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Drug Design Approaches to Manipulate the Agonist-Antagonist Equilibrium in Steroid Receptors

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1. Introduction

The steroid hormone receptors, the Androgen Receptor (AR), Estrogen Receptors (ERα and ERβ), Glucocorticoid Receptor (GR), Mineralocorticoid Receptor and Progesterone Receptor (PR), have been crucial targets for drug discovery even before their existence was known or understood. The drugs on the market for this sub-class of the nuclear hormone receptors constitute a significant pharmacopeia for the treatment of a vast array of conditions and ailments. Despite the breadth of drugs targeted toward this family, they remain an important target for the pharmaceutical industry.

Key considerations when designing drugs for any family, beyond the on-target pharmaceutical action and safety, is to ensure specificity against related targets, exploration of the most appropriate routes of administration and desirable pharmacokinetic (PK) profiles. Developing non-steroidal modulators for the steroid receptor family has been a key strategy employed to achieve these goals, although there appears to be growing consensus that not being steroidal is insufficient to justify new drugs on its own (Hermkens et al, 2006). Unlike targeting many families, steroid hormone receptor drug discovery also has to balance the need to elicit either agonistic or antagonistic responses depending on the desired indication.

The history of drug discovery for the steroid hormone receptors has tended to follow a common path, beginning with the application of purified endogenous hormone and followed by the application of the first synthetic analogs with improved PK properties or selectivity. For some of the receptors this period was followed by the design of antagonists, including non-steroidal structures. More recently, steroid hormone drug discovery has been

dominated by the search for ligands characterized by partial agonistic or partial antagonistic responses, the so called selective modulators. It is hoped and expected that partial agonists and antagonists for the various receptors will provide improved therapeutic profiles. For example, selective GR modulators (SGRMs) could provide their anti-inflammatory action without the undesirable side-effects, including osteoporosis and diabetes, currently associated with oral glucocorticoids (Hudson, Roach, and Higuchi, 2008). Selective ER modulators (SERMs) hold the promise of being active on bone but not breast or endometrial tissue (Shelly et al, 2008;Silverman, 2010), whereas a desirable profile for a selective AR modulator (SARM) would likely have a greater action in bone and muscle compared to the prostate (Gao and Dalton, 2007).

2. Molecular basis for partial agonism

The shared domain structure of steroid receptors includes a variable N-terminal domain, a highly conserved DNA-binding domain and a moderately conserved ligand-binding domain (LBD). The LBD domain tends to be the primary target for drug-design. The LBD combines a number of functions, including hormone binding, receptor dimerization and binding to other co-modulating proteins that play a role in the control of transcription. These functions have the ability to influence each other, with ligand-binding, as an example; influencing the pattern of co-modulator recruitment. Specifically, gene activation requires the recruitment of co-modulating proteins to a region of the surface of the LBD formed by helices 3/4, 5 and 12. The position of helix-12, as we will discuss, can be influenced by the nature of the ligand bound to the receptor allowing drugs to influence the binding of co-modulators and consequently gene activation and the resulting biological effects (Bourguet, Germain, and Gronemeyer, 2000;Egea, Klaholz, and Moras, 2000;Kumar and Thompson, 1999;Weatherman, Fletterick, and Scanlan, 1999).

Understanding the molecular basis for partial agonism is hampered by the difficulty in solving the X-ray structures of steroid-receptors in general and specifically complexes including partial active ligands (Nettles et al, 2008). Full agonists stabilize the receptor, and specifically helix-12, in a conformation suited to binding co-activating proteins and full antagonists stabilize the receptor in a conformation suited to binding co-repressing proteins. The apparent reason for the difficulty in co-crystallizing partial agonists is that they do not fully stabilize the receptor in either conformation, adopting some degree of equilibrium between the two (Nettles et al, 2008;Raaijmakers, Versteegh, and Uitdehaag, 2009). This equilibrium allows partially active compounds to bind unique patterns of co-modulators compared to full agonists and antagonists, resulting in their potentially interesting biological effects. Unfortunately as a result it also renders them poorly suited to co-crystallization studies.

The degree of partial activity (how far from either a full agonist or antagonist response) will go some way to determining the profile of co-modulators which will bind. Additionally, the ratio of co-activators compared to co-repressors in each cell type will influence the biological effect of a partial compound. In cells with a high co-activator concentration we would expect partial compounds to show a greater degree of agonistic activity compared to the same ligand in a cell with a high co-repressor concentration. The limitless combination of ligand partiality and co-modulator distribution appears to be a major contributor to the tissue selective responses of partial compounds.

