Progestagenic Effects of Tibolone on Human Endometrial Cancer Cells


Tibolone, a synthetic steroid acting in a tissue-specific manner and used in hormone replacement therapy, is converted into three active metabolites: a Δ4 isomer (exerting progestagenic and androgenic effects) and two hydroxy metabolites, 3α-hydroxytibolone (3α-OH-tibolone) and 3β-OH-tibolone (exerting estrogenic effects). In the present study an endometrial carcinoma cell line (Ishikawa PRAB-36) was used to investigate the progestagenic properties of tibolone and its metabolites. This cell line contains progesterone receptors A and B, but lacks estrogen and androgen receptors.

When tibolone was added to the cells, complete conversion into the progestagenic/androgenic Δ4 isomer was observed within 6 d. Furthermore, when cells were cultured with tibolone or when the Δ4 isomer or the established progestagen medroxyprogesterone acetate was added to the medium, marked inhibition of growth was observed. Interestingly, 3β-OH-tibolone also induces some inhibition of growth. These growth inhibitions were not observed in progesterone receptor-negative parental Ishikawa cells, and progestagen-induced growth inhibition of PRAB-36 cells could readily be reversed using the antiprogestagen Org-31489. Upon measuring the expression of two progesterone-regulated genes (fibronectin and IGF-binding protein-3), tibolone, the Δ4 isomer and medroxyprogesterone acetate showed similar gene expression regulation.

These results indicate that tibolone, the Δ4 metabolite, and to some extent 3β-OH-tibolone exert progestagenic effects. Tibolone and most likely 3β-OH-tibolone are converted into the Δ4 metabolite. (J Clin Endocrinol Metab 88: 2327–2334, 2003)

With menopause, the ovaries stop ovulating, and circulating levels of progesterone and estrogen decrease. Because of reduced levels of estrogens, women often experience adverse effects (hot flushes, vaginal dryness, increased bone loss, etc.). To relieve climacteric symptoms and prevent osteoporosis, hormone replacement therapy may be prescribed. At present, hormone replacement therapy usually contains separate estrogenic and progestagenic components. The estrogenic component substitutes for the decreased circulating estrogens; the progestagen component reduces the adverse effects of estrogens on the endometrium (1).

Tibolone is a synthetic steroid that is commonly used for the treatment of climacteric complaints and prevention of osteoporosis (2). The compound itself does not bind with high affinity to any of the known steroid receptors (3); however, depending on the activity of different enzymes during passage through the intestine and liver, tibolone will be converted by 3β-hydroxysteroid dehydrogenase (3βHSD) into its Δ4 isomer (Δ4-tibolone) or its 3β-reduced derivative [3α-hydroxytibolone (3β-OH-tibolone)] and/or by 3αHSD into its 3α-reduced derivative (3α-OH-tibolone) (4). In contrast to tibolone itself, these metabolic products show higher affinity binding to several steroid receptors; Δ4-tibolone binds to progesterone and androgen receptors, and 3α-OH-tibolone and 3β-OH-tibolone bind to estrogen receptors (3).

Upon measurement of the metabolites of tibolone in several animal tissues (5), it was observed that estrogenic metabolites were formed (3α-OH-tibolone and 3β-OH-tibolone). These metabolites, however, are quickly sulfated by sulfotransferases in the liver and intestine and will only become active as estrogens upon removal of the sulfate group by tissue-specific sulfatases (5). In breast cancer cell lines, sulfatase activity is inhibited by tibolone, and as a result of this, sulfated 3α-OH-tibolone, sulfated 3β-OH-tibolone, and sulfated endogenous estrogens will no longer act as biologically active estrogens (6–9). For the human endometrial cancer cell line HEC-1A, De Gooyer et al. (8) could show that sulfatase activity was also inhibited by tibolone. Furthermore, Falany et al. (10) found that sulfotransferases in the human endometrium are probably up-regulated by progestagens. Together with the finding that the progestagenic metabolite of tibolone (Δ4-tibolone) can locally be formed in the endometrium (3) and with observations from clinical trials (11–15), these data seem to indicate that the estrogenic activities of tibolone metabolites on the endometrium are balanced by progestagenic and other activities of tibolone and its metabolites on the endometrium.

