The oestrogenic effects of gestodene, a potent contraceptive progestin, are mediated by its A-ring reduced metabolites

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Abstract

Gestodene (17 α -ethynyl-13 β -ethyl-17 β -hydroxy-4,15-gonadien-3-one) is the most potent synthetic progestin currently available and it is widely used as a fertility regulating agent in a number of contraceptive formulations because of its high effectiveness, safety and acceptability. The observation that contraceptive synthetic progestins exert hormone-like effects other than their progestational activities, prompted us to investigate whether gestodene (GSD) administration may induce oestrogenic effects, even though the GSD molecule does not interact with intracellular oestrogen receptors (ER).

To assess whether GSD may exert oestrogenic effects through some of its neutral metabolites, a series of experimental studies were undertaken using GSD and three of its A-ring reduced metabolites. Receptor binding studies by displacement analysis confirmed that indeed GSD does not bind to the ER, whereas its 3 β ,5 α -tetrahydro reduced derivative (3 β GSD) interacts with a relative high affinity with the ER. The 3 α ,5 α GSD isomer (3 α GSD) also binds to the ER, though to a lesser extent. The ability of the A-ring reduced GSD derivatives to induce oestrogenic actions was evaluated by the use of two different molecular bioassays: (a) transactivation of a yeast system co-transfected with the human ER α (hER α) gene and oestrogen responsive elements fused to the β -galactosidase reporter vector and (b) transactivation of the hER α -mediated transcription of the chloramphenicol acetyl transferase (CAT) reporter gene in a HeLa cells expression system. The oestrogenic potency of 3 β GSD was also

assessed by its capability to induce oestrogen-dependent progestin receptors (PR) in the anterior pituitary of castrated female rats.

The results demonstrated that 3 β GSD and 3 α GSD were able to activate, in a dose-dependent manner, the hER α -mediated transcription of both the β -galactosidase and the CAT reporter genes in the yeast and HeLa cells expression systems respectively. In both assays the 3 β derivative of GSD exhibited a significantly greater oestrogenic effect than its 3 α isomer, while unchanged GSD and 5 α GSD were completely ineffective. Neither 3 β GSD nor 3 α GSD exhibited oestrogen synergistic actions. Interestingly, the pure steroidal anti-oestrogen ICI-182,780 diminished the transactivation induced by 3 β GSD and 3 α GSD in the yeast expression system. Furthermore, administration of 3 β GSD resulted in a significant increase of oestrogen-dependent PR in the anterior pituitaries of castrated rats in comparison with vehicle-treated animals. The characteristics of the 3 β GSD-induced PR were identical to those induced by oestradiol benzoate.

The overall results demonstrate that 3 β GSD and its 3 α isomeric alcohol specifically bind to the ER and possess a weak intrinsic oestrogenic activity, whereas unmodified GSD does not. The data contribute to a better understanding of the GSD mechanism of action and allow the hypothesis to be advanced that the slight oestrogen-like effects attributable to GSD are mediated by its non-phenolic, tetrahydro reduced metabolites.

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Introduction

The availability of novel, very potent synthetic progestins has allowed the development of the so-called third

generation contraceptive formulations, representing a breakthrough in the field of fertility regulation (Kuhl 1996). The most effective of these progestins is gestodene (GSD), a synthetic 19-nor steroid that has been widely

used in low dose combined oral contraceptives (Fotherby & Caldwell 1994, Wilde & Balfour 1995). Recently, a series of studies (WHO 1995a,b, Jick *et al.* 1995, Lewis *et al.* 1996) have suggested that low dose, combined oral contraceptives pills, containing highly potent progestins, such as GSD, probably carry a small risk of venous thromboembolic disease (VTD) beyond that attributable to combined oral formulations containing less potent progestins such as norethisterone or levonorgestrel. The origin of VTD associated with the use of hormonal contraceptives is multifactorial and a number of mechanisms has been involved (Inman & Vessey 1968, Vessey *et al.* 1986, Helmrich *et al.* 1987, WHO 1989, Vandenbroucke *et al.* 1994, WHO 1995b, Spitzer *et al.* 1996, Winkler 1998). The unexpected association between the use of GSD containing oral contraceptives and an increased risk of VTD raised the controversial question as to whether GSD may exert potent oestrogen agonistic or synergistic effects (WHO Scientific Group 1998), particularly since the GSD molecule does not interact with intracellular oestrogen receptors (ER) (Düstenberg *et al.* 1987).

