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Mini review

New lead compounds in the search for pure antiglucocorticoids and the dissociation of antiglucocorticoid effects

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ABSTRACT

Antiglucocorticoids that act as antagonists at the glucocorticoid receptor (GR) level may be used to block or modulate the undesirable effects of glucocorticoid excess (from endogenous or exogenous origin). RU486 developed in the early 80s, is an antiglucocorticoid but also a potent antiprogestin and abortifacient, nevertheless it still remains as the only GR antagonist drug in the market. Further on, in view of the variety of physiological processes in which glucocorticoids are involved, selective antiglucocorticoids that can block only some of these processes (eventually with tissue specificity) would be highly desirable. The bridged pregnane 21-hydroxy-6,19-epoxyprogesterone, was developed as an alternative lead being an antagonist of the GR with no affinity for mineralocorticoid and progesterone receptors. Antagonistic activity was evidenced by partial blocking of dexamethasone induction of tyrosine aminotransferase (TAT) and thymocyte apoptosis. Replacement of the oxygen bridge by a sulfur bridge gave a less bent, more flexible molecule. 21-Hydroxy-6,19-epithioprogesterone exhibited improved antiapoptotic activity on thymocytes but was not effective blocking TAT induction. This selectivity was improved further by oxidation to the sulfone. The sulfone but not the reduced compound also reverted the dexamethasone-mediated inhibition of NFκB activity in HeLa cells. Blocking of the apoptotic effect of TNFα by dexamethasone in the L929 cell line (mouse fibroblasts), was only reverted partially by the sulfone which exhibited a mild agonistic/antagonistic activity in this assay. None of these compounds showed antiprogestin activity. Similar overall molecular shapes but more lipophylic and with higher metabolic stability were obtained by introduction of a methylene bridge (6,19-methanoprogesterone) or by a direct bond between C-6 and C-19 (6,19-cycloprogesterone and its 21-hydroxy derivative). The latter highly bent steroids showed affinity for the GR. Recently we performed molecular dynamics simulations of GR–ligand complexes to investigate the molecular basis of the passive antagonism exhibited by 21-hydroxy-6,19-epoxyprogesterone. On the basis of our findings, we proposed that the passive antagonist mode of action of this antiglucocorticoid analog resides, at least in part, in the incapacity of GR–21-hydroxy-6,19-epoxyprogesterone complex to dimerize, making the complex unable to activate gene transcription.

Contents

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1. Introduction

Since world war II, pregnane derivatives (C-21 steroids) have acquired an unusual medical and social importance as hormonal agonists and antagonists, mainly due to the growing exposure of mankind to physical and psychological stress and the explosive increase of world population. As appropriately affirmed by Jensen in 1995, fundamental to an elucidation of hormone antagonism is the understanding of agonist action itself [1]. The most widely studied system for steroidal hormone action is that in which the steroid converts the native receptor to a functional transcription factor that enhances expression in target genes. Although progress has since been achieved, procedures and mechanisms discriminating between hormones (agonists) and antihormones (antagonists) are still tentative or a matter of hypotheses. Now as before, antagonists to a receptor quite often arise as "serendipity" during the empirical screening of molecules with high receptor-affinity.

Beyond the initial effect, the main agonistic biological endpoints for pregnane derivatives are the maintenance of gestation and avoidance of ovulation in progestins, sodium retention, volume expansion and potassium elimination in mineralocorticoids and a host of suppressive and activating properties in glucocorticoids. The latter comprise, among others, the expression of gluconeogenic and glycogenic enzymes, the suppression of native and acquired immunity and the maturation of lung surfactants. Natural progestins, mineralocorticoids and glucocorticoids participate in common biosynthetic pathways and as mentioned above, are related not only by their affinity to a receptor superfamily for steroids and retinoids, but also structurally because they share the pregnane skeleton.

Due to the many processes in which glucocorticoids participate their excess, be it from iatrogenic or endogenous origin, leads to serious consequences to the individual. The most relevant clinical manifestations of glucocorticoid excess are rounded "moon" faces with a plethoric appearance, truncal obesity with prominent supraclavicular and dorsal cervical fat pads ("buffalo hump"), and usually quite slender distal extremities and fingers. Muscle wasting and weakness are present, the skin is thin and atrophic with poor wound healing and easy bruising and purple striae may appear on the abdomen. Hypertension, renal calculi, osteoporosis, glucose intolerance, reduced resistance to infection, and psychiatric disturbances are also common. Compounds that could selectively block some or all of these effects, have many potential applications. Confirmed and suggested applications of the antiglucocorticoid property of currently studied molecules include presurgical treatment of Cushing's Syndrome, secondary to ectopic ACTH secretion or primary due to an adrenal tumor or adrenal hyperplasia. Also, treatment of glucocorticoid dependent hypertension, glucocorticoid induced immunosuppresion, central depression and anxiety, and ocular pressure and glaucoma, as well as for the accelerated healing of wounds and burns. Some of these have been evaluated with the available glucocorticoid blocking drugs although not always successfully [2,3].

