21-hydroxy-6,19-epoxyprogesterone: a Promising Therapeutic Agent and a Molecular Tool for Deciphering Glucocorticoid Action

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Abstract: Glucocorticoids are steroid hormones that exert most of their effects through their binding to the glucocorticoid receptor (GR), a ligand regulated transcription factor. Although glucocorticoids are widely used in the clinic, their usage in chronic therapies provokes severe adverse reactions. In the quest for safer glucocorticoids a dissociated model was established that proposes a disconnection between GR activated pathways responsible of desired pharmacological effects and pathways involved in adverse GR reactions. Under this model, a myriad of steroidal and non-steroidal compounds has been characterized, with most of them still producing side effects. X-ray crystallographic studies followed by molecular dynamics analysis led research to insights on the receptor Ligand Binding Domain (LBD), which undergoes specific ligand dependent conformational changes that influence receptor activities. In this sense, the flexibility of the ligand structure would contribute to the final GR outcome. Here, we review different data of 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP), a rigid steroid with potential pharmaceutical interest due to its anti-inflammatory and immunosuppressive activities, lacking several GR adverse reactions. The rigid structure endows this compound with an enhanced selectivity towards GR. Molecular characterization of the GR/21OH-6,19OP complex revealed specific intermediate conformations adopted by the receptor that would explain the influence on GR dimerization and the recruitment of a specific set of GR transcription modulators. We summarize recent data that will contribute to understand the complexity of glucocorticoid response.

Keywords: 21-hydroxy-6,19-epoxyprogesterone; Dissociated Ligands; Glucocorticoid Mechanism of Action; Glucocorticoid Receptor; Molecular Dynamics; Rigid Steroids.

INTRODUCTION

Glucocorticoids (GCs) are essential substances for survival. These steroid hormones exert diverse effects on virtually all tissues and organs mainly through their binding to the Glucocorticoid Receptor (GR), a member of the nuclear receptor superfamily [1]. When GCs levels are low (non-stress concentrations), they mainly regulate the expression of enzymes involved in metabolism. On the other hand, at high or stress concentrations of GCs, a network of anti-inflammatory and stress coping mechanisms becomes activated [2]. This last aspect of GC action is the main reason for their use in the clinic [3].

From a pharmacological perspective, synthetic GCs such as dexamethasone (Dex) (Figure 1a) are among the most prescribed drugs worldwide. They are used in almost all medical specialties for systemic therapies, as well as topical treatments and represent the standard therapy for reducing inflammation and immune activation in asthma, allergies, rheumatoid arthritis, inflammatory bowel, collagen, vascular, dermatological and other systemic diseases [4]. Unfortunately, cumulative doses of GCs are linked to severe side effects such as osteoporosis [5], diabetes mellitus, psychoses and mood disorders, atrophy of skin and impairment of wound healing, hypertension, atrophy of muscle, disorder of fat metabolism and redistribution of adipose tissue, glaucoma and cataracts, suppression of the HPA axis and increased susceptibility to infection [6]. In this sense, the holy grail of the pharmaceutical industry in the glucocorticoid field is to find dissociated ligands capable of uncoupling the desired anti-inflammatory effects from the metabolic side effects [7]. To this date, there is no synthetic glucocorticoid that can be used chronically without presenting severe adverse reactions [8, 9]. On the other side of the spectrum, antiglucocorticoids can also be used in the clinic to treat excessive endogenous GCs production as that occurring in Cushing syndrome, GC induced hypertension or depression and anxiety, among others [7, 10-12]. Given the fact that steroid hormone receptors (in particular androgen,

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mineralocorticoid and progesterone receptors) share a significant sequence identity, the design of specific drugs able to selectively interact with only one of the steroid receptors has proved quite challenging [13]. For example, the antihormone mifepristone (RU486, Figure 1a) is an effective antiglucocorticoid but also an antiprogestin licensed for use in medical abortion over 30 countries [14].