Evidence has been accumulated over the last years indicating that contraceptive synthetic progestins exert potent hormone-like effects other than their progestational activities (Vilchis *et al.* 1986, Pérez-Palacios *et al.* 1992, Lemus *et al.* 1997). The hormonal agonist, synergistic and even antagonistic effects of synthetic progestins are mediated either by their interaction with the wrong receptors (Bardin 1983, Chávez *et al.* 1985, Lemus *et al.* 1992) or by their metabolic conversion products (Larrea *et al.* 1987, Lemus *et al.* 1992).

This study was aimed at elucidating the role of the GSD metabolites in determining oestrogen-like effects. A series of experiments were undertaken to assess whether non-phenolic A-ring reduced derivatives of GSD may be involved in mediating its oestrogenic activity, taking advantage of the fact that GSD, after its administration, undergoes extensive metabolism in a similar manner to other 19-nor synthetic progestins (Düstenberg *et al.* 1987). The *in vivo* formation of 3 β ,5 α GSD (3 β GSD) and 3 α ,5 α GSD (3 α GSD) has been demonstrated after the administration of ^{14}C -labelled GSD to normal women (Düstenberg *et al.* 1987). Assessment of the intrinsic oestrogenic potency of GSD and its derivatives was done by the use of ER binding studies, oestrogen-induced transactivation systems in yeast and HeLa cells previously co-transfected with human ER α (hER α) expression vectors and their cognate reporter vectors, and induction of oestrogen-dependent progestin receptors (PR) in the rat anterior pituitary.

Additional interest in conducting these studies derived from the observation in our laboratory (Vilchis *et al.* 1986, Morali *et al.* 1990, Pérez-Palacios *et al.* 1992) that norethisterone, a synthetic contraceptive progestin,

exerts potent oestrogen-like effects through its neutral tetrahydro reduced metabolites.

Materials and Methods

Steroids and chemicals

Authentic GSD was kindly provided by Schering AG (Berlin, Germany) and 5 α -dihydro GSD (5 α GSD) was synthesised by lithium–ammonia reduction of GSD, crystallised from ethyl acetate–hexane and purified by flash chromatography (Still *et al.* 1978). A 60% yield of the pure compound was obtained. The 3 α ,5 α GSD tetrahydro derivative (3 α GSD) was prepared from reduction of 5 α GSD with L-selectride under anhydrous conditions (Brown & Krifhnamurthy 1972). Sodium hydroxide and hydrogen peroxide were added, and following extraction with ethyl acetate, a mixture of 3 α - (98%) and 3 β - (2%) GSD derivatives were obtained (yield: 95%). Synthesis of 3 β ,5 α tetrahydro GSD (3 β GSD) was performed by sodium borohydride reduction (Bowers *et al.* 1958) of 5 α GSD (yield: 70%). The molecular structures of GSD and its metabolites are depicted in Fig. 1. Chemical purity of GSD and its derivatives was assessed by their melting points, HPLC behaviour, infrared absorption, and ^{13}C - and ^1H -nuclear magnetic resonance. The physical and spectroscopic constants of the A-ring reduced GSD derivatives were as follows:

5 α GSD: m.p. 168–170 °C; i.r. (KBr) 3362, 3258, 3047, 2948, 2924, 2857, 2086, 1697 cm^{-1} ; ^1H -NMR (300 MHz, CDCl_3) δ 5.92 (dd, $J=5.7$ and 1.5 Hz, 1H), 5.67 (dd, $J=5.7$ and 3.6 Hz, 1H), 2.60 (s, 1H), 0.867 (t, $J=7.5$ Hz, 3H); ^{13}C -NMR (75 MHz, CDCl_3) δ 11.11, 20.36, 25.60, 29.51, 30.30, 30.37, 33.70, 38.74, 41.21, 43.67, 45.59, 47.91, 48.53, 55.80, 56.50, 74.89, 83.06, 83.82, 132.01, 135.56, 211.51; MS (FAB) 313.

3 α GSD: m.p. 178–181 °C; i.r. (KBr) 3567, 3392, 3252, 3051, 2929, 2089 cm^{-1} ; ^1H -NMR (300 MHz, CDCl_3) δ 5.93 (dd, $J=5.7$ and 1.52 Hz, 1H), 5.65 (dd, $J=5.7$ and 3.6 Hz, 1H), 4.09 (m, 1H), 2.60 (s, 1H), 0.85 (t, $J=7.5$ Hz, 3H); ^{13}C -NMR (75 MHz, CDCl_3) δ 11.10, 20.38, 23.56, 25.18, 29.60, 30.74, 32.90, 33.45, 36.15, 39.05, 40.54, 46.96, 48.33, 55.87, 56.81, 66.33, 74.68, 83.31, 83.83, 132.30, 135.33; MS (FAB) 315.