In the present review we deal with a group of novel antiglucocorticoids characterized by subtle differences between their antagonism to certain immunosuppressive and gluconeogenic activities of the broad glucocorticoid spectrum, and lack of receptor and biological cross-reactivity with the progesterone receptor (PR) and the mineralocorticoid receptor (MR). At the onset of our research these characteristics seemed of interest since, even at present, the antiglucocorticoid of reference is RU486 (**1**) (see Fig. 1 for structures) known by the generic name mifepristone that was introduced over 20 years ago. This steroid was developed in the early eighties as an antiglucocorticoid but showed to be also a potent antiprogestin and abortifacient [4]. From a structural point of view, compared with natural glucocorticoids and progestins, this compound lacks the C-19 methyl and has an additional double bond between C-9 and C-10 that results in a very flexible molecule. Another characteristic is the bulky substituent at C-11 that, according to the crystal structure of the GR ligand binding domain (LBD) bound with RU486, protrudes out of the ligand binding pocket distorting the active position of helix 12 (H12). Helix 12 is the last helix of the GR-LBD at the carboxy-terminal side of the receptor, and the main determinant of the interaction interface with coactivators [5]. This molecular mode of action of RU486 in the GR and PR [6], termed active antagonism, has been demonstrated for many antagonists of nuclear receptors [7]. Several hundreds of analogs of RU486 have been synthesized since, trying to separate the antiprogestagenic activity from the antiglucocorticoid activity. In most cases where this separation was achieved the remaining activity was antiprogestagenic with eventually very weak antiglucocorticoid activity, e.g. onapristone (**2**) [2]. Most of these molecules retained the flexibility and the large 11β-substituents. Much fewer cases were biased towards antiglucocorticoid activity; for example RU43044 (**3**) is a pure antiglucocorticoid but with only a sixth of the activity of RU486. A distinct characteristic of compound **3** is the presence of C-19 (thus rendering a more rigid structure) and the shift of the bulky substituent from C-11 to C-19. Unfortunately RU43044 was active only in vitro, as it is heavily metabolized in vivo [8]. Thus over 20 years later RU486 still remains as the only antiglucocorticoid drug in the market. However, its antiprogestagenic activity and abortifacient properties have restricted its use and availability. Pure antiglucocorticoids need to be developed that have no effect on the menstrual cycle and early pregnancy, and hence can also be used in countries where a compound with dual antiglucocorticoid and antiprogestational activities may not be made available because of, for example, restrictive abortion legislation [3].

2. Ligand conformation in specific glucocorticoids/antiglucocorticoids

2.1. The lead molecule

Our approach was based on the observation that pregnane molecules with a bent structure at their A/B ring junction, exhibit optimal and specific binding to GR and many down-stream glucocorticoid properties. Our research originated in early reports by Weeks and Duax according to which an increasingly bent A/B conformation correlates with anti-inflammatory properties of natural and synthetic pregnanes [9].

As mentioned above, from a structural point of view glucocorticoids, mineralocorticoids and progestins share the pregnane

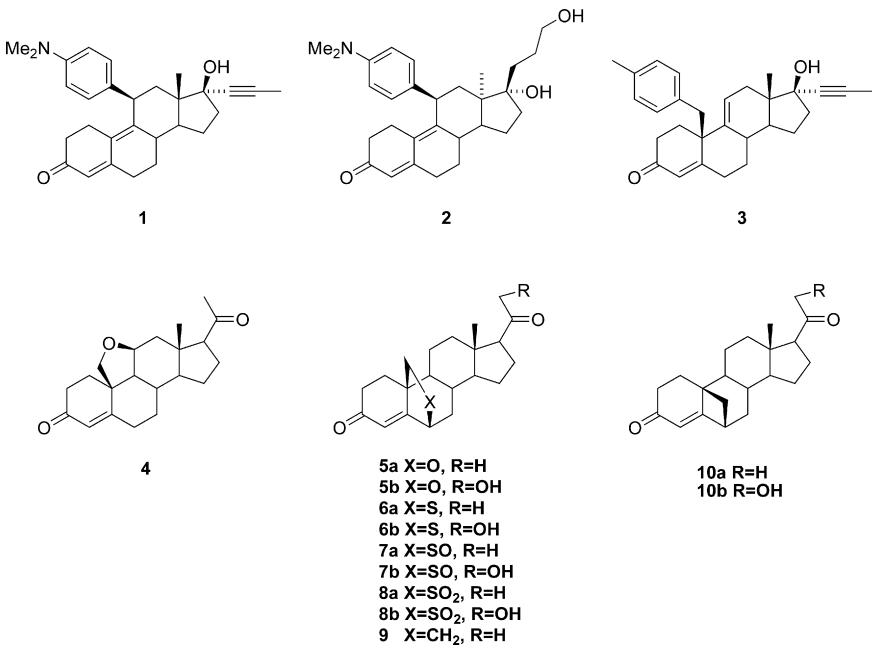

Fig. 1. Structures of RU486 (**1**), onapristone (**2**), RU43044 (**3**) and synthetic bridged-steroids.

skeleton, with its well defined conformation. However, due to its flexibility, the pregnane skeleton can be deformed upon introduction of substituents or bridges, resulting in reorientation of functional groups that can favor or disfavor interactions with specific receptors and give rise to substantial changes in activity. On the other hand, this flexibility allows the pregnane skeleton to "adapt" to different requirements when interacting with a receptor, and contributes to crosstalk between the specific receptors for each of these hormone types. So one goal we had at the start of this research was to select and lock certain conformations to increase specificity.

