



NON-PHENOLIC GESTODENE METABOLITES ENZYMATICALLY FORMED IN MCF-7 BREAST CANCER CELLS EFFICIENTLY INDUCE THE CELL PROLIFERATION

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ABSTRACT

Gestodene, a synthetic 19-norprogesterone has been used as contraceptive agent for millions of women. Epidemiological studies have raised concern over the relationship between contraceptive therapy with 19-norprogestins and a risk to develop breast cancer. Results from several studies have demonstrated that gestodene stimulates proliferation of estrogen-dependent MCF-7 breast cancer cells, effect that was inhibited by the presence of an antiestrogen, but not by an

antiprogesterone. A question arises because gestodene does not interact with estrogen receptors nor is able to be aromatized. Previous reports from our laboratory have shown that radiolabelled 19-norprogestins norethisterone and levonorgestrel, incubated with steroid target organs, undergo extensive enzymatic reductions on A-ring of their molecule, to form dihydro and tetrahydro metabolites which bind to estrogen receptor and were able to induce estrogen-like effects, in experimental models. The aim of the present study was to investigate the capacity of MCF-7 cells to metabolize [¹⁴C]-Gestodene, using an isotope dilution technique, as well as to determine the effect of gestodene and its reduced derivatives, to induce MCF-7 cell proliferation, in the presence or absence of steroid antagonists. Cell

proliferation was determined by fluorometric quantitation of cell DNA content. Our results demonstrated that MCF-7 cells were able to biotransform gestodene into 5α -dihydro, $3\alpha,5\alpha$ -tetrahydro and $3\beta,5\alpha$ -tetrahydro metabolites, which efficiently induced MCF-7 cell proliferation that was inhibited by the presence of an antiestrogen ICI 182,780, but neither by an antiprogesterin RU 486 nor antiandrogen Flutamide. Our findings can contribute to understand the mode of action of gestodene to induce estrogen-like effects, such as MCF-7 cell proliferation.

KEYWORDS: Gestodene, Breast cancer risk, Gestodene metabolism, Non-phenolic gestodene metabolites, MCF-7 cell proliferation.

INTRODUCTION

Oral contraceptives have been used for more than 50 years by millions of women. Gestodene (GSD; 17α -ethynyl- 13β -ethyl- 17β -hydroxy-4,15-gonadien-3-one) is a potent third generation 19-nor synthetic progestin widely used in combined oral contraceptives, because of its high effectiveness, safety and acceptability. ^[1,4] However, a series of studies have associated oral contraceptives containing GSD with an increased venous thromboembolism risk, as compared with those containing second generation progestins, such as Levonorgestrel. ^[5,6] Results from the Women's Health Initiative study ^[7] showed an enhancement of breast cancer risk in postmenopausal women using, as hormone replacement therapy, a combination of estrogen and progestin, such as GSD. ^[8-12] Moreover, some reports have also shown that the addition of GSD to estrogen-dependent MCF-7 breast cancer cells, induced cell proliferation, effect that was inhibited by an antiestrogen but not by an antiprogesterin. ^[13-19] All these unexpected findings, that strongly suggest estrogen-like activities exerted by GSD, raised the controversial question as to whether GSD may induce estrogenic effects, particularly since the GSD molecule neither interacts with intracellular estrogen receptors, ^[20-21] nor is able to be aromatized. ^[13, 22-23] Previous reports from our laboratory have shown that radiolabelled 19-norprogestins incubated with steroid target organs (hypothalamus, pituitary and ventral prostate), were extensively biotransformed to their corresponding $3\alpha,5\alpha$ - and $3\beta,5\alpha$ -tetrahydro metabolites, ^[24-26] which exert estrogen-like activities mediated by their interaction with intracellular estrogen receptors, though with a significantly lower affinity than estradiol (E_2). ^[24, 27-31] All these findings could explain the mode of action by which GSD induces estrogen-like effects on MCF-7 cell proliferation. However, to our knowledge, there is no evidence that MCF-7 cells metabolize GSD to its A-ring reduced metabolites, therefore the

aim of the present study was to investigate the enzymatic capacity of MCF-7 breast cancer cells to biotransform [^{14}C]-labelled GSD to its 5α -reduced metabolites, under different experimental conditions. The effect of GSD and its dihydro and tetrahydro derivatives on MCF-7 cell proliferation, in the presence or absence of steroid receptors antagonists, will also be studied.