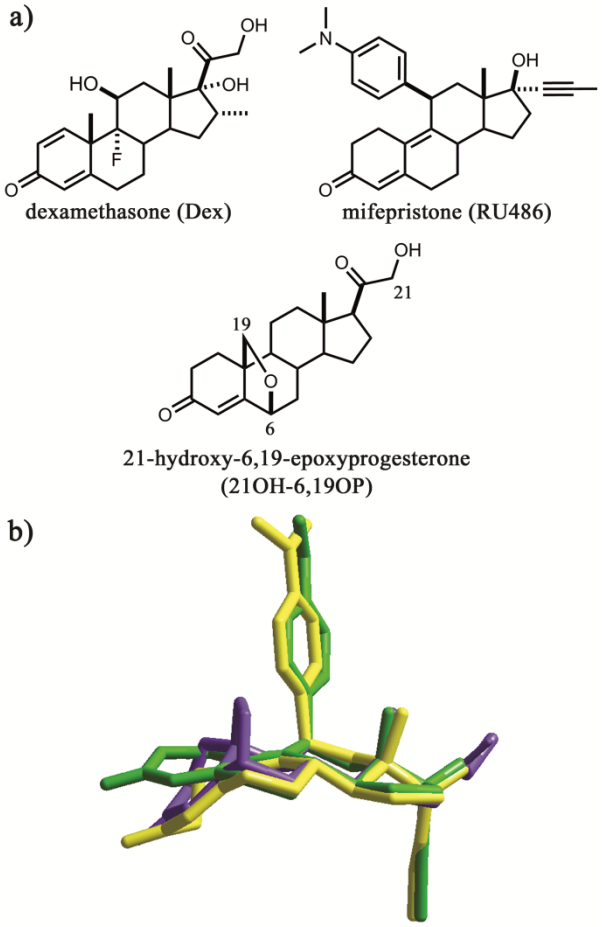

Figure 1. a) Structures of the synthetic glucocorticoids; b) Superposition of HF/6-31G** optimized structure of 21OH-6,19OP (violet) with RU486 as bound to GR (yellow, pdb:1NHZ) and with RU486 as bound to PR (green, pdb:2W8Y).

The GR is a ligand regulated transcription factor. In the absence of ligand it remains primarily cytoplasmic but upon hormone binding, a conformational change in the receptor triggers the almost complete translocation to the nucleus, where it produces most of its biological functions [15]. A direct mode of action (often and inaccurately referred as transactivation) involves the dynamic binding of GR homodimer to specific DNA elements named Glucocorticoid Response Elements (GREs) [16]. Only a fraction of all possible GREs are bound by GR in a specific tissue or cellular context. This binding selectivity appears to be coded by the chromatin structure landscape; where pre-programmed or pre-open chromatin allows GR accessibility to certain GREs [17, 18]. Nevertheless, in a fraction of binding events the GR has also the ability to open closed-chromatin and bind to the so-called *de novo* sites [17]. DNA itself appears to allosterically change the conformation of the receptor allowing interaction with a variety of co-regulators that will eventually enhance or repress gene expression of target genes [19, 20]. The GR also regulates gene transcription by an indirect mechanism (traditionally named transrepression), which involves protein-protein interaction with other transcription factors such us AP-1, STATs or NFκB [21]. In these cases, activated GR controls gene expression by modulating the transcriptional activities of those transcription factors, without direct binding to DNA.

During the search for safer glucocorticoids, a dissociated model of GR action was established. Under this paradigm, suppression of inflammation by GR is mainly mediated by the transrepression mechanism, and therefore is independent of GR transcriptional regulation through its direct binding to DNA. Accordingly, side effects of GCs were suggested to be dependent on GR-GRE interaction and the downstream consequence on gene regulation [22]. This dissociated model owns its genesis to the functional characterization of a single point mutation (A458T in humans, A465T in mice) in the GR, commonly known as GRdim [23]. The GRdim mice became a relevant and powerful physiological tool to characterize and dissect the GR effects and adverse reactions [24] . At its core, the rationale for the search of safer GCs goes as follows. First, a mutation that abrogates GR dimerization and GR binding to DNA -exemplified by the GRdim- eliminates the capacity of GR to transactivate but not to transrepress [25]. Second, the GRdim fully supports anti-inflammatory responses to GCs *in vivo* but does not mediate GC-induced side effects [26]. Hence, GR ligands that preferentially induce the transrepression rather than the transactivation pathway should retain the anti-inflammatory effects of GCs, but will lack the undesired adverse reactions. This model has been the guiding principle in the search for new compounds with dissociated glucocorticoid properties [23].

Twenty years have passed since the first characterization of the GRdim mutant but until recently no experiment was ever performed to evaluate its oligomeric state [25, 26]. Although still deeply used as a molecular tool in the community, it has been recently reported that this mutant – contrary to what it was believed- fully dimerizes *in vivo* [24, 27, 28]. Moreover, the GRdim binds to DNA and it is even able to transactivate at least some specific genes [19, 20, 27, 29-31]. No wonder that today this binary characterization of GR action (transactivation=side effects; transrepression=beneficial effects) is deeply criticized [6, 23, 32]. Nowadays, the GR response is starting to be visualized in a more analogic way, where unfortunately there is no clear cut between adverse effects and beneficial pharmaceutical action. In this sense, as the excellent review by Clark and Belvisi affirmed so elegantly [23]: "From an evolutionary perspective, it seems unlikely that we should discover a straightforward division along mechanistic lines between one action of GR that is therapeutically desirable and others that are less helpful to the patient and physician". Hence, the dissociated model of GR action needs major revisions and probably it is time to change the way we are searching for safer glucocorticoids.