3. Mechanisms for ligand-induced partial agonist design

In the absence of a complete record of X-ray structures of steroid receptors bound to agonists, antagonists and partially active compounds, we have to fill in the knowledge gaps with mutation studies and ligand-based structure-activity relationships (SAR). Even with this extra information, our understanding of the mechanisms underpinning the repositioning of helix-12 and the resulting spectrum of partial responses remains relatively naive, but there do appear to be a small number of approaches available to the drug designer who wishes to rationally influence the degree of agonism elicited by their compound series.

1. Sterically impede the agonistic orientation of Helix-12
2. Disrupt the function of other indirect stabilizing interactions.
3. Influence the position of Helix-12 by modulating the end of Helix-11 and the loop between Helices 11 & 12.
4. Reduce the stabilizing interactions between the ligand and Helix-12.
5. Straighten Helix-3, and/or disrupt interactions between Helices 3 & 5.

Incorporating these approaches into the optimization of steroid receptor ligands allows the drug-designer to modulate the degree of agonistic and antagonistic response their compounds induce. Pharmacologically it remains difficult to define *a priori* the precise agonistic or antagonistic efficacy (percentage effect or intrinsic activity) required for any desired indication, but it is now possible to generate a series of ligands with tuned efficacies to cover a broad range and then utilize molecular profiling approaches to select the most desirable.

The five basic approaches for generating partially active compounds have been deduced by numerous studies from all members of the steroid receptors and nuclear receptor family in total. For the purposes of this review we present a single receptor case study to demonstrate each of the five mechanisms, but wish to stress that to a greater and lesser degree all mechanisms should be applicable to all steroid receptors.

3.1 Sterically impede the agonistic orientation of helix-12
3.1.1 Case study: the progesterone receptor

Steroidal anti-progestins are typically differentiated structurally from progestins by the presence of a bulky attachment at their position 11 (Madauss, Stewart, and Williams, 2007). Recent publications of the anti-progestin Mifepristone (Raaijmakers, Versteegh, and Uitdehaag, 2009) and the SPRM Asoprisnil (Madauss et al, 2007) clearly demonstrate that the role of this bulky attachment is to clash with helix-12 and preclude it from adopting its required agonistic position. Both studies also demonstrate an important role specifically for Met909 in the agonism/antagonism balance. Met909 sits within helix-12 at the C-terminal end of the ligand binding domain (LBD), and in the classic agonist conformation of the receptor, is oriented toward the ligand binding pocket. Met909 is typically the only helix-12 residue directly in contact with ligands. The nature of the ligand-Met909 interactions appears to be a key determinant of the receptors function (Petit-Topin et al, 2009). Clashes between Met909 and ligands are likely to destabilize helix-12 (Raaijmakers, Versteegh, and Uitdehaag, 2009), which results in a reduced agonistic response. It has even been suggested that the degree of clash with Met909 might correspond directly to the reduction in agonism (Madauss, Stewart, and Williams, 2007), but this has yet to be shown categorically.

Introducing bulky groups onto PR modulating non-steroidal scaffolds has also been demonstrated to result in partial agonists on a number of occasions (Jones et al, 2005;Kallander et al, 2010;Thompson et al, 2009;Washburn et al, 2009).

