

21-Hydroxy-6,19-oxidoprogesterone: A Novel Synthetic Steroid with Specific Antigluco-corticoid Properties in the Rat

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SUMMARY

In the rat, the conformationally highly bent steroid 21-hydroxy-6,19-oxidoprogesterone efficiently displaces [³H]corticosterone from thymus-glucocorticoid receptors and blocks type II receptors in kidney cytosols but competes with neither [³H]aldosterone for kidney-mineralocorticoid receptors nor [³H]progesterone for uterus-progesterone receptors. It evokes Na⁺ retention only at very high doses (~100 μg/100 g of rat weight)

and is unable to induce tyrosine aminotransferase or to increase glycogen deposits in rat liver. When coincubated with corticosterone or dexamethasone, 2.5 μM 21OH-6OP inhibits 80% of tyrosine aminotransferase induction. It may therefore be used experimentally as an antigluco-corticoid virtually lacking mineralocorticoid or glucocorticoid properties as well as affinity for mineralocorticoid or progesterone receptors.

The GR, cloned in 1985 (1), is a member of a protein super-family of closely related intracellular receptors that function as ligand-activated transcription factors (2). Steroid ligands that are able to activate GRs and to trigger a biological response exhibit a slightly torsioned steroid nucleus at the A/B-ring junction for optimal GC activity (3, 4). On the other hand, MC agonists require an overall flat conformation to acquire sodium-retaining activity (5). The MR, cloned by Arriza *et al.* (6) in 1987, have been shown to be highly homologous with GR. Because of that considerable homology, it was not too surprising that natural and even synthetic steroids exhibited cross-reaction between GR and MR. The PR is the third member of a subfamily of these highly related steroid receptors. Its natural ligand, P₄, exhibits cross-reaction with both MR and GR.

Employing a systematic application of strategies to increase activity and decrease cross-reactivity and undesirable side effects, impressive progress has been reported in the development of new antihormonal agents with greater potency and selectivity, especially in the antiestrogen and antiandrogen fields (see Ref. 7 for a recent review).

The development of selective anticorticoids (of the spironolactone family, which are used in therapeutics) is more restricted. Those inhibitors seem to belong to the class of rapidly dissociating anti-MCs (7). Another synthetic steroid described as an anti-MC, ZK91587, shows specific binding

properties for kidney (8) and hippocampus type I MR (9), but not for type II GR. It may therefore be conveniently used as a tool in the investigation of MR function in tissues containing both receptor systems.

A long-lasting search for a GC antagonist finally succeeded with the development of the 11β-aminophenyl-substituted 19-norsteroid RU38486, later shortened to RU486, in the early 1980s (10). It soon became apparent, however, that this compound also possessed strong antiprogesterin activity, which led to its applications as a contraceptive/contragestive agent (see Ref. 11 for a review). Avoiding cross-reactions between GR and PR is still an unachieved goal for pharmacologists.

The present work is the culmination of several studies on a novel synthetic steroid, 21OH-6OP, that suggest that this compound is a selective antigluco-corticoid that is unable to cross-react with uterus-PR or kidney-MR. The new structure has been developed as a result of our systematic studies on MC potencies on a series of 21-deoxysteroids, of which the highly bent 6OP (conformation of the skeleton is shown in Fig. 1) exhibited practically no Na⁺-retaining properties (5). Preliminary studies also showed lack of GC properties for this steroid. For the current work, we introduced a 21-hydroxyl group into its structure to ascertain the possibility of conferring GC or anti-GC properties to the 6,19-oxidopregnane skeleton.

Materials and Methods

Reagents. ALDO, corticosterone, P₄, and DXM were purchased from Sigma Chemical (St.Louis, MO). 6OP and its 21-hydroxylated

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ABBREVIATIONS: GR, glucocorticoid receptor; GC, glucocorticoid; MC, mineralocorticoid; MR, mineralocorticoid receptor; PR, progesterone receptor; P₄, progesterone; 21OH-6OP, 21-hydroxy-6,19-oxidoprogesterone; 6OP, 6,19-oxidoprogesterone; ALDO, aldosterone; DXM, dexamethasone; TEGM, Tris/EDTA/glycerol/mercaptoethanol; CBG, transcortin; RBA, relative binding affinity; TAT, tyrosine aminotransferase.

