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Regulation of activities of steroid hormone receptors by tibolone and its primary metabolites

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ABSTRACT

This work was undertaken (i) to study deeply the estrogen, androgen and progestative activities of tibolone and its metabolites (ii) to determine whether tibolone and its metabolites present glucocorticoid or mineralocorticoid activity. For this purpose, we used human cell lines bearing a luciferase gene with a responsive element under the control of human estrogen receptor α (ERα) or estrogen receptor β (ERβ) or androgen receptor (AR) or chimeric Gal4 fusion with progesterone receptor (PR), glucocorticoid receptor (GR) or mineralocorticoid receptor (MR). The major tibolone metabolites, the two hydroxymetabolites, bind and activate ER with a preference for ERα. Tibolone and the Δ^4-tibolone are agonists for AR and PR and surprisingly 3α- and 3β-OH-tibolone are antagonists for them. Moreover we showed for the first time that tibolone and its primary metabolites are GR and MR antagonists with a stronger affinity for MR than for GR. In conclusion, tibolone by these actions on different receptors and by this capacity to transform in different metabolites, has more complex effects than initially supposed.

1. Introduction

Tibolone (Org OD14), a synthetic 19-norsteroid related to norethynodrel (NED) is an interesting alternative for hormonal treatment of menopause. In randomized studies, tibolone has been shown to improve menopausal symptoms to a similar degree of combined estrogen/progestogen hormonal therapy [1–3]. Moreover, clinical trials showed its interest on bone density [4,5], since tibolone acts as a bone resorption inhibitor in a similar way to estrogens. In two cohort studies in the UK a small increase in endometrial cancer was observed [6,7], but the results of a randomized controlled trial (the THEBES trial) does not show any stimulation of endometrial proliferation [8]. In this study, tibolone shows less breast pain than conjugated equine estrogen (CEE) plus medroxyprogesterone acetate (MPA). Tibolone acts as a pro-drug and is rapidly converted into 3 main metabolites. In plasma, the 3α-OH-tibolone is the main metabolite [9] followed by the 3β-OH-tibolone. Tibolone and the Δ^4 isomer are only present during the first 6 h after oral intake.

The Δ^4 isomer has progestin and androgen properties while the 3α- and the 3β-OH-tibolone have estrogenic properties [10]. The tissue selective action of tibolone is supposed to be a combination of receptor activation and steroid metabolism [11].

Using *in vitro* transactivation measurements with Chinese Hamster Ovary (CHO) cells, it has been demonstrated that tibolone and Δ^4-tibolone were agonistic ligands of progesterone receptor (PR), androgen receptor (AR) and estrogen receptor (ERs) whereas 3α- and 3β-hydroxytibolone were only active on ERs [10]. CHO cells were chosen because the metabolism of the major sex steroids was very limited and the intrinsic hormonal properties of synthetic steroids could be accurately determined [12]. Potential disadvantages of the CHO cells are that they contain a different pallet of co-activators and corepressors than the human target cells. Ideally the receptor–reporter systems should be transfected in a cell line derived from the target tissue, but unfortunately such cell lines

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are not always accessible. Here we have used the Hela human cell line transfected with human ERs, PR, GR or MR and a reporter system. ERα and ERβ reporter cells were obtained by transfecting HeLa cells (ER negative) successively by the ERE-βGlob-Luc-SVNeo plasmid (HELN cell line) and pSG5puro plasmids expressing ERα or ERβ (HELN ERα and HELN ERβ cells). Since the ERs cell lines are derived from the same HELN cell line, the effect of both ERs is determined by the same ERE. PR, GR and MR recognize the same hormone responsive element. As HeLa cells express GR, we constructed chimeric receptors in which each part ensures to obtain the required hormonal specificity: one part is the hormone binding domain of PR, GR or MR, the other part is the DNA binding domain of yeast Gal4 protein. After binding with the steroid hormone these chimeric receptors are able to interact with the Gal4 responsive element placed upstream the luciferase gene. Thus, PR, GR and MR reporter cells were obtained by transfecting HeLa cells successively by the Gal4RE5-bGlob-Luc-SVNeo plasmid (HG5LN cell line) and the pSG5puro plasmids fused with the Gal4 DBD and the LBD of PR, MR or GR (HG5LN Gal4-PR, -MR and -GR cells). This assay format eliminated background activity from endogenous receptors allowing quantification of relative activity for the three steroid receptors with the same reporter gene. This strategy could not be used for AR since the A/B domain deleted receptor does not transactivate. Thus we developed a PC3 cell line expressing the complete human AR and the same responsive reporter gene as used for GR, MR and PR.

In the present study, we used these different bioluminescent reporter cell lines to confirm that tibolone and its primary metabolites are ligands for human PR, AR and/or ER. Interestingly, our data indicate that these compounds are also able to bind glucocorticoid receptor (GR) and mineralocorticoid (MR) and exhibit low antiglucocorticoid and moderate antimineralocorticoid activities. Altogether, our study point out that tibolone and its primary metabolites exert multiple and complex effects on all steroid hormone receptors.

