Selective Tissue Distribution of Tibolone Metabolites in Mature Ovariectomized Female Cynomolgus Monkeys after Multiple Doses of Tibolone

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ABSTRACT:

Tibolone is a selective tissue estrogenic activity regulator (STEAR). In postmenopausal women, it acts as an estrogen on brain, vagina, and bone, but not on endometrium and breast. Despite ample supporting in vitro data for tissue-selective actions, confirmative tissue levels of tibolone metabolites are not available. Therefore, we analyzed tibolone and metabolites in plasma and tissues from six ovariectomized cynomolgus monkeys that received tibolone (0.5 mg/kg/day by gavage) for 36 days and were necropsied at 1, 1.25, 2.25, 4, 6, and 24 h after the final dose. The plasma and tissue levels of active, nonsulfated (tibolone, 3β-hydroxytibolone, and Δ4-tibolone), monosulfated (3α-sulfate,17β-hydroxytibolone and 3β-sulfate,17β-hydroxytibolone), and disulfated (3α,17β-disulfated-tibolone and 3β,17β-disulfated-tibolone) metabolites were measured by validated gas chromatography with mass spectrometry and liquid chromatography with tandem mass spectrometry. Detection limits were 0.1 to 0.5 ng/ml (plasma) and 0.5 to 2 ng/g (tissues). In brain tissues, estrogenic 3α-hydroxytibolone was predominant with 3 to 8 times higher levels than in plasma; levels of sulfated metabolites were low. In vaginal tissues, major nonsulfated metabolites were 3α-hydroxytibolone and the androgenic/progestagenic Δ4-tibolone; disulfated metabolites were predominant. Remarkably high levels of monosulfated metabolites were found in the proximal vagina. In endometrium, myometrium, and mammary glands, levels of 3-hydroxymetabolites were low and those of sulfated metabolites were high (about 98% disulfated). Δ4-Tibolone/3-hydroxytibolone ratios were 2 to 3 in endometrium, about equal in breast and proximal vagina, and 0.1 in plasma and brain. It is concluded that tibolone metabolites show a unique tissue-specific distribution pattern explaining the tissue effects in monkeys and the clinical effects in postmenopausal women.

Tibolone (Livial, Organon, Roseland, NJ and Oss, The Netherlands) is used for the management of postmenopausal symptoms: it relieves hot flushes and vaginal dryness, improves mood and libido, and prevents osteoporosis (Kloosterboer, 2004; Kenemans and Speroff, 2005; Landgren et al., 2005; Swanson et al., 2006). It does not act as an estrogen on endometrium, resulting in a low incidence of vaginal bleeding (Morris et al., 1999). It has little effects on breast as shown by no increase in mammographic density or tenderness (Lundström et al., 2002). Thus, it differs from estrogen-progestagen combinations. These effects of tibolone on different tissues are explained by the hypothesis that it is a selective tissue estrogenic activity regulator (STEAR).

In postmenopausal women, 3α-hydroxytibolone (3αOH-tib) is the predominant nonsulfated, free metabolite in blood, followed by 3β-hydroxytibolone (3βOH-tib) (for structures, see Fig. 1). Both 3-hydroxymetabolites bind to estrogen receptors (de Gooyer et al., 2003; Kloosterboer, 2004) and are responsible for the estrogenic activity. Serum levels of tibolone and the Δ4-isomer of tibolone (Δ4-tib), which bind to progestagenic and androgenic receptors (de Gooyer et al., 2003), are low and become undetectable after 4 to 6 h (Timmer and Houwing, 2002; Timmer et al., 2002). More than 75% of the metabolites in blood are sulfated (Vos et al., 2002). Tibolone and its metabolites have no antagonistic effect on estrogen receptors, rendering it different from selective estrogen receptor modulators (Kloosterboer, 2004). To explain why tibolone is estrogenic in some tissues but not in others, it was postulated that tibolone and its active metabolites are converted into sulfated metabolites and/or into the androgenic/progestagenic Δ4-tib, as has been shown in vitro (Kloosterboer, 2004). Tibolone can be converted to Δ4-tib in endometrial explants (Tang et al., 1993); enzymes of the aldoketoreductase family (AKR1C) are able to reduce tibolone to 3-hydroxymetabolites (Steckelbroeck et al., 2006) and back again (Schatz et al., 2005). In addition, 3αOH-tib and 3βOH-tib are substrates for sulfotransferases (SULT) expressed in different tissues (Chetrite et al., 1999a; Falany et al., 2004; SULT2A1 has been reported to be the major endogenous enzyme responsible for sulfation of tibolone metabolites in postmenopausal human tissues (Wang et al., 2006). Although sulfated metabolites are not active at receptor levels, sulfatases are able to readily convert 3-monosulfated (mono-S), but not 17-sulfated, steroids into active estrogenic metabolites (de Gooyer et al., 2001). In breast cell lines, tibolone and its nonsulfated and sulfated metabolites inhibit