From a structural perspective, the GR is organized into three major domains: a poorly conserved N- terminal ligand-

independent activation function-1 domain (AF-1), a highly conserved central DNA-binding domain (DBD) involved in GRE recognition -plus a dimerization region-, and a C-terminal ligand-binding domain (LBD) [33, 34]. The LBD contains 11 α–helices and four small β–strands that fold into ten α-helices that in turn fold into a globular structure, described as a net enclosing a central hydrophobic ligand-binding pocket (LBP). The LBP allows the binding of ligands with quite different geometries [35-40]; thus, bulky groups located in different positions of the steroid fit into the LBD without affecting its global conformation. In addition to the LBP, the LBD also contains a dimerization interface and a hydrophobic domain (AF-2), involved in the interaction with certain cofactors [40]. In this way, ligand structure may influence GR conformational states that would regulate its oligomerization state and/or modulate the recruitment of either coactivators or corepressors. Hence, the understanding of how specific ligands influence the GR-LBD conformation could be a key start-point in the rational design of new selective glucocorticoids.

In a previous work we have reviewed the biological activity of 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP, Figure 1a), a rigid steroid developed by our group, that behaves as a selective GR ligand with no affinity for mineralocorticoid and progesterone receptors [41]. Due to promising results obtained recently using this compound in several *in vitro* and *in vivo* biological assays, here we will update and summarize the extensive information accumulated to date related to this compound. Moreover, we will revise these results on the basis of the molecular mechanism involved in 21OH-6,19OP activity, which were investigated using molecular modeling techniques, biophysical tools and biochemical studies. Remarkably, this molecule not only appears promising as a therapeutic agent but also as a molecular tool to decipher the complexity of glucocorticoid action.

THE 21OH-6,19OP MOLECULE

The 21OH-6,19OP molecule is a progesterone analogue with a hydroxyl group at C-21 and an intramolecular bridge connecting C-6 and C-19 atoms [42]. Both X-ray and *ab initio* optimized structures demonstrated that the presence of the 6,19-epoxy bridge generates a rigid molecule with a pronounced bending of the steroid skeleton at the A/B ring junction, where the A ring is heavily torsioned towards the alpha face. This results in a global conformation very different to that adopted by classical glucocorticoids such as Dex. Remarkably, according to the crystal structure of the GR LBD/RU486 complex (pdb:1NHZ) [36], the antiglucocorticoid RU486 also presents the ring A torsioned towards the alpha face. In fact, the superposition of 21OH-6,19OP and this RU486 structure shows a very good overall coincidence in steroidal skeletons (Figure 1b). However, while the rigid structure of 21OH-6,19OP locks the conformation of the ring A, RU486 is a flexible molecule whose conformation depends on the environment. This is evidenced, for example, in the crystal structure of the PR LBD/RU486 complex, where the ligand exhibits a flat overall structure (pdb:2W8Y) (Figure 1b). Therefore, the GR selectivity of 21OH-6,19OP could be explained, at least in part, by its rigid torsioned structure.

Although both 21OH-6,19OP and RU486 behave as antagonists on the direct mode of GR action (transcription regulated by the GR bound to GREs) [40], their molecular basis of action might not be the same. This is because the antagonist action of RU486 has been specifically assigned to the bulky dimethylaniline substituent at C11. Crystal structures of the GR LBD/RU486 complex clearly show that the presence of the side chain in this ligand alters the AF-2 domain of the GR LBD, physically preventing helix 12 (H12) to adopt the characteristic agonist position that allows coactivators recruitment [43]. Thus, the impediment of the GR/coactivator complex assembly would result in the inability of the receptor to initiate the transcription of those target genes regulated by GREs. This type of GR inhibition, common among NRs antagonists, is usually dubbed active antagonism. Notably, lacking the bulky substituent at this steroidal position, the passive antagonism of 21OH-6,19OP might be explained by a different molecular basis of action. In the cue of those molecular determinants, molecular modeling methods were used to predict the influence of this ligand on GR LBD conformational changes. As will be shown in the following sections, the simulations not only generated a hypothesis to explain the passive antagonism of 21OH-6,19OP but also opened the door to get some insights on GR activity modulation.

MOLECULAR MODELING. CONFORMATIONAL DYNAMICS OF THE GR/21OH-6,19OP COMPLEX

Molecular Dynamics (MD) simulation, a powerful computational technique widely used to study the dynamical behavior of proteins, was applied with the purpose of obtaining structural information on the GR LBD/21OH-6,19OP complex. Describing the receptor-ligand system by using a classical force field, the MD yields the temporal evolution of each atomic coordinate from a determined initial structure. MD was used at several opportunities to study nuclear receptors behavior at the nanosecond scale, obtaining valuable information on their molecular basis of action [44-46]. Taking into account that the available crystal structure of the GR LBD/Dex complex (pdb:1m2z) includes a peptide corresponding to the LxxLL motif of the Nuclear Receptor Coactivator 2 (NCoA2) also known as Transcription Interacting Factor 2 (TIF2) [35] and knowing that 21OH-6,19OP induces GR translocation into the nucleus [47], this GR LBD/Dex structure was considered appropriate as a starting point for the MD simulations. Thus, MD studies were performed on two different systems: the agonist GR LBD/Dex and the antagonist GR LBD/21OH-6,19OP complexes. Auspiciously, comparison of 6 ns-MD between both systems showed significant alterations in the dynamics