MATERIALS AND METHODS

Steroids and chemicals

[^{14}C -ethynyl]-gestodene ([^{14}C]-GSD; 13β -ethyl- 17α -ethynyl- 17β -hydroxy-4,15-gonadien-3-one) specific activity (sp. act.) 43.75 mCi/mmol and authentic radioinert gestodene (GSD) were kindly provided by Schering (Berlin, Germany) and Schering Mexicana, S.A. de C.V. (Mexico City, Mexico), respectively. [^{14}C]-testosterone ([^{14}C]-T) sp. act. 57 mCi/mmol was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Chemical purity of radiolabelled steroids was assessed by their mobility on paper and thin layer chromatography in two systems of different polarity.^[32,33,34] Non-radioactive testosterone (T), 5α -dihydrotestosterone (5α -DHT), $3\alpha,5\alpha$ -androstenediol ($3\alpha,5\alpha$ -diol), $3\beta,5\alpha$ -androstenediol ($3\beta,5\alpha$ -diol), androstenedione ($\Delta^4\text{A}$) and estradiol (E_2) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA) and their chemical purity was assessed by crystallization and melting points determination. 5α -dihydrogestodene (5α -GSD) was synthesized by lithium-ammonia reduction of GSD.^[35] The $3\alpha,5\alpha$ -tetrahydro derivative of GSD ($3\alpha,5\alpha$ -GSD) was prepared by reduction of 5α -GSD with L-Selectride, under anhydrous conditions,^[35] whereas the $3\beta,5\alpha$ -tetrahydrogestodene ($3\beta,5\alpha$ -GSD) was synthesized by sodium borohydride reduction of 5α -GSD.^[35] The chemical purity of the synthesized GSD derivatives was assessed by their melting points determination, HPLC behavior, infrared absorption, and [^{13}C]- and [^1H]-nuclear magnetic resonance. The physical and spectroscopic constants of the A-ring reduced GSD derivatives have been previously reported.^[35] ICI 182,780 was purchased from Zeneca Pharma de México, S.A. de C.V. (Mexico City, Mexico), Mifepristone and Flutamide were purchased from Sigma-Aldrich Co. All reagents and solvents used were of analytical grade.

Cell culture

Estrogen receptor-dependent human breast cancer cells MCF-7, kindly provided by Dr. A. Zentella (Instituto Nacional de Ciencias Médicas y Nutrición S. Zubirán, Mexico City, Mexico) were cultured in a humidified 5 % CO_2 in air atmosphere, at 37°C , in T-45 flasks with Dulbecco's modified Eagle medium (DMEM) supplemented with 100 μM non-essential

aminoacids, 2 mM L-glutamine, 10 % fetal bovine serum (FBS), antibiotic-antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin-B) (Gibco BRL, Gaithersburg, MD, USA) and 100 pM E₂.