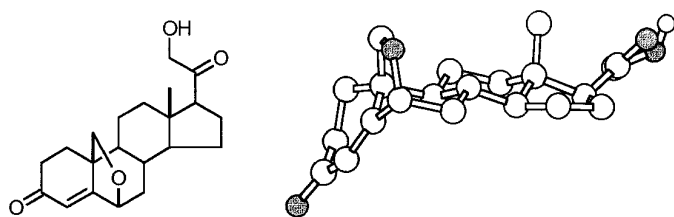


Fig. 1. Planar structure and most stable conformer of 21OH-6OP. Geometry optimizations were carried out with AMPAC 5.0 (Semichem, Shawnee, KS) using the AM1 semiempirical method.

derivative 21OH-6OP (Fig. 1) were synthesized as described previously (Refs. 12 and 13, respectively). [^3H]Corticosterone (specific activity, 80 Ci/mmol), [^3H]ALDO (specific activity, 59 Ci/mmol), [^3H]P₄ (specific activity, 90 Ci/mmol), and [^3H]ZK91587 (specific activity, 86.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA). RU28362 was a kind gift from Roussel-Uclaf (Romainville, France). All reagents used were of analytical grade.

Binding assays. Adrenalectomized male Sprague-Dawley rats were bled to death by heart puncture. Cold 0.9% NaCl was injected profusely through the aorta until organs were completely blanched. Thymuses were used as a source of GR and kidneys as a source of MR. Homogenization and incubation conditions were performed as described previously (Refs. 14 and 15, respectively). Briefly, homogenates performed in cold TEGM buffer (0.1 M Tris-HCl, 10 mM EDTA, 10 mM β -mercaptoethanol, 20 mM Na₂MoO₄, 25% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 2.0 TIU/ml aprotinin and 1 mg/100 ml leupeptin) at pH 7.4 were centrifuged at $37,000 \times g$ for 30 min at 0°. Supernatants from this centrifugation are referred to as "cytosol." Thymus or kidney cytosols were incubated 12 hr at 0° in the presence of 5 nM [^3H]corticosterone or [^3H]ALDO, and increasing amounts of unlabeled steroids. [^3H]P₄ binding to uterus PR was assayed on immature female rats according to the method of Jordan *et al.* (16).

RU28362 (1.0 μM) was added to kidney cytosols to impede [^3H]ALDO cross-reacting with GR, and 1 μM cortisol, to uterus cytosols to prevent [^3H]P₄ cross-reactions with GR and CBG. RBA for partially purified CBG was determined by steroid competition for [^3H]corticosterone binding as described previously (14).

Bioassays. Liver glycogen deposition was measured on adrenalectomized male Sprague-Dawley rats (180–220 g) as follows: steroids were dissolved in ethanol/propylene glycol/0.9% NaCl solution (3:3:34) and 100 μg of steroid/100 g of body weight were injected intramuscularly on the previous night. On the morning of the experiment, this dose was reinjected intraperitoneally. Three hours later, the rats were killed by cervical dislocation and livers were removed immediately. Glycogen purification and quantification were carried out according to the method of Krisman (17). Na⁺-retaining properties were measured as described (5). TAT was induced in rat hepatocytes isolated by the procedure of Fry *et al.* (18). TAT activity was determined according to the method of Granner and Tomkins (19).

Results

Fig. 2 shows the binding properties of 21OH-6OP to GR from rat thymus (Fig. 2A), CBG partially purified from rat plasma (Fig. 2B), and MR from rat kidney (Fig. 2C). We have included the displacement curve of 6OP for comparative purposes.

This steroid exhibits a moderate RBA for GR ($K_d = 125$ nM) and a low RBA for CBG ($K_d = 1.27$ μM). It does not compete efficiently with [^3H]ALDO for MR, even when a thousandfold excess was added. The introduction of a functional hydroxyl group at 21 increased binding properties of 6OP to GR by more than 40-fold and binding to CBG by 100-fold. This introduction does not improve the binding of the 6,19-oxido-pregnane skeleton to MR (Fig. 2C).