3 β GSD: m.p. 139–141 °C; i. r. (KBr) 3373, 3281, 2919, 2859 cm^{-1} ; ^1H -NMR (300 MHz, CDCl_3) δ 5.84 (dd, $J=5.5$ and 1.2 Hz, 1H), 5.57 (dd, $J=5.5$ and 3.6 Hz, 1H), 3.47 (m, 1H), 2.53 (s, 1H), 0.768 (t, $J=7.2$ Hz, 3H); ^{13}C -NMR (75 MHz, CDCl_3) δ 10.83, 20.21, 25.37, 28.12, 29.42, 30.60, 33.22, 35.27, 38.86, 41.17, 42.86, 45.95, 48.11, 55.68, 56.60, 70.05, 74.18, 83.32, 131.99, 135.25; MS (FAB) 315.

[2,4,6,7- ^3H]oestradiol (^3H E $_2$), specific activity (sp. act.) 97 Ci/mmol was purchased from Amersham

Figure 1 Molecular structures of GSD and three of its A-ring reduced metabolites, 5 α -dihydrogestodene (5 α GSD), 3 α ,5 α -tetrahydrogestodene (3 α ,5 α GSD) and 3 β ,5 α -tetrahydrogestodene (3 β ,5 α GSD).

International plc (Bucks, UK) and its radiochemical purity was established by its thin-layer chromatographic behaviour and by repeated crystallisation of an aliquot to constant specific activity. Other natural and synthetic steroids were supplied by Sigma Chemical Co. (St Louis, MO, USA). The anti-oestrogen ICI-182,780 was obtained from Zeneca Farma (Mexico City, Mexico). All reagents and solvents used were analytical grade.

Oestradiol receptor binding studies

All procedures were performed in accordance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare and approved by the Research Ethics Board of the Universidad Autonoma Metropolitana. Immature female, intact Wistar rats (body weight: 100–200 g) without oestrogen priming were used for these studies. Animals were kept under a 14 h light : 10 h darkness cycle, maintained on food and water and allowed

to feed *ad libitum*. Animals were killed by decapitation and uteri were immediately removed, blotted and weighed; thereafter all procedures were carried out at 4 °C. Uterine tissues were homogenised in chilled TEDLM buffer (20 mM Tris-HCl, pH 7.4 at 4 °C, 1.5 mM EDTA, 0.25 mM dithiotreitol, 10 µg/ml leupeptine, and 10 mM sodium molybdate) in a ratio (w/v) 1:6, with three 10 s bursts.

The homogenate was centrifuged at 180 000 *g* for 1 h at 2 °C in an SW 50.1 rotor (Beckman Instruments, Palo Alto, CA, USA). Cytosol protein was determined by the Bradford's dye binding method (Bradford 1976) using bovine serum albumin as a standard.

Stereospecificity of the binding of GSD and its derivatives to ER was assessed by displacement analysis as previously reported (Chávez *et al.* 1985). Uterine cytosol aliquots (2.0–3.4 mg protein/ml) were incubated with [³H]E₂ in the absence or presence of increasing concentrations (1–500 nM) of radioinert E₂ at 2–4 °C for 18–20 h. The relative binding affinities (RBA) of GSD

and its derivatives to cytosol ERs were evaluated by their capability to displace bound [^3H]E₂ from the ER. Radioactive content in the samples was determined in a Packard Tri-Carb liquid scintillation spectrometer, Model 1900 TR (Packard, Downers Grove, IL, USA) using Insta-Gel Plus as counting solution. The counting efficiency for ^3H was 65% and quenching was corrected in all samples by external standardisation. The RBA and the inhibition constant (K_i) of GSD and its metabolites were calculated according to procedures previously described (Cheng & Prusoff 1973, Reel *et al.* 1979).