2. Materials and methods

2.1. Chemicals and materials

Materials for cell culture came from Life Technologies (Cergy-Pontoise, France) except 96-well Cellstar plates, which were obtained from Greiner labortechnic (Poitiers, France). Luciferin (sodium salt) and geneticin were purchased from Promega (Charbonnières, France). R1881 was purchased from NEN Life Science Products (Paris, France). [^{3}H]-aldosterone (39 Ci/mmol specific activity) and [^{3}H]-dexamethasone (84 Ci/mmol) were purchased from Amersham-GE Healthcare Europe GmbH (Orsay, France). R5020 (promegestone) was a gift from Sanofi-Aventis (Romainville, France). 17β-Estradiol (E2), testosterone, aldosterone (ALDO), dexamethasone (DEX), puromycin and aminoglutethimide (AG) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Tibolone [(7α,17α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one]; Δ^4-tibolone [(7α,17α)-17-hydroxy-7-methyl-19-norpregn-4-en-20-yn-3-one]; 3α-OH-tibolone [(3α,7α,17α)-7-methyl-19-norpregn-5(10)-en-20-yne-3,17-diol]; 3β-OH-tibolone [(3β,7α,17α)-7-methyl-19-norpregn-5(10)-en-20-yne-3,17-diol] were supplied by the department of Medicinal Chemistry of Organon, part of Schering-Plough, Oss, The Netherlands. These ligands were dissolved in dimethyl sulfoxide (DMSO) at 10^{-2} M and dilutions from this stock solution were prepared in culture medium.

2.2. Reporter cell lines for human ER, AR, PR, GR and MR

The HELN ERα and HELN ERβ reporter cell lines were previously described [13,14] and cultured in DMEM without phenol red, supplemented with 6% dextran-coated, charcoal-treated FCS, 1 mg/ml G418 and 0.5 μg/ml puromycin.

To study the potential activity of tibolone and its metabolites *via* the AR, we used the PALM cell line obtained from PC3 cells stably transfected with the complete human AR and a luciferase gene under transcriptional control of MMTV [15]. HG5LN cells were obtained by transfecting HeLa cells successively by the Gal4RE5-βGlob-Luc-SVNeo plasmid and by pSG5puro plasmids fused with the Gal4 DBD and the LBD of PR, MR or GR) [16]. HG5LN Gal4-PR, Gal4-MR and Gal4-GR were cultured in DMEM with phenol red, supplemented with 5% FCS, 1 mg/ml G418 (geneticin) and 0.5 μg/ml puromycin. In some experiments, aminogluthetimide (AG) was added to the culture medium at a concentration of 50 μM to inhibit the intrinsic aromatase activity in HeLa cells. PALM cells were obtained as already described [15] and cultured in Ham's F12, supplemented with 5% FCS, 1 mg/ml G418 and 1 μg/ml puromycin.

2.3. Luciferase assays: stable gene expression assays for ERs, AR, PR, GR and MR

Reporter cells were seeded at a density of 2×10^4 cells per well, in 96-well white opaque tissue culture plates and maintained in DMEM without phenol red, supplemented with 6% FCS treated with dextran-coated charcoal (DCC) (except for PALM cells, which were maintained in Ham's F12, supplemented with 6% DCC–FCS). Tibolone and its metabolites were tested at concentrations range (see legends) in at least three independent experiments performed in quadruplicate. The assays were used for testing both agonistic and antagonistic activities of tibolone and its metabolites. The reference compounds for ERs, AR, PR, GR and MR were E2, R1881, R5020, DEX and ALDO, respectively. For testing the antagonistic activity the cells were stimulated with a concentration (see text and legends) of the reference compound and co-incubated with a dose range of tibolone or one of its metabolites.

The luciferase substrate was added after 8 h, 16 h or 40 h (40 h only with PALM cell line) of treatment, the medium containing effectors was removed and replaced by culture medium (DMEM without phenol red or Ham's F12, supplemented with 6% DCC FCS) containing 0.3 mM luciferin. At this concentration, luciferin diffuses into the cell and, about 5 min later, produces a stable luminescent signal which was quantified using a Microbeta Wallac luminometer (integration during 2 s).

2.4. Ligand binding assays for GR and MRy

HG5LN-GalGR or HG5LN-GalMR cells were seeded at a density of 10^5 cells/well in 24-well tissue culture plates and grown in 6% DCC–FCS. For ligand competition experiments, tibolone and its metabolites were tested at least three times. Cells were labeled with 3 nM of [1,2,4,6,7-^{3}H] Dexamethasone or 1 nM of [1,2-^{3}H]-aldosterone at 37 °C for 3 h in the absence or presence of increasing concentrations of non-radioactive competitive compounds.

