

## **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 activity 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  $\alpha$  (ER  $\alpha$ ) or estrogen receptor  $\beta$  (ER  $\beta$ ) 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 ER $\alpha$ . Tibolone and the  $\Delta^4$ -tibolone are agonists for AR and PR and surprisingly 3 $\alpha$  - and 3  $\beta$ -OH-tibolone are antagonists for them. Moreover we showed for the first time that tibolone and its primary metabolites bind to GR and MR and are all antagonists for them. 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.

Key words: tibolone, estrogen receptors, androgen receptor, progesterone receptor, glucocorticoid receptor, mineralocorticoid receptor, reporter cell lines.

## 1. Introduction

Tibolone (Org OD14), a synthetic 19-norsteroid related to norethynodrel (NED) is an interesting alternative for hormonal treatment of menopause. In randomised studies, tibolone has been shown to improve menopausal symptoms to a similar degree of combined estrogen/progestogen hormonal therapy (Landgren et al., 2002; Modelska and Cummings, 2002; Trevoux et al., 1983). Moreover, clinical trials showed its interest on bone density (Gallagher et al., 2001; Moore, 1999) 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 (Corinne et al, 2005; Beral et al, 2005 ), but the results of a randomized controlled trial (the THEBES trial) does not show any stimulation of endometrial proliferation (Archer et al., 2007). 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  $3\alpha$ -OH-tibolone is the main metabolite (Timmer and Houwing, 2002) followed by the  $3\beta$ -OH-tibolone. Tibolone and the  $\Delta^4$  isomer are only present during the first 6 hours after oral intake.

The  $\Delta^4$  isomer has progestin and androgen properties while the  $3\alpha$ - and a  $3\beta$ -OH-tibolone have estrogenic properties (De Gooyer et al., 2003). The tissue selective action of tibolone is supposed to be a combination of receptor activation and steroid metabolism (Kloosterboer, 2001).

Using *in vitro* transactivation measurements with Chinese Hamster Ovary (CHO) cells, it has been demonstrated that tibolone and  $\Delta^4$ -tibolone were agonistic ligands of progesterone receptor (PR), androgen receptor (AR) and estrogen receptor (ERs) whereas  $3\alpha$ - and  $3\beta$ -hydroxytibolone were only active on ERs (De Gooyer et al., 2003). 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 be determined (Dijkema et al 1998). 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 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. ER $\alpha$  and ER $\beta$  reporter cells were obtained by transfecting HeLa cells (ER negative) successively by the ERE- $\beta$ Glob-Luc-SVNeo plasmid (HELN cell line) and pSG5puro plasmids expressing ER $\alpha$  or ER $\beta$  (HELN ER $\alpha$  and HELN ER $\beta$  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 antiglucocorticoid and 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 labortechnik (Poitiers, France). Luciferin (sodium salt) and geneticin were purchased from Promega (Charbonnières, France). R1881 was purchased from NEN Life Science Products (Paris, France). [ $^3\text{H}$ ]-aldosterone (39 Ci/mmol specific activity) and [ $^3\text{H}$ ]-dexamethasone (84 Ci/mmol) were purchased from Amersham- GE Healthcare Europe GmbH (Orsay, France). R5020 (promegestone) was a gift from Sanofi-Aventis (Romainville, France).  $17\beta$ -estradiol (E2), testosterone, aldosterone (ALDO), dexamethasone (DEX), puromycin and aminogluthetamide (AG) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Tibolone [(7 $\alpha$ ,17 $\alpha$ )-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one];  $\Delta^4$ -tibolone [(7 $\alpha$ ,17 $\alpha$ )-17-hydroxy-7-methyl-19-norpregn-4-en-20-yn-3-one]; 3 $\alpha$ -OH-tibolone [(3 $\alpha$ ,7 $\alpha$ ,17 $\alpha$ )-7-methyl-19-norpregn-5(10)-en-20-yne-3,17-diol]; 3 $\beta$ -OH-tibolone [(3 $\beta$ ,7 $\alpha$ ,17 $\alpha$ )-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 ER $\alpha$  and HELN ER $\beta$  reporter cell lines were previously described (Balaguer et al., 1999a; Escande et al., 2006b) and cultured in DMEM without phenol red, supplemented with 6% dextran-coated, charcoal-treated FCS, 1mg/ml G418 and 0.5  $\mu\text{g/ml}$  puromycin.