Oestrogen-induced yeast transactivation system

Oestrogen-like activity of GSD and its A-ring reduced derivatives was assessed in the yeast expression system as previously reported (Lyttle *et al.* 1992), where *Saccharomyces cerevisiae* hyperpermeable yeast strain RS 188 N was co-transfected with an expression vector containing the hER α , under the control of a yeast copper metallothionein promoter and a β -galactosidase reporter vector, expression of which was under the control of oestrogen response elements (EREs). Yeast cultures were grown overnight, as described by Sherman *et al.* (1982), diluted to an optical density (o.d.) of 0.5 at 660 nm and allowed to grow for 1 h, after which ER α synthesis was induced with 100 μM copper sulphate for an additional hour and incubated for 4.5 h with varying concentrations of GSD, its derivatives, and/or other naturally occurring and synthetic steroids including the pure anti-oestrogen ICI-182,780. Oestradiol served as the positive control, while dihydrotestosterone (DHT), progesterone (P₄) and pregnenolone (P₅) were the negative controls. Yeast were harvested by centrifugation, washed with Z buffer (50 mM NaH₂PO₄, pH 7.0, 10 mM KCl, 1 mM MgSO₄, 5 mM β -mercaptoethanol) and the cytosolic fraction was obtained by vortexing with glass beads in Z buffer followed by centrifugation at 10 000 g. The supernatant was assayed for β -galactosidase activity using *o*-nitrophenyl- β -D-galactoside (4 mg/ml) as substrate, and the o.d. at 420 nm was registered after 10 min.

Oestrogen-induced HeLa cells transactivation system

The oestrogen-like actions of GSD and its A-ring reduced derivatives were also assessed in the HeLa cells co-transfected with the mammalian expression vector for human ER α (PCR 3.2-hER α) and its cognate reporter vector EREe1b-chloramphenicol acetyl transferase (CAT) (Cooney *et al.* 1992, 1993). HeLa cells were plated the day before transfection in a six-well plate at a density of 3.0×10^5 cells/well in DMEM-high glucose (HG) without phenol red, which was supplemented with 5% stripped fetal bovine serum (FBS) and 10 000 U of penicillin and streptomycin. Cells were maintained in 5% CO₂ atmosphere at 37 °C. The next day, cells were visualised to assure cell density between 30 and 50%.

Transfections were performed in triplicate using SuperFect (Qiagen, Inc., Valencia, CA, USA). In a sterile tube, 100 μl of serum-free media was aliquoted and the DNA was added, after vortexing 10 μl of SuperFect reagent were added and vortexed for 10 s. The samples were incubated at room temperature for 5–10 min and afterwards 600 μl of supplemented DMEM-HG were added to each sample and the mixture was pipetted up and down twice. The medium containing the transfection complexes was added to the cell monolayer previously rinsed with PBS. The plates were incubated for 3 h at 37 °C with 5% CO₂. After incubation, the plates containing the transfection complexes were rinsed with PBS, and 3 ml of complete growth medium were added to each well. Twenty-four hours later, the medium in each well was replaced with complete medium containing different concentrations of GSD, 5 α GSD, 3 α GSD, 3 β GSD and E₂. Dimethyl sulphoxide (DMSO) or ethanol (EtOH) were used as steroid vehicles. The next day the plates were harvested for CAT assays as previously described (Cooney *et al.* 1992, 1993). Briefly, the medium was aspirated from each well and the plates were rinsed with PBS, and 1 ml of cold TEN (40 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl) buffer was added to each well. The cells were removed from the monolayer with a cell lifter, and the contents of each well were transferred to an 1.5 ml tube. The tubes were centrifuged for 5 min at 4 °C. The supernatant was removed and 80 μl of cold 0.25 M Tris-HCl, pH 8.0 were added to each sample which was kept on ice. Samples went through three freeze-thaw cycles and centrifugation at 4 °C. The liquid CAT assay as described by Seed & Sheen (1998) was used to determine the CAT activity, employing 5 μg protein, 25 μg of butyryl coenzyme-A (Sigma Chemical Co., St Louis, MO, USA), 2×10^5 c.p.m. of xylene-extracted [^3H]chloramphenicol (Dupont NEN Research Products, Boston, MA, USA) and 0.25 M Tris-HCl, pH 8.0 for 1 h incubation at 37 °C.