The final incubation volume was 400 μl and each dilution was performed in duplicate. After incubation, unbound material was aspirated and cells washed three times with 400 μl of cold PBS. Then, 250 μl lysis buffer (400 mM NaCl, 25 mM Tris phosphate pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% triton X-100) was added and plates were shaked for 5 min. Total cell lysate (200 μl) was mixed with 4 ml of LSC-cocktail (Emulsifier-Safe, Packard BioScience) and [^{3}H] bound radioactivity was liquid scintillation counted (LS-6000-SC, Beckman-Coulter, Roissy, France). Protein concentrations were measured by Bio-Rad protein assay and used to normalize bound radioactivity values expressed in dpm. Results were plotted as measured dpm versus concentration of tested compound. IC_{50} values were defined as compound concentration required decreasing maximum [^{3}H]-compound binding by 50%.

2.5. Data analysis

For each test, independent experiments were performed in triplicate for each concentration and data are shown as mean ± SD. Individual concentration–response curves, for the agonistic and antagonistic assays, were fitted using the sigmoidal dose–response function of a graphics and statistics software (Graph-Pad Prism, version 4.0, 2003, Graphpad Software Incorporated, San Diego. CA). Agonistic transactivation data are presented as EC_{50} values, effective concentration for half-maximal luciferase activity and antagonistic transactivation data as IC_{50}, half-maximal inhibitory concentration for each compound tested. Binding data are presented as IC_{50} values, effective concentration to decrease maximum [^{3}H]-compound binding by 50%.

3. Results

3.1. In vitro estrogen receptors studies

We used HELN ERα and HELN ERβ cell lines [13,14] to evaluate the estrogenic activities of tibolone and its primary metabolites. In HELN parental cells, neither E2 nor tibolone and its metabolites induced luciferase expression. On the contrary, a small decrease was observed for tibolone and its metabolites at concentrations higher than 1 μM. This is probably due to toxicity (data not shown). In HELN ERα and HELN ERβ cell lines, the EC_{50} value of E2 was respectively 0.018 and 0.08 nM (Fig. 1A and B). Tibolone and its metabolites were full agonists for ERs but their potency appeared very low compared to E2 (Fig. 1A and B). In HELN ERα cells, 3α-, 3β-OH-tibolone and tibolone displayed higher activity on ERα transactivation (EC_{50} of 1.7, 2.4 and 4.3 nM, respectively) than Δ^4-tibolone (EC_{50} of 26 nM). By contrast, tibolone and its derivatives were less active on ERβ. 3 compounds have the same potency to activate ERβ (EC_{50} of 3α-hydroxytibolone 100 nM, EC_{50} of tibolone 105 nM, EC_{50} of 3β-hydroxytibolone 115 nM) compared to Δ^4-tibolone (EC_{50} of 300 nM).

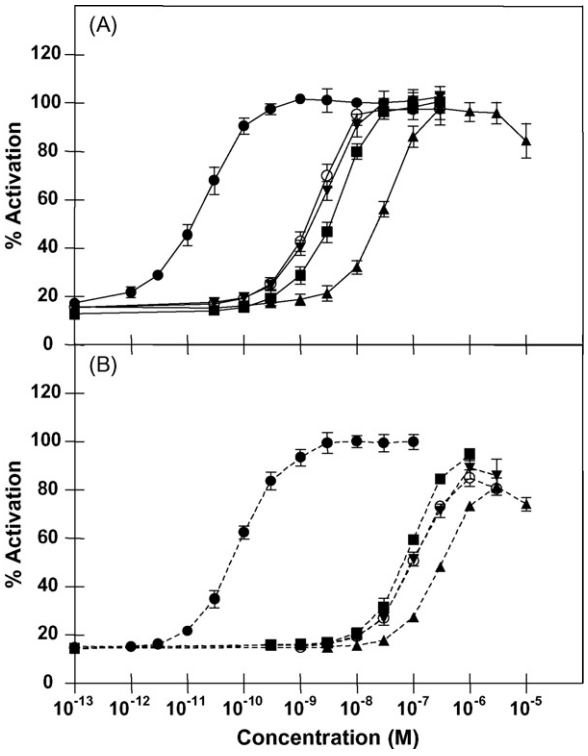

Fig. 1. Transcriptional activity as % activity of ERα and ERβ in response to tibolone and metabolites. HELN-ERα (A) and HELN-ERα (B) cell lines were treated with E2 (●), tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) at the indicated concentrations for 16 h. Maximal activity (100%) corresponds to the activity obtained with 10 nM E2. Values are mean ± S.D. from three independent experiments.