ABBREVIATIONS: 3αβOH-tib, 3αβ-hydroxytibolone; Δ4-tib, Δ4-isomer of tibolone; AKR1C, aldoketoreductase 1C family; SULT, sulfotransferase(s); mono-S, monosulfated; di-S, disulfated; AUC, area(s) under the curve.
sulfatases (Chetrite et al., 1997, 1999a; Purohit et al., 2002). In addition, one study showed that tibolone and its metabolites inhibit sulfatase in endometrium-derived cell lines, whereas no inhibition is found in a bone-derived cell line (de Gooyer et al., 2001). These tissue-specific differences in the balance between sulfation and de-sulfation may contribute to tissue-selective effects of tibolone.

Although the mechanisms involved in the tissue-selective effects of tibolone have been shown in vitro, it is not known whether these mechanisms play a role in vivo. Confirmation by measuring tissue levels of tibolone and its nonsulfated and sulfated metabolites is not yet available. To obtain these tissue levels, six ovariectomized cynomolgus monkeys were treated p.o. with 0.5 mg/kg/day tibolone, and the tissue levels were analyzed. These monkeys have been selected in view of their marked similarity to humans, as found in a previous study with monkeys fed an atherogenic diet, showing protection by tibolone against bone loss, without stimulation of breast and endometrium (Clarkson et al., 2002, 2004; Cline et al., 2002). The dose of 0.5 mg/kg/day was chosen to be able to measure tissue levels of tibolone metabolites. This study presents the levels of tibolone and its nonsulfated, 3-mono-S, and 3- and 17β-disulfated (di-S) metabolites in efficacy (brain, vagina), safety (uterus, breast), and control (heart) tissues, and in plasma from ovariectomized monkeys treated with 0.5 mg/kg/day tibolone for 36 days.

**Materials and Methods**

**Study Design.** A study in mature, healthy, female, ovariectomized cynomolgus monkeys (n = 6; weight 2.0–2.8 kg; supplied by Primate Products, Miami, FL) was approved by the Institutional Animal Care and Use Committee, performed at Huntington Life Sciences (East Millstone, NJ and Huntingdon, Cambridgeshire, UK), and complied with the Animal Welfare Act Regulations and Good Laboratory Practice standards as described elsewhere (Verheul et al., 2007). After at least 1 month postovariectomy, the monkeys received a single dose, and after a washout of 1 week, they received repeated daily p.o. doses of tibolone (Org OD14; 0.5 mg/kg/day) from days 8 through 44 via nasogastric gavage. Blood, urine, and feces were collected for 7 days after the doses on days 1 and 36 for the assessment of the pharmaco kinetic parameters of the sulfated and nonsulfated tibolone metabolites. These results are reported elsewhere (Verheul et al., 2007). At day 44, one monkey was necropsied at each of the following times: 1, 1.25, 2.25, 4, 6, and 24 h after the final dose. This design was chosen to establish concentration-time curves in plasma and tissues, rather than to determine the concentrations at one time point in six animals. Blood was collected into tubes containing K₃EDTA and centrifuged for 10 min at 2000g within 1 h, and plasma was stored below −20°C until analysis. The following tissues were washed free of blood and stored at −20°C until further processing and analysis: brain (cortex, hypothalamus, brainstem, cerebellum, hippocampus, midbrain, corpus callosum, and corpus striatum), vagina (distal and proximal), uterus (endometrium and myometrium), breast (mammary glands and fat), and heart.