Oestrogen-induced rat anterior pituitary PR

The induction of PR in the anterior pituitary (AP) of castrated rats was used as previously described (Vilchis *et al.* 1986) to further assess the oestrogenic potency of the 3 β GSD derivative. Adult female Wistar rats (body weight 200–250 g) were ovariectomised under ether anaesthesia and kept under a 14 h light : 10 h darkness cycle with free access to water and food. Three weeks after ovariectomy, groups of 20 rats each were daily injected for 6 consecutive days with 100 μl of propyleneglycol containing either 500, 1000 or 1500 μg of 3 β GSD. Animals treated with 5 $\mu\text{g}/\text{day}$ for 6 days oestradiol benzoate (E₂B) or with vehicle alone were used as experimental controls. After completion of the treatment, animals were killed by decapitation and the AP were immediately removed, rinsed with ice-cold TEDM buffer (20 mM Tris-HCl, pH 7.4 at

4 °C, 1.5 mM EDTA, 0.25 mM dithiothreitol, and 10 mM sodium molybdate), blotted and weighed. Thereafter all procedures were carried out at 4 °C. Tissues were homogenised in a glass homogeniser with a Teflon pestle with TEDM buffer supplemented with 10% glycerol (v/v) and 10 µg/ml leupeptine in a tissue:buffer ratio of 1:6 (w/v). Pituitary cytosol preparations were obtained as described for binding studies. Aliquots of cytosol (300–400 µl; 10–14 mg protein/ml) were incubated with 4 nM [6,7-³H]ORG 2058 (promegestone) for 4 h at 4 °C and layered on the top of linear sucrose gradients (20–35% in TEDM buffer supplemented with 10% (v/v) glycerol). An excess (125 ×) of radioinert ORG 2058 was added to an incubation set of cytosols from animals treated with 3βGSD. BSA, on a parallel gradient, was used as an external marker. Gradients were centrifuged at 398 000 *g* for 2 h and 30 min at 2 °C in a VTi 65 rotor (Beckman Instruments, Palo Alto, CA, USA). Fractions of 150 µl each were collected from the bottom of the gradients and their radioactive content determined.

Statistical analysis

The results of transactivation studies in the yeast and HeLa cells expression systems are expressed as mean ± S.D. for each experimental group. Significance of differences was evaluated by Student's *t*-test. Group differences were considered to be significant when $P \leq 0.05$.

Results

Competition of GSD and its metabolites for ER

The effects of increasing concentrations of non-radioactive GSD and its A-ring reduced derivatives upon the [³H]E₂ binding to uterine cytosolic ER obtained from immature rats are shown in Fig. 2. The RBAs and the inhibition constant (*K_i*) for each steroid are shown in Table 1. The only striking competitor for the ER binding sites was the 3βGSD derivative (RBA: 1.34%; *K_i*: 40 nM), though the 3αGSD isomer also induced a slight competitive effect (RBA: 0.67%; *K_i*: 80 nM). Unmodified GSD and 5αGSD were completely ineffective. Naturally occurring E₂ used as positive control was the most potent inhibitor.

Transactivation of the yeast expression system by GSD metabolites

The oestrogen-like effects of GSD and its A-ring reduced metabolites, as assessed in the hyperpermeable yeast expression system, are shown in Fig. 3. The 3β,5α tetrahydro derivative of GSD and its 3α,5α isomer were able to induce the ERα-dependent transactivation of the yeast system in a dose-response manner, similar to that induced by naturally occurring E₂, yet to a significantly

Figure 2 Competition of GSD and three A-ring reduced GSD metabolites for the rat uterine [³H]-ER binding sites. Oestradiol receptors were labelled *in vitro* by overnight incubations of uterine cytosol preparations from prepubertal animals, with 1 nM [³H]E₂, at 4 °C (final incubation volume=200 µl). Increasing concentrations of the following radioinert steroids were added to the incubations: E₂ (◆), GSD (■), 5αGSD (●), 3αGSD (✱) and 3βGSD (▲). Bound and free fractions were separated by the addition of a dextran-coated charcoal suspension. Each point represents the mean of five experiments in triplicate. The results show that 3βGSD was the most effective synthetic steroid competitor for ER binding sites, though 3αGSD also induced a slight competitive effect. Unmodified GSD and 5αGSD were completely ineffective. Naturally occurring E₂, used as positive control, was the most potent competitor.

lesser extent. At steroid concentrations that induced the highest β-galactosidase transactivation (Fig. 3), the oestrogen-like effect of 3βGSD (50 nM) was 100-fold less potent than E₂ (0.5 nM), whereas 3αGSD (500 nM) was 25-fold less potent than 3βGSD (20 nM). The 5α dihydro derivative of GSD had very little, if any, effect on the oestrogen-mediated transactivation of the yeast expression system, even at 1000 nM dose, whereas unmodified GSD was completely devoid of oestrogen-like effects as depicted in Fig. 3. A lack of oestrogenic potency of DHT, P₄ and P₅, used as negative controls, was observed on this expression system. When 1 µM of the pure anti-oestrogen