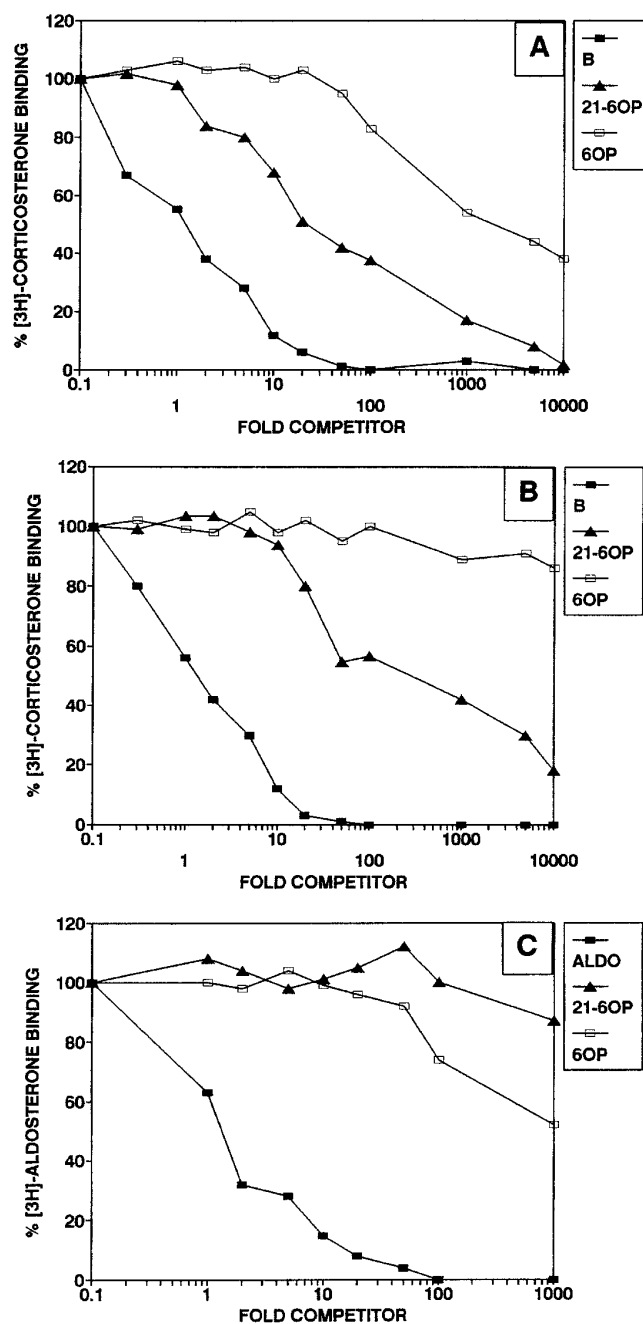


Fig. 2. Binding properties of 21OH-6OP. Displacement curves of 5 nM [^3H]corticosterone and increasing amounts of unlabeled steroid were performed for 12 hr at 0° using the following binding proteins: A, thymus cytosol (6.0 mg protein/ml). B, partially purified CBG (1 mg protein/ml) (see Materials and Methods). C, competition of unlabeled steroids with 5 nM [^3H]ALDO for kidney cytosol (10 mg protein/ml) in the presence of 1.0 μM RU28362 to prevent cross-reactions with GRs. Results are expressed as mean \pm standard error of five experiments. Hundred percent and nonspecific binding (i.e., in the presence of a 1000-fold excess of radioinert tracer) were $19,613 \pm 594$ and 543 ± 67 cpm (five experiments) for A, $18,818 \pm 853$ and 272 ± 35 cpm (five experiments) for B, and $12,626 \pm 477$ and $2,718 \pm 167$ cpm for C (five experiments). B, corticosterone.