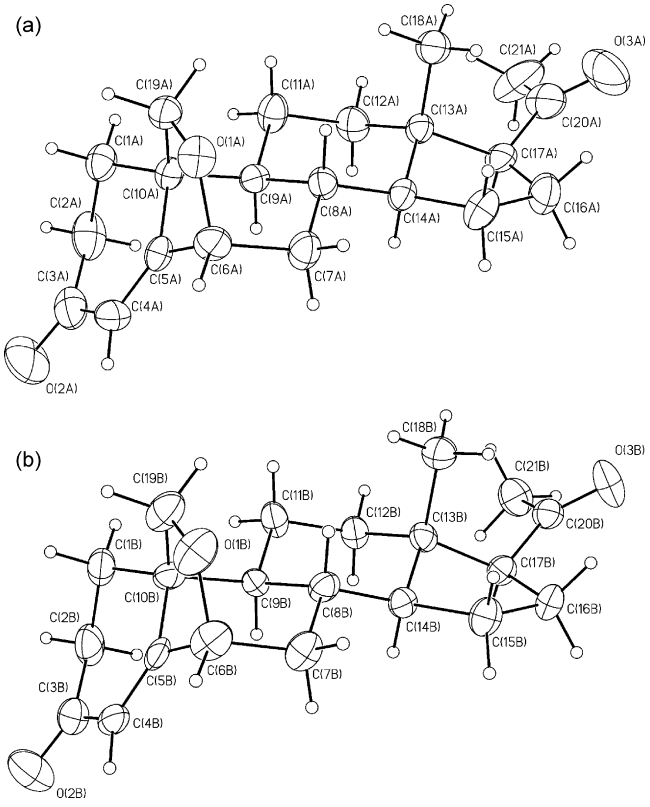

Fig. 2. Single crystal X-ray diffraction structures of 6,19-epoxyprogesterone less bent (a) and more bent (b) forms [11].

The use of bridges or fused rings to lock specific conformations is a well known strategy in medicinal chemistry; our initial studies showed that introduction of an 11,19-epoxy bridge in the progesterone molecule gives a flat rigid structure (**4**) with potent sodium-retaining properties and affinity for the mineralocorticoid receptor (MR) [10]. On the other hand, introduction of a 6,19-epoxy bridge into progesterone, results in a structure with a pronounced bending at the A/B ring junction (compound **5a**). In this compound, the A ring is heavily torsioned towards the alpha face and has an inverted half-chair conformation compared to progesterone and natural corticoids (i.e. 1β,2α). The X-ray crystal structure of **5a** is shown in Fig. 2 [11]. Two similar but independent molecules are present in the crystal, slightly differing in the bending at the A/B ring junction; the least bent conformer matches the AM1 calculated structure. This suggests that the more bent conformer may arise due to crystal packing forces. This steroid proved to be *per se* devoid of glucocorticoid or mineralocorticoid activity but the introduction of a 21-hydroxyl (21OH-6,19OP, **5b**) resulted in a pronounced improvement of glucocorticoid receptor (GR) binding and, most important, antiglucocorticoid properties. Neither affinity for MR nor the progesterone receptor (PR) was observed for this compound [12]. Downstream, the antagonistic activity was evidenced by partial blocking of dexamethasone and corticosterone induction of tyrosine aminotransferase (TAT) in rat hepatocytes. More recently, the groups of Funder and Challis showed that 21-OH-6,19OP (**5b**) reversed the blocking effect of cortisol on 15-hydroxyprostaglandin dehydrogenase activity, an important prostaglandin-activating event leading to parturition at term of gestation [13].

Interestingly, RU486 also presents ring A in an inverted chair conformation in its crystal structure, with the A ring bent towards the alpha face [2]. In the crystal structure of the GR LBD–RU486 complex, the bound RU486 molecule is heavily distorted with an exaggerated bending of ring A towards the alpha face [5]. This structure has very good overall coincidence with that of **5a** (and **5b**) regarding A ring conformation and bending (Fig. 3). Thus compound **5b** appears to present the A/B ring conformation of GR-bound