Table 1 Relative binding affinities (RBAs) and inhibition constants (*K_i*) of natural and synthetic steroids for cytosol ERs. [³H]E₂ was used as radioligand. RBAs were determined according to Reel *et al.* (1979) and *K_i* values were calculated according to Cheng & Prusoff (1973)

Steroid competitors	RBA (%)	<i>K_i</i> (nM)
GSD	—	—
5αGSD	—	—
3αGSD	0.67	80
3βGSD	1.34	40
E ₂	100	0.53

Figure 3 Activation of hER α -mediated β -galactosidase activity by GSD and its A-ring reduced metabolites in a yeast expression system. The yeast strain RS 188 N was co-transfected with an expression vector containing the hER α and oestrogen responsive elements fused to the β -galactosidase reporter vector. Yeast were grown in the presence of increasing concentrations of GSD and its derivatives, as well as naturally occurring steroids. The results are expressed as β -galactosidase activity. Each point represents the mean $\pm$ s.d. of three experiments in triplicate (for details see the text). The results show that 3 β GSD and 3 α GSD induced the hER α -mediated β -galactosidase activity in a dose-dependent manner, similar to that observed with naturally occurring E₂ used as positive control. 5 α GSD exhibited very little, if any, oestrogen-like effect, while unchanged GSD was completely ineffective. Dihydrotestosterone (DHT), progesterone (P₄), pregnenolone (P₅) and the vehicle (Veh), used as negative controls, were also ineffective.

ICI-182,780 was added to the incubations of co-transfected yeast cultures with either 5 μ M E₂, 100 μ M 3 β GSD or 500 μ M 3 α GSD, a significant diminution of the β -galactosidase activity induced by E₂ and the two GSD tetrahydro metabolites was observed, as depicted in Fig. 4. To investigate whether GSD and its derivatives may exert synergistic oestrogenic effects, yeast cultures were incubated with 100 nM GSD, 100 nM 3 β GSD and 500 nM 3 α GSD in the presence of 0.5 nM E₂. The results (Fig. 5) showed that neither GSD nor its two tetrahydro-reduced derivatives exhibit synergistic effects with E₂ in this system. Oestradiol exerted additive effects with the GSD tetrahydro metabolites.

Transactivation of the HeLa cells expression system by GSD metabolites

The oestrogen-like effects of GSD and its derivatives as assessed in the HeLa cells expression system are shown in Fig. 6. In general, the results were similar, though not identical, to those obtained in the transactivation yeast expression system. The 3 β GSD derivative exhibited

oestrogen agonistic effects, inducing transactivation of the hER α -mediated CAT activity, while its 3 α isomer also induced the transactivation of the CAT system to a significant lesser extent. Unmodified GSD was completely ineffective to activate the hER α -mediated CAT activity, thus indicating a lack of oestrogen agonistic effect (Fig. 6).

3 β GSD induction of rat anterior pituitary PR

Incubations of AP cytosol from vehicle-treated castrated rats with [³H]ORG 2058 exhibited very little, if any, progestin binding activity as disclosed by the employed gradient labelling technique (Fig. 7), whereas oestradiol benzoate (E₂B) administration (5 μ g/day) fully restored the pituitary content of oestrogen-dependent PR. Daily administration of 3 β GSD for 6 consecutive days to castrated animals successfully restored anterior pituitary PR in a dose-dependent manner; thus the dose of 500 μ g/day was almost ineffective, whereas the daily doses of 1000 and 1500 μ g were able to induce the presence of PR in the rat AP. The [³H]ORG 2058 complexes in the

Figure 4 Effect of the pure anti-oestrogen ICI-182,780 (ICI) on the hER α -mediated β -galactosidase activity in the yeast expression system described in Fig. 3. Yeast cultures were exposed to 5 nM E₂, 100 nM 3 β GSD and 500 nM 3 α GSD in the absence or presence of 1 μ M ICI. Results are expressed as the β -galactosidase activity and each point represents the mean $\pm$ S.D. of three experiments in triplicate. E₂ and ICI alone were used as controls. The results show that ICI diminished the oestrogen-like effects of 3 β GSD and 3 α GSD, indicating that these effects are indeed hER α -mediated.

rat pituitary cytosol sedimented at 7–9 S in linear sucrose gradients, whether induced by E₂B or by 3 β GSD (Fig. 7). Addition of an excess of radioinert ORG 2058 completely abolished the PR labelling induced by the administration of 3 β GSD (data not shown). These results demonstrated that 3 β GSD exhibits, in this model, oestrogenic effects similar to those observed after E₂B administration with a significantly lower potency.

