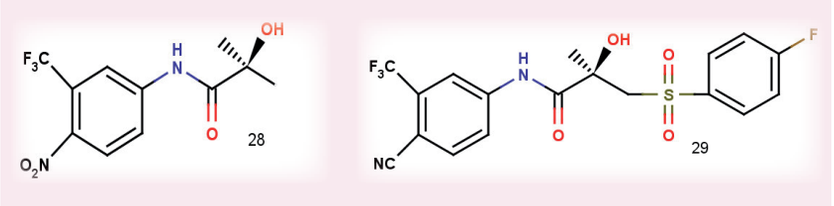
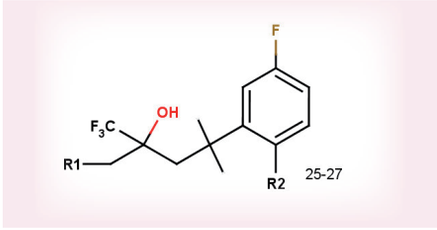


Figure 9. Hydroxyflutamide (compound 28) and bicalutamide (compound 29).

Table 1. Model 1: structure–activity relationship focused on an A-ring mimetic and modification of the D-ring.



Compound	R1	R2	Binding affinity	Transrepression
25	Quinazolinone	OMe	Low	Active
26	Naphthyridin-4-one	OMe	Moderate	Active
27	Quinolone	Hydroxyl	High	Active

is dependent upon the substitution pattern on the phenyl ring and an appropriate choice of an A-ring mimetic.

In the same group, Betageri *et al.*focused on the methyl and hydroxyl quaternary center of the GR agonists (compounds **23** and **24**) [64]. This quaternary center is common to the androgen receptor antagonists hydroxyflutamide and bicalutamide **(Figure 9; compounds 28 & 29)** and proved to be a crucial pharmacophore feature for biological activity [68]. The replacement of the methyl group with a CF_3 group altered the functional effect from antagonist to partial agonist at the AR. Therefore, aiming to conduct a SAR study on GR ligands to assess the role played by CF_3 and the hydroxyl group, two analogues with a phenyl ring and with a 2-hydroxy-5-fluoro phenyl ring at the R2 position (compound **30** and **31**), were prepared **(Table 2)**. Molecules with the substituted phenyl ring are generally more potent than the unsubstituted analogues, suggesting that the R2 group may be involved in HB interactions that could explain the higher binding affinity. Substitutions of the CF_3 group led to the conclusion that it is likely embodied in a lipophilic pocket with encouraging results for phenyl or benzyl derivatives. To estimate the importance of the central hydroxyl group in the GR ligands, it was replaced with a hydrogen atom, leading to a dramatic loss of activity, suggesting that the presence of a central hydroxyl group is a requirement to guarantee a satisfactory binding affinity. However, it was speculated that the CF_3 group could increase the HB potential of the central hydroxyl group by enhancing its acidity [69], while alkyl or aryl groups decreased the ability of the central hydroxyl moiety to engage in HB interactions.

In conclusion, the hydroxyl group coupled with an appropriate substituent is involved in important interactions with GR and, together, work as essential pharmacophore features. The dissociative profile of this series is complex as, again, no linear correlation was found between the binding affinity and agonist activity in an IL-6 TR assay and antagonist behavior in the luciferase TA assay.

Docking studies of compound **32** into a GR agonist co-complex x-ray structure suggested that replacing the CF_3 group with the larger benzyl substituent clashed with the agonist conformation of helix 12, thus enhancing the antagonistic activity of these ligands. Docking of compound 32 into the RU486 antagonist crystal structure displayed a good overlay between the positions of the two ligands. A second docking study performed with compound **31** in the x-ray co-complex crystal structure GR-LBD/fluticasone propionate [70] indicated that a similar docking pose to the crystallized ligand could be obtained and that there was no steric clash with helix 12. This is in line with SAR data where substitution of the 2-hydroxy-5-fluorophenyl in the R3 position with phenyl or 2-methoxy-5-fluorophenyl results in a loss in GR binding.