To study the progestagenic effects of tibolone treatment on the endometrium in more detail, a progesterone-sensitive, estrogen- and androgen receptor-negative, well differen-
TABLE 1. Measurement of different metabolites of tibolone

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>% 3H(tibolone)</th>
<th>% Δ4-tibolone</th>
<th>% 3α-OH-tibolone</th>
<th>% 3β-OH-tibolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRAB-36 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input: 3H(tibolone)</td>
<td>0 d</td>
<td>80</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 d</td>
<td>81</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 d</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input: 3H(Δ4-tibolone)</td>
<td>0 d</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 d</td>
<td>100</td>
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<tr>
<td></td>
<td>6 d</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input: 3H(3α-OH-tibolone)</td>
<td>0 d</td>
<td>100</td>
<td></td>
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<tr>
<td></td>
<td>3 d</td>
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<tr>
<td></td>
<td>6 d</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input: 3H(3β-OH-tibolone)</td>
<td>0 d</td>
<td>100</td>
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<td>3 d</td>
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PRAB-36 cells were cultured for 0, 3, or 6 d in medium containing 5% DCC-FBS, in the presence of 50 nM 3H(tibolone), 3H(Δ4-tibolone), 3H(3α-OH-tibolone), or 3H(3β-OH-tibolone). HPLC analysis was performed to measure conversion of the input compound into its possible metabolic products.

Materials and Methods

Cell culture

Ishikawa cells are derived from a well differentiated endometrial adenocarcinoma and were a gift from Dr. Masato Nishida (Tsukuba, Japan) (16). The cells were negative for mycoplasma contamination, determined using the Mycoplasma-Plus-PCR-Primer-Set (Stratagene, La Jolla, CA). The cells were maintained in DMEM/Ham’s F-12 (Invitrogen, Paisley, Scotland) and 5% fetal bovine serum (FBS; Perbio Science, Helsingborg, Sweden) supplemented with penicillin/streptomycin in a 37°C incubator at 5% CO2. The cells were transfected with human progesterone receptor-A (hPRA) and hPRB (17) and were maintained under similar culture conditions, with continuous selection pressure using neomycin (500 μg/ml; G418, Invitrogen, Breda, The Netherlands) and hygromycin (250 μg/ml; Invitrogen).1

Compounds

Tibolone [(7α,17α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one], 3α-OH-tibolone [(3α,7α,17α)-7-methyl-19-norpregn-5(10)-en-20-yn-3,17-diol], 3β-OH-tibolone [(3β,7α,17α)-7-methyl-19-norpregn-5(10)-en-20-yn-3,17-diol], and Δ4-tibolone [(7α,17α)-17-hydroxy-7-methyl-19-norpregn-4-en-20-yn-3-one] were provided as crystalline powder containing less than 0.6% impurities by N.V. Organon (Oss, The Netherlands). Tritiated compounds were also provided by N.V. Organon; the radiochemical purity of these tritiated compounds was above 97%, and specific activity ranged between 38–53 Ci/mmol. Medroxyprogesterone acetate (MPA) was obtained from Sigma-Aldrich Corp. (St. Louis, MO).

at 4°C. The proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH). The PRA/B (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was incubated with the membrane as follows. The membrane was rinsed with PBS-Tween (0.5%) and blocked for 1 h with blocking solution. The PRA/B antibody was diluted 1:2000 in blocking solution and incubated with the membrane for 1 h. The membrane was washed four times for 15 min each time with PBS-Tween. Antibody-peroxidase conjugate was diluted in blocking solution and incubated with the membrane for 1 h. The membrane was washed four times for 15 min each time with PBS-Tween. Antibody-peroxidase conjugate was diluted in blocking solution and incubated with the membrane for 1 h. The membrane was washed four times for 15 min each time with PBS-Tween. The PRA and PRB bands were detected using the Luminochemiluminescence procedure (NEN Life Science Products, Boston, MA) and was visualized by exposing the blot to x-ray film (Kodak X-Omat, Eastman Kodak Co., New Haven, CT) for at least 1 min.