Discussion

The results presented herein provide evidence that GSD, a widely used contraceptive synthetic progestin, exhibits limited oestrogen-like effects mediated by two of its A-ring reduced metabolites. Receptor binding studies demonstrated that GSD does not interact at all with ER, confirming and extending previous reports (Düstenberg *et al.* 1987, Wilde & Balfour 1995). Interestingly however, the 3 β ,5 α tetrahydro GSD derivative specifically binds to ER, though with a significantly lower binding affinity than naturally occurring E₂ (Fig. 2; Table 1). The 3 α ,5 α tetrahydro GSD derivative also interacts with ER, yet to

Figure 5 Effects of naturally occurring E₂ on the β -galactosidase activity stimulated by GSD and its A-ring reduced derivatives in the yeast expression system. Yeast were grown in the presence of 100 nM GSD, 100 nM 3 β GSD and 500 nM 3 α GSD. E₂ was added to incubations at 0.5 nM concentration in ethanol. All determinations were performed at least in triplicate on three separate experiments. The results (mean $\pm$ S.D.) demonstrate that GSD and its tetrahydro A-ring reduced derivatives, when incubated simultaneously with E₂, does not exhibit oestrogen synergistic effects. The data show an additive effect of E₂.

a significantly lesser extent than its 3 β isomeric alcohol (Fig. 2; Table 1). These results are similar but not identical to those reported for norethisterone (NET), another 19-nor synthetic contraceptive progestin; indeed, the 3 β ,5 α and the 3 α ,5 α reduced NET metabolites specifically interact with ER (Chávez *et al.* 1985) though with a higher relative binding affinity than the corresponding GSD tetrahydro metabolites. The 5 α dihydro derivative of GSD was unable to bind ER (Fig. 2), a finding that correlates with the previous observation that 5 α dihydro NET is not recognised by ER (Chávez *et al.* 1985).

To assess whether the interaction of 3 β GSD and 3 α GSD with the ER results in genomic oestrogen-like effects, a transactivation yeast expression system was used. This system has proved to be a suitable model to evaluate hormone agonistic, synergistic and antagonistic activities (Lyttle *et al.* 1992). The results indicated that 3 β GSD was able to induce hER α -mediated β -galactosidase activity in a dose–response manner (Fig. 3) thus demonstrating a clear oestrogen-agonistic effect in this system. Indeed, the 3 β GSD-induced transactivation of the β -galactosidase

Figure 6 Induction of human ER α -mediated transactivation of CAT by GSD and its A-ring reduced metabolites in a HeLa cell expression system. Cells were co-transfected with the expression vector for hER α (PCR 3'-2-hER α) and its cognate reporter vector (EREe1bCAT). Cells were exposed to GSD, 3 β GSD, 3 α GSD and E₂ at 10 nM concentration. DMSO and EtOH were used as vehicles. Results are expressed as the CAT activity (c.p.m. × 10⁻³) and represent the mean ± S.D. of triplicates. The results demonstrate that unmodified GSD was unable to induce hER α -mediated CAT activity in this system, whereas 3 β GSD exhibited oestrogen agonistic effects. 3 α GSD exhibited also oestrogen-like effects, though to a lesser extent than 3 β GSD.

gene was similar to that exhibited by E₂ though with a significantly lower potency, an observation that is in line with their corresponding binding affinities for the ER. The 3 α GSD isomer also induced hER α -mediated β -galactosidase activity in a dose-dependent manner, indicating an oestrogen agonistic effect. The oestrogen-like potency of 3 α GSD was significantly lower than that of 3 β GSD. The lack of oestrogen-like effects of unmodified GSD and 5 α GSD correlates with their lack of binding to ER.