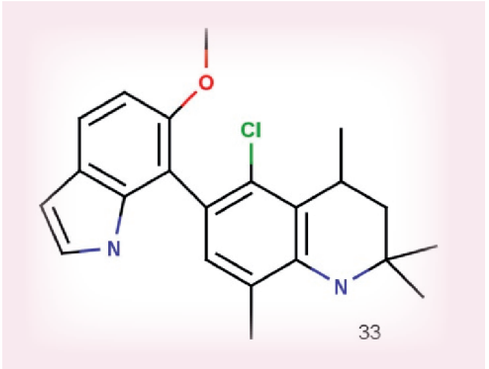


Figure 10. Glucocorticoid receptor-selective ligand (compound 33).

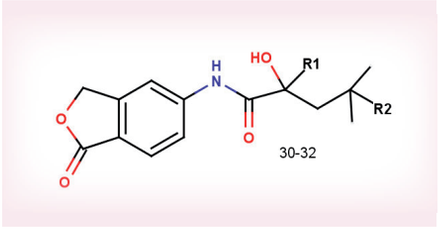
Finally, Roach *et al.* also reported a series of nonsteroidal GR ligands based on a 6-indole-1,2,3,4-tetrahydroquinoline scaffold **(Figure 10)** [71]. SAR around the indole group identified compound **33**, which exhibits good GR binding affinity (K_i = 1.5 nM) and 100- to 1000-fold selectivity over MR, PR and AR while showing good activity in a TR assay [71]. Significant NR cross-reactivity can be characteristic of new nonsteroidal GR ligands; thus, such optimizing of receptor selectivity is of critical importance.

Quantitative SAR

Quantitative SAR are frequently employed in medicinal chemistry projects, both to rationalize SAR for known series of compounds and to help in the design of innovative structures endowed with desired pharmacological properties. In the 3D QSAR approach, structural descriptors are calculated from molecular models of the ligands as interaction fields within a 3D lattice of points surrounding the ligand structures.

Human cytochrome P450 enzyme CYP3A4 induction is regulated by one or more of the steroid-activated NRs [72,73]. Following this observation, it was postulated that the GR could act as a mediator between the steroid's activation and CYP3A4 induction [74]. A homology model of hGR LBD was built from the human ERα using the genetic computer group package [75]. Energy minimization was subsequently applied to obtain a low-energy conformation and relieve steric clashes in the protein structure. Subsequently, the GR homology model was used to perform a docking procedure with ten suitable compounds using the Sybyl molecular modeling package. Biological data for ligands known to induce CYP3A4 through GR were used to validate the ligand-binding interaction data obtained from docking with the GR homology model. The analysis focused on the relationship between ligand physiochemical properties and GR-mediated CYP3A4 induction, which was measured through a reporter gene assay. While the biological data on the reporter gene was collected from the literature [76], structural and physiochemical parameters were either collected from the literature or computed in Sybyl and the Pallas software packages [77]. The observation that the number of HBs (NHB) played a central role in the GR functional profile together with hydrophobic interactions was confirmed by the QSAR analysis, which highlighted the relationship between the NHB and the degree of induction of CYP3A4 expression

Table 2. Modifications at the methyl and hydroxyl quaternary center and the phenyl ring of the glucocorticoid receptor agonists.



Compound	R1	R2	IC_{50} nM
30	CF3	Phenyl	79 ± 8
31	CF3	2-hydroxy-5-fluorophenyl	6 ± 2
32	Benzyl	2-hydroxy-5-fluorophenyl	60 ± 1

with a correlation factor of r^2 of 0.95. However, further QSAR models, including molecular planarity (area/depth^2) and rectangularity (length/width) descriptor terms, enhanced the correlation between the NHB and fold induction of CYP3A4 to r^2 = 0.98. Moreover, two of the four equations that contained frontier orbital energies E_{LUMO} and E_{HOMO} achieved a correlation coefficient of r^2 of 0.98 for eight of the ten compounds. The molecular dipole moment μ is also a common factor among the equations. It appears that the equations developed in this paper were able to correlate computational data and the biological information regarding GR-mediated CYP3A4 induction.