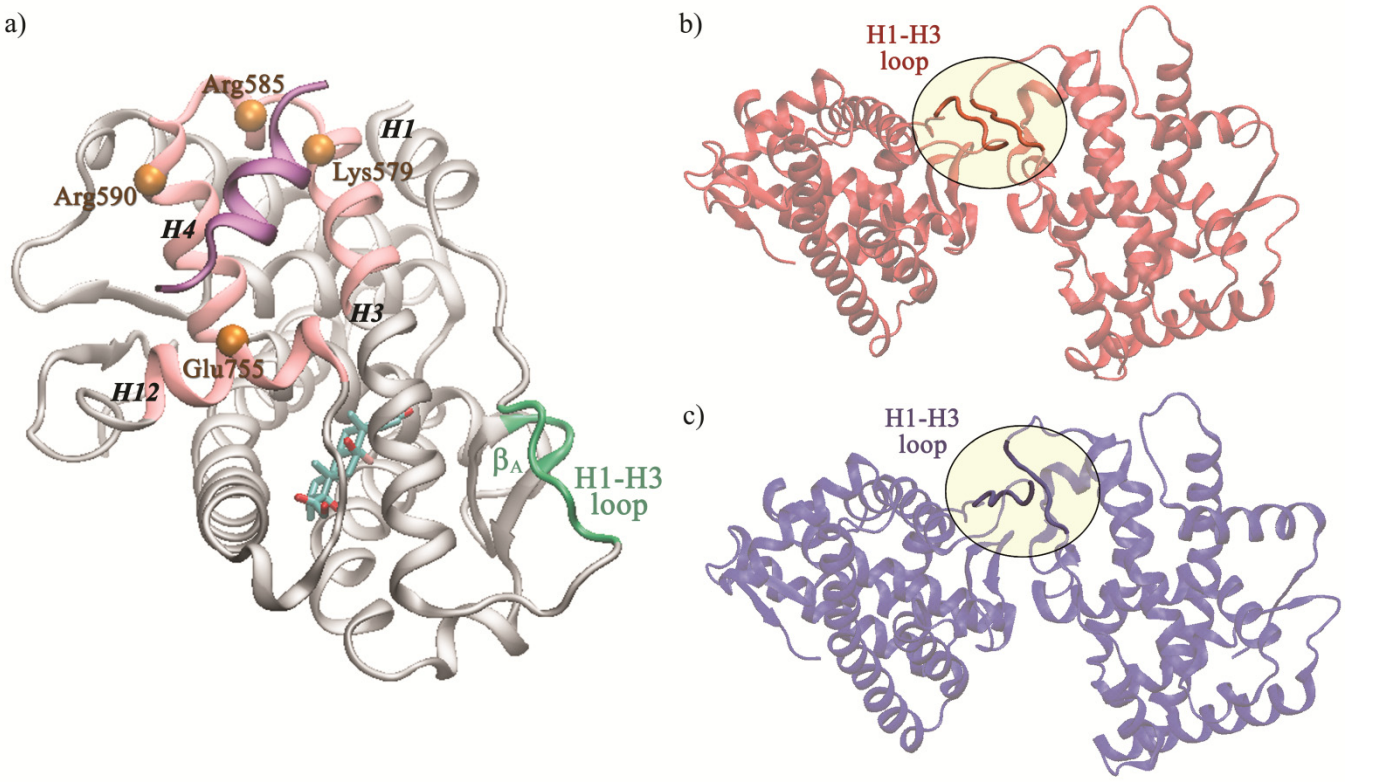

Figure 2. a) The 3D structure of the GR LBD/Dex complex (pdb:1M2Z) where the two fundamental regions to receptor function are highlighted: the dimerization interface in green (formed by residues of the βA strands and the H1-H3 loop) and the AF-2 domain involved in the coactivator recognition in pink (formed by residues of H3, H4 and H12 helices). Cα atoms of polar receptor residues (Glu755, Lys579, Asp590 and Arg585) participating in the charge clamp interaction with the coactivator TIF-2 peptide (in violet) are shown as orange balls. b) Schematic view of the plausible GR LBD/Dex dimer and c) the lost interaction in a fictional GR LBD/21OH-6,19OP dimer highlighting the relative disposition of H1-H3 loops. These representations were obtained by superposition of two average MD structure of each system on each crystal monomer of the GR LBD/Dex complex (pdb:1M2Z).

behavior of two fundamental regions: the H1-H3 loop and the AF-2 domain, which are involved in the GR dimerization and coactivator recruitment, respectively (Figure 2a) [40].