To study the potential activity of tibolone and its metabolites *via* the androgen receptor (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 (Terouanne et al., 2000). HG5LN cells were obtained by transfecting HeLa cells successively by the Gal4RE5- $\beta$ Glob-Luc-SVNeo plasmid and by pSG5puro plasmids fused with the Gal4 DBD and the LBD of PR, MR or GR) (Molina Molina et al., 2006). 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  $\mu$ g/ml puromycin. In some experiments, aminogluthetimide (AG) was added to the culture medium at a concentration of 50  $\mu$ M to inhibit the intrinsic aromatase activity in HeLa cells. PALM cells were obtained as already described (Terouanne et al., 2000) and cultured in Ham's F12, supplemented with 5% FCS, 1mg/ml G418 and 1 $\mu$ g/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  $2 \times 10^4$  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 activity 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 minutes later, produces a stable luminescent signal which was quantified using a Microbeta Wallac luminometer (integration during 2seconds).

#### *2.4. Ligand binding assays for GR and MR $\gamma$*

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 1nM of [1,2- $^3$ H] Aldosterone at 37 C for 3h in the absence or presence of increasing concentrations of non-radioactive competitive compounds.

The final incubation volume was 400  $\mu$ l and each dilution was performed in duplicate. After incubation, unbound material was aspirated and cells washed three times with 400  $\mu$ l of cold PBS. Then, 250  $\mu$ l 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 shaken for 5 min. Total cell lysate (200  $\mu$ l) 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<sub>50</sub> 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  $\pm$  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<sub>50</sub> values, effective concentration for half-maximal luciferase activity and antagonistic transactivation data as IC<sub>50</sub>, half-maximal inhibitory concentration for each compound tested. Binding data are presented as IC<sub>50</sub> values, effective concentration to decrease maximum [<sup>3</sup>H]-compound binding by 50%.

### 3. Results

#### 3.1. *In vitro* estrogen receptors studies

We used HELN ER $\alpha$  and HELN ER $\beta$  cell lines (Balaguer et al., 1999; Escande et al., 2006) 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  $\mu$ M. This is probably due to toxicity (data not shown). In HELN ER $\alpha$  and HELN ER $\beta$  cell lines, the EC<sub>50</sub> value of E2 was respectively 0.018 nM and 0.08 nM (Fig 1A and 1B). Tibolone and its metabolites were full agonists for ERs but their potency appeared very low compared to E2 (Fig 1A and 1B). In HELN ER $\alpha$  cells, 3 $\alpha$ - and 3 $\beta$ -OH-tibolone displayed higher activity on ER $\alpha$  transactivation (EC<sub>50</sub> of 1.7 and 2.4 nM, respectively) than tibolone and  $\Delta^4$ -tibolone (EC<sub>50</sub> of 4.3 and 26 nM, respectively). By contrast, tibolone and its derivatives were less active on ER $\beta$ . 3 compounds have the same potency to activate ER $\beta$  (EC<sub>50</sub> of 3 $\alpha$ -hydroxytibolone 100 nM, EC<sub>50</sub> of tibolone 105 nM, EC<sub>50</sub> of 3 $\beta$ -hydroxytibolone 115 nM) compared to  $\Delta^4$ -tibolone (EC<sub>50</sub> of 300 nM).

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 presence of aminoglutethimide (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 ER $\alpha$  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 and (De Gooyer et al., 2003). Tibolone and its metabolites did not show antagonistic activity for the ERs.

