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IN VITRO EVALUATION OF ESTROGENIC, ESTROGEN ANTAGONISTIC AND PROGESTAGENIC EFFECTS OF A STEROIDAL DRUG (Org OD-14) AND ITS METABOLITES ON HUMAN ENDOMETRIUM

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Summary—The human endometrial model for *in vitro* evaluation of estrogenic, estrogen antagonistic, and progestagenic effects of endogenous steroids, natural products or synthetic drugs was applied to the study of Org OD-14, an analog of norethynodrel developed by Organon International, Oss, The Netherlands, and some of its metabolites. Estrogen antagonistic actions of Org OD-14 and its 4-ene isomer were evident from their ability to suppress the enhancement of $PGF_{2\alpha}$ output elicited by estradiol on fragments of secretory endometrium and to decrease the rate of output of the prostaglandin by proliferative tissue, already stimulated by endogenous estrogens. These inhibitory effects were similar to those obtained with progesterone and do not appear to involve competition for the estrogen receptor since the antiestrogen 4-hydroxyamoxifen was not active in parallel incubations of proliferative endometrium. The progestagenic effects of Org OD-14 and its 4-ene isomer were also evident from their capability to enhance estradiol 17β-dehydrogenase activity and glycogen accumulation in specimens of proliferative endometrium. Estrogenic effects of the 3α- and 3β-hydroxy metabolites of Org OD-14 were demonstrated by their stimulatory actions on $PGF_{2\alpha}$ output during incubations of secretory endometrium. The estrogenic and progestagenic actions of these compounds are in general agreement with their relative affinity for binding to the estradiol and progesterone receptors, although their actions may be influenced by intracellular metabolism in the endometrial tissue. For instance, the similarity in progestagenic activity of Org OD-14 and the 4-ene isomer, contrasting with their different affinities for the progesterone receptor, may result from *in situ* isomerization of Org OD-14 to the 4-ene metabolite.

INTRODUCTION

Human *secretory* endometrium in culture responds to the addition of estradiol (E_2) to the medium by markedly increasing both $PGF_{2\alpha}$ and PGE_2 output [1–3]. The effect of E_2 can be counteracted with either progesterone (P) or *trans*-4-hydroxytamoxifen (OHTam) [1–4] but these estrogen antagonists appear to act through different mechanisms since only OHTam significantly competes with E_2 for binding to the estrogen receptor. The relatively large output of $PGF_{2\alpha}$ by *proliferative* endometrium during the first days in culture cannot be further stimulated by E_2 and can be suppressed by P but not by OHTam. Other effects on the human endometrium are specific to progestins: enhancement of 17β-hydroxysteroid dehydrogenase activity in proliferative endometrium in culture [5] and accumulation of glycogen in the epithelial cells of endometrial glands [6].

These findings led us to propose [7] the use of human endometrial tissue, obtaining as surgical specimens from uterine curettages or hysterectomies, for the *in vitro* evaluation of estrogenic, antiestrogenic, estrogen antagonistic and progestagenic actions of endogenous compounds, natural products or synthetic drugs. This approach offers the obvious advantage of providing information on actions in a human target tissue, directly relevant to subsequent clinical studies on women, avoiding animal experimentation of questionable relevance.

We have already reported results obtained with this model system on the effects of C_{19}-steroids of adrenal origin [8] and on the effects of clomiphene citrate [9]. We are now presenting results obtained with Org OD-14 and its metabolites (structures shown in Fig. 1). Org OD-14 is a product of Organon International B.V., Oss, The Netherlands, and is marketed in Europe for the treatment of climacteric patients. It is reported to have weak estrogenic, androgenic and progestational activity, to prevent bone loss and to alleviate vasomotor symptoms in perimenopausal women [10]. The pharmacologic importance of the drug, the availability of clinical data, the reported complexity of its hormonal actions, and the