3.1.2 Additional examples

The existence of a clash between antagonists and helix-12 was first demonstrated for ER by studies comparing the X-ray structures of Estradiol to Raloxifene (Brzozowski et al, 1997) and Diethylstilbestrol to Tamoxifen (Shiau et al, 1998). Numerous reviews of these two studies have been published (Hubbard et al, 2000;Kong, Pike, and Hubbard, 2003;Mueller-Fahrnow and Egner, 1999;Pike et al, 2000;Pike, Brzozowski, and Hubbard, 2000) as have many further studies on the X-ray structures of SERMs, full antagonists and full agonists bound to the ERs (Blizzard et al, 2005;Dykstra et al, 2007;Heldring et al, 2007;Kim et al, 2004;Renaud et al, 2003;Renaud et al, 2005;Tan et al, 2005;Vajdos et al, 2007).
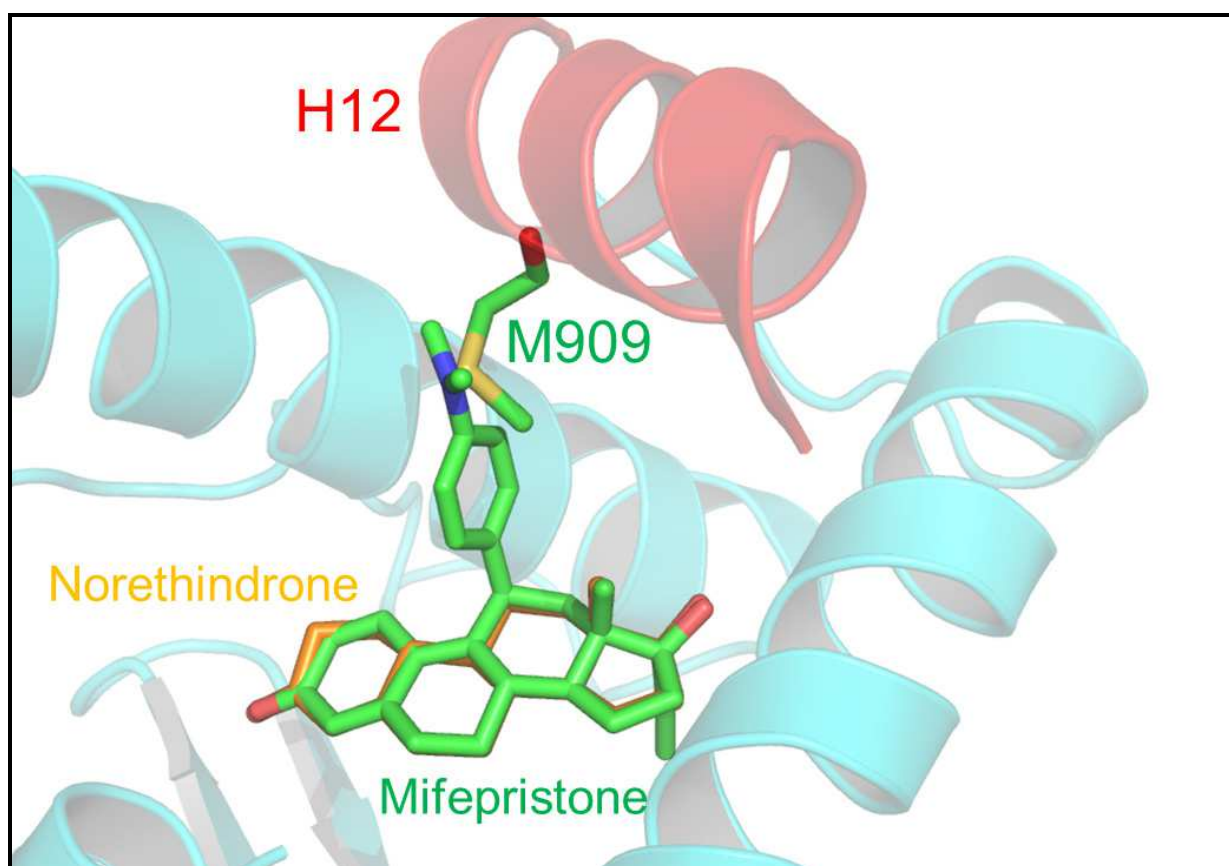

Fig. 1. Binding of PR agonist Norethindrone (orange) from X-ray structure compared to PR antagonist Mifepristone (green) demonstrating clash between antagonists and Met909 in helix-12.

The same helix-12 clash has also been demonstrated for AR (Cantin et al, 2007) and GR (Schoch et al, 2010) in recent X-ray structure determination studies. It was also suggested for GR by a mutagenesis study (Hillmann et al, 2002) that showed that mutating Leu753 (equivalent to Met909 in PR) to a phenylalanine results in a receptor defective in transactivation. We can conclude that the reason for this loss of activation is that an increase in the size of the residue at this position prevents helix-12 from adopting its agonistic conformation due to a clash with the ligand.

3.2 Disrupt the function of direct stabilizing interactions
3.2.1 Case study: the androgen receptor

The binding of testosterone and dihydrotestosterone to AR demonstrate the existence of crucial receptor stabilizing interactions mediated by agonistic ligands. As we will discuss later, the loop between helix-11 and helix-12 is a key region for mediating partial agonism. As shown in figure 2, AR is stabilized by a ligand mediated hydrogen-bond network from Thr877 in helix-11 to the 17β-OH group in the endogenous steroidal agonists to Asn705 in helix-3 and finally to the backbone of Asp890 in the loop itself (Matias et al, 2000).