### 3.2. *In vitro* androgen receptor studies

To study the potential activity of tibolone and its metabolites *via* the androgen receptor (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 (Terouanne et al., 2000). As shown in Figure 3A, the EC<sub>50</sub> value for the synthetic androgen R1881 in PALM cells was 0.1 nM. Tibolone and the  $\Delta^4$  isomer behaved as full agonists for AR (Fig 3A), the  $\Delta^4$  isomer displaying the best transactivation potency (EC<sub>50</sub> 0.2 nM vs 1.05 nM). The activity of 3 $\beta$ -OH-tibolone was variable (see error bars), which may be caused by metabolism. We tested therefore 3 $\beta$ -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). 3 $\beta$ -OH-tibolone was only partially active after 8h of incubation but it became more active after 16h and 40h of treatment (Fig 3B). These results suggest that 3 $\beta$ -hydroxylated tibolone can be progressively transformed in an active androgen which might be tibolone or the  $\Delta^4$  isomer. Interestingly, this did not occur with the 3 $\alpha$ -hydroxylated tibolone but acted surprisingly as an antagonist (IC<sub>50</sub> 135 nM in the presence of 1nM R1881) (Fig 3C). Tibolone,  $\Delta^4$  isomer and 3 $\beta$ -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  $\beta$ -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) (Molina-Molina et al, 2006). 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<sub>50</sub> value for the progestin R5020 was 5 nM. Tibolone and the  $\Delta^4$  isomer were full PR agonists (Fig 4A). The  $\Delta^4$  isomer displayed the highest potency to transactivate AR (EC<sub>50</sub> 46 nM) as compared to tibolone which was less active (EC<sub>50</sub> 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<sub>50</sub> 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, Fig 4C).

We then tested the antagonistic activity of tibolone and its metabolites on reference stimulated PR, GR and MR activity. In the presence of 5nM R5020, the 3-hydroxylated compounds showed a weak activity of complete PR antagonist (IC<sub>50</sub> corresponding to 20  $\mu$ M for 3 $\beta$ -OH-tibolone and 2.4  $\mu$ M for 3 $\alpha$ -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<sub>50</sub> value was 1.7  $\mu$ M for the  $\Delta^4$  isomer, 10  $\mu$ M for tibolone, and not measurable for 3 $\alpha$ -OH-tibolone and 3 $\beta$ -OH-tibolone (Fig 5B). Finally, as shown in Figure 5C, tibolone and its metabolites were antagonists for MR (activated by aldosterone at 0.5 nM concentration). Again, the  $\Delta^4$  isomer was the better antagonist (IC<sub>50</sub> of 30 nM) followed by tibolone (IC<sub>50</sub> of 170 nM) and the 3-OH-tibolones (IC<sub>50</sub> corresponding to 1.2  $\mu$ M for 3 $\alpha$ -OH-tibolone and 3.3  $\mu$ M for 3 $\beta$ -OH-tibolone) (Fig 5C).

### 3.5. 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 [ $^3\text{H}$ ]-dexamethasone as a tracer.  $\Delta^4$ -tibolone was the most effective compound, inhibiting the binding of dexamethasone at a 10  $\mu\text{M}$  concentration (Fig. 6A). The binding affinity of tibolone was very low but was able to significantly displace dexamethasone from GR at 10  $\mu\text{M}$  (Fig 6A). The least effective compounds were the 3-hydroxylated ligands, which showed low binding affinity for GR.

Binding to MR was assessed using 1 nM [ $^3\text{H}$ ]-aldosterone as a tracer. Tibolone and  $\Delta^4$  isomer inhibited [ $^3\text{H}$ ]-aldosterone binding to MR in a dose dependent manner and complete inhibition was achieved at 1 and 10  $\mu\text{M}$  respectively for tibolone and  $\Delta^4$ -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, ER $\alpha$  and ER $\beta$ ) using HeLa or PC3 cells stably transfected with a given receptor and a luciferase reporter gene. We show that tibolone and its  $\Delta^4$ -isomer predominantly bind and activate PR and AR whereas 3 $\alpha$  and 3 $\beta$ -OH-tibolone predominantly bind and activate ER $\alpha$  (Table 1). The hydroxyl metabolites are full agonists for both ERs and bind preferentially ER $\alpha$ . 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 (Bodine et al., 2002 , Wiegratz et al., 2002) 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 (De Gooyer et al., 2003) is due to direct binding to ERs, because tibolone does not contain the required hydroxyl-group. Our data demonstrating that 3 $\alpha$ - and 3 $\beta$ -OH-tibolone have a better affinity for ER $\alpha$  than tibolone and its  $\Delta^4$  derivative are consistent with previous studies showing that compounds with a 3 keto  $\Delta^4$  configuration do not bind to ER and require at least a 3 hydroxyl group (Tanenbaum et al., 1998). Most likely the cell lines contain aldo-ketoreductases, which can convert tibolone to the 3-hydroxymetabolites ( Steckelbroeck et al., 2004). Larrea et al., (2001) have shown that 19-nortestosterone derived progestagens can be converted to A-ring reduced metabolite, which are agonists for ER $\alpha$ .