First, the last residues belonging to the H1-H3 loop presented an important difference in their fluctuation pattern [47]. Thus 21OH-6,19OP substantially diminished the mobility of these receptor residues, generating a conformation of the H1-H3 loop quite more rigid than that observed in the GR LBD/Dex complex. Additionally, in the GR LBD/21OH-6,19OP system the H1-H3 loop was heavily shifted from its original position and placed further away from the rest of the protein compared to the GR LBD/Dex loop [47]. Since the crystal structure of the GR LBD/Dex dimer indicates that the H1-H3 loop forms part of the dimerization interface [35], the observed conformational changes could compromise the GR dimerization process, explaining in this sense, the incapacity of the GR/21OH-6,19OP complex to activate GRE promoters/enhancers. If two MD average structures of GR LBD/Dex or GR LBD/21OH-6,19OP were superposed on each crystal monomer in order to form a dimer with the simulated monomers, an estimation may be obtained of how the global geometry of the dimerization interface is affected in each case. In the case of the GR LBD/Dex system, the antiparallel arrangement of the H1-H3 loops would allow an adequate interaction between monomers through the formation of a hydrogen bond network involving residues 547–551 of each monomer (Figure 2b). In contrast, the conformational changes of the GR LBD/21OH-6,19OP system produce a perpendicular arrangement of H1-H3 loops, which would prevent the formation of this fundamental inter-monomer interaction (Figure 2c). According to these observations, the antagonist activity of 21OH-6,19OP would reside, at least in part, in the inability of its GR LBD complex to homodimerize. One interesting result that supports this hypothesis was obtained with MD simulations of the GR LBD/21HS-6,19OP, a hemisuccinate derivative of 21OH-6,19OP that behaves as a GR agonist in the direct GRE mode of action [47]. Notably, the binding of this compound, which only differs in the C-21 moiety, reversed the modification of the H1-H3 loop observed in the GR LBD/21OH-6,19OP antagonist system, resulting in an average conformation very similar to that adopted by the loop in the GR LBD/Dex complex.

Regarding the AF-2 domain, less pronounced but still significant modifications were observed in the GR LBD/21OH-6,19OP complex. On one hand, the H12, main determinant of AF-2 conformation, becomes longer by five residues compared to the GR LBD/Dex complex [47]. On the other, the relative position among the four GR LBD residues that participate of the polar interaction with coactivators (termed "charge clamp" residues, Figure 2a) resulted slightly different between both simulated systems [47]. Since these residues determine the accessible opening dimension of the AF-2 groove, a deviation in their position could then influence GR's ability to recruit a coactivator. In fact, results from the LBD/ligand/TIF2 complexes support this hypothesis, since they clearly showed that at variance with the GR LBD/Dex system, hydrogen bonds between Glu755 (a "charge clamp" residue located at H12) and the TIF2 peptide were rapidly lost in the GR LBD/21OH-6,19OP complex. In this sense, a stable GR LDB-TIF2 interaction could not be established when 21OH-6,19OP was bound to the receptor [48].

In summary, the MD simulations suggested that two non-mutually exclusive possibilities would explain the inability of the GR/21OH-6,19OP complex to regulate the expression of target genes throughout the direct activation mechanism: impaired GR LBD dimerization and/or compromised coactivator recruitment.

BIOPHYSICAL STUDIES. NUCLEAR BEHAVIOR OF THE GR/21OH-6,19OP COMPLEX

The characterization of the quaternary structure of the GR is a matter of continuous controversy. In the absence of ligand GR forms a heterocomplex with different proteins like Hsp90, Hsp70 and immunophilins, among others [15, 49]. It is believed that the receptor remains monomeric in this heterocomplex; however GR overexpression may also induce ligand independent cytoplasmic dimerization [50]. The accepted model of GR activation states that after ligand binding, an undetermined equilibrium between monomers and homodimers exists in the nucleus, and while the dimeric GR would be responsible for the direct activation mechanism, the monomeric GR would be exclusively involved in the indirect transrepression one [2, 4, 7, 8, 51]. Since this model has been mostly based on the GRdim paradigm, which is currently profoundly revised, a clearly distinction between monomeric and dimeric activities has not still been properly established [27, 52]. In fact, GR appears to be completely dimeric inside the nuclear compartment when it is bound to either Dex or the natural ligand corticosterone, suggesting that both the direct GRE dependent- and the indirect pathways can be performed by the dimeric GR [52]. Moreover, which protein domains are involved in GR dimerization is also a matter of intense debate. The unfounded notion that the GRdim was exclusively monomeric led most of the community to believe that the DBD was the major contributor to the receptor's dimerization [53, 54], even though there is compelling evidence that the LBD participates as well in this process [35, 52, 55].