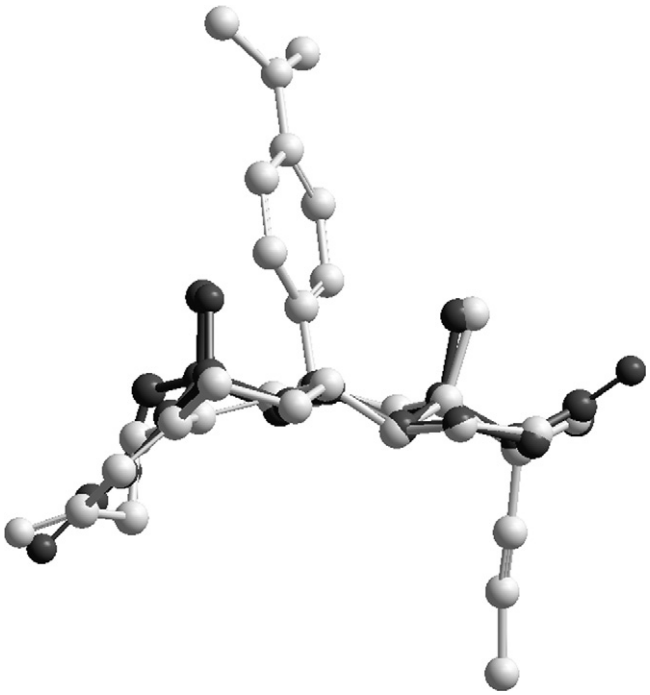

Fig. 3. Superposition of RU486 as bound to GR (white) [5] and less bent form of 6,19-epoxyprogesterone (dark gray) [11].

RU486, but without the need for a flexible structure or a bulky substituent at C-11 to acquire it. Interestingly, this conformational characteristic appears sufficient to confer antiglucocorticoid but not antiprogestagenic properties. It should be noted that a previous report on the antiglucocorticoid activity of cyproterone acetate also suggested that a bulky substitution at C-11β was not mandatory to attain this antagonistic effect [14]. In contrast with RU486, ligands such as 21OH-6,19OP and cyproterone acetate, that lack the C-11 bulky substituent, are termed passive antagonist.

2.2. Sulfur and carbon-bridged analogs

Focusing our attention on the conformational characteristics of 21OH-6,19OP (**5b**), we then envisaged the possibility of substituting the oxygen-bridge by either less tensioned and more flexible bridges or more tensioned less flexible ones, in order to achieve a degree of bending that would maximize the antiglucocorticoid activity maintaining the lack of antigestagenic properties. The first alternative we considered was a 6,19-sulfur bridge. Indeed, the longer C–S bonds in 6,19 epithiopregnanes (**6a** and **6b**) were expected to result in a less tensioned structure, according to semiempirical calculations (AM1) that predicted a less bent A ring compared to their oxygen-bridged counterparts. This was confirmed by single crystal X-ray diffraction, **6a** crystallized as a single conformer with a less bent A ring compared to **5a** and this structure showed excellent coincidence with that obtained from AM1 calculations [11]. Furthermore, oxidation of the sulfur atom allowed the straightforward synthesis of sulfoxide (**7a**,**b**) and sulfone (**8a**,**b**) bridges exhibiting increased steric and polar interactions on the steroid β-face and reduced lipophylicities. ^{1}H NMR data indicated that in the sulfoxides, the oxygen atom is oriented towards the A ring which results in an asymmetrical distribution of electrostatic potential around the 6,19-bridge. Electrostatic potential distribution for **6a**,**b** is similar to that of the sulfones (**8a**,**b**) and more disperse around the 6,19-bridge compared to **5a** (Fig. 4).

Other variations introduced on the 6,19-bridge include the replacement of the oxygen atom by a methylene group (**9**) [15] or

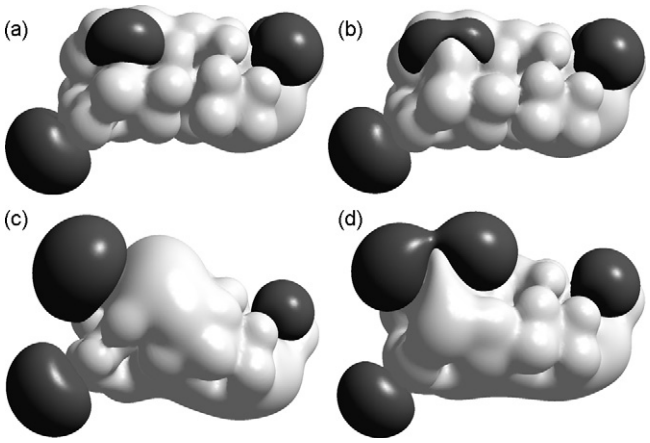

Fig. 4. Electrostatic potential isosurfaces at 0.02 a.u. on AM1 calculated structures of (a) **5b**, (b) **6b**, (c) **7b** and (d) **8b** (white: positive, dark gray: negative).

the removal of the bridging atom to give a direct bond between C-19 and C-6 (**10a**) [16]. The latter compounds are more lipophylic than compounds with a heteroatom bridge and are expected to have higher metabolic stability. AM1 calculations predict for **9** a conformation similar to that of **5a** with almost identical bending of ring A, while for **10a** the higher tension introduced by the smaller 4-membered ring should lead to a more bent conformation with a less hindered β face.