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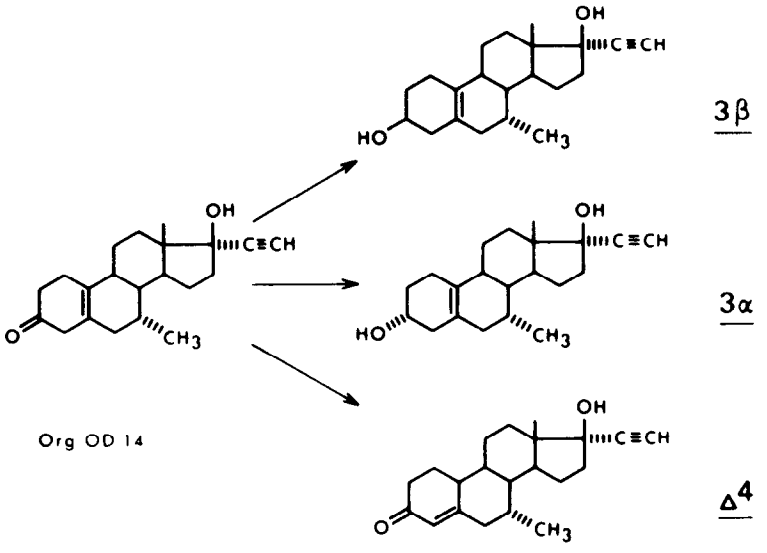

Fig. 1. Chemical structures and abbreviated notation of Org OD-14 and metabolites (reprinted from Ref. [10], with permission).

possibility of studying the effects of some of its metabolites justifies its selection for a practical application of the model system.

MATERIALS AND METHODS

Tissue collection

Specimens of histologically normal endometrium were obtained from uteri of patients undergoing hysterectomy for reasons other than endometrial neoplasia. Immediately after excision, the uterus was opened and endometrial tissue, removed with a scalpel from the fundal region, was transferred to the laboratory in sterile minimum essential medium (MEM) containing 1% of an antibiotic–antimycotic mixture (Grand Island Biological Co., Grand Island, N.Y., GIBCO). Under a laminar flow hood, the tissue was trimmed, washed with Hank's Balanced Salt Solution (HBSS, GIBCO), and cut into small fragments (approx. 1 mm^3). Randomized fragments were fixed in formalin for histologic dating according to the method of Noyes *et al.*[11].

Evaluation of $PGF_{2\alpha}$ production

Fragments of endometrium were placed on lens paper resting on stainless steel grids in various 6 cm-diameter polystyrene culture dishes (Falcon Plastics, Los Angeles, Calif.) containing 3.5 ml of Ham's F10 medium (GIBCO), supplemented with 10 ng/ml insulin, 4 mg/ml glucose, 1% antibiotic–antimycotic mixture, and 10% bovine calf serum (CS, Flow Labs, McLean, Va.) pretreated with dextran-coated charcoal to remove endogenous steroids as previously described [8]. The tissue was only partially immersed in the medium in order to facilitate oxygenation. The dishes, each holding 4–10 mg tissue, were kept in an incubator at 37°C in a humidified atmosphere of 95% air–5% CO_2. After a 24 h "settling period", the medium was replaced by medium containing the following test compounds, alone or in combination: E_2 (Sigma Chemical Co., St Louis, Mo.), P (Sigma), OHTam (a gift from Stuart Pharmaceuticals, Division of Imperial Chemical Industries, Wilmington, Del.), and Org OD-14, its 4-ene isomer and the 3α- and 3β-reduced metabolites (provided by Dr L. Tax, Organon International B.V.). The vehicle-control dishes contained 0.1% ethanol. Incubations were carried out in parallel for another 24 h. At the end of the incubation period, the medium from each dish was collected and centrifuged, storing the supernatants at −70°C for $PGF_{2\alpha}$ radioimmunoassay. Tissue from each dish was recovered, washed thoroughly, homogenized and analyzed for protein content, using the method of Lowry *et al.*[12].

Prostaglandin $F_{2\alpha}$ radioimmunoassay

Levels of $PGF_{2\alpha}$ were measured by RIA performed on duplicate aliquots and at 2 dilutions of unextracted culture medium, as previously described [2]. A rabbit anti-$PGF_{2\alpha}$ antibody was kindly supplied by Dr Franco Bolelli, Servizio di Fisiopatologia della Reproduzione, Policlinico S. Orsola, Universita degli Studi, Bologna.