Hydroxyflutamide is the active metabolite of the androgen receptor antagonist flutamide. Its antagonism appears to be a result of its inability to complete the entire network of stabilizing hydrogen-bonds (Bohl et al, 2005) also shown in figure 2. The result is that Thr877 is left buried in a predominately hydrophobic pocket, destabilizing the receptor and shifting the agonist-antagonist equilibrium.
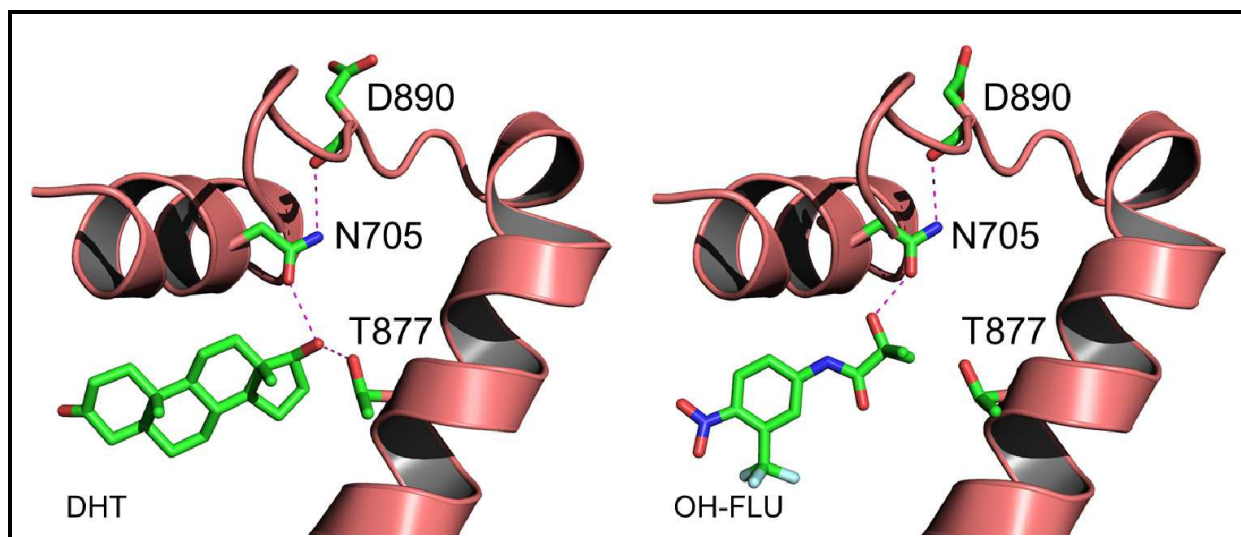

Fig. 2. Left shows the X-ray structure of DHT bound to AR including full hydrogen-bond network. Right shows a model of hydroxyflutamide bound to AR based on the X-ray structure of hydroxyflutamide bound to an AR-T877A mutant.

3.2.2 Additional examples

The residue equivalent to AR residue Asn705 in MR is Asn770. Extensive X-ray, SAR and mutation studies have been conducted on Asn770 which demonstrate clearly the existence of a ligand-mediated hydrogen bonding network which is critical for the activation of MR in a similar fashion to the one described for AR (Bledsoe et al, 2005;Hellal-Levy et al, 2000).

Agonistic steroidal ligands for GR and MR are typified by 11β-hydroxyl groups which hydrogen bond to Asn564 in GR and Asn770 in MR respectively. Despite the similarity between MR and PR, the endogenous PR agonist progesterone behaves as an antagonist of PR. This appears to at least in part be due to a lack of an 11β-hydroxyl group on progesterone. It is interesting how the lack of the hydroxyl group doesn't disturb the agonistic activity of PR but does MR.

Another important example of disrupting the function of stabilizing interactions can be seen in the estrogen receptors. In addition to their role in sterically precluding helix-12, SERM side-chains also contain an important basic amine function which is almost ubiquitous

amongst this drug class. The role of this nitrogen is to form a salt-bridge to Asp351 in helix-3 of ERα (Asp303 in ERβ). The importance of this salt-bridge is that it requires Asp351 to adopt a new conformation and prevents it from undertaking is usual function of stabilizing the agonistic position of helix-12 by hydrogen-bonding to backbone residues in the helix. It also appears that the exact nature of the interaction between the basic amine and Asp351, including angle, distance and perhaps pKa can influence the biological effect of the ligands.

3.3 Modulate the end of helix-11 & the loop between helices-11 & 12
3.3.1 Case study: the glucocorticoid receptor

Due to the difficulty in crystallizing partial agonists in complex with steroid-receptors much of the evidence to support these mechanisms has to be inferred from other indirect sources. Some of the most valuable evidence comes from mutagenesis studies including those that indicate that the loop between helix-11 and helix-12 is a hotspot that is crucial to the agonism/antagonism balance in GR.