**Assay Methods.** The assay procedures for the determination of tibolone and its nonsulfated and sulfated metabolites in plasma and tissue homogenates have been described elsewhere (Verheul et al., 2007).

After weighing, tissues were homogenized with internal standards in 70% ethanol for 5 to 15 min using an ultraturrax. The homogenates were divided into aliquots and stored for at least 24 h before further sample processing. The aliquots were centrifuged; the supernatant was evaporated, redissolved in water, and analyzed. In one aliquot, tibolone, 3αOH-tib, and 3βOH-tib were determined by gas chromatographic and mass spectrometric procedures (ABL, Assen, The Netherlands). The second aliquot was subjected to protein precipitation for analysis of the di-S metabolites: 3αS,17β-tib and 3βS,17β-tib. Liquid-liquid extraction was performed on the third aliquot; Δ4-tib was determined in the organic phase; and the mono-S metabolites (3αS,17βOH-tib and 3βS,17βOH-tib) were extracted by solid-phase extraction from the water phase. All the analytes were determined by liquid chromatography followed by tandem mass spectrometry procedures (Xendo, Groningen, The Netherlands). It was decided to develop assays for the 3α-mono-S metabolites only because 1) the supply of most tissues was very limited; 2) the levels of the 17β-mono-S metabolites were shown in a pilot experiment to be very low in myometrium and liver; and 3) the 17β-mono-S metabolites cannot readily be reconverted to the nonsulfated metabolites again (Goldzieher et al., 1988; de Gooyer et al., 2001; Takahashi et al., 2003; Simoncini et al., 2004). In a previous study (Vos et al., 2002) with radiolabeled tibolone, 17β-mono-S metabolites constituted less than 10% of the radiolabel administered. We have also determined the 3α- and 3β-di-S metabolites because their levels were very high in the pilot study, and these di-S metabolites were considered as end-product for sulfation.

Gas chromatography with mass spectrometry procedures have been validated for human serum, and liquid chromatography/tandem mass spectrometry procedures have been validated for human serum, myometrium, and breast tissue with regard to selectivity, sensitivity, calibration curves, accuracy, precision, stability, dilution, and carryover. The procedures have been used for monkey plasma and tissues without further validation because no monkey control tissue was available for validation. Detection limits were 0.1 to 0.5 ng/ml (plasma) and 0.5 to 2 ng/g (tissues). Analytes in monkey plasma and tissues were determined with acceptable precision (coefficient of variation <20% for overall, within batch, and between batch) and accuracy (bias...
representative brain tissue. The predominant free metabolite in brain tissues was the estrogenic 3αOH-tib, with peak levels at 1 to 2.25 h and decreasing to 10 to 20% at 24 h (Fig. 3A). The predominant sulfated metabolite in most brain regions was 3αS,17βS-tib, with peak levels later and lower (3–20 times) than the corresponding 3αOH-tib. AUC of 3βS,17βS-tib were lower than those of 3αOhtib (Table 2), and AUC of mono-S metabolites were relatively high. Levels of all the sulfated metabolites decreased with time and were less than 1 ng/g at 24 h (data not shown). Assuming that plasma levels (ng/ml) and tissue levels (ng/g) may be compared, AUC in most brain areas were higher for 3αOhtib, 3βOhtib, and Δ4-tib than in plasma. In contrast, AUC of di-S metabolites in most brain tissues were considerably lower, ranging from 1/25 times in midbrain to <1/100 times in other brain regions.

Vagina. The predominant free metabolite in distal vagina was 3αOhtib. In proximal vagina, the androgenic/progestagenic Δ4-tib metabolite had the largest AUC of the three nonsulfated metabolites (Table 2), although the difference with 3αOhtib is not large. In both distal and proximal vaginal tissues, 3αS,17βS-tib was the predominant inactive sulfated metabolite, followed by 3βS,17βS-tib (Table 2). The Tmax were 4 and 1 to 2.5 h for di-S metabolites and for mono-S and free metabolites, respectively. AUC of mono-S metabolites were considerably (6 times) higher in proximal vagina. Compared with plasma, AUC in vagina tissues were lower, except for 3βOhtib and Δ4-tib and for mono-S metabolites (proximal vagina only). Metabolite patterns in vaginal tissues are different from the metabolite patterns in brain and plasma.