Estradiol 17β dehydrogenase activity

Fragments of proliferative endometrium were placed in 6 cm culture dishes (15–20 mg/dish) resting on lens paper over a stainless steel grid, as described above. Incubations were continued for 2–3 days in media containing test compounds or vehicle, changing medium every 24 h. At the end of the incubation period the tissue was collected and stored at −70°C. Estradiol 17β-dehydrogenase activity was measured using a radiometric method previously described [5]. The reaction mixture consisted of an 800 *g* supernatant of tissue homogenate in 50 mM Tris

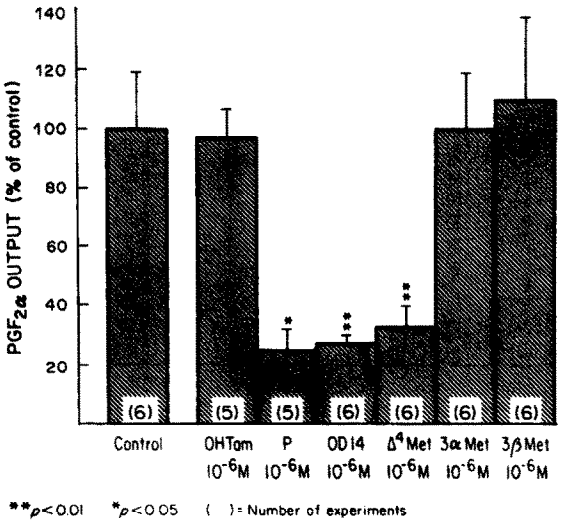

Fig. 2. Inhibitory effects of Org OD-14 and its 4-ene isomer on $PGF_{2\alpha}$ output by proliferative endometrium during the first 24 h after addition of test compounds ($\bar{X} + SE$). Average $PGF_{2\alpha}$ output in controls: 300 ng/(mg protein × day) ± 57, $n = 6$. P values relative to control.

buffer, pH 8.0, containing about 0.8 mg protein/ml, 18 μM [^{3}H]E_2 (SA approx. 500 dpm/pmol) and 1.4 mM NAD^+. The reaction was carried out in a shaking water bath kept at 37°C. Aliquots were taken at different time intervals between 2 and 8 min and added to methanol containing [^{14}C]estrone (E_1) indicator to evaluate losses and a mixture of E_1 and E_2 carriers (500 μg each). After centrifugation and evaporation of the supernatants to dryness, the residues were subjected to TLC using silica gel plates (Analthech Inc., Newark, Del.) to isolate E_1. Amounts of [^{3}H]E_1 formed were calculated from $^3H/^{14}C$ ratios in eluted E_1 and rates of conversion of E_2 to E_1 were estimated from the slopes of regression lines corresponding to plots of amounts of E_1 formed as a function of incubation times. Enzymatic activities were expressed as nmol E_1 formed per mg protein per h.

Glycogen accumulation

Effects on glycogen accumulation were evaluated by incubating for 72 h fragments of proliferative endometrium in Ham's F10-10%CS containing test compounds or vehicle. Formation of vesicles containing glycogen was visualized by hematoxylin-eosin staining, as described elsewhere [13].

Statistics

Statistical significance of differences between means were estimated by paired Student's t-test.

RESULTS

Antiestrogenic and estrogen antagonistic effects of Org OD-14 and metabolites

On proliferative endometrium. Figure 2 shows that both Org OD-14 and the 4-ene metabolite, added to the medium at 1 μM concentration, reduce the output of $PGF_{2\alpha}$ during the first 24 h of incubation as strongly as P at the same concentration. The 3α- and 3β-metabolites have no significant effects on $PGF_{2\alpha}$ output. It can then be concluded that Org OD-14 and its isomer can act as estrogen antagonists by mechanisms similar to those of P.

Figure 3 shows a concentration dependence of the estrogen antagonistic effects of Org OD-14 in proliferative endometrium, statistically significant at 100 nM and 1 μM but not at 10 nM.

On secretory endometrium. Figure 4 shows that Org OD-14 and its 4-ene isomer practically eliminate the marked increase in $PGF_{2\alpha}$ output produced by addition of E_2 to the culture medium, when the drugs are mixed with E_2 in a 100-fold molar excess. These effects of Org OD-14 and the 4-ene isomer are similar to those of P or OHTam at equimolar concentration.