Hammer *et al.* performed a homology model-based 3D QSAR analysis in an attempt to explain side effects of GC double esters such as prednisolone 17-ethylcarbonate (P17EC) and prednisolone 21-ethylcarbonate (P21EC) used in skin disease **(Figure 11)** [78]. An interaction field between the ligand and a methyl probe was calculated and the GOLPE software was then applied to reduce the resulting variables. A

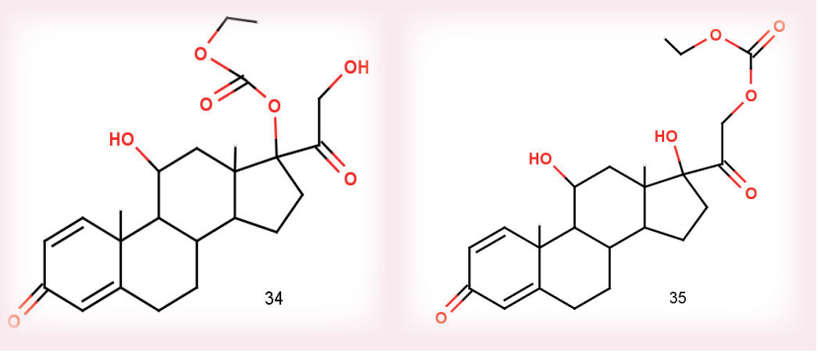


Figure 11. The glucocorticoid double esters. Prednisolone 17-ethylcarbonate (P17EC) (compound 34) and prednisolone 21-ethylcarbonate (P21EC) (compound 35).

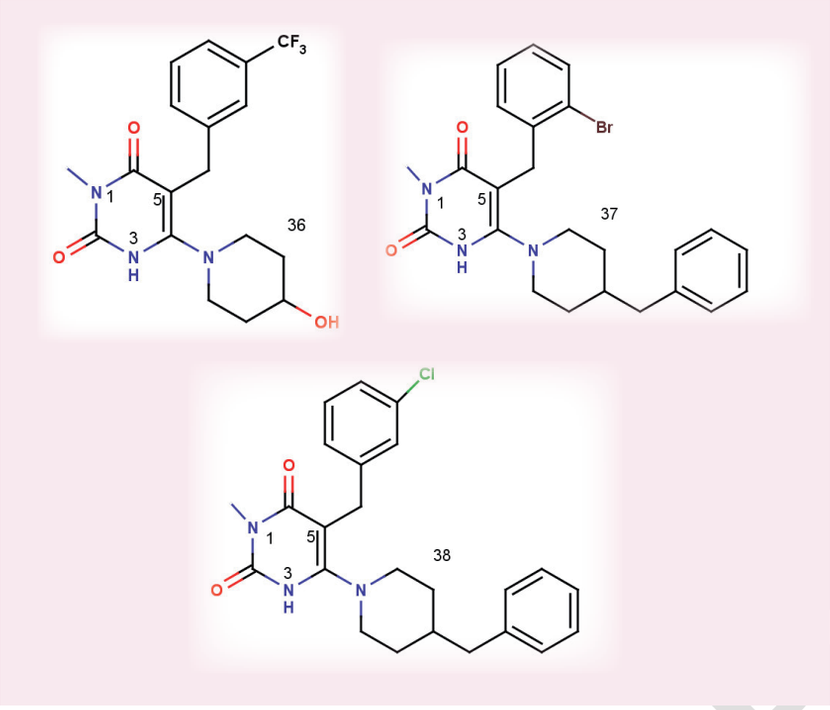


Figure 12. Compounds 36–38 from the study by Ray *et al.* [86].