Mutation of **Ile747**, which sits in the middle of the helix-11 to helix-12 loop, to methionine results in GR having a reduced transactivation potential without affecting the binding of classic glucocorticoids (Vottero et al, 2002). Presumably, the increased size of the residue prevents the correct packing of the loop and therefore destabilizes helix-12.

Tyr735 at the end of helix-11 is a surface residue whose role is poorly understood, but it has been shown that various mutations (W735F, W735V and W735S) result in a receptor with significant reduction in transactivation activity without affecting ligand binding (Ray et al, 1999;Stevens et al, 2003).

Thr739 is the last residue in helix-11 whose mutation to alanine has no effect on the binding of triamcinolone acetonide, but does result in a 16-fold reduction in transactivation (Lind et al, 2000).

In addition to these mutation studies, as discussed already, there is also overwhelming evidence across the family to support the hypothesis that **Asn564** is crucial for the agonistic activity of GR and related receptors (Bledsoe et al, 2005;Bledsoe, Stewart, and Pearce, 2004;Fagart et al, 1998;Hellal-Levy et al, 2000;Necela and Cidlowski, 2003;Rafestin-Oblin et al, 2002). The role of Asn564 (Asn705 in AR, Asn770 in MR) was previously discussed.

Tyr735, Thr739 and Ile747, as shown in Fig 3, are all located at the end of helix-11 or in the following loop. Asn564 has an important role in stabilizing the loop. The studies associated with each of these residues indicate how sensitive this region to influencing the agonism/antagonism balance and therefore the potential to modify its function by ligand design.

The helix-11 to helix-12 loop in steroid-receptors is well suited to drug-design intervention as it forms around the 17β group of steroids and is therefore likely to be in close proximity to most ligands.

Bledsoe and colleagues recognized the importance of this region when solving the first GR-Dexamethasone structure (Bledsoe et al, 2002;Bledsoe, Stewart, and Pearce,2004) as did the group of Kauppi when solving GR complexed with Dexamethasone and RU486, including noting the flexibility of this loop (Kauppi et al, 2003).

3.3.2 Additional examples

The importance of the loop region between helix-11 and helix-12 has also been demonstrated by X-ray structure studies for ERα (Pike et al, 1999;Shiau et al, 1998), and mutagenesis studies on MR also support the conclusion that this region of the steroid-receptors is crucial for the agonism/antagonism balance (Fagart et al, 2005).
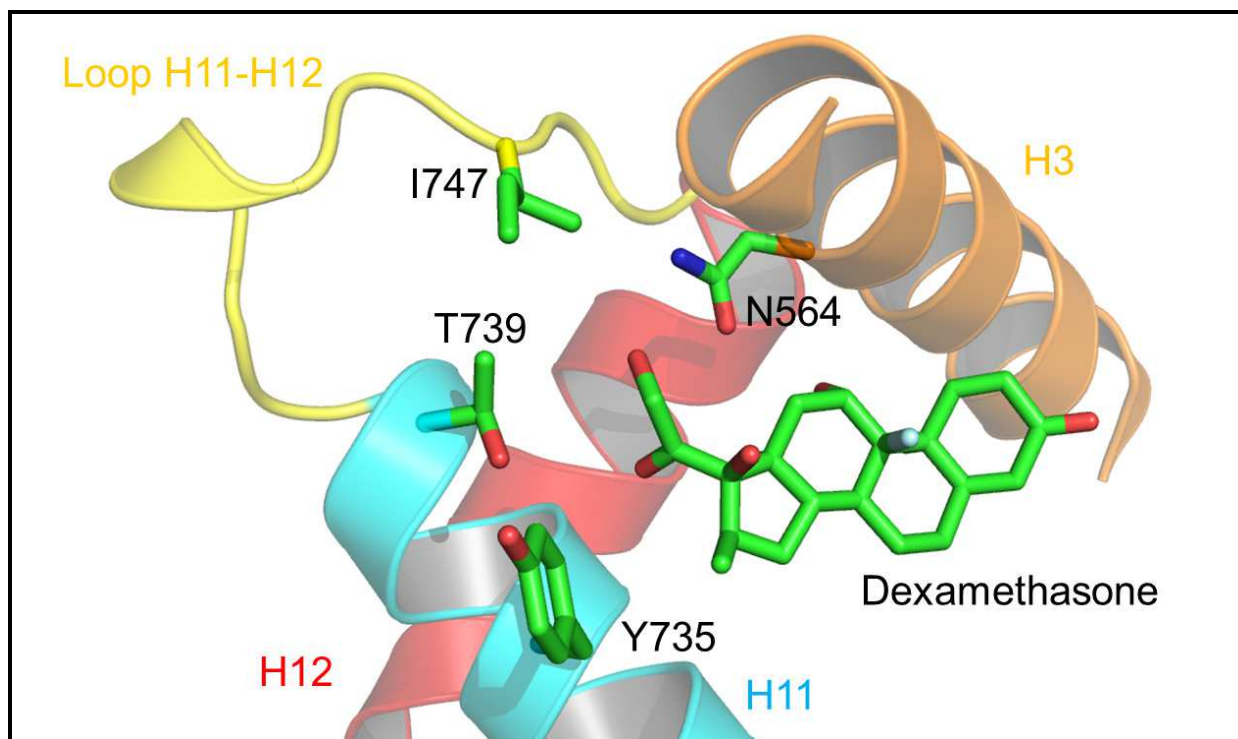
Fig. 3. The loop between helix-11 and helix-12 illustrating key residues believed to influence GR function