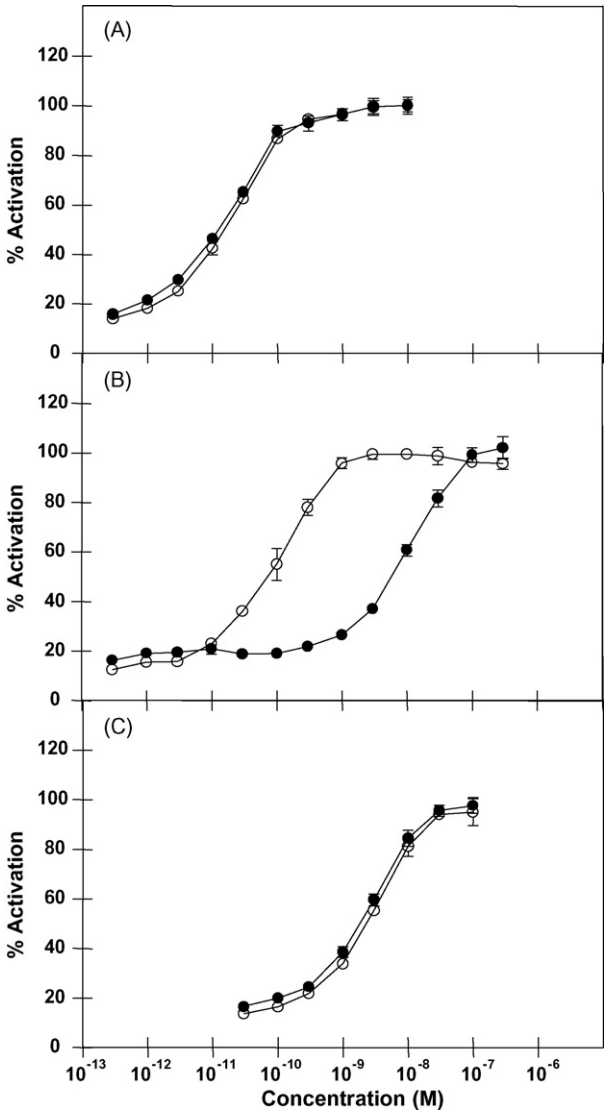

Fig. 2. Transcriptional activity % activity of ERα in response to tibolone and metabolites with or without aminogluthetimide (AG). HELN-ERα cell lines was treated with E2 (A), testosterone (B) or tibolone (C) without (○) or with (●) aminogluthetimide, at indicated concentrations for 16 h. Maximal activity (100%) corresponds to the activity obtained with 10 nM E2. Values are mean ± S.D. from three independent experiments.

Parental HELN cells contain aromatase activity. In order to demonstrate that the estrogenic activity of tibolone was not dependent on its aromatization, we measured its activity in the presence of aminogluthetimide (AG), an inhibitor of aromatase. The positive control, testosterone (which is transformed in estradiol by aromatase) was found to be less active in stimulating HELN ERα cells treated with AG than in cells untreated with AG (Fig. 2B). On the contrary, the estrogenic activity of estradiol was not modified in the presence of AG (Fig. 2A). As shown in Fig. 2C, the estrogenic activity of tibolone was not modified in the presence of AG indicating that it was not due to its aromatization. Furthermore, tibolone is able to bind ER with an affinity which reflects its transcriptional activity [data not shown, 10]. Tibolone and its metabolites did not show antagonistic activity for the ERs.