satisfactory correlation between the biological activity and the interaction field was obtained using a model of three partial least squares regression components. The partial least squares method provided a satisfactory prediction of IC_{50} values for each ligand of the training set with a correlation coefficient of r^2 of 0.98. A leave-one-out (LOO) procedure was then used to cross-validate the model leading to a correlation Q^2_{LOO} of 0.79 with a standard deviation of error prediction (SDEP) of 0.36. Aiming to test the efficacy of the model, IC_{50} values of a validation set of six highly correlated GCs were predicted and compared with the IC_{50} experimentally determined in the COS-7 system. The authors claimed to have produced a model with a reasonable agreement between the predicted and observed IC_{50} values for which only the SDEP value of 0.33 is given. A Q^2_{LOO} of 0.84 with a SDEP of 0.29 were achieved.

However, both these attempts to validate a QSAR model using such a small training set even in combination with the validation set goes against the basics of accepted statistical procedure. The wider applicability of such a model is therefore questionable.

Buchwald applied a linearized bi-exponential (linBiExp) method to QSAR studies in order to describe data showing bilinear-type distribution as he observed for GR ligands [79]. Buchwald highlighted a bilinear distribution when considering the log of the receptor-binding affinity (logRBA) against molecular volume (MV) of these compounds. Due to the size limitation of the GR-binding pocket, he found a strong dependency between this descriptor and RBA: the ligand's logRBA increased linearly with the MV, up to a maximum, after which the activities decreased linearly again. Importantly, the two sides of the maximum were not symmetrical (bilinear distribution). The linBiExp QSAR model developed to interpret these observations was able to predict the increased activity observed when large 17α substitutions on the main scaffold were introduced, as opposed to other NRs where the binding pocket cannot accommodate such large side chains. The optimum MV was therefore identified around 353.3 ± 4.1 Å, a value very close to fluticasone propionate (FP; 357.8 Å) and mometasone furoate (MF; 367.9 Å).

Recently, Spreafico *et al.* obtained experimental binding affinity data from a variety of sources on 110 GR-binding compounds composed of four different chemical classes: steroids, quinoline derivatives, fluorophenyl indazole derivatives and spirocyclic dihydropyridine derivatives [80]. They identified the binding modes to the GR (PDB 1M2Z, chain A) using flexible docking (software Yeti [81]) and then split the data into 88 training and 22 test compounds. The Quasar [82] and Raptor [83] technologies were utilized in consensus scoring mode to establish a multidimensional QSAR (mQSAR) in a mixed-modeling approach. The Quasar model family converged at a cross-validated r^2 of 0.702 for the 88 training compounds and a predictive r^2 of 0.719 for the 22 test ligands. A variety of scramble tests were performed, which indicated that the GR model was sensitive to the biological data and therefore was a reliable tool for predicting the binding affinities. In a consensus approach, a Raptor simulation using the same ligand alignment and selection yielded an r^2 of 0.680 and a predictive r^2 of 0.519. Compared with the Quasar result, the authors considered the Raptor simulation as only having modest predictive power. However, given the chemical diversity of the compounds and the different literature sources of the binding information, they deemed it to be of acceptable quality. Additionally, the model was tested on an external validation set of eight compounds with a resultant low predictive r^2 of 0.538. However, the model was incorporated

into VirtualToxLab [83] and used to study the potential interactions of 24 psychotropic drugs with the GR. A full conformational search was followed by flexible docking and calculation of the binding affinity using 6D QSAR. The authors mentioned that the model is limited by its sensitivity to formal charge and the molecular weight of the compound to be tested.