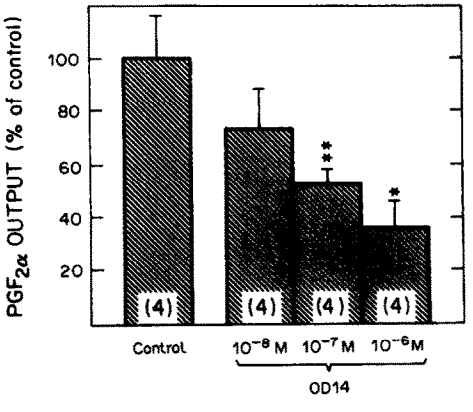

Fig. 3. Dose–dependence of Org OD-14 on $PGF_{2\alpha}$ output by proliferative endometrium ($\bar{X} + SE$). Average $PGF_{2\alpha}$ output in controls: 200 ng/(mg protein × day) ± 33, $n = 4$. P values relative to control.

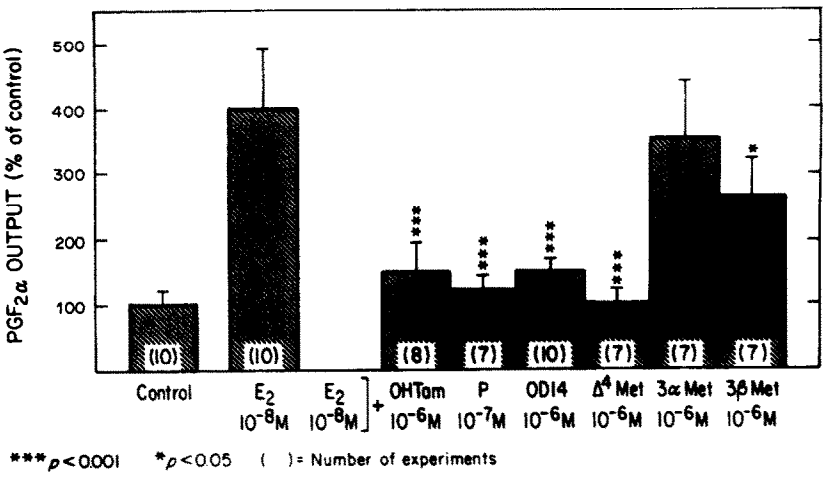

Fig. 4. Estrogen antagonistic effects of Org OD-14 and metabolites on $PGF_{2\alpha}$ output by secretory endometrium ($\bar{X}$ + SE). Average $PGF_{2\alpha}$ output in controls: 160 ng/(mg protein × day) ± 37, $n = 10$. P values relative to E_2.

In order to test for the possibility that isomerases present in serum could have converted Org OD-14 to the 4-ene metabolite before it entered endometrial cells, 5 experiments similar to those reported in Fig. 4 were carried out on secretory endometrium in the presence or absence of serum. It was found (data not shown) that under serum-free conditions, as well as in the presence of serum, Org OD-14 completely counteracted the E_2 effect on $PGF_{2\alpha}$ output.

As shown in Fig. 4, the 3-hydroxy metabolites are either very weakly antiestrogenic (3β) or ineffective (3α).

Estrogenic effects of Org OD-14 metabolites

These studies were conducted on secretory endometrium since E_2 does not increase prostaglandin output by proliferative tissues *in vitro*

Figure 5 shows that Org OD-14 and the 4-ene isomer are devoid of estrogenic activity in this system, whereas the 3β and, with a lower statistical significance, the 3α-reduced metabolites are estrogenic. For the purpose of comparison, the figure also includes effects of E_2 and P in parallel incubations.

Progestagenic effects of Org OD-14 and metabolites

Estradiol 17β-dehydrogenase activity in proliferative endometrium. Figure 6 shows that Org OD-14 and the 4-ene isomer are as effective as P at the same concentration in enhancing the activity of estradiol 17β-dehydrogenase, a typical and specific endometrial response to natural and synthetic progestagens [5].

Glycogen accumulation in proliferative endometrium. Figure 7 shows that Org OD-14 and the 4-ene isomer at 1 μM levels provoke *in vitro* an accumulation of glycogen comparable to that produced by P at the same concentration, the 3α-metabolite did not induce glycogen accumulation and the 3β-metabolite had a very weak effect.