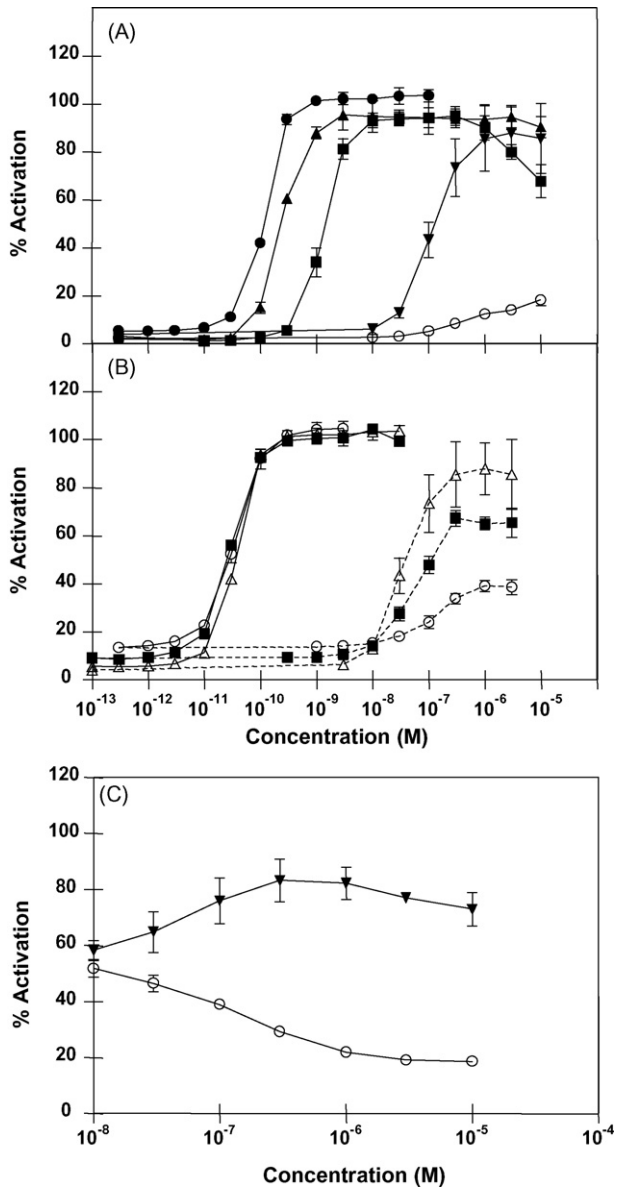

Fig. 3. Transcriptional activity % activity of AR in response to tibolone and metabolites in different condition treatments. PALM cell line (A) was treated with R1881 (●), tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) at the indicated concentrations for 40 h. Maximal activity (100%) corresponds to the activity obtained with 100 nM R1881. Values are mean ± S.D. from three separate experiments. PALM cell line (B) was treated with R1881 (----) or 3β-OH-tibolone (- - - - - -) at the indicated concentrations for 8 h (○), 16 h (■) and 40 h (△). PALM cell line (C) was treated with 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) in the presence of R1881 1 nM. Values are mean ± S.D. from three separate experiments.

3.2. In vitro androgen receptor studies

To study the potential activity of tibolone and its metabolites *via* the AR, we used the PALM cell line obtained from PC3 cells stably transfected with human AR and a luciferase gene under transcriptional control of MMTV [15]. As shown in Fig. 3A, the EC_{50} value for the synthetic androgen R1881 in PALM cells was 0.1 nM. Tibolone and the Δ^4 isomer behaved as full agonists for AR (Fig. 3A), the Δ^4 isomer displaying the best transactivation potency (EC_{50} 0.2 nM vs 1.05 nM). The activity of 3β-OH-tibolone was variable (see error bars), which may be caused by metabolism. We tested therefore 3β-OH-tibolone during shorter incubation times. R1881 showed the same transactivation for all three times of incubations, but the AR transactivation increased with exposure time (Fig. 3B). 3β-OH-tibolone was only partially active after 8 h of incubation but it became more active after 16 h and 40 h of treatment (Fig. 3B). These results suggest that 3β-hydroxylated tibolone can be progressively transformed in an active androgen which might be tibolone or the Δ^4 isomer. Interestingly, this did not occur with the 3β-hydroxylated tibolone but acted surprisingly as an antagonist (IC_{50} 135 nM in the presence of 1 nM R1881) (Fig. 3C). Tibolone, Δ^4 isomer and 3β-OH-tibolone expressed no antagonistic AR activity.

3.3. In vitro progesterone, glucocorticoid and mineralocorticoid receptors studies

HeLa cells were chosen as the host cell line for the luciferase reporter gene driven by a pentamer of the GAL4 recognition sequence in front of the β-globin promoter. This reporter system is insensitive to endogenous receptors which cannot recognize the GAL4 binding site. These cells called HG5LN cells were then transfected with the DNA binding domain of the yeast transactivator GAL4 fused to the EF domain (which contain the LBD and the AF-2 activation function) of human PR (GAL4-PR), human GR (GAL4-GR) or human MR (GAL4-MR) [16]. R5020, dexamethasone, aldosterone, tibolone or its metabolites did not induce luciferase expression in the parental cell line HG5LN (data not shown).

In HG5LN Gal4-PR, the EC_{50} value for the progestin R5020 was 5 nM. Tibolone and the Δ^4 isomer were full PR agonists (Fig. 4A). The Δ^4 isomer displayed the highest potency to transactivate AR (EC_{50} 46 nM) as compared to tibolone which was less active (EC_{50} 123 nM). By contrast, the 3-hydroxylated metabolites of tibolone did not activate PR (Fig. 4A).

Using respectively HG5LN Gal4-GR and HG5LN Gal4-MR cells, we determined that the EC_{50} values were 6 nM for dexamethasone (Dex) (Fig. 4B) and 0.36 nM for aldosterone (Aldo) (Fig. 4C). By contrast, using these reporter cell lines, we found that tibolone and its metabolites did not exhibit glucocorticoid or mineralocorticoid activity (Fig. 4B and C).

We then tested the antagonistic activity of tibolone and its metabolites on reference stimulated PR, GR and MR activity. In the presence of 5 nM R5020, the 3-hydroxylated compounds showed a weak activity of complete PR antagonist (IC_{50} corresponding to 20 μM for 3β-OH-tibolone and 2.4 μM for 3α-OH-tibolone) (Fig. 5A). All the tibolone compounds were also antagonists on GR upon activation using 10 nM Dex, but not completely (Fig. 5B). The IC_{50} value was 1.7 μM for the Δ^4 isomer, 10 μM for tibolone, and not measurable for 3α-OH-tibolone and 3β-OH-tibolone (Fig. 5B). Finally, as shown in Fig. 5C, tibolone and its metabolites were antagonists for MR (activated by aldosterone at 0.5 nM concentration). Again, the Δ^4 isomer was the better antagonist (IC_{50} of 30 nM) followed by tibolone (IC_{50} of 170 nM) and the 3-OH-tibolones (IC_{50} corresponding to 1.2 μM for 3α-OH-tibolone and 3.3 μM for 3β-OH-tibolone) (Fig. 5C).

