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

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Abstract

Gestodene (17α-ethyl-13β-ethyl-17β-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 3β,5α-tetrahydro reduced derivative (3βGSD) interacts with a relative high affinity with the ER. The 3α,5α GSD isomer (3αGSD) 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 ERα (hERα) gene and oestrogen responsive elements fused to the β-galactosidase reporter vector and (b) transactivation of the hERα-mediated transcription of the chloramphenicol acetyl transferase (CAT) reporter gene in a HeLa cells expression system. The oestrogenic potency of 3βGSD was also assessed by its capability to induce oestrogen-dependent progesterin receptors (PR) in the anterior pituitary of castrated female rats.

The results demonstrated that 3βGSD and 3αGSD were able to activate, in a dose-dependent manner, the hERα-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 3β derivative of GSD exhibited a significantly greater oestrogenic effect than its 3α isomer, while unchanged GSD and 5αGSD were completely ineffective. Neither 3βGSD nor 3αGSD exhibited oestrogen synergistic actions. Interestingly, the pure steroidal anti-oestrogen ICI-182,780 diminished the transactivation induced by 3βGSD and 3αGSD in the yeast expression system. Furthermore, administration of 3βGSD 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 3βGSD-induced PR were identical to those induced by oestradiol benzoate.

The overall results demonstrate that 3βGSD and its 3α 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, Vandenburgoucke 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 3β,5α-GSD (3βGSD) and 3α,5α-GSD (3αGSD) has been demonstrated after the administration of 14C-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 ERα (hERα) 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 5α-dihydro GSD (5αGSD) 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 3α,5αGSD tetrahydro derivative (3αGSD) was prepared from reduction of 5αGSD with t-selectride under anhydrous conditions (Brown & Krillhammurthy 1972). Sodium hydroxide and hydrogen peroxide were added, and following extraction with ethyl acetate, a mixture of 3α- (98%) and 3β- (2%) GSD derivatives were obtained (yield: 95%). Synthesis of 3β,5α tetrahydro GSD (3βGSD) was performed by sodium borohydride reduction (Bowers et al. 1958) of 5αGSD (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 13C- and 1H-nuclear magnetic resonance. The physical and spectroscopic constants of the A-ring reduced GSD derivatives were as follows:

5αGSD: m.p. 168–170 °C; i.r. (KB) 3362, 3258, 3047, 2948, 2924, 2857, 2086, 1697 cm−1; 1H-NMR (300 MHz, CDCl3) 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); 13C-NMR (75 MHz, CDCl3) 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.

3αGSD: m.p. 178–181 °C; i.r. (KB) 3567, 3392, 3252, 3051, 2929, 2089 cm−1; 1H-NMR (300 MHz, CDCl3) δ 5.93 (dd, J=5.7 and 1.5 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); 13C-NMR (75 MHz, CDCl3) 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.

3βGSD: m.p. 139–141 °C; i. r. (KB) 3373, 3281, 2919, 2859 cm−1; 1H-NMR (300 MHz, CDCl3) 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); 13C-NMR (75 MHz, CDCl3) 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.

13C- and 1H-nuclear magnetic resonance. The physical and spectroscopic constants of the A-ring reduced GSD derivatives were as follows:

\[ [4,6,7-^3H] \text{oestradiol (} [^3H] \text{E2}) \], specific activity (sp. act.) 97 Ci/mmol was purchased from Amersham
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 molibdate) 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.