Similarity search

A structural similarity search among small molecules is a standard tool used in molecular classification and *in silico* drug discovery. Ray *et al.* utilized a 3D similarity search in the development of a novel class of nonsteroidal GR modulators based on the RU-43044 and CP-409069 queries **(Figure 3)** [84]. The queries were analyzed with MacroModel [85] to identify low-energy conformers utilizing MMFF. FlexS was then used to flexibly superimpose a screening database on the low-energy queries [103]. The initial 718,000 screening database was previously filtered and clustered using the properties of known GR antagonists as a guide to obtain a focused library of 862. Hits, which scored the best with FlexS and did not contain undesirable features, were then clustered through a similarity search based on Tanimoto (0.85 similarity threshold) and Daylight fingerprints [202] to remove redundant compounds. In total, 18 selected compounds were tested in a GR-binding assay and the most active molecule, compound **36**, had a K_i of 4.5 μM, **(Figure 12)** was used as a 2D query for a substructure and similarity search of the full 718,000 compound database, leading to the discovery of compound **37**, displaying an activity of 33 nM in the GR-binding assay **(Figure 12)**.

The most potent compound, compound **38**, with a K_i of 16 nM in the GR-binding assay and 135 nM in a GR-functional assay **(Figure 12)**, was discovered after performing a final similarity search around compound **37**. SAR analysis around compound **38** demonstrated that bulky lipophilic substituents such as phenyl or benzyl groups are preferred at the 4-piperidyl position, while the insertion of a methyl group causes a drop in activity. Furthermore, introducing a substitution at N-1 does not lead to any improvement in activity while, at the N-3 position, it is detrimental. No benefit was observed to GR binding and functional potencies after the introduction of polar substitutions in the 5-benzyl ring. The rational similarity search approach pursued in this work led to the identification of a novel scaffold for GR, with compound **38** being the most potent.

Synthetic chemistry efforts subsequently investigated decoration around the 5-unsubstituted benzyl scaffold. It was determined that more polar groups were not tolerated, with 2- and 3-substitutions favored over 4-substitution. Similar probing of the substitution on the 5-([4-phenyl]-1-piperidyl) ring demonstrated little tolerance for 4-phenyl substitution and none of the explored substitution patterns gave improved binding or functional potency over the nonsubstituted analogue. The selectivities of key compounds against the other nuclear hormone receptors PR, MR, ER and AR were examined, with little or no activity demonstrated for ER or AR. Selectivity of more than 500-fold against PR and of more than 40-fold against MR was obtained.

In conclusion, this approach successfully led to the identification of a selective nonsteroidal GR antagonist active in both binding and functional assays.

Discussion

Until recently (with the advent of a number of novel GR crystal structures in 2008), more data has been available for LBVS approaches than for SBVS, making LBVS methods more commonly and successfully used on GR [25]. The abundance of GR actives along with their characterization on TR/TA assay and cross-reactivity studies afford a productive environment for the design of new compounds. The information derived from a set of compounds able to bind to the GR target but with different biological profiles can be exploited in building pharmacophore and SAR models. The early identification of the A- and B-ring of the steroid core as essential features for binding GR resulted in a pharmacophore-guided SAR moving in two distinct directions. In the first case, the idea was to explore the 'right hand side' of the steroid molecule (C-ring) while, in the second approach, A-ring mimetics were widely explored. Both resulted in the development of GR binders that are now under patent. However, as Regan *et al.* highlighted 'often there is a lack of correlation between GR binding affinity and trans-repression activity', making GR LBVS approaches especially challenging [65].

Pharmacophore models should embody the key features required for receptor binding. However, building a cross-chemotype (nonsteroidal) pharmacophore can be very challenging when molecules are structurally distinct from

the co-crystallized steroidal GR ligands, particularly in terms of generating the ligand alignment. No such pharmacophore model utilized in virtual high-throughput screening (vHTS) has been published to date.

Additionally, computer-aided pharmacophore and SAR studies appear to be appropriate when applied to GR lead optimization. The aforementioned approaches disregarded information regarding the binding pocket coming from x-rays while focusing on the key features involved in the ligand–protein interaction. This is because GR undergoes a complex tertiary structure rearrangement upon ligand binding, as in the case of the other NRs, which often leads to unpredictable switches from agonist to antagonist activities. Thus, an intensive SAR study should always be performed with docking studies or MD simulations, if feasible with the purpose of rationalizing the protein response to small molecular changes.