3.4. Binding of tibolone to GR and MR

To determine whether the antagonistic effects of tibolone as observed in the GR and MR transactivation experiments reflected their binding to the receptors, we performed "whole-cell" competition binding assays using HG5LN-Gal4-GR and HG5LN-Gal4-MR cells. The binding affinities of tibolone and its metabolites for GR were assessed using 3 nM [^{3}H]-dexamethasone as a tracer. Δ^4-Tibolone was the most effective compound, inhibiting the binding of dexamethasone at a 10 μM concentration (Fig. 6A). The binding affinity of tibolone was very low but was able to significantly displace dexamethasone from GR at 10 μM (Fig. 6A). The least effective compounds were the 3-hydroxylated ligands, which showed low binding affinity for GR.

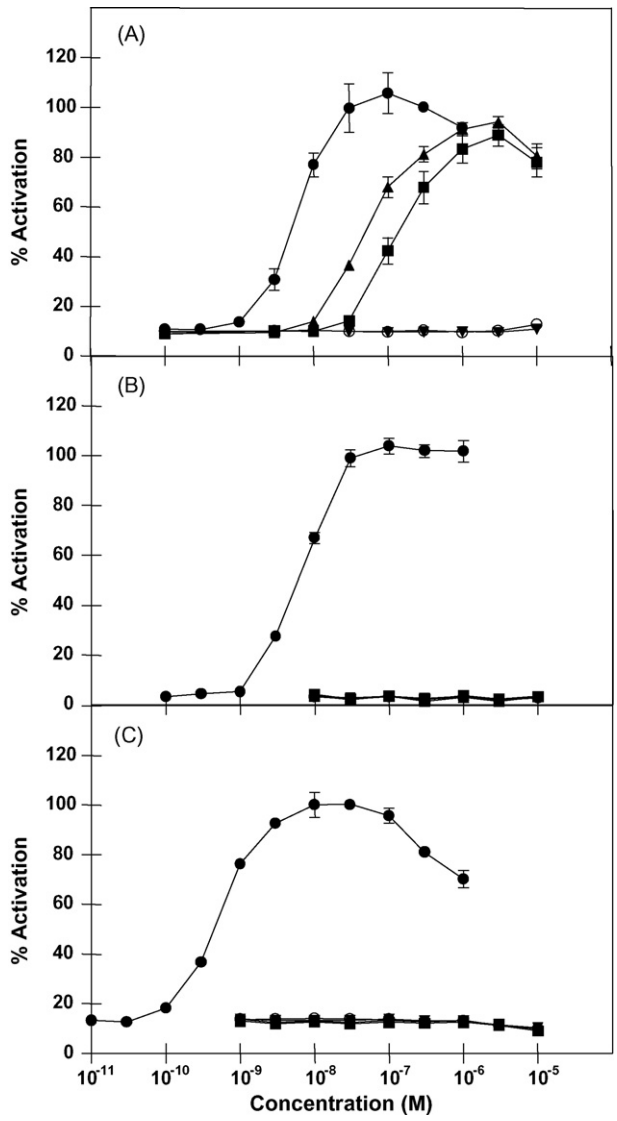

Fig. 4. Transcriptional activity % activity of PR, GR, MR in response to tibolone and metabolites. HG5LN-GalPR (A) was treated with R5020 (●), tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) at the indicated concentrations for 16 h. Maximal activity (100%) corresponds to the activity obtained with 100 nM R5020. HG5LN-GalGR (B) was treated with dexamethasone (●), tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) at the indicated concentrations for 16 h. Maximal activity (100%) corresponds to the activity obtained with 100 nM dexamethasone. HG5LN-GalMR (C) was treated with aldosterone (●), tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) at the indicated concentrations for 16 h. Maximal activity (100%) corresponds to the activity obtained with 10 nM aldosterone. Values are mean ± S.D. from three independent.