In this context, 21OH-6,19OP played a highly relevant role to gain insights on the domains involved in GR dimerization [52]. The Number and Brightness (N&B) assay is a fluorescence fluctuation spectroscopy technique that allows the determination of the oligomerization state of fluorescent particles with high spatial resolution [56]. The *in vivo* quantification of GR's oligomeric state performed by the N&B assay showed that most –if not all- of GR/21OH-6,19OP complexes remain dimeric in the nucleus [48]. In this way, 21OH-6,19OP is still able to induce dimerization of the wild type receptor to a similar extent as Dex. Although at first sight this experimental result may seem contradictory with MD results that predicted an impaired ability of the GR-LBD/21OH-6,19OP complex to dimerize [47, 48], since the DBD participates in receptor dimerization as well, this domain might be responsible for the observed GR/21OH-6,19OP dimerization. The latter explanation was further supported by the fact that mostly monomeric GR/21OH-6,19OP complexes were observed with the GRdim mutant that has an impaired DBD dimerization domain [52]. Thus, the incapacity of 21OH-6,19OP to induce the dimerization of the GRdim mutant not only validates the MD simulation predictions but also implies that GR dimerization depends on both DBD and LBD domains (Figure 3). This very important conclusion was confirmed by the fact that simultaneous point mutations in both DBD and LBD dimerization interfaces (A465T/I634A mutant) are necessary to completely abrogate GR dimerization [52]. Thus, even though 21OH-6,19OP does not change the dimerization state of wild type GR it is reasonable to speculate that the conformational changes in the H1-H3 loop provoked by this compound may generate impaired GR dimers, not fully able to undergo the downstream direct GREs mediated activation pathway.

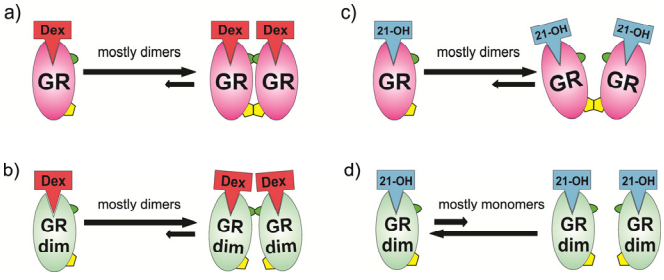

Figure 3. Schematic representation of monomer-dimer equilibrium for the GRwt (a and b) and the GRdim mutant (c and d) bound to Dex (a and c) or 21OH-6,19OP (21-OH) (b and d). The DBD and LBD homodimerization interfaces are represented by the yellow and green connectors, respectively.

GR ability to induce transcription may also depend, at least in part, on the ligand-induced interaction with transcription cofactors [57]. As mentioned above, the MD simulation predicts conformational changes at the AF-2 domain in the GR LBD/21OH-6,19OP complex that could weaken the GR LBD-TIF2 interaction [48]. Co-immunoprecipitation studies performed between GR and TIF2 confirmed that the

GR/21OH-6,19OP complex failed to recruit the TIF2 cofactor [48]. The above results taken together lead to the conclusion that 21OH-6,19OP induces a transactivation-deficient GR dimer hampered in its ability to recruit TIF2 and probably other transcription coactivators.

BIOLOGICAL ACTIVITY. POTENTIAL PHARMACOLOGICAL USE OF 21OH-6,19OP

The glucocorticoid response is not easy to define, and simplicity can be deceiving. Although therapeutic GR activity modulation has been occasionally achieved through a judicious combination of sub-optimal doses of synthetic glucocorticoids with antiglucocorticoids, the actual goal in pharmacology is focused on the development of new drugs with potential specificity for each glucocorticoid effect.

21OH-6,19OP has demonstrated to be a highly selective GR ligand devoid of mineralocorticoid and progestational activities [42]. As was mentioned above, the structural rigidity of this compound would be responsible for the efficient displacement of [^{3}H]corticosterone from glucocorticoid receptors and for the lack of competition with either [^{3}H]progesterone from progesterone receptors (PR) or [^{3}H]aldosterone from mineralocorticoid receptors [42]. This GR selectivity was not observed in RU486 that contains a flexible steroidal skeleton.

Complementary to the above observations are the gene expression reporter assays performed to evaluate the reporter MMTV-luciferase induction. 21OH-6,19OP blocked Dex mediated luciferase induction in a concentration-dependent manner in Cos-1 cells transfected with the human GR construct [47], while it was unable to inhibit MMTV activation triggered by progestins in cells transfected with the human PR (unpublished result).

The specific antiglucocorticoid properties of 21OH-6,19OP without PR effects were also observed in pregnant mice; at variance with RU486, 21OH-6,19OP showed no abortive effects when injected in pregnant mice [58]. Moreover, in chorion and placental trophoblast cells, the co-incubation of the rigid steroid with Dex but not with progesterone reversed glucocorticoid expression of prostaglandin dehydrogenase [59]. 21OH-6,19OP was also used as a potent and selective GR antagonist to demonstrate the minimum role that plays the GR in the progesterone dependent reduction of the myogenic tone of the uterine artery during pregnancy [60]. Taking together these results support the specificity of this compound towards the GR.