3.4 Reduce the stabilizing interactions between the ligand and helix-12
3.4.1 Case study: the estrogen receptors alpha & beta

Methods for antagonizing or reducing the agonism of steroid receptors that do not involve direct steric clashes with the receptor are often referred to as "passive antagonism". This term was coined by the group of Geoffrey Greene to explain their observations when studying the binding of tetrahydrochyrsene (THC) and its interactions with ERα and ERβ (Shiau et al, 2002).

THC is an ERα agonist and an ERβ antagonist. The group of Greene was able to conclude, after generating X-ray structures of both complexes that THC stabilizes ERα in its agonist conformation but ERβ is in an antagonist conformation. This difference on its own is of significant interest, but the study also demonstrated that the reason for ERβ not adopting an agonist conformation was due to missing stabilizing interactions between the receptor and the ligand. They observe that in ERβ residues Leu476 and Met479 are not positioned correctly by the ligand to form interactions with relevant residues in helix-12 to stabilize its agonist conformation. The result is a failure of THC to stabilize the agonist conformation of helix-12 and therefore a shift in the agonist-antagonist equilibrium. The fact that THC has such differing effects on two such similar receptors illustrates the challenge when following this or any of the five described approaches in drug-design.

3.5 Straighten helix-3, and/or disrupt interactions between helices-3 & 5
3.5.1 Case study: the mineralocorticoid receptor

It is generally accepted that steroid-receptor activation is facilitated by interactions between helix-3 and helix-5. The correct positioning of the basic component of the charge clamp (Lys579 in GR and Lys785 in MR) and the formation of the hydrophobic pocket in which co-

activators bind is dependent on a bend forming in the middle of helix-3. That bend in helix-3 is induced by a ligand mediated hydrogen bond to helix-5 via the 3-keto group of steroidal ligands. It was initially believed that the importance of the classic interactions between the 3-keto group of steroids and the Glutamine (Glutamate in ERα and ERβ) and Arginine residues in steroid hormones was purely to ensure potent binding of the steroids, but the work of Bledsoe (Bledsoe et al, 2005) and Huyet (Huyet et al, 2007) have demonstrated that is also has a role in the agonism-antagonism balance. Huyet *et al* demonstrated that mutation of either Gln776 or Arg817 in MR to alanine results in previously ligand-mediated agonistic responses being lost.

Bledsoe *et al* have further demonstrated the importance of this bend in helix-3 by characterizing the S810L mutation in MR. This mutation has the effect of stabilizing the agonist conformation of MR, rendering some antagonistic ligands to have an increased agonistic response. Their analysis shows that the role of the S810L mutation is to increase the hydrophobic stabilization between helix-3 and helix-5.

3.5.2 Additional example

A recent X-ray structure publication from our group suggests that PR antagonism seen in a compound series can in part be explained by a loss of these same interactions (Lusher et al, 2011).