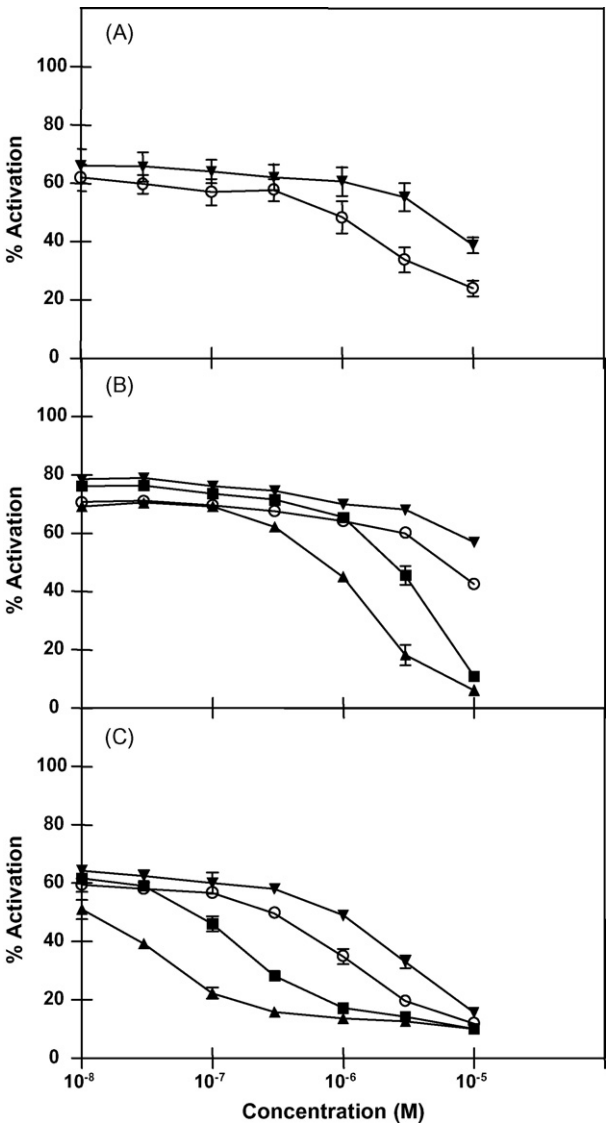

Fig. 5. Antagonistic transcriptional effects of tibolone and metabolites on PR, GR, and MR.
HG5LN-GalPR (A) was treated with 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) in the presence of R5020 5 nM. HG5LN-GalGR (B) was treated with tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) in the presence of Dexamethasone 10 nM. HG5LN-GalMR (C) was treated with tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▼) in the presence of aldosterone 0.5 nM. Values are mean ± S.D. from three independent.

Binding to MR was assessed using 1 nM [^{3}H]-aldosterone as a tracer. Tibolone and Δ^4 isomer inhibited [^{3}H]-aldosterone binding to MR in a dose dependent manner and complete inhibition was achieved at 1 and 10 μM respectively for tibolone and Δ^4-tibolone (Fig. 6B). On the contrary, the binding affinities of 3-hydroxylated compounds were very low (Fig. 6B).

4. Discussion

In this study, we have assessed the potential hormonal profile of tibolone and its primary metabolites on all human steroid receptors (PR, AR, GR, MR, ERα and ERβ) using HeLa or PC3 cells stably transfected with a given receptor and a luciferase reporter gene. We show that tibolone and its Δ^4 isomer predominantly bind and activate PR and AR whereas 3α and 3β-OH-tibolone predominantly bind and activate ERα (Table 1). The hydroxyl metabolites are full agonists for both ERs and bind preferentially ERα. The estrogenic activity of tibolone as shown here is not due to a conversion on a potent estrogenic metabolite by aromatase as it was suggested by Bodine et al. [17] and Wiegratz et al. [18] because an aromatase inhibitor does not influence the percentage of activation at different tibolone concentrations. In contrast, testosterone shows a clear effect of the aromatase inhibitor confirming that these cells contain the aromatase enzyme. The estrogenic activity of tibolone is therefore not dependent on aromatization. It is also not possible that the estrogenic activity of tibolone as shown here and by others [10] is due to direct binding to ERs, because tibolone does not contain the required hydroxyl-group. Our data demonstrating that 3α- and 3β-OH-tibolone have a better affinity for ERα than tibolone and its Δ^4 derivative are consistent with previous studies showing that compounds with a 3 keto Δ^4 configuration do not bind

Table 1
EC_{50} and IC_{50} values of tibolone and its metabolites on estrogen, androgen, progesterone glucocorticoid and mineralocorticoid receptors.

Receptors						
Compounds	ERα (nM) ± S.D.	ERβ (nM) ± S.D.	AR (nM) ± S.D.	PR (nM) ± S.D.	GR (nM) ± S.D	MR (nM) ± S.D.
Estradiol	0.018 ± 0.002	0.08 ± 0.01				
R1881			0.1 ± 0.035			
R5020				5 ± 3		
Dexamethasone					6 ± 2.5	
Aldosterone						0.36 ± 0.26
Tibolone	4 ± 1	105 ± 25	1.05 ± 0.65	123 ± 60	*2410 ± 1000**	*170 ± 60**
Δ^4-Tibolone	26 ± 10	300 ± 155	0.2 ± 0.07	46 ± 22	*1760 ± 760**	*30 ± 10**
3α-OH-tibolone	1.7 ± 0.5	100 ± 40	*135 ± 25**	*2400 ± 1200**	*ND**	*1160 ± 280**
3β-OH-tibolone	2.4 ± 0.4	115 ± 35	*ND**	*20000 ± 16600**	*ND**	*3310 ± 850**

EC_{50} values were concentrations required to produce half-maximal induction in different cell lines, value are indicated in black characters. IC_{50} values were concentrations required to produce half-maximal inhibition in different cell lines, value are indicated in black slanting characters with asterisk (*).

to ER and require at least a 3 hydroxyl group [19]. Most likely the cell lines contain aldo-ketoreductases, which can convert tibolone to the 3-hydroxymetabolites [20]. Larrea et al. [21] have shown that 19-nortestosterone derived progestagens can be converted to A-ring reduced metabolite, which are agonists for ERα.