Fig. 3 shows biological properties using *in vitro* and *in vivo* methodologies. Compared with adrenalectomized controls, DXM or corticosterone produce 3–4-fold more liver glycogen deposits and increases TAT activity by a 5–6-fold. Despite the increase in RBA for GR, the 21-hydroxylation was unable

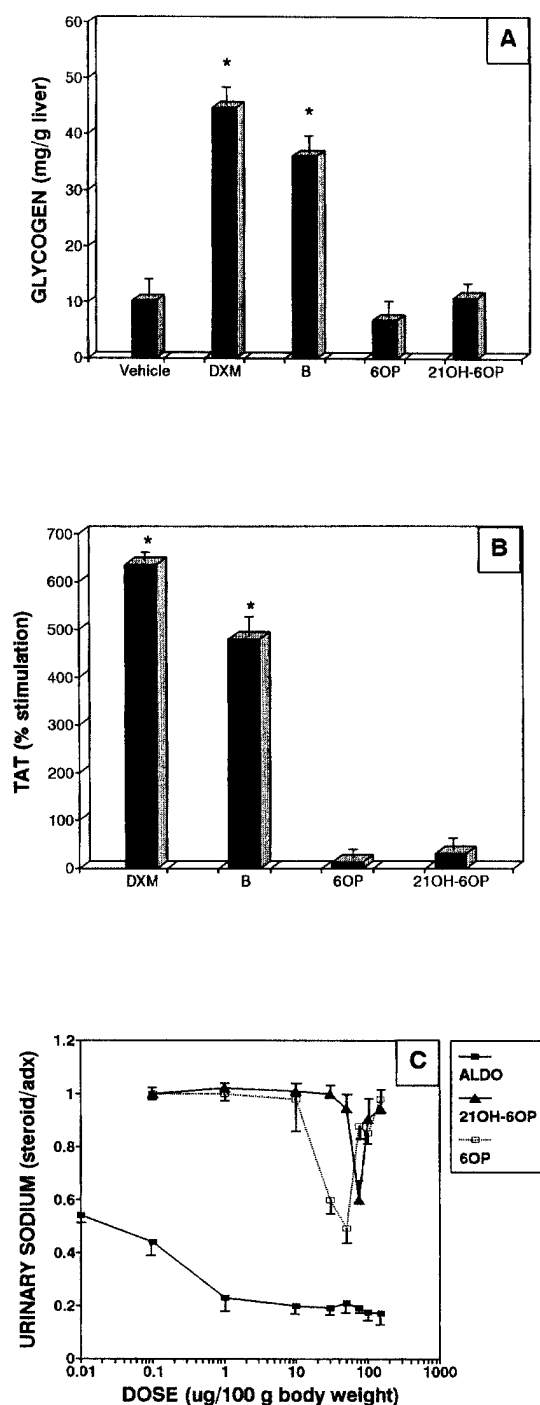


Fig. 3. Biological properties of 21OH-6OP. A, Glycogen storage *in vivo*: 100 μ g of steroid/100 g of body weight of male rats were injected as indicated in Materials and Methods. Liver glycogen was purified and quantified with Krisman's reagent (17). Controls were injected with vehicle. Results are expressed as mean \pm standard error of six experiments. *, different from controls $p < 0.02$. B, TAT induction *in vitro*. Rat hepatocytes were incubated for 5 hr in Ham's/F12 medium in the presence of 100 nM steroid and TAT activity was measured. Results are expressed as percentage of enzyme activity (mean \pm standard error of four experiments) over controls incubated in the presence of vehicle. *, different from controls $p < 0.01$. C, Sodium-retaining activity. Adrenalectomized rats were treated with the indicated dose of steroids. Results are expressed as the ratio of sodium-excretion rates of steroid-injected animals to the excretion rates of rats injected with vehicle. The figure shows one representative experiment performed with five rats per dose assayed.

to trigger GC responses. In effect, Fig. 3 shows that neither 6OP nor 21OH-6OP evoke these responses.

21OH-6OP is almost devoid of MC properties compared with the already weak Na^+ -retainer 6OP (Fig. 3C). Like other weak Na^+ retainers, 6OP and 21OH-6OP exhibit a parabolic dose-response function with maxima of 40% retention for 21OH-6OP and 50% for 6OP at high doses, then lose activity at still higher doses (also see Ref. 5).

To study the binding of ALDO to kidney MR without the competitive influence of the relatively more abundant GR, we removed GR with the specific ligand RU28362 (7). This avoided the cross-reaction of ALDO with GR; hence, a two-slope Scatchard plot was created (20–22). Both situations can be seen in Fig. 4.