3. Competition assays of the synthetic steroids

Competition assays of these compounds for dexamethasone-GR, R5020-PR, and aldosterone-MR were determined initially. However, as these assays do not necessarily predict the nature or degree of steroid hormone receptor signaling activity, we also examined the ability of these compounds to function as agonists or antagonists of gene transcription mediated by GR or PR [17]. Fig. 5 summarizes the [^{3}H]-dexamethasone displacement assays on GR from rat liver, obtained with compounds **5b**, **6b**, **7b** and **8b**. RU486 was used as a positive control. Results show that only **5b** was moderately active displacing dexamethasone in this system. Preliminary data indicate that compounds **10a** and **10b** also present affinity for GR with the former being more active [16]. On the other hand none of these compounds showed affinity for PR ([^{3}H]-R5020 displacement), nor MR ([^{3}H]-aldosterone displacement).

To complement the above observations, BHK (baby hamster kidney cells) that express high amount of GR, were used for the stan-

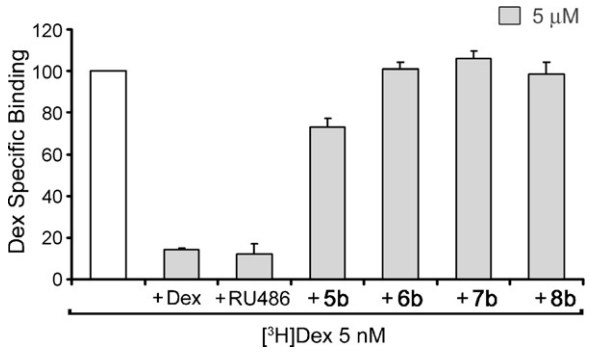

Fig. 5. [^{3}H]-dexamethasone displacement assays on glucocorticoid receptor (GR) from rat thymus. An enriched thymic proteic fraction of GR was used. Competition was measured by displacement of 5 nM [^{3}H]-dexamethasone (dex) with 5 μM unlabelled competitors (600 μg of protein, 16 h at 4 °C). Displacement by 5 μM RU486 was used as positive control. Each treatment was done by triplicate. Means ± SD from a representative experiment (n = 3) are shown (data taken in part from ref. [16]).

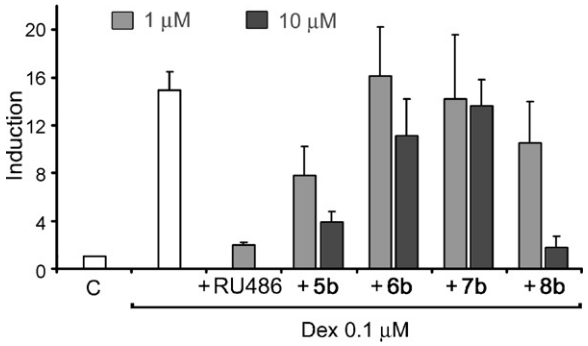

Fig. 6. Inhibiting effects of RU486, **5b**, **6b**, **7b** and **8b** at final concentrations of 1 μM or 10 μM, on the stimulation of the luciferase/β-galactosidase ratio by dexamethasone (dex) 0.01 μM in BHK cells transfected with the MMTV-LUC reporter gene. C: control (untreated cells). Results are expressed as mean ± SD of three independent experiments.

dard transactivation assay of antiglucocorticoid effects. Results are summarized in Fig. 6. Thus **5b** blocked the dexamethasone induction of MMTV-luciferase reporter in BHK cells, attaining maximum effect at 10 μM, while the sulfone **8b** displayed a strong antiglucocorticoid activity at 10 μM (unpublished results). In Cos-1 cells transfected with PR, none of the steroids assayed blocked progesterone induction of luciferase [11].

4. Glucocorticoid and antiglucocorticoid properties

Besides suggesting improved structures for antiglucocorticoid activity and specificity, the results with 21-hydroxy-6,19-epoxyprogesterone paved the way to the search for molecules with a selective glucocorticoid spectrum favoring clinically useful over harmful properties. The search for anti-inflammatory, anti-leukemic, anti-lymphomic, anti-autoimmune or anti-arthritic treatments devoid of an often fatal tendency to immunodeficiency-dependent opportunistic infections is a long cherished goal for glucocorticoids which has as yet not been attained.

4.1. Effect on the induction of tyrosine aminotransferase by dexamethasone

Induction of TAT is a typical transactivation event triggered by glucocorticoids [18]. 21-Hydroxy-6,19-epoxyprogesterone **5b** but not the 21-deoxy analog **5a** has been shown to block corticosterone induction of TAT in rat hepatocytes [12]. In rat hepatoma cells HTC 10 μM **5b** partially blocked induction of TAT by 10 nM dexamethasone while the 21-hydroxy sulfur-bridged analogs **6b**–**8b** were inactive at that concentration [11].