Tibolone and its  $\Delta^4$  metabolite are relatively strong agonists for AR. The effect of tibolone on androgen sensitive parameters is often thought to be contributed to the  $\Delta^4$  metabolite of tibolone. However, Verheul et al (2007) have shown that tibolone and the  $\Delta^4$  metabolite are

present in target tissues in very low concentration, except in the liver. The direct androgenic effects of tibolone and  $\Delta^4$  metabolite has therefore little impact on target tissues. The possible androgenic effect in tibolone users on libido (Nathorst-Boos and Hammar, 1997) may well be explained by the observed decreased levels of SHBG, which cause increased levels of free testosterone (Doren et al., 2001). Surprisingly the  $3\beta$ -OH-tibolone stimulates the AR and this stimulation increases with prolonged incubation times. This indicates that the  $3\beta$ -OH-tibolone is most likely metabolized to the  $\Delta^4$  metabolite. Similarly, Schatz et al have shown in human stromal cells that  $3\beta$ -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  $3\beta$ -OH-tibolone to  $\Delta^4$  metabolite (Steckelbroeck et al, 2004). The anti-androgenic activity of the  $3\alpha$ -OH-tibolone is weak and occurs only at very high concentrations.

As shown by binding and transactivation experiments PR, GR and MR bind preferentially tibolone and its  $\Delta^4$  metabolite than  $3\alpha$  and  $3\beta$ -OH-tibolone. Furthermore, 3-hydroxymetabolites were antagonists on these three receptors while tibolone and its  $\Delta^4$  metabolite were agonists for PR. This is due to the fact that these receptors bind preferentially and are activated by 3-keto steroids (Tanenbaum et al., 1998). The antagonism of tibolone and  $\Delta^4$  isomer on GR and MR might be due to the absence of hydroxyl in position 11. Several studies showed that the presence of  $11\beta$ -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 (Fagart et al., 1998; Kauppi et al., 2003; Rafestin-Oblin et al., 2002). A role of the 17-hydroxyl group is also possible. Takeda et al (2007) demonstrated, using several mutant MRs, that the contact of  $17\beta$ -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 17 $\alpha$ -OH-Progesterone has a lower affinity than progesterone for the MR but a much lower mineralocorticoid agonist activity. and expresses antagonistic activity (Quinkler et al, 2002). Whether tibolone and its  $\Delta^4$  metabolite, are able to disrupt the complex ligand-MR contacts in the loop L11-12 and helix 12 region leading to anti mineralocorticoid action (Fagart et al., 1998) needs further investigations. However, the IC50s of tibolone and the  $\Delta^4$  metabolite to the MR are far higher than that is seen for progesterone (Quinkler et al, 2002) 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.

## References

- D.F. Archer, S. Hendrix, A. Ferenczy., J. Felix., J.C. Gallagher, J.Rymer, S.O.Skouby., W. den Hollander, V.Stathopoulos. F.A. Helmond, THEBES Study Group.. Tibolone histology of the endometrium and breast endpoints study: design of the trial and endometrial histology at baseline in postmenopausal women. *Fertil Steril.* 88 (2007) 866-878.
- Balaguer, P., Francois, F., Comunale, F., Fenet, H., Boussioux, A. M., Pons, M., Nicolas, J. C., Casellas, C.,. Reporter cell lines to study the estrogenic effects of xenoestrogens. *Sci.Total Environ.* 233 (1999) 47-56.
- Beral V, Bull D, Reeves G; Million Women Study Collaborators. Endometrial cancer and hormone-replacement therapy in the Million Women Study. *Lancet.* ;365 (2005) 1543-51.
- Bodine, P.V., Harris, H.A., Lyttle, C.R., Komm, B.S.,. Estrogenic effects of 7 $\alpha$ -methyl-17 $\alpha$ -ethynylestradiol: a newly discovered tibolone metabolite. *Steroids.* 67 (2002) 681-686.