Safety Tissues. Uterus. The AUC of the estrogenic metabolite, 3αOhtib, in endometrium was 8-fold higher than that of 3βOhtib (Table 3; Fig. 3B). After their peak at 1 h, both decreased rapidly. In contrast, Δ4-tib levels initially increased for about 4 h and became higher than those of 3αOhtib and 3βOhtib (Fig. 3B). The predominant sulfated metabolite in endometrium was 3αS,17βS-tib, whereas the mono-S metabolites in endometrium were 40- to 50-fold lower than those of the di-S. The metabolite pattern in myometrium was comparable with the endometrial pattern. Compared with plasma, AUC of free and sulfated metabolites were lower in uterine tissues, except those of Δ4-tib, which were considerably (8- to 10-fold) higher. Compared with brain and vagina, the AUC of di-S and Δ4-tib in uterus were higher. The metabolite pattern in uterus is different from those in plasma, brain, and vagina.

Breast. The predominant metabolites in glandular breast tissue were di-S metabolites (Table 3) with peak levels at 4 h. AUC of 3αOhtib and 3βOhtib were 30 times and >100 times lower (Table 3). The AUC of Δ4-tib was comparable with that of 3αOhtib. As in the
endometrium, levels of 3αOH-tib (T\text{max} = 1 h) in the breast rapidly decreased, whereas those of Δ4-tib (T\text{max} = 2.25 h) appeared to decrease later in time. Compared with fat breast tissue, AUC of mono-S and di-S metabolites in glandular tissue were higher (1.5-fold), whereas those of free metabolites were lower (0.5-fold). Compared with plasma, AUC in glandular and fat tissue of S-metabolites were lower (3-fold); those of 3αOH-tib and 3βOH-tib were comparable; and those of Δ4-tib were higher. Compared with vaginal tissue, in particular proximal vagina, the AUC of the mono-S in breast tissues were lower. In both breast tissues and uterus, the AUC of the di-S metabolites were large, but the AUC of the Δ4-tib were lower in mammary glands and higher in mammary fat tissues. The metabolite pattern in breast tissues is different from those in the efficacy tissues, brain, and vagina and from that in uterus.
about 2 in vagina. The ratio of tibolone is rapidly hydrolyzed by AKR1C isoenzymes (Steckelbroeck et al., 2004). Sulfation renders compounds inactive at receptors. Sulfation converts tibolone and its metabolites to monosulfates and disulfates (Falany et al., 1998). The high degree of sulfation in monkey tissues, suggesting that in vitro data should be extrapolated with caution to in vivo. The time-dependent concentration-time curves in tissues have unique tibolone metabolite patterns, which, in turn, are different from that in plasma. As in humans, the predominant nonsulfated metabolite in monkeys is 3αOH-tib, whereas levels of tibolone and Δ4-tib are low and rapidly decrease over time. A quantitative difference from humans is the ratio of 3αOH-tib/3βOH-tib in plasma (2- to 35-fold versus a constant 3-fold in humans). This may be attributed to differences in species, formulation, or design (e.g., dose). The 0.5-mg/kg/day dose was used to enhance the chance to detect metabolites in plasma and tissues and also at longer intervals. Based on body weight, this is 10- to 15-fold, to 2- to 3-fold higher than the human dose. Thus, it would appear that—despite single measurements at necropsy—these results are a good reflection of the actual situation. The time-dependent concentration-time curves in tissues also support this conclusion.

**Discussion**

This report presents levels of tibolone and its free and sulfated metabolites in plasma and, for the first time, in tissues. The summary in Fig. 4 shows that tissues have unique tibolone metabolite patterns, which, in turn, are different from that in plasma.