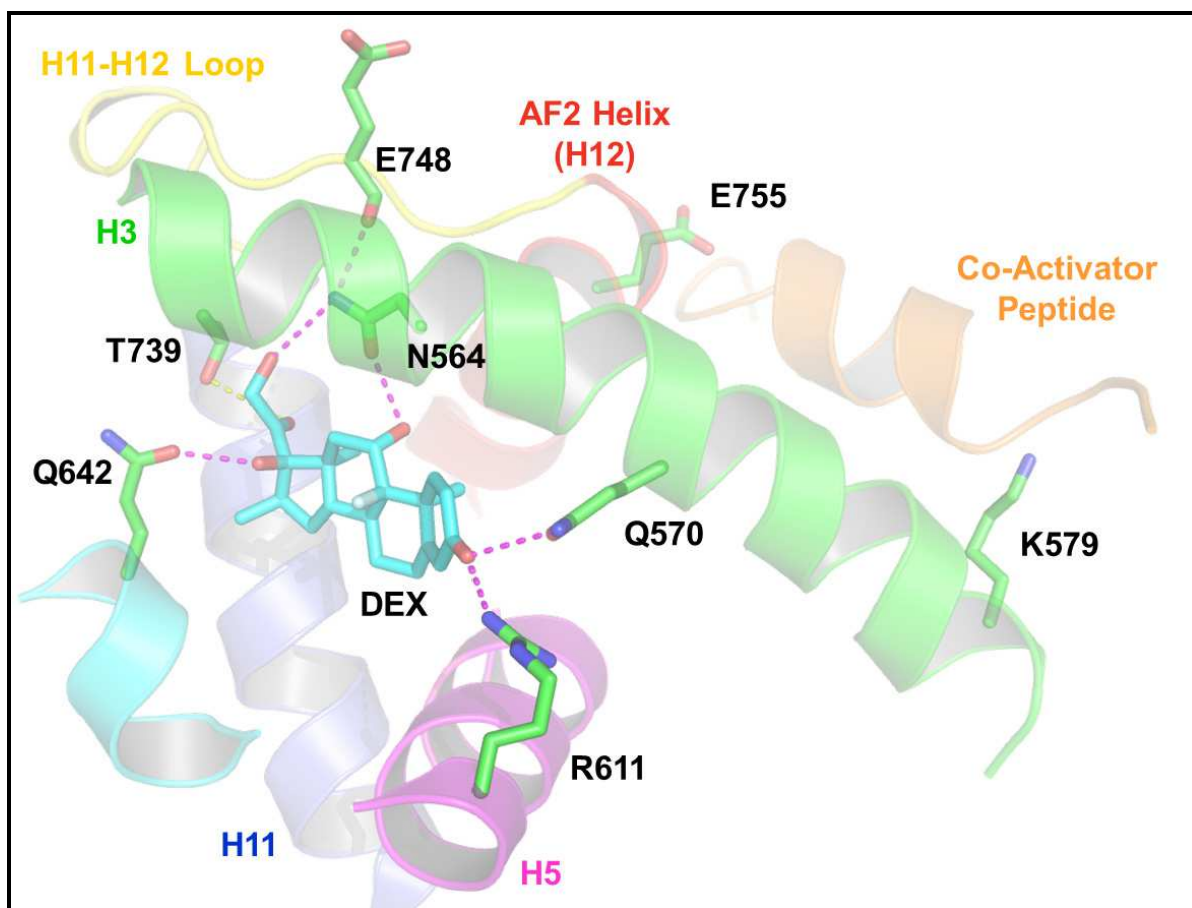## 4. Pictorial summary of five drug design approaches

Fig. 4. Binding of Dexamethasone (DEX) to GR

4.1 Binding mode of DEX to GR illustrates each of the five design approaches

The binding of Dexamethasone to the Glucocorticoid Receptor (GR) is shown to illustrate the five major routes for reducing agonist efficacy in steroid receptors via the destabilisation of the binding of co-activating proteins. Co-activating proteins bind in a hydrophobic pocket on the surface of the ligand-binding domain (LBD) stabilised by a charge-clamp formed by residues Lys579 in helix-3 and Glu755 in helix-12. [1] Direct clashes between ligands and helix-12 prevent Glu755 from adopting its necessary position and thus prevent the formation of the charge clamp. It has been shown for some receptors that clashes with helix-12 result in the helix adopting a new orientation actually precluding the binding of co-activators by binding in the required hydrophobic pocket. [2] It is probably therefore not a surprise that the positioning of helix-12 can be influenced by the residues that directly precede it. The loop before helix-12 influences its position and is clearly a hotspot that can influence degree of agonism by modifying the ligand. [3] Other interactions also help stabilise helix-12 in its agonist position. For example, in GR, there is a hydrogen-bond network from the ligand to Asn564 in helix-3 to Glu748 in the loop before helix-12. Disruption of this network, by perhaps removing the hydrogen-bonding function in the ligand, can influence the stabilisation of helix-12. [4] In a number of nuclear receptors Helix-12 also makes direct hydrophobic interactions to the ligand. Loss of these interactions, by changing the properties of the ligand, can decrease the stabilisation of helix-12 and therefore alter the agonistic capability of the complex. [5] Finally, the first four approaches are directly or indirectly related to ensuring Glu755, as half of the charge-clamp, is correctly positioned. The second residue in the charge-clamp, Lys579, should not be overlooked. Lys579 is part of helix-3 which itself bends midway along its length. This bend is crucial for ensuring that Lys579 is in the correct position to form the charge-clamp. The bend in helix-3 is partly as a result of its interaction with helix-5. For GR this is largely mediated by a hydrogen-bond network between Gln570 in helix-3, the ligand and Arg611 in helix-5. Disrupting this network by modifying the ligand may influence the distortion in helix-3 and therefore the correct formation of the charge-clamp and therefore co-activator binding.