**Northern blotting**

For total RNA isolation, cells were cultured in DMEM/Ham’s F-12 and 5% DCC-FBS for the indicated time in the presence or absence of 1 nM MPA, 100 nM tibolone, or 100 nM Δ⁴-tibolone. Total RNA was isolated by lysing the cells with 3 M lithium chloride/6 M urea (18); subsequently, the RNA was purified and separated as described by Blok et al. (19). As a probe for fibronectin, a 361-bp fragment of fibronectin cDNA was used. As a probe to detect IGF-binding protein-3 (IGFBP-3) mRNA on the Northern blot, a 2.4-kb EcoRI cDNA fragment containing the complete mouse IGFBP-3-coding sequence was used. This cDNA was provided by Dr. S. L. S. Drop (Erasmus Medical Center, Rotterdam, The Netherlands).

**Statistics**

Statistical analysis was performed using SPSS software version 10.05, patched with version 10.07 (SPSS, Inc., Chicago, IL). The experiments described in Figs. 2 and 3 were repeated three times, and paired-sample t tests were performed. Differences between control and treatments were considered significant at P < 0.01.

**Results**

**Tibolone and its metabolites**

Depending on the tissue or cell line to which tibolone is administered, the compound can be metabolized differently. There are three derivatives to which tibolone can be converted: 3α-OH-tibolone (Org-4094), 3β-OH-tibolone (Org-30126), and Δ⁴-tibolone (Org-OM38). These compounds were used with tibolone and MPA in our studies. MPA was chosen as the progestagen because this compound is used in clinical practice.

To determine to what extent tibolone is converted to the two estrogenic metabolites (3α-OH-tibolone and 3β-OH-tibolone) or the progestagenic/androgenic metabolite (Δ⁴-tibolone) in the progesterone receptor-expressing PRAB-36 cell line, HPLC measurements were performed. PRAB-36 cells were cultured in the presence of [³H]tibolone, [³H]3α-OH-tibolone, [³H]3β-OH-tibolone, or [³H]Δ⁴-tibolone for 0, 3, or 6 d. Subsequent conversion to tibolone...
metabolites was measured by HPLC analysis (Table 1). It was observed that handling (evaporating the dissolvent and dissolving the resulting powder in culture medium) of tibolone in itself caused some conversion to $\Delta^4$-tibolone. It was also observed that after 6 d of culture, all tibolone was converted to the $\Delta^2$ isomer, explaining the clear progestagenic activity of tibolone in culture. At 3 d, however, about 20% of the original amount of tibolone is metabolized into one of the estrogenic metabolites, $3\beta$-OH-tibolone. Despite the fact that $3\beta$-OH-tibolone, when administered alone, seems very stable in culture, 3$\beta$-OH-tibolone, which was produced from tibolone, could no longer be detected in culture after 6 d. The HPLC analysis allows for detection of sulfated forms of tibolone and its metabolites. However, no sulfated forms of $3\alpha$-OH-tibolone and $3\beta$-OH-tibolone were detected. Our explanation is that sulfotransferase activity needs to be induced or that $3\alpha$-OH-tibolone and $3\beta$-OH-tibolone have a lower affinity to sulfotransferase than estrone.

**PRAB-36 cells cultured in the presence of tibolone are inhibited in growth**

To measure the progestagenic effects of tibolone, parental Ishikawa cells, or Ishikawa cells that had been stably transfected with PRA and PRB (PRAB-36; Fig. 1) were cultured in the presence or absence of tibolone or its metabolites in 0.1-$\mu$g concentrations. As a control for growth inhibition, cells were also cultured in the presence of MPA (1 nm).