Additionally, QSAR studies on GR retrieved from the literature successfully identified a good correlation between experimental and computational data. However, to date, the QSAR method has failed to play a major role in advancing drug discovery for GR. In contrast to the other methods, Spreafico *et al.* included induced fit, a key mechanism for ligand binding explicitly in both the docking and the QSAR simulations [80].

Finally, similarity searches have had some success when starting from known GR-modulators. A target focused library search resulted in novel scaffold discovery that was optimized through further SAR studies. This is the first published LBVS vHTS to determine novel scaffolds with subsequent refinement steps resulting in more potent hits.

Challenges & future perspective

In this work, we have focused on the ligand-based computational and SAR techniques that have been applied to the discovery and optimization of novel GCs. A safer GC should have full efficacy in anti-inflammatory activity, but reduced efficacy and potency in one or more side effects. In addition to TR/TA selectivity, there are also the challenges of discovering novel GR ligands that do not possess cross-reactivity with other NRs [66]. The ideal dissociated GR is expected to bind to GR, but with reduced activity in inducing GRE-mediated transcription. So far, no dissociated GC has been identified that completely separates the anti-inflammatory activity (desirable effect) from the GRE-mediated gene activation (undesirable side effects). Molecular modeling and cheminformatics are becoming an essential component of drug discovery throughout the pharmaceutical industry and have been successfully applied in a number of studies on the GR LBD. A diverse range of computational tools, both structure- and ligand-based, have been implemented to attempt to tackle the problem of producing safer GCs.

For viable LBVS approaches, it is absolutely essential to have a good dataset of actives, while diverse structures, well-characterized potencies and a range of activities are mandatory for QSAR. Most of the reported successes in LBVS have been in the area of lead optimization rather than hit discovery. However, there have been some successes in similarity searches (LBVS vHTS) in the discovery of novel scaffolds. In 5–10 years, it is envisaged that there will be more information available regarding ligands that bind to different sites and more selectivity information available. To meet this challenge, hybrid approaches may be utilized that bridge ligand-based and receptor-based design domains such as that recently implemented by Spreafico *et al.* [80].

Despite the benefits of utilizing rational drug-design approaches, drug discovery will remain a multidisciplinary process with diverse scientific teams working together towards a common goal. Receptor-binding assays, cell-based assays and animal models are routinely used to evaluate the binding potency of compounds, the compound's TA/TR activity and *in vivo* anti-inflammatory activity and side effects. The benefit of using a rational design approach is to accelerate the process by speeding up the discovery of new chemical entities that may become new drugs. Hence, in the next 10 years, we envisage the commencement of clinical trials for dissociated nonsteroidal GR modulators.

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Executive summary

- Glucocorticoids are widely used to treat a variety of inflammatory and immune diseases such as asthma and allergy conditions. The current pharmaceutical challenge is to find compounds that preserve the immune effects of glucocorticoids without the concurrent detrimental side effects.
- Ligand-based computational approaches in drug-design efforts utilize the available information on known glucocorticoid receptor (GR)-active ligands towards the determination of novel GR modulators.
- Today's medicinal chemist can apply molecular modeling and computational techniques to drug-design problems to reduce the time and expense of the drug discovery and development process. To date, ligand-based virtual screening (LBVS) approaches have been more successful than structure-based virtual screening on the GR.
- There has been considerable success in pharmacophore-driven LBVS structure–activity relationship generation and lead optimization studies for the GR.
- A mixed-model quantitative structure–activity relationship model has been used to predict the binding mode and affinity of drugs, which was validated on an external test set ligands.
- A recent virtual high-throughput screening similarity search was successful in developing a novel series of nonsteroidal GR antagonists.
- Future trends towards integrated GR ligand design incorporating ligand- and structure-based methodologies are inevitable.

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No writing assistance was utilized in the production of this manuscript.

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