The presence of 1.0 μM RU28362 reduces the plot to a single line corresponding to type I sites, as does the selective ligand for MR, ZK91587. The calculated K_d for type I sites in different conditions ranges between 0.4 and 1 nM (Table 1), which is at least 80-fold higher than the affinity for type II sites. When we added the novel synthetic steroid 21OH-6OP to incubations performed in the presence of [^3H]ALDO, a single binding site with kinetic parameters compatible with MR was again observed. This suggests that 2.5 μM 21OH-6OP is able to mask the cross-reaction of [^3H]ALDO with GR. Hill coefficients (n_H) calculated for each condition suggest a lack of allosteric interactions (Table 1).

In agreement with these binding properties, an antagonistic effect of 21OH-6OP against GC could be expected. Indeed, Fig. 5 shows that the novel pregnanesteroid antagonizes TAT induction by corticosterone and dexamethasone. The basal TAT activity in hepatocytes (Fig 5, *control*) was measured by adding vehicle (final concentration, 0.2% ethanol) to the culture. Neither 6OP nor 21OH-6OP facilitate TAT induction (▨) but 100 nM corticosterone (□) and 100 nM DXM (■) stimulate enzyme activity 5–6-fold. 6OP (2.5 μM) does not affect this stimulation. However, 2.5 μM 21OH-6OP inhibits 80% of TAT induction when coincubated with corticosterone or DXM.

Fig. 6 shows that 21OH-6OP displaces [^3H]P₄ from uterus receptors as weakly as the negative control 17 β -estradiol (E_2). Unlabeled P₄, in contrast, was able to displace the tracer efficiently.

Discussion

The data presented in this article demonstrate that 21OH-6OP may be a useful tool for studying steroid-receptor interactions in systems in which more than one type of receptor is present. The RBA for GR refers to the mean affinity of [^3H] corticosterone in thymus cytosols (125 nM). In spite of this moderate affinity, 21OH-6OP showed no ability to interact with MR or PR. It was also able to block type II receptors in kidney cytosols, where GR coexists not only with MR but also with a high level of CBG of dual origin [an unavoidable contamination by plasma (22, 23) and biosynthesis by the kidney (24)]. Fig. 2B indicates that 2.5 μM 21OH-6OP can simultaneously block almost 60% of the CBG binding sites. Fig. 2A shows only 75% of tracer displacement instead of the 95% saturation predicted according to mass action, perhaps because the CBG of dual origin was acting as a competitor for GR. (22, 23).

The induction of TAT activity in hepatocytes, triggered by both corticosterone and DXM, could be significantly inhibited