Despite the fact that many reports claim the opposite (16, 20, 21), in the parental Ishikawa cell line, using Western blotting (Fig. 1), Northern blotting, and an $[^3H]$ligand binding assay (17), we could not detect progesterone receptor expression (Fig. 1) or androgen or estrogen receptor expression (not shown). Furthermore, no growth regulation by MPA, tibolone, or its derivatives was observed (Fig. 2A). Only in the very early passages of the parental Ishikawa cells were we sometimes able to show progesterone regulation of growth, which makes it possible that during culture in our laboratory the parental Ishikawa cells lost their steroid receptors. Using these unexpected findings, however, an Ishikawa cell line expressing high levels of PRA and PRB (PRAB-36) could be created next to an Ishikawa cell line lacking progesterone receptor expression (IK-par) (17).

When PRAB-36 cells were cultured in the presence of tibolone or $\Delta^4$-tibolone, a significant inhibition of growth could be measured (Fig. 2B). The extent of the inhibition was comparable, with maximal growth inhibition observed during culture in the presence of MPA. Culture in the presence of $3\beta$-OH-tibolone also resulted in a small, but significant, inhibition of cell growth in the PRAB-36 cell line. This inhibition was markedly less than that observed when the PR-containing cells were cultured in the presence of MPA (Fig. 2B). Using another Ishikawa subline that expressed only PRB receptors (PRB-59), very similar results were obtained (data not shown).

To measure the ED$_{50}$ of tibolone and $\Delta^4$-tibolone compared with that of MPA, dose-response experiments were performed. The ED$_{50}$ values for both tibolone and $\Delta^4$-tibolone were approximately 1 nm, while the ED$_{50}$ for MPA was less than 0.1 nm (Fig. 3). These data are comparable to observations in other reports (3).

A time-course experiment was performed to determine when growth inhibition became first visible. Cells were cultured in the presence of tibolone, 3a-OH-tibolone, 3$\beta$-OH-tibolone $\Delta^4$-tibolone, or MPA for 3, 6, 10, and 14 d. After 6 d of culture in the presence of tibolone, $\Delta^4$-tibolone, and MPA,
a clear growth inhibition was observed (Fig. 4). Furthermore, during growth inhibition, cell growth did not come to a complete standstill; rather, the growth rate became severely inhibited (Fig. 4).

**Tibolone acts as a progestagenic compound on PRAB-36 endometrial cancer cell lines**

Incubation of a progesterone-responsive endometrial cancer cell line in the presence of tibolone, Δ⁴-tibolone, or 3β-OH-tibolone results in significant growth inhibition (Fig. 2B). Because progesterone receptor-negative parental Ishikawa cells do not show this growth inhibition (Fig. 2A), it is likely that the progesterone receptor is involved. To investigate this more thoroughly, we made use of the pure antiprogestagen Org-31489 to inhibit the progestagenic effects of tibolone, Δ⁴-tibolone, and MPA on cell growth. Tibolone-, Δ⁴-tibolone-, and MPA-induced growth inhibition could readily be reversed by administration of the pure antiprogestagen Org-31489 (Fig. 5).

**FIG. 4. Time course of growth inhibition by MPA, tibolone, and its derivatives.** PRAB-36 cells were cultured for different periods (3, 6, 10, and 14 d) in the absence (Con) or presence of tibolone (Tibol; 0.1 μM), Δ⁴-tibolone (delta-4; 0.1 μM), 3α-OH-tibolone (3α-OH; 0.1 μM), 3β-OH-tibolone (3β-OH; 0.1 μM), or MPA (1 nM). Cells were harvested in 1 N NaOH, and OD₂₆₀ nm measurements were performed to measure cell growth. Growth is expressed as micrograms of DNA per well. Each figure represents a single experiment, and each point in the curve represents the mean ± SD of four wells.