De Gooyer, M. E., Deckers, G. H., Schoonen, W. G., Verheul, H. A., Kloosterboer, H. J.,  
Receptor profiling and endocrine interactions of tibolone. *Steroids* 68 (2003) 21-30.

Corinne S. de Vries,<sup>1</sup> Susan E. Bromley,<sup>1</sup> Hilary Thomas<sup>2</sup> and Richard D.T. Farmer<sup>1</sup>.  
Tibolone and Endometrial Cancer. A Cohort and Nested Case-Control Study in the UK. *Drug Safety*; 28 (2005) 241-249

Dijkema R, Schoonen WG, Teuwen R, van der Struik E, de Ries RJ, van der Kar BA, Olijve W. Human progesterone receptor A and B isoforms in CHO cells. I. Stable transfection of receptor and receptor-responsive reporter genes: transcription modulation by (anti)progestagens. *J Steroid Biochem Mol Biol.* ;64 (1998):147-56.

Dören M, Rübig A, Coelingh Bennink HJ, Holzgreve W. Differential effects on the androgen status of postmenopausal women treated with tibolone and continuous combined estradiol and norethindrone acetate replacement therapy. *Fertil Steril.* 2001;75:554-9.

Escande, A., Pillon, A., Servant, N., Cravedi, J. P., Larrea, F., Muhn, P., Nicolas, J. C., Cavailles, V., Balaguer, P., Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. *Biochem.Pharmacol.* 71 (2006) 1459-1469.

Fagart, J., Wurtz, J. M., Souque, A., Hellal-Levy, C., Moras, D., Rafestin-Oblin, M. E.,  
Antagonism in the human mineralocorticoid receptor. *EMBO J.* 17 (1998) 3317-3325.

Gallagher, J. C., Baylink, D. J., Freeman, R., McClung, M., Prevention of bone loss with tibolone in postmenopausal women: results of two randomized, double-blind, placebo-controlled, dose-finding studies. *J.Clin.Endocrinol.Metab* 86 (2001) 4717-4726.

Kauppi, B., Jakob, C., Farnegardh, M., Yang, J., Ahola, H., Alarcon, M., Calles, K., Engstrom, O., Harlan, J., Muchmore, S., Ramqvist, A. K., Thorell, S., Ohman, L., Greer, J., Gustafsson, J. A., Carlstedt-Duke, J., Carlquist, M., The three-dimensional structures of antagonistic and agonistic forms of the glucocorticoid receptor ligand-

binding domain: RU-486 induces a transconformation that leads to active antagonism. *J.Biol.Chem.* 278 (2003) 22748-22754.

Kloosterboer, H. J., Tibolone: a steroid with a tissue-specific mode of action. *J.Steroid Biochem.Mol.Biol.* 76 (2001) 231-238.

Landgren, M. B., Bennink, H. J., Helmond, F. A., Engelen, S., Dose-response analysis of effects of tibolone on climacteric symptoms. *BJOG.* 109 (2002), 1109-1114.

Larrea, F., Garcia-Becerra, R., Lemus, A. E., Garcia, G. A., Perez-Palacios, G., Jackson, K. J., Coleman, K. M., Dace, R., Smith, C. L., Cooney, A. J., A-ring reduced metabolites of 19-nor synthetic progestins as subtype selective agonists for ER alpha. *Endocrinology* 142(2001) 3791-3799.

Molina-Molina, J. M., Hillenweck A., Jouanin I., Zalko D., Cravedi J. P., Fernandez M. F., Pillon A., Nicolas J. C., Olea N., Balaguer P., Steroid receptor profiling of vinclozolin and its primary metabolites. *Toxicol Appl Pharmacol* 216 (2006) 44-54.

Modelska, K., Cummings, S., Tibolone for postmenopausal women: systematic review of randomized trials. *J.Clin.Endocrinol.Metab* 87 (2002) 16-23.

Moore, R. A., Livial: a review of clinical studies. *Br.J.Obstet.Gynaecol.* 106 Suppl 19 (1999) 1-21.

Nathorst-Boos, J., Hammar, M., Effect on sexual life--a comparison between tibolone and a continuous estradiol-norethisterone acetate regimen. *Maturitas* 26(1997) 15-20.