Tibolone and its Δ4 metabolite are relatively strong agonists for AR. The effect of tibolone on androgen sensitive parameters is often thought to be contributed to the Δ4 metabolite of tibolone. However, Verheul et al. [22] have shown that tibolone and the Δ4 metabolite are present in target tissues in very low concentration, except in the liver. The direct androgenic effects of tibolone and Δ4 metabolite has therefore little impact on target tissues. The possible androgenic effect in tibolone users on libido [23] may well be explained by the observed decreased levels of SHBG, which cause increased levels of free testosterone [24]. Surprisingly the 3β-OH-tibolone stimulates the AR and this stimulation increases with prolonged incubation times. This indicates that the 3β-OH-tibolone is most likely metabolized to the Δ4 metabolite. Similarly, Schatz et al. have shown in human stromal cells that 3β-OH-tibolone stimulates progesterone sensitive parameters and they suggested that this can be explained by the presence of aldo-ketoreductases of which have been shown that they can convert 3β-OH-tibolone to Δ4 metabolite [20]. The anti-androgenic activity of the 3α-OH-tibolone is weak and occurs only at very high concentrations.

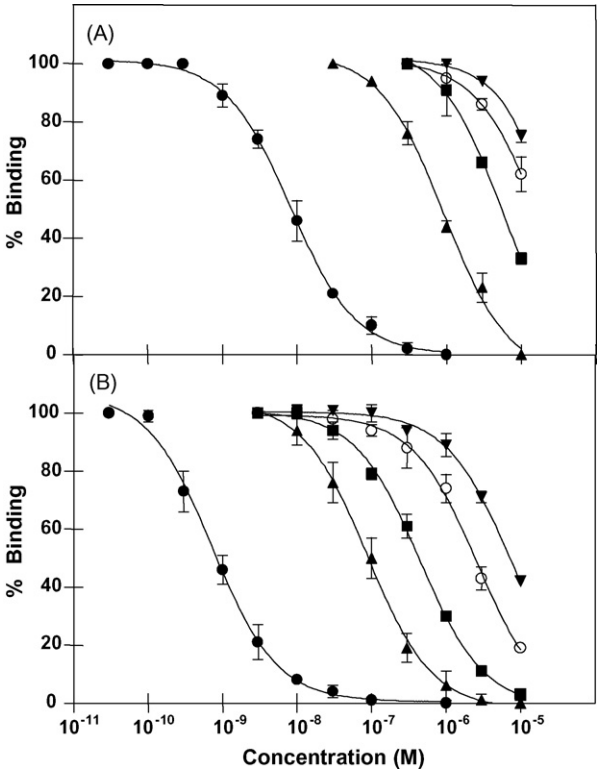

Fig. 6. Binding of tibolone and metabolites on GR and MR. HG5LN-GalGR (A) was treated with Dexamethasone (●), tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▲) in the presence of [1,2,4,6,7-^{3}H]Dexamethasone 3 nM. HG5LN-GalMR (B) was treated with aldosterone (●), tibolone (■), Δ4-tibolone (▲), 3α-OH-tibolone (○) and 3β-OH-tibolone (▲) in the presence of [1,2-^{3}H] aldosterone 1 nM. Values are mean ± S.D. from three independent.

As shown by binding and transactivation experiments PR, GR and MR bind preferentially tibolone and its Δ^4 metabolite than 3α- and 3β-OH-tibolone. Furthermore, 3-hydroxymetabolites were antagonists on these three receptors while tibolone and its Δ^4 metabolite were agonists for PR. This is due to the fact that these receptors bind preferentially and are activated by 3-keto steroids [19]. The antagonism of tibolone and Δ^4 isomer on GR (low) and MR (moderate) might be due to the absence of hydroxyl in position 11. Several studies showed that the presence of 11β-hydroxyl substitute on different MR and GR agonist ligands is required to adopt a conformation competent for ligand binding and is critical for stabilizing the active receptor conformation [25–27]. A role of the 17-hydroxyl group is also possible. Takeda et al. [28] demonstrated, using several mutant MRs, that the contact of 17β-hydroxyl group of androgens with the Asn 770, Cys942 and Thr945 residues of the ligand binding cavity of the MR stabilize binding complexes but is unable to stabilize the receptor in active state. The same group has also shown that 17α-OH-Progesterone has a lower affinity than progesterone for the MR but a much lower mineralocorticoid agonist activity and expresses antagonistic activity [29]. Whether tibolone and its Δ^4 metabolite are able to disrupt the complex ligand–MR contacts in the loop L11–12 and helix 12 region leading to anti mineralocorticoid action [25] needs further investigations. However, the IC_{50}s of tibolone and the Δ^4 metabolite to the MR are far higher that that is seen for progesterone [29] and a clinical effect is therefore unlikely.

In conclusion, tibolone by these actions on different receptors and by this capacity to transform in different metabolites, has more complex effects than initially supposed.

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