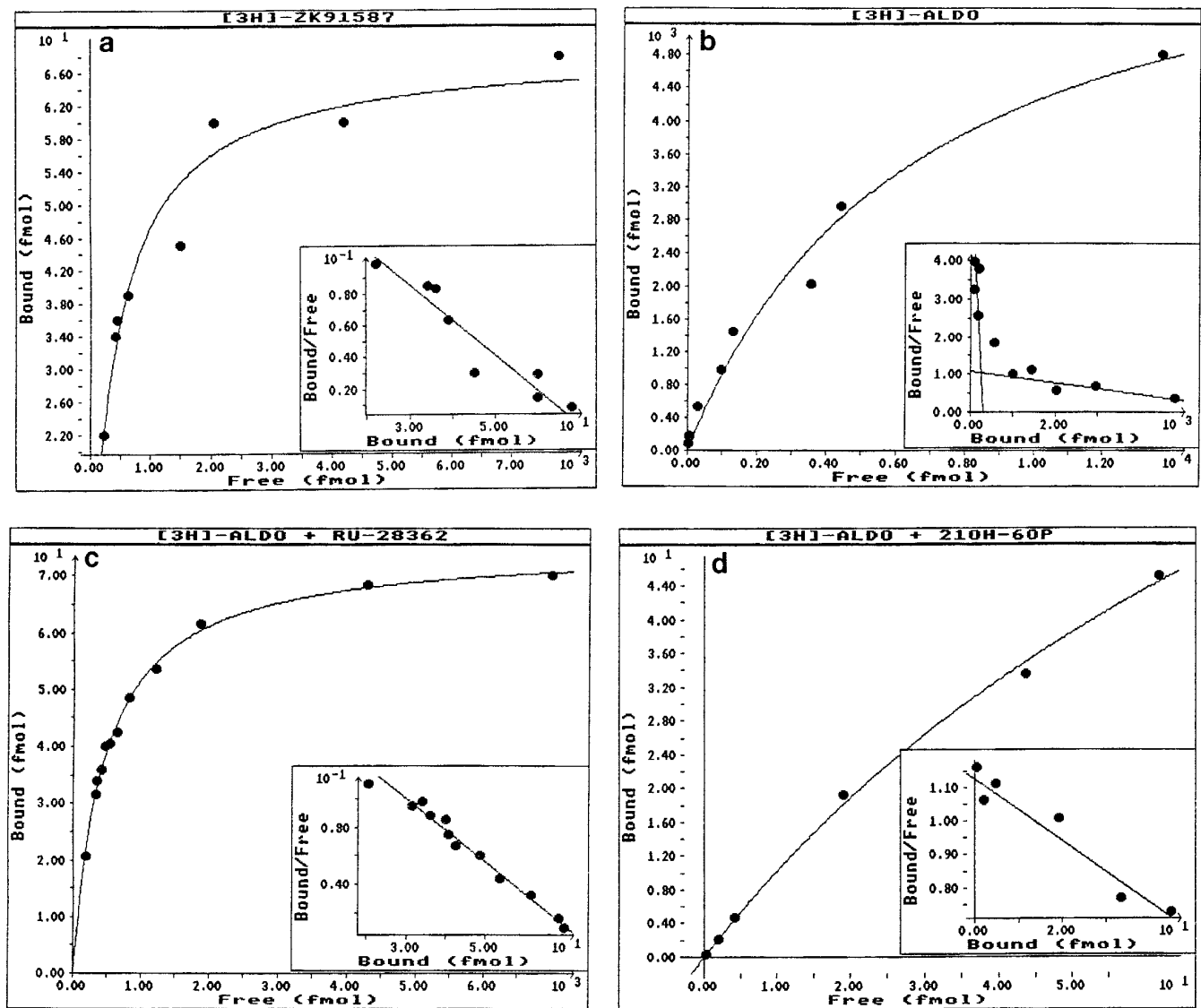


Fig. 4. 21OH-60P is an efficient blocking steroid for kidney-type II sites. Scatchard plots were performed on kidney cytosols (8 mg protein/ml) in TEGM buffer. Incubations for 4 hr at 0° were performed using [³H]ZK91587 as specific ligand for type I sites (A), [³H]ALDO alone (B), [³H]ALDO plus 1.0 μM RU28362 to mask type II binding sites (C), and [³H]ALDO plus 2.5 μM 21OH-60P (D). Values calculated for the binding constants are shown in Table 1.

TABLE 1
Dissociation constants, apparent number of sites and Hill coefficients for type I receptors in the presence of RU-28362 and 21OH-60P.
For methods, see Figure 4.

Tracer	Receptor type	In the presence of	Kd	Q	n _H
			nm	fmol/mg	
[³ H]ZK91587	I		0.42	98	0.91
[³ H]ALDO	I	1.0 μM RU-28362	0.44	104	0.97
[³ H]ALDO	I	2.5 μM 21OH-60P	0.72	124.4	0.90
[³ H]ALDO	I		0.80	110	0.88
	II		64.4	2900	0.86

by 21OH-60P, indicating that this novel steroid may be a potential GC antagonist. Because it does not cross-react with PR (Fig. 6), the oxidopregnanesteroid could be interesting as a potential specific GR blocking agent. To our knowledge, a pure anti-GC has still not been developed. In effect, most of

the antiprogestins and antiglucocorticoids available cross-react with other receptors (7).