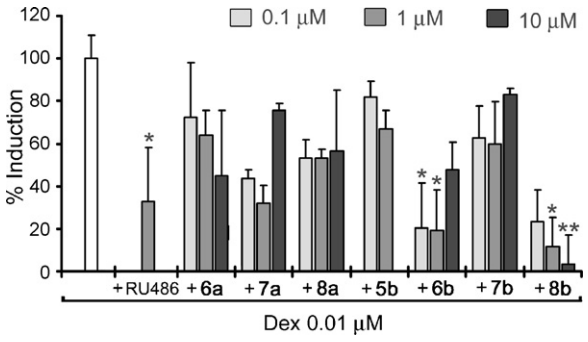

Fig. 7. Inhibition of dexamethasone (dex)-induced apoptosis in thymocytes by 21-deoxy analogs **6a**, **7a**, **8a** and 21-hydroxy analogs **5b**, **6b**, **7b**, **8b** at 0.1 μM, 1 μM or 10 μM final concentrations. Inhibition by 1 μM RU486 is shown for comparison purposes. Thymocytes were incubated during 4 h at 37 °C. A fluorescein isothiocyanate (FITC) conjugate of annexin V was used to detect apoptosis by flow cytometry [8]. Results are expressed as the mean percentage induction relative to controls ± SD; control: dexamethasone 0.01 μM, no steroids added. $^{*}p < 0.05$ *vs.* control; $^{**}p < 0.01$ *vs.* control (data taken from ref. [11]).

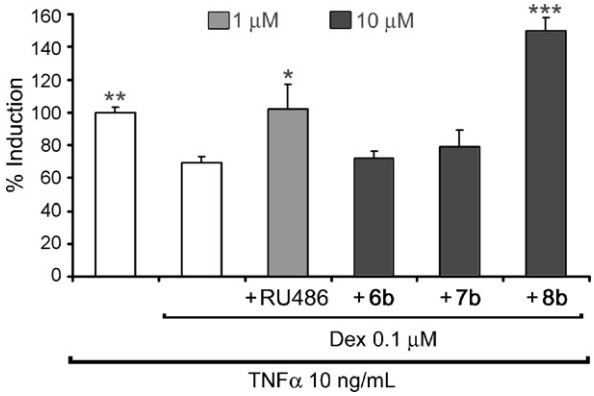

Fig. 8. Blocking effects of 21-hydroxy-epithiopregnanes **6b**, **7b** and **8b** at 10 μM final concentration, on the inhibition of TNFα 10 ng/mL mediated luciferase/β-galactosidase ratio by dexamethasone (dex) in HeLa cells. 1 μM RU486 was used as positive control. Results are expressed as mean ± SD of three independent experiments. $^{*}p < 0.05$ *vs.* dex 0.1 μM; $^{**}p < 0.01$ *vs.* dex 0.1 μM; $^{***}p < 0.001$ *vs.* dex 0.1 μM (data taken from ref. [11]).

4.2. Effect on the immunosuppressive actions of dexamethasone

Historically, a classical albeit limited approach to immunosuppressive assessments of glucocorticoids are thymus masses in immature rodents [19,20]. The modern, cytological counterpart of this parameter is apoptosis of thymocytes or, by extension, thymus-

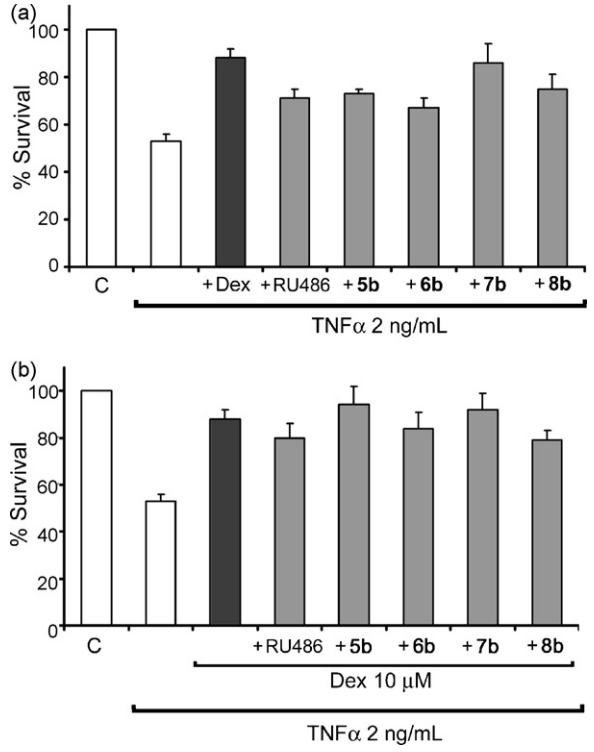

Fig. 9. (a) Protection effect of dexamethasone (dex), RU486, **5b**, **6b**, **7b** and **8b** at 10 μM final concentration, on TNFα 2 ng/ml mediated apoptosis in L929 cells; (b) antiglucocorticoid effect of RU486, **5b**, **6b**, **7b** and **8b** at 10 μM final concentration, on the inhibition of TNFα mediated apoptosis by dexamethasone (dex) 10 μM in L929 cells. L929 cells were incubated during 24 h at 37 °C with the different compounds. Viable cells were detected by crystal violet stainning. C: control (untreated cells).