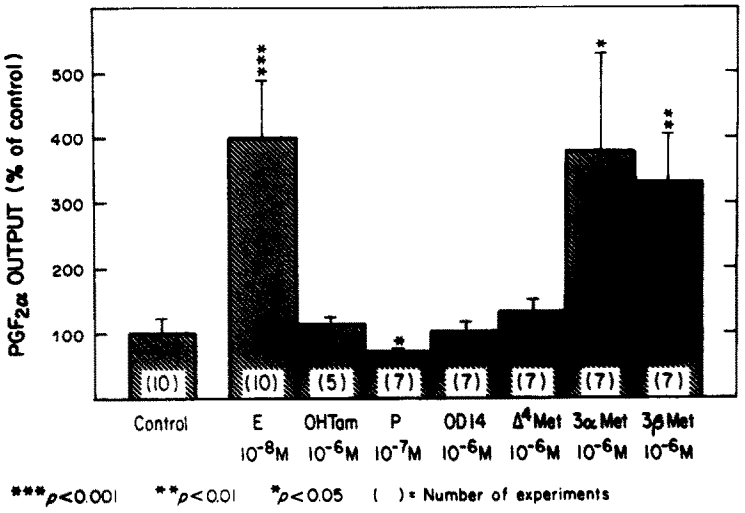

Fig. 5. Estrogenic effects of 3α- and 3β-hydroxy metabolites of Org OD-14 on $PGF_{2\alpha}$ output by secretory endometrium ($\bar{X}$ + SE). Average $PGF_{2\alpha}$ output in controls: 160 ng/(mg protein × day) ± 37, $n = 10$. P values relative to control.

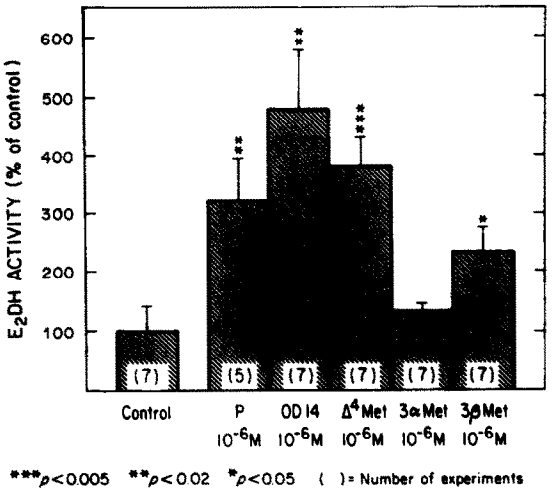

Fig. 6. Progesterone-like stimulation of estradiol 17β-dehydrogenase on proliferative endometrium by Org OD-14 and its 4-ene isomer ($\bar{X}$ + SE). Estradiol 17β-dehydrogenase in controls: 0.67 nmol E1/(mg protein × h) ± 0.25, $n = 7$. P values relative to control.

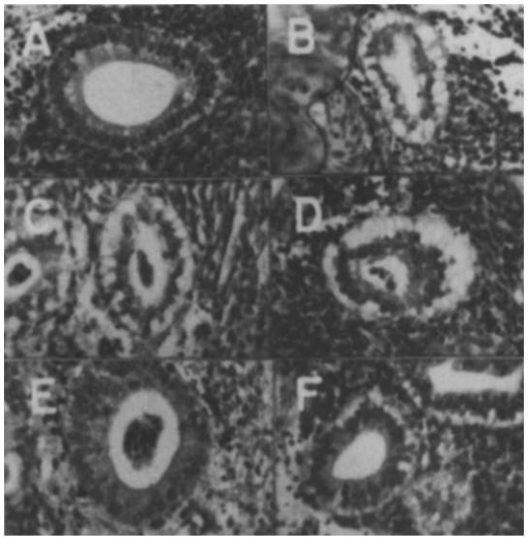

Fig. 7. Effects of Org OD-14 and metabolites on sub-nuclear glycogen accumulation in proliferative endometrium after 72 h in culture in medium alone (A), or in the presence of 1 μM progesterone (B), Org OD-14 (C), 4-ene isomer (D), 3α-hydroxy metabolite (E) or 3β-hydroxy metabolite (F).

DISCUSSION

The progestin-like and estrogen antagonistic *in vitro* effects of Org OD-14 and its 4-ene metabolite (7α-methyl analogs of norethinodrel and norethindrone, respectively) were demonstrated in this series of experiments by their inhibitory actions of $PGF_{2\alpha}$ output and stimulatory effects on estradiol 17β-dehydrogenase activity and glycogen accumulation. The estrogen antagonistic effects were of the progestin type, rather than the competitive OHTam type, since they were also observed in proliferative endometrium.