Quinkler M, Meyer B, Bumke-Vogt C, Grossmann C, Gruber U, Oelkers W, Diederich S, Bahr V. Agonistic and antagonistic properties of progesterone metabolites at the human mineralocorticoid receptor. *Eur J Endocrinol.* 146 (2002) :789-99.

Rafestin-Oblin, M. E., Fagart, J., Souque, A., Seguin, C., Bens, M., Vandewalle, A., 11beta-hydroxyprogesterone acts as a mineralocorticoid agonist in stimulating Na<sup>+</sup> absorption

in mammalian principal cortical collecting duct cells. *Mol.Pharmacol.* 62 (2002) 1306-1313.

Steckelbroeck, S., Jin, Y., Oyesanmi, B., Kloosterboer, H.J., Penning, T.M., Tibolone is metabolized by the 3 $\alpha$ /3 $\beta$ -hydroxysteroid dehydrogenase activities of the four human isozymes of the aldo-keto reductase 1C subfamily: inversion of stereospecificity with a  $\Delta^5(10)$ -3-ketosteroid. *Mol Pharmacol.* 66 (2004) 1702-1711.

Takeda AN, Pinon GM, Bens M, Fagart J, Rafestin-Oblin ME, Vandewalle A. The synthetic androgen methyltrienolone (R1881) acts as a potent antagonist of the mineralocorticoid receptor. *Mol Pharmacol.* ;71(2007):473-82.

Tanenbaum, D. M., Wang, Y., Williams, S. P., Sigler, P. B., Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc.Natl.Acad.Sci.U.S.A* 95 (1998) 5998-6003.

Taskin, O., Muderrisoglu, H., Akar, M., Simsek, M., Mendilcioglu, I., Kursun, S., Comparison of the effects of tibolone and estrogen replacement therapy on echocardiographic basic cardiac functions in post-menopausal women: a randomized placebo controlled study. *Maturitas.* 48 (2004) 354-359.

Terouanne, B., Tahiri, B., Georget, V., Belon, C., Poujol, N., Avances, C., Orio, F., Jr., Balaguer, P., Sultan, C., A stable prostatic bioluminescent cell line to investigate androgen and antiandrogen effects. *Mol.Cell Endocrinol.* 160 (2000) 39-49.

Timmer, C. J., Houwing, N. S., Dose proportionality of three different doses of tibolone. *Pharmacotherapy* 22 (2002) 6-13.

Trevoux, R., Dieulangard, P., Blum, A., Efficacy and safety of Org OD 14 in the treatment of climacteric complaints. *Maturitas* 5 (1983), 89-96.

Verheul H.A., Blok L.J., Burger C.W., Hanifi-Moghaddam P., Kloosterboer H.J., Levels of tibolone and estradiol and their nonsulfated and sulfated metabolites in serum, myometrium, and vagina of postmenopausal women following treatment for 21 days with tibolone, estradiol, or estradiol plus medroxyprogesterone acetate. *Reprod Sci* 14 (2007)160-168.

Wiegratz I, Sanger N, Kuhl H. Formation of 7 alpha-methyl-ethinyl estradiol during treatment with tibolone. *Menopause*. 9 (2002):293-5.

## Figure legends

### **Fig. 1. Transcriptional activity as % activity of ER $\alpha$ and ER $\beta$ in response to tibolone and metabolites .**

HELN-ER $\alpha$  (A) and HELN-ER $\beta$  (B) cell lines were treated with E2 (◆), Tibolone (■),  $\Delta$ 4-Tibolone (▲), 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) at the indicated concentrations for 16 h. Maximal activity (100%) corresponds to the activity obtained with 10 nM E2. Values are mean  $\pm$  S.D. from three independent experiments.

### **Fig. 2. Transcriptional activity % activity of ER $\alpha$ in response to tibolone and metabolites with or without aminogluthetimide (AG).**

HELN-ER $\alpha$  cell lines was treated with E2 (A) without (◆) or with (◇) aminogluthetimide, with testosterone (B) without (✱) or with (✕) aminogluthetimide and with 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  $\pm$  S.D. from three independent experiments.