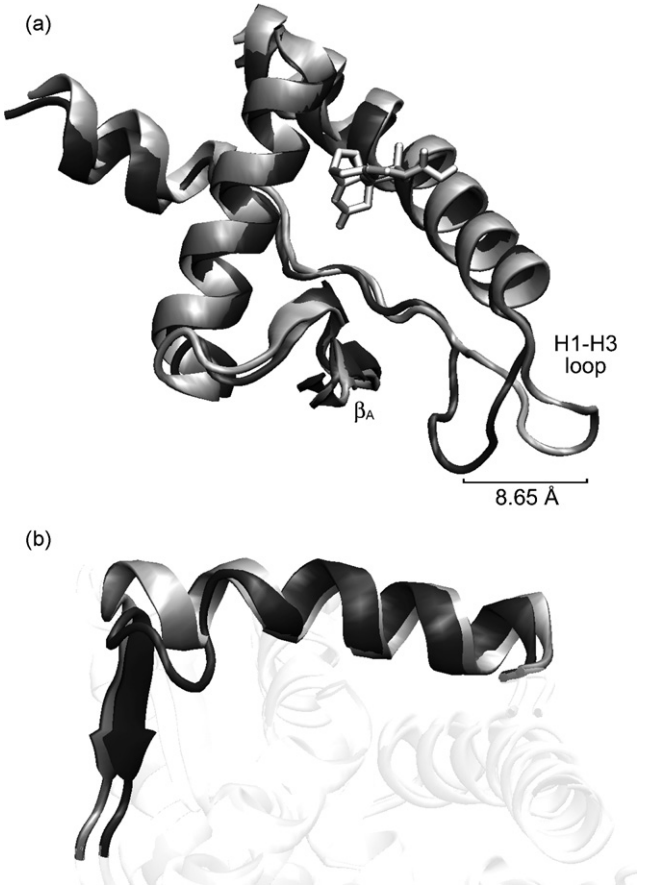

Fig. 10. H1–H3 loop average structure (a) and H-12 average structure (b) of the systems GR-dexamethasone (dark gray) and GR-21OH-6,19OP (light gray) taken over the last 4 ns of MD simulation. The ligand shown is 21OH-6,19OP in GR-21OH-6,19OP (data taken from ref. [30]).

derived blood cells. Although the physiological nexus and many molecular mechanisms mediating hormone-induced apoptosis remain still obscure (see below), apoptosis of thymocytes is increasingly used as a parameter of glucocorticoid-antiimmune properties. We employed two different criteria to evaluate apoptosis: DNA-fragmentation and externalization of phosphatidyl-serine.

Apoptotic DNA fragments of thymocytes from glucocorticoid-treated mice can be visualized under UV light following electrophoresis in agarose gels that contain ethidium bromide. 21-hydroxy-6,19-epithioprogesterone (**6b**) and the 6,19-sulfone analog (**8b**) were equally effective in preventing dexamethasone-induced DNA fragmentation, but they were so only at high concentrations (10^{-4} M). The sulfoxide **5b** was ineffective. In this same assay the 21-deoxy sulfone **8a** was marginally active at 10^{-4} M [11].

On the other hand, upon apoptosis, rapid alterations occur in the localization of membrane phospholipids leading to exposure of phosphatidylserine at the cell surface. Recognition of this phospholipid by phagocytes in vivo, leads to the removal of cells programmed to die [21]. Experimentally, externalized phosphatidylserine can be detected in vitro by its reaction with the anticoagulant annexin V. We adopted this procedure in a second approach to apoptosis based on an entirely different intermediate mechanism, which employs a fluorescein–isothiocyanate conjugate of annexin V for quantification of apoptosis by flow cytometry–fluorometry [22].

Fig. 7 shows results obtained with the 21-deoxy and the 21-hydroxy sulfur-bridged analogs (**6a**–**8a**, **6b**–**8b**) in blocking the apoptosis induced by dexamethasone 10^{-8} M. Interestingly although 6,19-epoxyprogesterone **5a** is inactive (data not shown), the 21-deoxy sulfur analogs showed moderate activity. In particular the sulfoxide **7a** had almost the same activity as RU486 at 10^{-6} M although activity decreased at higher concentrations. For the 21-hydroxy analogs, 21-hydroxy-6,19-epithioprogesterone (**6b**) and the sulfone (**8b**), were more active than the lead molecule 21-hydroxy-6,19-epoxyprogesterone (**5b**) and that the reference compound RU486 [11].

4.3. Blockage of dexamethasone mediated inhibition of the activation of the NFκB transcription factor by TNFα

The potent immunosuppressant activity of glucocorticoids is largely derived from the GR's ability to repress the transcription of many pro-inflammatory molecules. For this goal, the GR interferes with transcription factors such as NFκB, AP-1 and members of the STAT family [23,24,25]. We focused our attention on NFκB and connected pro-inflammatory cytokines to gain more insight into the antiapoptotic activity described above. It is well established by now, that the GR can "transrepress" promoters in this process without binding to a GRE [24,26]. It is also accepted that pro-inflammatory cytokines mediate the activation process of glucocorticoid-inactivated NFκB and that the latter responds to signals from cytokines such as TNFα, IL-1β and others. The same cytokines also stimulate various steps of the hypothalamus–pituitary–adrenal axis [27]. Taken together, these sequences signify a regulatory network between glucocorticoids, cytokines and proinflammatory transcription factors such as NFκB. In this network glucocorticoids inhibit the synthesis, release or activity of the proinflammatory cytokines [28].