**FIG. 5. Inhibition of progestagenic effects of tibolone and its derivatives by the antiprogestagen Org-31489.** PRAB36 cells were cultured for 10 d under control conditions (Con) or in the presence of the antiprogestagen Org-31489 (10 nM Anti or 100 nM Anti), tibolone (top panel; 1 nM Tibol, 10 nM Tibol), Δ⁴-tibolone (middle panel; 1 nM delta-4, 10 nM delta-4), MPA (bottom panel; 0.1 nM MPA, 1 nM MPA), or a combination of hormones and antihormones as indicated. Cells were harvested in 1 N NaOH and OD₂₆₀ nm measurements were performed to measure cell growth. Growth is expressed as micrograms of DNA per well. The figure represents a single representative experiment, and each point in the curve represents the mean ± SD of four wells.
Using microarray technology, several progesterone-regulated genes were identified (Ref. 17; see Footnote 1). Two of those genes (fibronectin and IGFBP-3) were rapidly regulated by progesterone (8–72 h) and were used in the current studies to investigate the progestagenic properties of tibolone. It was observed that regulations of these genes by tibolone, Δ⁴-tibolone, and MPA were similar (Fig. 6), indicating that tibolone and Δ⁴-tibolone also have a progestagenic effect on gene regulation in the endometrial cancer cell lines used in this study.

Discussion

The presence of estrogens and the absence of progestagens results in growth of the endometrium, which may lead to endometrial hyperplasia. When this hormonal regime is maintained, hyperplasia will progress into endometrial adenocarcinoma in about 25% of the cases (22). Because of this, unopposed estrogens are usually not administered to relieve climacteric problems or to prevent osteoporosis in women who still have a uterus, but the hormone is always provided in combination with progesterone. Progesterone opposes the activity of estrogens in several ways: progesterone decreases the expression of estrogen receptors in the endometrium (23); progesterone increases the conversion of estradiol into the less potent estrone in the glandular epithelium (24, 25); and progesterone increases enzyme activity (sulfotransferase), which can result in inactivation of estrogens (10). Furthermore, it is likely that some genes that are regulated by progesterone oppose the growth-stimulating activities of estrogen-regulated genes.

Tibolone is used for the treatment of climacteric complaints and the prevention of osteoporosis (26). Tibolone exerts tissue-specific effects (4), showing in some tissues a more potent estrogenic activity; in other tissues, such as endometrium, the progestagenic properties of tibolone seem more pronounced (27). The effects and putative adverse effects of tibolone treatment on the endometrium have recently been discussed (27–30).

The current investigations were performed to dissect out the progestagenic effects of tibolone on the endometrium. Therefore, a human endometrial cancer cell line was used that expressed high levels of PRA and PRB, did not express estrogen or androgen receptors, and consequently did not respond to estrogen or androgen treatment (17). Using this cell line, a profound growth inhibitory effect of tibolone, Δ⁴-tibolone, and MPA was observed. Surprisingly, when progesterone-responsive cells were cultured in the presence of the estrogenic 3β-OH metabolite of tibolone, cell growth was also slightly inhibited. This effect of 3β-OH-tibolone was unexpected, because HPLC analyses failed to show any conversion of 3β-OH-tibolone into the progestagenic/androgenic metabolite Δ⁴-tibolone. Differences in experimental conditions between the growth experiments and the HPLC experiments, or the sensitivity of the HPLC measurements may be at the basis of this. However, the observation that 3β-OH-tibolone exhibits weak progestagenic activities has also been made by others. Using human endometrium tissue cultures, Markiewicz and Gurpide (3) showed the regulation of expression of several progesterone-regulated proteins by tibolone, Δ⁴-tibolone, and 3β-OH-tibolone. These results indicate that 3β-OH-tibolone, most likely through conversion to the Δ⁴ metabolite of tibolone, may act as a weak progestagen.