The involvement of GR in the effects of medroxyprogesterone acetate (MPA) -a PR agonist usually used in hormone replacement therapy and as a contraceptive- was also demonstrated by co-incubating breast tumor cells with MPA and 21OH-6,19OP [61]. These authors showed that MPA acting by way of the GR endows tumor cells with an enhanced capacity to affect immunosurveillance. Interestingly, contrary to Dex, 21OH-6,19-OP was unable to induce *in vivo* chemoresistance in mammary tumor cells treated with the antineoplastic drug paclitaxel [62]. In this sense, the use of 21OH-6,19OP would have potential implications in the control of breast cancer incidence.

The selective GR antagonism of 21OH-6,19OP on Dex-dependent MMTV promoter activation was observed not only in Cos-1 cells overexpressing GR but also in other cell lines expressing high amounts of endogenous receptor, as the fibroblast L929 cells [47] and the baby hamster kidney (BHK) cells [41]. In all cases, 21OH-6,19OP attained a maximum inhibitory effect at a 10μM concentration. When cells were treated with 21OH-6,19OP alone, no activation of MMTV was observed [47]. Furthermore, 21OH-6,19OP *per se* was also unable to induce the expression of tyrosine aminotransferase (TAT) [42], a gene regulated throughout a direct binding of the GR to specific GREs [63]; whereas its co-incubation with corticosterone showed 80% inhibition of TAT expression [42]. The lack of certain metabolic effects of 21OH-6,19OP, were also reflected through its incapacity to increase glycogen deposits in rat liver [42]. These results are consistent with the proposed inability of the GR/21OH-6,19OP complex to acquire an active dimer conformation or to recruit certain specific coactivators, impairing the expression of genes driven by GREs.

Glucocorticoids control apoptosis and cell survival in a tissue-specific manner. They induce cell death in monocytes, macrophages, and T lymphocytes [64], whereas they protect against apoptotic signals in mammary epithelial cells [65, 66], endometrium [67], ovarian follicle [68], hepatocytes [69] and fibroblasts [70]. One of the key genes mediating glucocorticoid effects is bcl-X, a member of the Bcl-2 family, which plays a critical role in the regulation of cell death. Up- and down-regulation of the antiapoptotic isoform bcl-XL, respectively correlates with apoptosis prevention of epithelial cells [67, 71, 72] and apoptosis induction of lymphocytes [72, 73]. When the apoptotic activity of 21OH-6,19OP was evaluated in thymocytes, this compound behaved as a strong antiglucocorticoid, blocking the Dex mediated cell death induction [47]. In parallel, this steroid antagonized Dex protection on cell death in tumor mammary cells treated with paclitaxel [62]. Of note, 21OH-6,19OP was unable to modulate bcl-XL expression [62]. Therefore, since most of the apoptotic effects of glucocorticoids mainly encompass the direct GR mode of action, the antagonism of 21OH-6,19OP would also be explained by the GR/21OH-6,19OP nuclear behavior.

Inhibitions of inflammation and immunosuppressant activities are the main desired responses to GCs from a pharmacological point of view. These effects mostly involve several simultaneous pathways triggered by the activated GR, which include the tethering of transcription factors (such as NFκB and AP-1); the down-regulation of p38 activity through the induction of MKP-1 phosphatase [74] and the induction of anti-inflammatory factors involved in controlling both gene expression [75] and mRNA stability [76]. However, the precise contribution of the direct and indirect mode of action on these pathways has not been clearly determined.

Remarkably, 21OH-6,19OP has the potential behavior of an anti-inflammatory agent since it inhibits NFκB and AP-1 dependent reporter gene induction in BHK and Cos-1 cells overexpressing pRelA (the active subunit of NFκB) or pC-jun (a component of the AP-1 transcription factor) [48]. Similar to other GR agonists [77-79] this compound reduced the LPS/IFN-γ mediated nitrite formation in a concentration-dependent manner and repressed NOS-2 gene expression in RAW264.7 cells [62]. Furthermore, 21OH-6,19OP completely inhibited COX-2 expression in peritoneal macrophages from mice injected with LPS, confirming the *in vivo* anti-inflammatory effects of this compound.

Similar to Dex, 21OH-6,19OP inhibited cellular death triggered by TNF-α in L929 fibroblasts [47], whereas in human lung cancer A549 cells, it decreased TNF-α-induced COX-2 and IL-8 expression. Interestingly, COX-2 downregulation observed in this cell line would be mainly mediated by the inactivation of the p38 pathway through the increase of MKP-1 phosphatase levels. However, contrary to Dex, 21OH-6,19OP was ineffective in repressing pRelA and pC-Jun mediated COX-2 induction [62].