Enzymes involved in tibolone’s metabolism were described previously. Tibolone is rapidly hydrolyzed by AKR1C isoenzymes (Steckelbroeck et al., 2006). The liver-specific AKR1C4 preferentially forms 3αOH-tib, accounting for the predominance of 3αOH metabolites in plasma. AKR1C1 and AKR1C2 preferentially forming 3βOH-tib were shown in tissues resulting in 3βOH-tib predominance in tissues in vitro. However, our data show that 3αOH-tib is predominant in monkey tissues, suggesting that in vitro data should be extrapolated with caution to in vivo. The high degree of sulfation in plasma may be explained by the SULT2A1 enzyme in the stomach, liver, and intestine (Wang et al., 2006), which is able to convert tibolone and its metabolites to monosulfates and disulfates (Falany et al., 2004). Sulfation renders compounds inactive at receptors. Sulfation at the 17-position seems to be irreversible (de Gooyer et al., 2001) as shown in rats with double-labeled estradiol sulfate (Takanashi et al., 2003). Two studies suggest, however, that 17-sulfation may, to some degree, be reversible (Goldzieher et al., 1988; Simoncini et al., 2004).

Both AUC and concentration-time curves of tibolone metabolites in plasma at necropsy from one monkey per time point were similar to AUC and concentration-time curves obtained on day 36, based on the mean of six monkeys. Thus, it would appear that—despite single measurements at necropsy—these results are a good reflection of the actual situation. The time-dependent concentration-time curves in tissues also support this conclusion.

Our results show that tibolone metabolites have unique patterns in various tissues. Brain tissues contain high levels of 3-hydroxytibolone and low levels of di-S metabolites and a considerable percentage of mono-S metabolites (Fig. 4). One explanation may be that only more lipophilic, nonsulfated metabolites can pass the blood-brain barrier. However, this would not explain the higher levels of free metabolites in brain compared with plasma. Recently, organic anion transporter proteins have been found in various tissues, including the brain (Kullak-Ublick et al., 1998; Steckelbroeck et al., 2004). These proteins can transport 3-sulfated steroids, such as estrone sulfate and dehydroepiandrosterone sulfate, across membranes. Assuming that these organic anion transporter proteins described in human temporal lobe are expressed in monkey brain and that they act on other 3-sulfated steroids, it would explain the relatively high levels of the 3-mono-S tibolone metabolites in brain tissues. Mono-S metabolites entering the brain can then readily be desulfated by sulfatases (Gold-
gende may thus serve as an estrogenic reservoir. The androgenic/progestagenic predominance in the proximal vagina can be desulfated to active estrogenic hydroxy metabolites and sulfated estrogenic metabolites in the distal vagina. These high levels of 3-monosulfated metabolites in the proximal vagina is high (39%) compared with the distal vagina. Interestingly, in the proximal vagina, the percentage of mono-S metabolites is high, compared with the distal vagina. These high levels of 3-monosulfated metabolites in the proximal vagina can be desulfated to active estrogenic hydroxy metabolites and sulfated estrogenic metabolites in the distal vagina. This difference in sulfation is due to the high degree of sulfation and the presence of the progestagenic metabolite. The vaginal balance is only slightly toward estrogenic metabolites, but the observed estrogenic effects on the brain, in this case, a reduction in hot flushes as shown in ovariec-tomized monkeys (Jelinek et al., 1984) and postmenopausal women (Landgren et al., 2005). However, estrogenic effects of tibolone on the pituitary seem not to be very strong because tibolone only partly reduces follicle-stimulating hormone levels in postmenopausal women (Doeren et al., 2001) and cynomolgus monkeys (Gibbs et al., 2002), in contrast to continuous combined regimens resulting in near-complete suppression. Unfortunately, the pituitary was too small for assessment of hormone levels.