To provide additional support to the observation that oestrogenic effects of 3 β GSD and 3 α GSD are indeed hER α -mediated, a study using ICI-182,780, a pure anti-oestrogen (Wakeling *et al.* 1991), was conducted. The results showed that ICI-182,780 significantly diminished the E₂-, 3 β GSD- and 3 α GSD-induced transactivation of the β -galactosidase gene in the yeast expression system (Fig. 4), thus confirming that the oestrogenic actions of GSD tetrahydro derivatives are mediated through the hER α . Since GSD-containing oral contraceptive formulations are combined with ethynyl oestradiol, it was of interest to investigate whether GSD and/or its A-ring reduced metabolites may exert oestrogen synergistic effects. The results indicated that neither GSD nor its derivatives exhibited oestrogen synergism, rather an

Figure 7 Induction of oestrogen-dependent PRs in the anterior pituitary of ovariectomised adult rats by 3 β GSD. PRs were assessed by a sucrose gradient labelling technique using [³H]ORG 2058. BSA (arrow) was used as an external marker. Results are expressed as the sedimentation profile of PR (7–9 S) in cytosol pituitary preparations from animals treated for 6 consecutive days with different doses, 1000 μ g (◆) and 1500 μ g (▲) of 3 β GSD. Animals treated with 5 μ g oestradiol benzoate (E₂B)/day for 6 days served as positive controls (■), whereas rats treated with vehicle alone (*) were the negative controls. Administration of E₂B fully restored the pituitary content of PR in castrated animals. 3 β GSD was effective in restoring oestrogen-dependent PR in a dose-dependent manner, though it was ineffective at the 500 μ g/day dose. Addition of an excess of radioinert ORG 2058 abolished the PR labelling induced by 3 β GSD (data not shown).

additive effect of GSD and its metabolites was observed when they were simultaneously incubated with E₂ in the yeast expression system (Fig. 5).

To further evaluate the oestrogen-like potency of 3 β GSD and 3 α GSD in a mammalian expression system, HeLa cells co-transfected with the hER α and CAT vectors were used. The results demonstrated that unmodified GSD was unable to induce the hER α -mediated activation of CAT, thus indicating a lack of oestrogen agonistic effect. The 3 β ,5 α reduced metabolite of GSD induced the transactivation of CAT in a similar manner to that of naturally occurring E₂ (Fig. 6). The 3 α ,5 α reduced

GSD derivative also was able to induce the hER α -mediated CAT activation, though with a significantly lower potency than that exerted by 3 β GSD. These results are similar to those obtained in the yeast expression system, indicating that both 3 β GSD and 3 α GSD exert oestrogen actions mediated by the hER α , whereas their parent compound, GSD is completely devoid of these effects. Whether GSD and its derivatives also exert oestrogenic actions through the oestrogen receptor β (ER β) activation (Kuiper *et al.* 1996, Mosselman *et al.* 1996), can not be ascertained from these studies and deserve further investigation, particularly because of the relevant role that ER β may play on the cardiovascular effects of oestrogens (Kuiper *et al.* 1997, Grohé *et al.* 1998, Giguère *et al.* 1998).

To investigate whether the receptor-mediated oestrogenic activity of 3 β GSD, demonstrated in both expression systems, may be expressed in an *in vivo* model, its ability to induce oestrogen-dependent PR in the anterior pituitary was studied in castrated female rats. The results demonstrated that 3 β GSD restored, in a dose-dependent manner, the rat pituitary content of PR in a similar fashion to that of E₂B, though with a significantly lower potency (Fig. 7). This finding is similar to that previously reported for the 3 β ,5 α derivative of NET (Vilchis *et al.* 1986); however, the oestrogen agonistic potency of 3 β ,5 α GSD was lower than that of the corresponding 3 β ,5 α NET derivative, an observation that correlates with their relative binding affinities for the ER.

This study demonstrates that two GSD derivatives, 3 β GSD and 3 α GSD, possess weak oestrogenic activity and lack oestrogen synergistic actions. The weak oestrogen-like potency of the GSD metabolites, as assessed by their low RBA for the ER and the high concentration of 3 β GSD required to induce *in vivo* PR in the rat pituitary, does not appear to be related to some clinical effects attributed to GSD use. This observation is in line with the latest case-control studies and analyses which have found no difference in the VTD risk between users of oral contraceptives containing GSD or older 19-nor progestins, particularly when the data were controlled for age and duration of contraceptive use (Farmer & Preston 1995, Spitzer *et al.* 1996, Farmer *et al.* 1997, WHO Scientific Group 1998, IFFS 1999).

The overall results provide a plausible explanation for those studies which have suggested that high concentrations of 19-nor progestins, including GSD, stimulate MCF-7 breast cancer cell growth by activating the ER (Van der Burg *et al.* 1992, Catherino *et al.* 1993, Kalkhoven *et al.* 1994, Schoonen *et al.* 1995). All in all, this study contributes to a better understanding of the mode of action of GSD.

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