Gestodene metabolism

To assess the *in situ* conversion of GSD to A-ring reduced derivatives in human breast cancer cells, the metabolism of [¹⁴C]-GSD in cultured MCF-7 cells was studied. MCF-7 cells were plated at a density of 2X10⁶ cells/culture dish in phenol red-free DMEM (culture medium) containing 5 % FBS, previously treated with charcoal-Dextran to avoid the endogenous steroids (stripped FBS), and supplemented with 100 µM non-essential aminoacids, 2 mM L-glutamine and antibiotic-antimycotic solution and incubated at 37° C for 24 h (to allow cell adherence) in a humidified atmosphere of 5 % CO₂ in air. After incubation, the culture medium was removed and replaced by fresh medium containing 2 µM [¹⁴C]-GSD and incubated at 37° C for 15, 30, 45, 60, and 120 min, in 5 % CO₂ in air atmosphere. Concentration of the substrate [¹⁴C]-GSD was selected according to previous studies from our group, to obtain the highest radiolabelled progestin bioconversion to 5α-reduced metabolites. [32] Incubations with [¹⁴C]-T, as experimental control, and cell-free and boiled inactivated cells (negative controls), under identical experimental conditions, were also carried out. The final incubation volume was 5 ml. Protein content was determined by a protein-dye-binding method, using BSA as standard. [36] At the end of the incubation periods, the reaction was stopped by the addition of ethyl acetate. Radiolabelled steroids, formed during incubation, were extracted (4X), using three volumes of water-saturated ethyl acetate and 2.5 µg each of the following steroid carriers were added to the organic extracts: GSD, 5α-GSD, 3α,5α-GSD and 3β,5α-GSD or T, 5α-DHT, 3α,5α-diol, 3β,5α-diol and Δ⁴A. The separation, identification and radiochemical purity of the formed radiolabelled metabolites were established by a reverse-isotope dilution technique, as previously described by our group, [33, 34] which included identical behavior to that of the steroid carriers, in two different thin layer chromatographic systems (chloroform:acetone 9:1 and benzene:ethyl acetate 2:1) and recrystallizations to obtain a constant sp. act. Radiolabelled metabolites were located on chromatographic plates using a Packard InstantImager (Packard Instruments Company Inc., Downers Grove, IL, USA). Non-radioactive steroid carriers were detected on the plates using *p*-anisaldehyde-sulphuric-acetic acids reagent. Radioactivity was determined in a liquid scintillation spectrometer (Packard Tri-Carb model 1900 TR). The counting efficiency for [¹⁴C] was 86 % and quenching was corrected in all samples by external standardization. The

formation of the metabolic conversion products of [^{14}C]-GSD and [^{14}C]-T is expressed as picomol/mg protein.

Effect of GSD and its A-ring reduced derivatives on breast cancer cells proliferation

MCF-7 cells proliferation induced by GSD, 5 α -GSD, 3 α ,5 α -GSD and 3 β ,5 α -GSD, was determined by a fluorometric method based on cell DNA content, using the Cell Proliferation Assay Kit CyQUANTTM (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. Briefly: MCF-7 cells were seeded into 96-well plates at a density of 1×10^4 cells/well in 200 μl of phenol red-free and steroid-free DMEM medium containing 5 % stripped FBS, 2 mM L-glutamine, 100 μM non-essential aminoacids and the antibiotic-antimycotic solution and incubated at 37° C for 24 h to allow cell adherence. The culture medium was then removed and replaced by fresh medium containing increasing concentrations (1, 10, 100, and 1000 nM) of GSD, 5 α -GSD, 3 α ,5 α -GSD and 3 β ,5 α -GSD and incubated for 24, 48 and 72 h. Incubations with E₂ (1, 10, 100 and 1000 pM) and vehicle alone (ethanol 0.5 %), under identical conditions, were used as controls. At the end of the incubation periods, medium was removed and the plates were frozen at -70° C for 30 min, thawed at room temperature and 200 μl of the dye in lysis buffer were added to each well, incubating at 37° C for 30 min in complete darkness. The fluorescent signals were quantitated on a fluorometer Synergy HT (Biotek, Winoosky, UT, USA) at an excitation/emission wavelength of 480/530 nm, using purified salmon thymus DNA as standard. The content of cell DNA is expressed in nanograms.

To investigate the interaction of GSD and its 5 α -reduced metabolites with the intracellular steroid receptors, additional experiments were performed. MCF-7 cells (1×10^4) were incubated for 72 h with 1000 nM GSD or 5 α -GSD and 10 nM 3 α ,5 α -GSD and 3 β ,5 α -GSD, in the presence or absence of 50 nM the antiestrogen ICI 182,760 (ICI), Mifepristone (RU), a progesterone receptor antagonist or the non-steroidal antiandrogen Flutamide (FLU). Only cells, vehicle alone and 100 pM E₂ were used as controls. Cell DNA content is expressed in nanograms.

Statistical Analysis

Statistical differences between the experimental groups and controls were determined by one-way analysis of variance followed by appropriate post hoc tests using a SigmaStat Analysis System (Jandel Corporation, San Rafael, CA, USA). Differences were considered statistically significant when $p < 0.05$ was reached.