Sterespecificity 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

Figure 1 Molecular structures of GSD and three of its A-ring reduced metabolites, 5α-dihydrogestodene (5αGSD), 3α,5α-tetrahydrogestodene (3α,5αGSD) and 3β,5α-tetrahydrogestodene (3β,5αGSD).
and its derivatives to cytosol ERs were evaluated by their capability to displace \[^{3}H\]E_2 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 \(^{3}H\) was 65% and quenching was corrected in all samples by external standardisation. The RBA and the inhibition constant (K) 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\(\alpha\), under the control of a yeast copper metallothionein promoter and a \(\beta\)-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\(\alpha\) synthesis was induced with 100 \(\mu\)M 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 (P\(_4\)) and pregnenolone (P\(_3\)) were the negative controls. Yeast were harvested by centrifugation, washed with Z buffer (50 mM Na\(_2\)HPO\(_4\), pH 7.0, 10 mM KCl, 1 mM MgSO\(_4\), 5 mM \(\beta\)-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 \(\beta\)-galactosidase activity using o-nitrophenyl-\(\beta\)-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\(\alpha\) (PCR 3-2-hER\(\alpha\)) 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 \(3\times10^5\) 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% CO\(_2\) 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 \(\mu\)l of serum-free media was aliquoted and the DNA was added, after vortexing 10 \(\mu\)l of SuperFect reagent were added and vortexed for 10 s. The samples were incubated at room temperature for 5–10 min and afterwards 600 \(\mu\)l 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% CO\(_2\). 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\(\alpha\)GSD, 3\(\alpha\)GSD, 3\(\beta\)GSD and E\(_2\). 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 TREN (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 \(\mu\)l 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 \(\mu\)g protein, 25 \(\mu\)g of butyryl coenzyme–A (Sigma Chemical Co., St Louis, MO, USA), 2 \times 10^5 c.p.m. of xylene-extracted [\(^{3}H\)]chloroamphenicol (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\(\beta\)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 \(\mu\)l of propylene glycol containing either 500, 1000 or 1500 \(\mu\)g of 3\(\beta\)GSD. Animals treated with 5 \(\mu\)g/day for 6 days oestradiol benzoate (E\(_2\)B) 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
The oestrogen-like GSD metabolites Transactivation of the yeast expression system by tetrahydro derivative of 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 \(3\beta\)GSD derivative (RBA: 1·34%; \(K_i\): 40 nM), though the \(3\alpha\)GSD isomer also induced a slight competitive effect (RBA: 0·67%; \(K_i\): 80 nM). Unmodified GSD and \(5\alpha\)GSD were completely ineffective. Naturally occurring \(E_2\) 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 \(3\beta,5\alpha\) tetrahydro derivative of GSD and its \(3\alpha,5\alpha\) isomer were able to induce the ER-\(\alpha\)-dependent transactivation of the yeast system in a dose–response manner, similar to that induced by naturally occurring \(E_2\), yet to a significantly lesser extent. At steroid concentrations that induced the highest \(\beta\)-galactosidase transactivation (Fig. 3), the oestrogen-like effect of \(3\beta\)GSD (50 nM) was 100-fold less potent than \(E_2\) (0·5 nM), whereas \(3\alpha\)GSD (500 nM) was 25-fold less potent than \(3\beta\)GSD (20 nM). The \(5\alpha\) 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, \(P_4\) and \(P_5\), used as negative controls, was observed on this expression system. When 1 \(\mu\)M of the pure anti-oestrogen

4 °C, 1·5 mM EDTA, 0·25 mM dithiothreitol, and 10 mM sodium sulphate), homogenised 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 (w/v) and 10 \(\mu\)g/ml leupeptin 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 \(\mu\)l; 10–14 mg protein/ml) were incubated with 4 nM \([6,7-\text{3H}]\text{ORG }2058\) (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 \times \(\mu\)l) of radioinert ORG 2058 was added to an incubation set of cytosols from animals treated with \(3\beta\)GSD. 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 \(\mu\)l 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 \(P\leq0\cdot05\).

**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 \([\text{3H}]\text{E}_2\) 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 \(3\beta\)GSD derivative (RBA: 1·34%; \(K_i\): 40 nM), though the \(3\alpha\)GSD isomer also induced a slight competitive effect (RBA: 0·67%; \(K_i\): 80 nM). Unmodified GSD and \(5\alpha\)GSD were completely ineffective. Naturally occurring \(E_2\) used as positive control was the most potent inhibitor.

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ICI-182,780 was added to the incubations of co-transfected yeast cultures with either 5 µM E₂, 100 µM 3αGSD or 500 µM 3βGSD, 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 3αGSD and 500 nM 3βGSD 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 3βGSD derivative exhibited oestrogen agonistic effects, inducing transactivation of the hERα-mediated CAT activity, while its 3α isomer also induced the transactivation of the CAT system to a significant lesser extent. Unmodified GSD was completely ineffective to activate the hERα-mediated CAT activity, thus indicating a lack of oestrogen agonistic effect (Fig. 6).