Together, these results support the idea that GR/21OH-6,19OP dimers actually have an appropriate conformation to selectively participate in pathways involved in the GR anti-inflammatory responses. The slightly dissimilar behavior between GR/Dex and GR/21OH-6,19OP complexes observed in lung cancer epithelial cells makes this compound a useful tool to improve the understanding of GR anti-inflammatory mechanism of action.

CONCLUSION

Almost two decades have passed since 21OH-6,19OP was described as a specific antiglucocorticoid [42]. Nowadays, accumulated evidence obtained through diverse multidisciplinary methods has demonstrated that this steroid has distinctive glucocorticoids characteristics that turned out significant in glucocorticoid research. The GR is a flexible molecular machine whose outcome depends on several actors; from the specific conformations acquired by the activated GR/ligand complex that affects its ability to interact with other proteins, including itself, to the chromatin landscape of each cellular context. In this scenario, we speculate that restricting ligand flexibility may constrain the receptor to explore only certain conformations that might result in a better prediction of GR outcome. The use of a rigid steroid like the 21OH-6,19OP has thus provided valuable information to understand certain GR mediated pathways.

The GR oligomerization state has been evaluated in depth, finding high correlation between theoretical predictions obtained by molecular modeling and experimental results from biophysical studies [47, 48, 52]. A direct association among events occurring at different levels -the conformational change of the H1-H3 loop, the GR nuclear behavior and the global transcriptional response- was established by using the 21OH-6,19OP ligand. The usage of this compound also contributed to conclude that at least one of the two dimerization interfaces is sufficient to allow GR homodimer formation; although homodimerization through both interfaces is necessary to get a fully functional complex able to activate genes driven by GREs [52].

21OH-6,19OP passive antagonism model assumes that the H1-H3 loop participates in GR homodimerization, idea whose validity at first sight might be questioned, since it derives exclusively from data obtained from crystal structures of isolated GR LDBs. However, the fact that the GRwt/21OH-6,19OP dimerizes while the GRdim/21OH-6,19OP exist only as monomer [52] clearly indicates that somehow this ligand alters the receptor structure and impairs its dimerization though an interface different from the one located in the DBD. Notably, the ability of this ligand to manipulate the conformation of GR dimers could help to further understand which pathways would be triggered depending on the GR quaternary structure. Nevertheless, additional studies are still necessary to determine whether the H1-H3 loop is effectively involved in GR homodimerization and what is the relevance of this region in the interaction of GR with other regulatory proteins. In this sense, it was recently suggested that this loop is involved in the regulation of the FKBP51 and FKBP52 chaperones with the GR/Hsp90 heterocomplex [80].

As it is known, ligand intermediate conformations adopted by the AF-2 domain – in particular the H12 helix- would be directly related to the recruitment of GR transcription modulators. On this respect, according to theoretical results the GR LBD/21OH-6,19OP complex was unable to recognize the TIF-2 LxxLL motif, being this consistent with the incapacity of the GR/21OH-6,19OP to recruit this co-activator [47, 48]. Since the LxxLL motif is mostly conserved among a great variety of GR interacting proteins, it would be interesting to explore how this ligand affects the recruitment not only of other GR coactivators but also co-repressors, evaluating in this way the functional consequences of these interactions.

At variance to the GR direct mode of action, the molecular events involved in the indirect mode are poorly understood. In this way, the search for a correlation between GR conformational changes and global responses results far more challenging. More information will be necessary to find out those molecular determinants involved in the indirect regulation of GR target genes. Current data supports the hypothesis that GR monomers are not required for tethering certain transcription factors [52]. In particular, GR/NFκB interaction seems to be independent of GR oligomerization state; however, the recruitment of TIF2 by the activated GR would be necessary to repress certain NFκB target genes [81, 82]. This information would probably explain the inability of GR/21OH-6,19OP to inhibit Cox-2 expression induced by Rel-A. Nonetheless, further studies using this ligand would contribute to achieve a deeper panorama on the modulation of NFκB activity. In this sense, the precise role of each GR domain and the allosteric communication among them should be investigated to reach a detailed description. Promisingly, essential information about the AF-1 domain function has been recently described

[83], enlarging the knowledge on this fundamental, and many times unconsidered, GR region.

In summary, final clinical efficacy of GCs will depend on the relative contribution of multiple determinants involved in GR action. After several years, the dissociated paradigm is being gradually abandoned by the community members. Therefore, new approaches for mitigating the side effects of chronic glucocorticoid treatment should be explored. A systematic screening to identify differential GR/ligand nuclear behaviors that display favorable functional profiles is still necessary. The question is whether it will be possible to determine the contribution of each molecular determinant to get the desired glucocorticoid outcome. Continuing to improve our knowledge on the molecular basis of GR responses will be the best approach to the rational design of safer glucocorticoids.

LIST OF ABBREVIATIONS

HPA axis: hypothalamic-pituitary-adrenal axis
NRs: Nuclear Receptors

CONFLICT OF INTEREST

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