The administration of tibolone itself resulted in a significant reduction of cell growth. Because the affinity of tibolone for progesterone receptors is very low, local conversion into its progestagenic Δ⁴ isomer was expected. To investigate this, progesterone-expressing PRAB-36 cells were cultured for 0,
3, or 6 d in the presence of 50 nm $[^{3}H]$tibolone. It was observed that after 3 d of incubation in the presence of 50 nm $[^{3}H]$tibolone, the compound had been converted to $[^{3}H]$3β-OH-tibolone (19%) and $[^{3}H]$Δ4-tibolone (81%). Despite the fact that $[^{3}H]$3β-OH-tibolone, when administered alone, seems very stable in culture, $[^{3}H]$3β-OH-tibolone, which was produced from tibolone, could no longer be detected in culture after 6 d. This observation seems to indicate that administration of tibolone and conversion into its $\Delta^4$ isomer have a stimulatory effect on the activity of the enzymes involved in the conversion of 3β-OH-tibolone into $\Delta^4$-tibolone (3βHSD). In agreement with this, Tang et al. (31) showed that progestagens are indeed capable of inducing 3βHSD activity in the endometrium. Another interesting finding was that although sulfotransferase activity is documented for Ishikawa cells (32, 33), no sulfation of tibolone or its metabolic products was observed. Sulfation of tibolone, 3α-OH-tibolone, and 3β-OH-tibolone has been reported; however, it is possible that the specific enzymes responsible for sulfation of these compounds are not active in Ishikawa cells.

The experiments performed to date have indicated that tibolone acted mainly through its $\Delta^4$ metabolite to induce growth inhibition of progesterone-receptor-responsive endometrial cancer cells through activation of the hormone receptor. Using the antiprogestagen Org-31489 in combination with tibolone, $\Delta^4$-tibolone, or MPA indeed showed a reversion of the progestagenic effects. Furthermore, it was observed that 10 nm of the antiprogestagen was effective in reverting the effects of 1 nm MPA and 10 nm tibolone or $\Delta^4$-tibolone. This indicated that the affinity of MPA for the progesterone receptor is approximately 10-fold higher than the affinity of tibolone or $\Delta^4$-tibolone for the receptor. These results fit very well with the finding that the ED$_{50}$ for growth inhibitory effects on PRAB-36 cells for MPA is 10-fold lower than the ED$_{50}$ for tibolone and its $\Delta^4$ isomer.

Because tibolone, $\Delta^4$-tibolone, and MPA had comparable growth-inhibiting effects on the progesterone receptor-expressing endometrial cancer cell line PRAB-36, and because these effects could only be measured after several days of culture, it was decided to also study earlier and more direct effects of progestagens on PRAB-36 cells. Regulation of mRNA expression of IGFBP-3 and fibronectin was chosen for further evaluation, because IGFBP-3 and fibronectin had both been reported as early (effects were observed at 8 h) progesterone-regulated genes (Ref. 17; see Footnotes 1 and 2). In the current investigations it was shown that incubation of PRAB-36 cells in the presence of tibolone, $\Delta^4$-tibolone, and MPA resulted in a reproducible decline in the expression of fibronectin and IGFBP-3 mRNA. According to Dai et al. (34), inhibition of expression of the cellular adhesion molecule fibronectin could play a role in inhibiting endometrial cancer cell invasiveness.

In summary, using a progesterone-responsive endometrial cancer cell line it was shown that the $\Delta^4$ metabolite of tibolone displayed clear progestagenic effects on cell growth and gene regulation. Furthermore, it was shown that 3β-OH-tibolone had some minor, but significant, inhibitory effects on cell growth. These results are in good accordance with data from the literature and indicate that tibolone, the $\Delta^4$ metabolite of tibolone, and, to some extent, 3β-OH-tibolone, exert clear progestagenic effects on a progesterone-sensitive endometrial cancer cell line. Tibolone and most likely 3β-OH-tibolone are converted into the progestogenic $\Delta^4$ metabolite.

**Acknowledgments**

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