The observed progestagenic activity of the 4-ene isomer is in good agreement with its affinity for the progesterone receptor, as shown in Table 1. In contrast, Org OD-14 also showed progestagenic activity while its affinity for the receptor was much lower. On the basis of this paradox, we investigated the possibility of a conversion of Org OD-14 to the 4-ene metabolite by endometrial tissue. Incubation of tissue homogenates with Org OD-14 revealed an extensive shift of the 5–10 double bond in the substrate to the 4–5 position, as estimated from the decline of 206 nm absorption concurrent with the appearance of absorption at 240 nm (unpublished results). Since isomerase activity was also detected in human plasma, the possibility that a conversion of Org OD-14 to the 4-ene metabolite before entering the endometrial cells could account for the estrogen antagonistic effects of Org OD-14 shown in Fig. 4 required consideration. However, the equivalence of these results with those obtained during incubations in serum-free medium ruled out this possibility. These findings suggest the interesting possibility of a metabolic regulation of the activity of the drug at the target tissue level, which might account for tissue

Table 1. Relative binding affinities of Org OD-14 and metabolites for the progesterone and the estrogen receptors

	Relative affinity*			
	PR		ER	
Compound	Human myometrial cytoplasm	MCF-7 cells	Human myometrial cytoplasm	MCF-7 cells
Reference compounds				
Estradiol	—	—	100	100
Org 2058	100	100	—	—
Progesterone	18	—	—	—
Norethisterone	42	18.8	—	—
Norethynodrel	6	2.2	1.5	<0.5
Org OD-14 and metabolites				
Org OD-14	3	4.9	1.3	1.3
4-ene isomer	48	12.9	0.5	ND
3α OH metab.	<0.5	ND	3.9	3.2
3β OH metab.	<1	ND	3.0	1.7

ND = not detectable. *Data from Scientific Development Group, Organon International B.V., Oss, The Netherlands (unpublished), obtained as described by Bergink *et al.*[18, 19].

specificity in the relative estrogenic/progestagenic activities of Org OD-14. In fact, homogenates of secretory endometrium fragments washed with serum-free medium showed conversion of Org OD-14 to the 4-ene metabolite.

Clinical tests involving prolonged oral administration of Org OD-14 (2.5 mg/day) to climacteric patients revealed an atrophic endometrium in most (over 80%) of the treated patients and initial proliferation, shown by histologic patterns similar to those observed in the early proliferative phase, in others [10, 14, 15]. In the same studies, the vagina appeared to be a more sensitive target for estrogenic activity. It might be of interest to determine in short-term *in vivo* experiments whether the progestagenic effects of Org OD-14 observed *in vitro* could be duplicated. The concentrations used in the *in vitro* studies were higher than those expected during treatments since, assuming a metabolic clearance rate for Org OD-14 equal to that of norethindrone (about 500 l plasma/day [16]), a 2.5 mg daily dose would result in an average concentration of about only 20 nM.

The *in vitro* estrogenic effects of the 3α- and 3β-hydroxy metabolites, and the lack of such actions by Org OD-14 and the 4-ene metabolite, correspond to their relative affinity for the estrogen receptor (Table 1). The progestagenic properties of the 3β-hydroxyl metabolite, evident from its effects on estradiol 17β-dehydrogenase activity and glycogen accumulation, may result from the action of an endometrial 3β-hydroxysteroid dehydrogenase leading to its conversion to Org OD-14 and the 4-ene metabolite.

The expected overall effect of administration of Org OD-14 to human subjects would depend on the relative proportion of circulating drug and metabolites. It is recognized, however, that the activity of unconjugated 3-hydroxy metabolites observed *in vitro* may not correspond to that of their glucuronides or sulfates which, by similarity to the metabolites of endogenous steroids and ethisterone [17], are likely to be the forms in which they are present in blood.

The reported effects of Org OD-14 on perimenopausal vasomotor symptoms and its antiovulatory effects [10], indicate achievement of effective concentration of the drug or metabolites in the brain. The weak interaction of Org OD-14 with sex hormone binding globulin in blood may favor crossing of the brain–blood barrier, although interactions with other plasma proteins could be significant. Whether the pharmacologic actions of Org OD-14 or metabolites on the hypothalamus or pituitary correspond to their progestagenic actions, evident from these *in vitro* studies, remains to be elucidated.

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