One may imagine a number of possible mechanisms by which an antihormone may interfere with the agonist; the most common is interference with steroid binding by direct or indirect (allosteric) inhibition. However, recent findings relevant for the comprehension of receptor activation advise caution. Thus, dopamine and epidermal growth factor can affect the transformation of native hormone receptors to their active forms even though they do not seem to bind to the receptor (25). It was also suggested (26) that the insertion of the steroid between DNA strands plays an important role in transactivation. We are performing preliminary studies that suggest a competitive mechanism against glucocorticoid binding. However, there is no reason to believe that the antihormone binds to a given site similarly to the agonist. Because the binding is only the first event in a very sophisticated and still unclear cascade leading receptors to act as

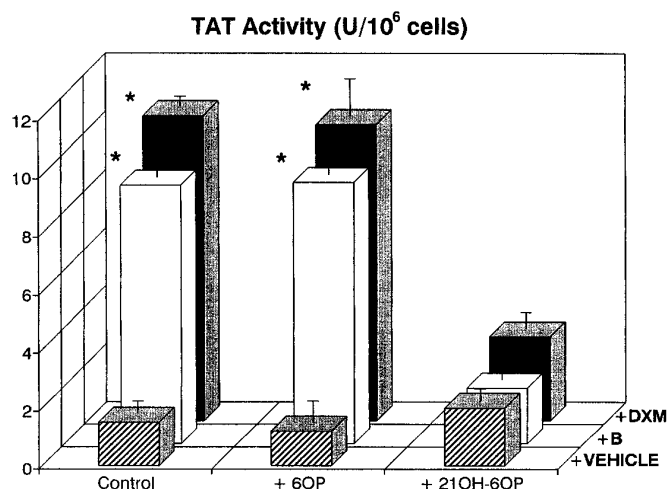


Fig. 5. 21OH-6OP shows antagonistic effect on TAT induction. TAT activity was induced in hepatocytes by 100 nM DXM (■) or 100 nM DXM (□), in the absence (control) or presence of 2.5 μ M 6OP (+ 6OP) or 21OH-6OP (+ 21OH-6OP). ▨, Incubations performed in the presence of vehicle instead of agonist; *, significantly different ($p < 0.01$) from basal activity. Results are presented as mean \pm standard error of four experiments.

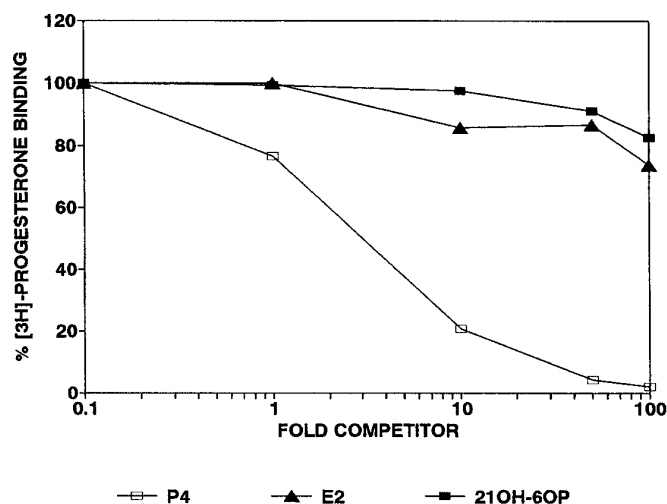


Fig. 6. 21OH-6OP does not cross-react with progesterone receptor. [³H]P₄ binding to uterus cytosols (2.5 mg protein/ml) in TEGM was performed according to the method of Jordan and Dix (16) for 12 hr at 0°, in the presence of different amounts of unlabeled competitors. Hundred percent and nonspecific binding (i.e., in the presence of a 1000-fold excess of radioinert progesterone) were 35,851 \pm 1,115 and 8,308 \pm 950 cpm (five experiments).

transcription factors, we still cannot speculate on a given molecular mechanism of action for 21OH-6OP.

In conclusion, this work indicates that 21OH-6OP binds almost exclusively to type II receptors but lacks biological activity and antagonizes GC responses. It may therefore be conveniently used as a tool for studying steroid-receptor interactions. The fact that 21OH-6OP, in contrast to other known antisteroids, is virtually devoid of affinity for MR and PR suggests some advantages for *in vitro* and even *in vivo* studies.

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