3βGSD 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 µg/day) fully restored the pituitary content of oestrogen-dependent PR. Daily administration of 3βGSD for 6 consecutive days to castrated animals successfully restored anterior pituitary PR in a dose-dependent manner; thus the dose of 500 µg/day was almost ineffective, whereas the daily doses of 1000 and 1500 µg were able to induce the presence of PR in the rat AP. The [³H]ORG 2058 complexes in the...
rat pituitary cytosol sedimented at 7–9 S in linear sucrose gradients, whether induced by E2B or by 3βGSD (Fig. 7). Addition of an excess of radioinert ORG 2058 completely abolished the PR labelling induced by the administration of 3βGSD (data not shown). These results demonstrated that 3βGSD 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 3β,5α tetrahydro GSD derivative specifically binds to ER, though with a significantly lower binding affinity than naturally occurring E2 (Fig. 2; Table 1). The 3α,5α tetrahydro GSD derivative also interacts with ER, yet to a significantly lesser extent than its 3β 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 3β,5α and the 3α,5α 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 5α dihydro derivative of GSD was unable to bind ER (Fig. 2), a finding that correlates with the previous observation that 5α dihydro NET is not recognised by ER (Chávez et al. 1985).

To assess whether the interaction of 3βGSD and 3αGSD 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 3βGSD was able to induce hERα-mediated β-galactosidase activity in a dose–response manner (Fig. 3) thus demonstrating a clear oestrogen-agonistic effect in this system. Indeed, the 3βGSD-induced transactivation of the β-galactosidase
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 3αGSD isomer also induced hERα-mediated β-galactosidase activity in a dose-dependent manner, indicating an oestrogen agonistic effect. The oestrogen-like potency of 3αGSD was significantly lower than that of 3βGSD. The lack of oestrogen-like effects of unmodified GSD and 5αGSD correlates with their lack of binding to ER.

To provide additional support to the observation that oestrogenic effects of 3βGSD and 3αGSD are indeed hERα-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-, 3βGSD- and 3αGSD-mediated 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 hERα. Since GSD-containing oral contraceptive formulations are combined with ethynyl oestadiol, 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 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 3βGSD and 3αGSD in a mammalian expression system, HeLa cells co-transfected with the hERα and CAT vectors were used. The results demonstrated that unmodified GSD was unable to induce the hERα-mediated activation of CAT, thus indicating a lack of oestrogen agonistic effect. The 3β,5α reduced metabolite of GSD induced the transactivation of CAT in a similar manner to that of naturally occurring E₂ (Fig. 6).

![Figure 6](image-url) **Figure 6** Induction of human ERα-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 hERα (PCR 3-2-hERα) and its cognate reporter vector (ERα-1³CAT). Cells were exposed to GSD, 3βGSD, 3αGSD 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 hERα-mediated CAT activity in this system, whereas 3βGSD exhibited oestrogen agonistic effects. 3αGSD exhibited also oestrogen-like effects, though to a lesser extent than 3βGSD.

![Figure 7](image-url) **Figure 7** Induction of oestrogen-dependent PRs in the anterior pituitary of ovariectomised adult rats by 3βGSD. 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 μg (●) and 1500 μg (▲) of 3βGSD. Animals treated with 5 μg 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. 3βGSD was effective in restoring oestrogen-dependent PR in a dose-dependent manner, though it was ineffective at the 500 μg/day dose. Addition of an excess of radioinert ORG 2058 abolished the PR labelling induced by 3βGSD (data not shown).
GSD derivative also was able to induce the hERα-mediated CAT activation, though with a significantly lower potency than that exerted by 3βGSD. These results are similar to those obtained in the yeast expression system, indicating that both 3βGSD and 3αGSD exert oestrogen actions mediated by the hERα, 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 (ERβ) 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 ERβ 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 3βGSD, 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 3βGSD 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 3β,5α derivative of NET (Vilchis et al. 1986); however, the oestrogen agonistic potency of 3β,5αGSD was lower than that of the corresponding 3β,5αNET derivative, an observation that correlates with their relative binding affinities for the ER.

This study demonstrates that two GSD derivatives, 3βGSD and 3αGSD, 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 3βGSD 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, Catnerino 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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