RESULTS

Metabolism of [^{14}C]-Gestodene in MCF-7 cells

After the extraction procedure, 95 % of the incubated radioactive material was recovered in the organic fractions of MCF-7 cells incubated with [^{14}C]-GSD or [^{14}C]-T, used as experimental control, and submitted to thin layer chromatography (TLC). Four radioactive zones were detected: the more polar zone, Zone 1 (R_F 0.41), corresponding to the major metabolic conversion product, had a chromatographic behavior identical to that of $3\beta,5\alpha$ -GSD carrier. Zone 2 (R_F 0.52) had the same chromatographic mobility than that of $3\alpha,5\alpha$ -GSD carrier. Zone 3 (R_F 0.61), associated with the carrier of GSD, was identified as unchanged substrate [^{14}C]-GSD and Zone 4 (R_F 0.70) behaved in an identical manner to that of 5α -GSD carrier. To establish the radiochemical purity of the isolated radiolabelled GSD metabolites, the different zones were eluted, radioactive content determined and representative aliquots of Zones 1, 2 and 4, were separately mixed with additional (10 mg) authentic radioinert $3\beta,5\alpha$ -GSD, $3\alpha,5\alpha$ -GSD, and 5α -GSD, respectively, and submitted to repeated crystallizations until constant specific activity was obtained. As can be seen in Table 1, the specific activities of [^{14}C]-labelled $3\alpha,5\alpha$ -GSD, $3\beta,5\alpha$ -GSD and 5α -GSD, remained constant in at least two successive crystallizations and were identical to that of mother liquors, thus confirming the identity of the above mentioned metabolites. When TLC plates of organic extracts from cell-free and boiled inactivated cells incubations, both used as negative controls, were analyzed, a single radioactive zone was detected, corresponding to unmodified [^{14}C]-GSD molecule.

The analysis of TLC plates of organic extracts from incubations with [^{14}C]-T, revealed the presence of four radioactive zones. Zone 1 (R_F 0.22) representing the major metabolic conversion product of [^{14}C]-T, had a chromatographic behavior identical to that of $3\alpha,5\alpha$ -diol and $3\beta,5\alpha$ -diol (Diols) carriers. Zone 2 (R_F 0.34), exhibiting the same chromatographic mobility than that of T carrier, was identified as unchanged [^{14}C]-T, while zones 3 (R_F 0.46) and 4 (R_F 0.55) were associated to 5α -DHT and $\Delta^4\text{A}$ carriers, respectively. After elution of Zone 1 (Diols), and because $3\alpha,5\alpha$ -diol and $3\beta,5\alpha$ -diol could not be separated by TLC, radioactive content was divided into two equal portions and crystallized separately with 10 mg each $3\alpha,5\alpha$ -diol and $3\beta,5\alpha$ -diol. Radiochemical purity data of these [^{14}C]-T metabolites are shown in Table 1. Zone 3 was eluted, mixed with 10 mg non-radioactive 5α -DHT and

submitted to crystallization (Table 1). After elution of Zone 4, only traces of radioactivity were detected, indicating very small formation of Δ^4 A.

Table 1. Representative radiochemical purity of isolated metabolites after *in vitro* incubations of MCF-7 cells with [14 C]-Gestodene ([14 C]-GSD) and [14 C]-Testosterone ([14 C]-T).

| Isolated metabolites from [14 C]-GSD incubations | Successive crystallizations | Specific activity (dpm/mg) | Isolated metabolites from [14 C]-T incubations | Successive crystallizations | Specific activity (dpm/mg) |
|--|------------------------------|----------------------------|--|-----------------------------|----------------------------|
| 3 α ,5 α -GSD | C ₁ ^a | 1069 | 3 α ,5 α -diol | C ₁ | 634 |
| | C ₂ | 1001 | | C ₂ | 581 |
| | C ₃ | 931 | | C ₃ | 473 |
| | ML ₃ ^b | 786 | | ML ₃ | 385 |
| 3 β ,5 α -GSD | C ₁ | 660 | 3 β ,5 α -diol | C ₁ | 370 |
| | C ₂ | 492 | | C ₂ | 351 |
| | C ₃ | 442 | | C ₃ | 319 |
| | ML ₃ | 472 | | ML ₃ | 334 |
| 5 α -GSD | C ₁ | 1424 | 5 α -DHT | C ₁ | 580 |
| | C ₂ | 1252 | | C ₂ | 529 |
| | C ₃ | 1073 | | C ₃ | 516 |
| | ML ₃ | 1101 | | ML ₃ | 580 |

^aCrystals.