Compared with the brain, the proximal vagina has a different metabolite pattern (Fig. 4) with higher progestagen/estrogen ratios. The vaginal balance is only slightly toward estrogenic metabolites, but this is apparently sufficient for favorable vaginal effects of tibolone (Kloosterboer, 2004). Interestingly, in the proximal vagina, the percentage of mono-S metabolites is high (39%) compared with the distal vagina. These high levels of 3-monosulfated metabolites in the proximal vagina can be desulfated to active estrogenic hydroxy metabolites and may thus serve as an estrogenic reservoir. The androgenic/progestagenic metabolite is likely not to interfere with tibolone’s estrogenic effects on the vagina: in rats (de Gooyer et al., 2003) progestagens do not interfere with estrogenic responses in vagina, and in early postmenopausal women addition of medroxyprogesterone acetate does not prevent estrogenic effects of conjugated equine estrogens on the vagina maturation index (Utian et al., 2001). The androgenic effects of 4-tib may also contribute to vaginal lubrication (Traish et al., 2002). These observations provide an explanation for favorable effects of tibolone on vaginal atrophy and dryness observed in postmenopausal women (Morris et al., 1999; Kloosterboer, 2004; Kenemans and Speroff, 2005).

Compared with efficacy tissues, metabolite patterns in safety tissues were different. In contrast to the brain, tibolone metabolites in the mammary glands were almost completely (98%) in the di-S form (Fig. 4), implying that the balance is toward sulfation. This is supported by in vitro data. Falany et al. (2004) have shown that of the three major human SULT, SULT2B1b is expressed in breast tissue, and that tibolone, 3a-OH-tib, and 3βOH-tib are substrates. In addition, tibolone and its metabolites inhibit sulfatase activity in breast cell lines (de Gooyer et al., 2001). These combined mechanisms shift the system toward sulfation. The AUC of the androgenic/progestagenic metabolite is higher in breast tissues than in plasma and the brain, and the AUC-based 4-tib/3-OH-tib ratios in mammary glands and fat are approximately 1, suggesting no androgenic/progestagenic or estrogenic predominance. Recently, Stute et al. (2006) showed that in monkeys the amount of breast sulfatase activity is lower after 2 years of tibolone treatment in tissues with a high fat content.

The potential stimulatory estrogenic effects of tibolone on the breast may be attenuated by the androgenic effects of 4-tib because physiological doses of testosterone inhibit estrogen-induced mammary epithelial proliferation in ovariectomized monkeys (Dimitrakakis et al., 2003). These observations suggest antiestrogenic effects of tibolone on breast tissue, in line with its neutral effects on mammographic density and its low incidence of breast tenderness in postmenopausal women (Lundström et al., 2002; Hofling et al., 2005).

In the endometrium, the percentage of sulfated metabolites is also high (>97%) (Fig. 4), indicating that comparable mechanisms may be operational to those in the breast. SULT3E1 is expressed in endometrium and modulated by progestagenic activity (Falany and Falurum, 1996; Chetrite et al., 1999a,b), and sulfatase activity in endometrial tissue-derivated cell lines is inhibited by tibolone and its non-sulfated and sulfated metabolites (de Gooyer et al., 2001). In addition to the high sulfation rate, levels of 4-tib in endometrium increase after about 1 h and remain high, exceeding levels of 3αOH-tib and 3βOH-tib after about 4 h. This increase in 4-tib at times when plasma 4-tib is undetectable strongly suggests local formation from the 3-hydroxy metabolites, possibly by AKR1C enzymes, as shown in vitro (Schatz et al., 2005; Steckelbroeck et al., 2006). The (tib + 4-tib)/3αOH-tib + 3βOH-tib ratios in the endometrium are toward the androgenic/progestagenic 4-tib. The presence of 4-tib is supported by the generation of progestagen-sensitive factors in endometrial cells (Tang et al., 1993; Schatz et al., 2005). It appears that at least two mechanisms preventing stimulation are operational in the endometrium: a high degree of sulfation and the presence of the progestagenic metabolite 4-tib. This corroborates with the lack of endometrial stimulation after tibolone treatment observed in cynomolgus monkeys (Cline et al., 2002) and in clinical studies (Kloosterboer, 2004). In conclusion, the different tibolone metabolite patterns in plasma and tissues of cynomolgus monkeys are the result of multiple mechanisms, including metabolism and enzymatic inactivation or activation, confirming that tibolone is a selective tissue estrogen activity regulator. The observed patterns explain the clinical effects in postmenopausal women.

References