5. Other structure-based design considerations

In addition to exploring the development of partial agonists, structure-based approaches continue to play an important role in the identification of new ligands via virtual screening approaches and other compound optimization tasks. An important lesson in this regard has been our change in understanding the dynamic nature of the steroid-receptor binding pocket. We have seen examples of extensive induced fits for amongst others the glucocorticoid receptor which is able to bind ligands beyond the conventional confines of its binding pocket whilst remaining in an agonistic conformation (Biggadike et al, 2009;Madauss et al, 2008;Suino-Powell et al, 2008). The pocket, behind the crucial helix-3 and helix-5 binding residues, Gln570 and Arg611, is normally water filled. It has already been demonstrated to be a viable ligand-binding region with the potential to improve ligand potency. An interesting note regarding the exploration of the pocket is that GSK report difficulty in combining the use of this pocket with the maintenance of partial agonism (Biggadike et al, 2009). PR has been shown to adapt to steroids baring bulky 17α groups (Madauss et al, 2004) and Trp741 in AR adapts to different ligands, adopting a new position to open an additional channel in the receptor (Bohl et al, 2005).

6. Conclusion

As we look to the future of rational and structure-based drug design for the steroid receptors there remain key areas and questions that will dominate research in the short to medium term:

1. Is each of the five described methods for generating partial compounds equally applicable for each of the receptors? It is generally considered true that ERβ is easier to antagonize than ERα. This is most likely due to the agonist conformation of ERβ being less intrinsically stable than ERα and therefore ensuring that ERβ is more sensitive than ERα in this respect (Pike et al, 1999).

2. Does the choice of the mechanism for instilling partiality affect the eventual biological activity? Does a compound with a 40% reduction in agonistic activity due to a clash with helix-12 have the same biological effect as a compound with a 40% reduction in agonism due to the loss of other stabilizing interactions?

3. As described earlier, partial agonists and antagonists are often poor candidates for co-crystallization Recently we have seen the first publications describing methods to circumvent this problem, either by introducing stabilizing mutations into the receptor (Bohl et al, 2007;Fagart et al, 2005;Nettles et al, 2008;Sack et al, 2001) or by generating stable crystals of the receptor using a receptor stabilizing ligand and then exchanging this compound with other compounds of interest via soaking (Raaijmakers, Versteegh, and Uitdehaag, 2009). Both approaches have the potential to dramatically increase our understanding of the biological mechanisms underpinning partial agonism.

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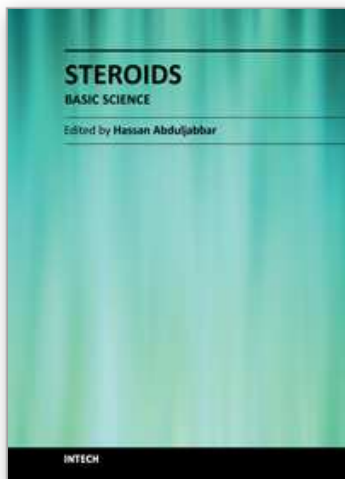
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This book explains the basic science of steroids and is targeted towards professionals engaged in health services. It should be noted that medical science evolves rapidly and some information like the understanding of steroids and their therapeutic use may change with new concepts quickly. Steroids are either naturally occurring or synthetic fat-soluble organic compounds. They are found in plants, animals, and fungi. They mediate a very diverse set of biological responses. The most widespread steroid in the body is cholesterol, an essential component of cell membranes, and the starting point for the synthesis of other steroids. Since the science of steroids has an enormous scope, we decided to put the clinical aspects of steroids in a different book titled "Steroids-Clinical Aspects". The two books complete each other. We hope that the reader will gain valuable information from both books and enrich their knowledge about this fascinating topic.

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