In our experiments, the immunosuppressive effect of glucocorticoids was also evidenced by the ability of dexamethasone to inhibit TNFα activation of NFκB. HeLa cells were transfected with a pκB-Luc plasmid which expresses luciferase enzyme under the control of the κB response elements from HIV promoter [11]. The blocking effects of 21-hydroxy sulfur-bridged pregnanes are summarized in Fig. 8. 21-Hydroxy-6,19-epithioprogesterone (**6b**) and the sulfoxide **7b** were not effective to counteract dexamethasone inhibition. Interestingly, the sulfone **8b** not only blocked completely the dexamethasone inhibition, but stimulated above dexamethasone free controls the TNFα mediated expression of NFκB.

Table 1
Summary of activities of relevant steroids.

	Competition assays			Anti-GC properties			
	GR	PR	MR	BHK (GR)	Thymocyte apoptosis	NF-κB/TNFα	TAT induction
5b	++	–	–	++	+	nd	++
6b	–	–	–	+	++	–	–
7b	–	–	–	–	±	–	–
8b	–	–	–	+++	+++	+++	–
RU486	+++	+++	–	+++	++	++	+++

nd: not determined; ± indicates loss of activity at higher doses.

4.4. Effect on cell death induced by TNFα on L929 mouse fibroblasts

The protection conferred by dexamethasone against TNFα cytotoxicity on L929 cells has been used as a model to study the protective mechanisms involved in steroid hormone dependent tumor resistance, a frequent characteristic in aggressive forms of breast and prostate cancer [29]. We determined the effect of the synthetic steroids in the protection against the TNFα induced cell death. Fig. 9a shows that all the steroids tested partially reverted the TNFα effect, the sulfoxide **7b** being as potent as dexamethasone. We also tested the ability of these same compounds to inhibit the dexamethasone effect on TNFα induced cell death in this cell line (Fig. 9b) (unpublished results). In this case, only the sulfone **8b** had a moderate activity, while the other steroids failed to revert the dexamethasone activity. Interestingly, in these assays the sulfone **8b** exhibited a behavior similar to that of RU486.

5. Molecular basis of action of 21OH-6,19OP

Recently, in order to evaluate the molecular determinants of the passive antagonism exhibited by 21OH-6,19OP (**5b**), we carried out a subcelullar localization experiment together with a set of Molecular Dynamics (MD) simulations [30]. Confocal microscopy of an immunofluorescence analysis of the GR–21OH-6,19OP complex, showed that 21OH-6,19OP is able to induce the transformation and translocation of the receptor to the nucleus. The overall conformations of GR LBD–dexamethasone and GR LBD–21OH6,19OP complexes were studied by MD simulations, a powerful computational technique. Our results showed that in the receptor bound to 21OH-6,19OP (**5b**) the average position of the loop between helices 1 and 3 (H1–H3 loop) adopts a markedly different conformation compared to the GR LBD–dexamethasone complex (Fig. 10a). Since the H1–H3 loop is a fundamental region of the homodimerization interface, and homodimerization of GR is necessary to induce the transcription of genes regulated by glucocorticoid response elements, we proposed that the antagonist activity of 21OH-6,19OP in standard transactivation assays would reside, at least in part, in the incapacity of the GR complex to homodimerize. Furthermore changes were also observed in the conformation of the AF-2 domain involved in corepressor and coactivator recognition, particularly in the length of helix 12 (Fig. 10b). These ligand induced changes could result in a fine regulation of the ability of the receptor to recruit specific coactivators or corepressors.

6. Conclusions

While this research was in progress, a latent discussion of years matured, concerning such fundamental basic aspects as the mechanisms underlying apoptosis as well as the activation of transcription-factors such as AP-1 and NFκB, the role of glucocorticoids in these processes, their physiological meaning and interconnections between these processes. This has opened the possibility of manipulating the steroid receptor activity with specifically designed ligands that can block or minimize unwanted activities while retaining others [31,32]. Bridged pregnanes derived from 21OH-6,19OP (**5b**) allow us to manipulate the GR without interacting with the MR or PR. The activities of the most relevant molecules presented above, compared with the original lead (21-hydroxy-6,19-epoxyprogesterone) and RU486 are summarized in Table 1. The selectivity displayed by the sulfur-bridged analogs **6b** and **8b**, indicates that these compounds may act at different stages of the sequence of events leading to immunosuppresion and antiinflammation. This is in line with reports showing that antiglucocorticoids can either block the capacity of the GR to interact with the hormone response elements or interfere with the subsequent processes linked to transcriptional activation [33,34].

Finally, our hypothesis that the passive antagonist mode of action of 21OH-6,19OP (**5b**) is related to the failure of the GR–21OH-6,19OP complex to dimerize and the possibility of fine regulation of the AF-2 domain conformation by other bridged pregnanes that retain the conformational characteristics of 21OH-6,19OP, may be used as a starting points for the design of dissociated antagonists and modulators of the GR.

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