### **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 (■),  $\Delta$ 4-Tibolone (▲), 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) at the indicated concentrations for 40 h. Maximal activity (100%) corresponds to the activity obtained with 100 nM R1881. Values are mean  $\pm$  S.D. from three separate experiments.

PALM cell line (B) was treated with R1881 (◆) or 3 $\beta$ -OHTibolone (▼) at the indicated concentrations for 8h (—), 16h (---) and 40h (·····).

PALM cell line (C) was treated with 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) in presence of R1881 1nM. Values are mean  $\pm$  S.D. from three separate experiments.

**Fig. 4. Transcriptional activity % activity of PR, GR, MR in response to tibolone and metabolites**

HG5LN-GalPR (A) was treated with R5020 (◆), Tibolone (■),  $\Delta$ 4-Tibolone (▲), 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) 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 (■),  $\Delta$ 4-Tibolone (▲), 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) 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 (■),  $\Delta$ 4-Tibolone (▲), 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) at the indicated concentrations for 16 h. Maximal activity (100%) corresponds to the activity obtained with 10 nM Aldosterone. Values are mean  $\pm$  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 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) in presence of R5020 5nM.

HG5LN-GalGR (B) was treated with Tibolone (■),  $\Delta$ 4-Tibolone (▲), 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) in presence of Dexamethasone 10nM.

HG5LN-GalMR (C) was treated with Tibolone (■),  $\Delta$ 4-Tibolone (▲), 3 $\beta$ -OHTibolone (▼) and 3 $\alpha$ -OHTibolone (●) in presence of Aldosterone 0.5nM. Values are mean  $\pm$  S.D. from three independent

**Fig. 6. Binding of tibolone and metabolites on GR and MR.**

HG5LN-GalGR (A) was treated with Dexamethasone (◆), Tibolone (■),  $\Delta$ 4-Tibolone (▲),  $3\beta$ -OHTibolone (▼) and  $3\alpha$ -OHTibolone (●) in presence of [1,2,4,6,7- $^3$ H]Dexamethasone 3nM.

HG5LN-GalMR (B) was treated with Aldosterone (◆), Tibolone (■),  $\Delta$ 4-Tibolone (▲),  $3\beta$ -OHTibolone (▼) and  $3\alpha$ -OHTibolone (●) in presence of [1,2- $^3$ H]Aldosterone 1nM. Values are mean  $\pm$  S.D. from three independent

**Table 1: EC 50 and IC 50 values of tibolone and its metabolites on estrogen androgen progesterone glucocorticoid and mineralocorticoid receptors.**

Receptors Compounds	ER $\alpha$ (nM) $\pm$ S.D.	ER $\beta$ (nM) $\pm$ S.D.	AR (nM) $\pm$ S.D.	PR (nM) $\pm$ S.D.	GR (nM) $\pm$ S.D.	MR (nM) $\pm$ S.D.
Estradiol	0.018 $\pm$ 0.002	0.08 $\pm$ 0.01				
R1881			0.1 $\pm$ 0.035			
R5020				5 $\pm$ 3		
Dexamethasone					6 $\pm$ 2.5	
Aldosterone						0.36 $\pm$ 0.26
Tibolone	4 $\pm$ 1	105 $\pm$ 25	1.05 $\pm$ 0.65	123 $\pm$ 60	10000 $\pm$ 3000*	170 $\pm$ 60*
$\Delta^4$ -tibolone	26 $\pm$ 10	300 $\pm$ 155	0.2 $\pm$ 0.07	46 $\pm$ 22	1760 $\pm$ 760*	30 $\pm$ 10*
3 $\alpha$ -OH-tibolone	1.7 $\pm$ 0.5	100 $\pm$ 40	135 $\pm$ 25*	2400 $\pm$ 1200*	ND*	1160 $\pm$ 280*
3 $\beta$ -OH-tibolone	2.4 $\pm$ 0.4	115 $\pm$ 35	ND*	20000 $\pm$ 16600*	ND*	3310 $\pm$ 850*

EC50 values were concentrations required to produce half maximal induction in different cell lines, value are indicated in black characters. IC50 values were concentrations required to produce half maximal inhibition in different cell lines, value are indicated in black slanting characters with asterisk.