^bMother liquors.

To determine the formation rate, as a function of time, of isotopically labelled 5 α -reduced GSD metabolites, incubations were carried out at 15, 30, 45, 60 and 120 min, and results are shown in Fig. 1(A). A highest conversion rate (838 ± 50 pmol/mg protein) of [14 C]-GSD to 5 α -GSD, representing 8.88 % of the original substrate, was reached after only 15 min of incubation, displaying a sustained drop along the subsequent incubation times. As can be seen, the major metabolic conversion product was 3 β ,5 α -tetrahydro GSD (9.62 %), with a conversion rate of 875 ± 87 pmol/mg protein, at 120 min of incubation. The bioconversion of [14 C]-GSD to 3 α ,5 α -GSD was similar to that observed for its 3 β epimer, but in minor proportion, with a conversion rate of 199 ± 50 pmol/mg protein (2.49 %). The maximal conversion rate of this reduced metabolite occurred at 30 and 45 min of incubation.

The formation rates of [14 C]-T metabolites in incubations with MCF-7 cells, at different time periods, are shown in Fig. 1(B). Bioconversion of [14 C]-T to 5 α -DHT (2.9 %) with a conversion rate of 249 ± 41 pmol/mg protein, followed by a rapid decrease, was observed. The maximal formation of Diols (7.37 %) with a formation rate of 694 ± 43 pmol/mg protein occurred at 120 min of incubation. Only small formation of Δ^4 A (0.83 %) was found.

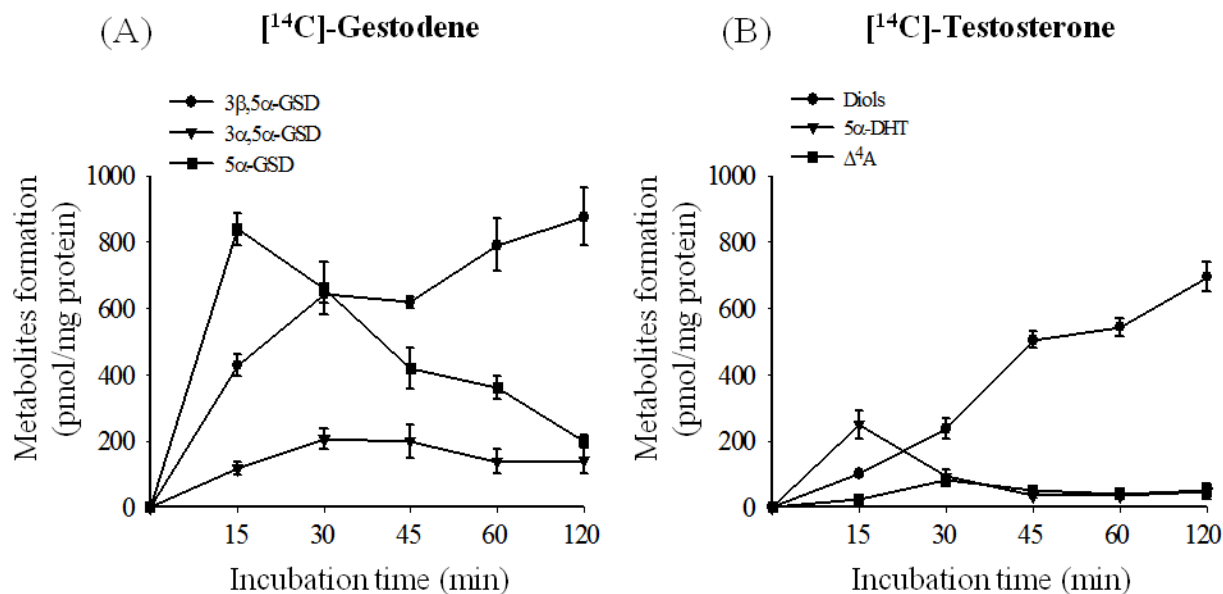


Fig. 1. Bioconversion of radiolabelled Gestodene and Testosterone to their 5 α -reduced metabolites in MCF-7 cells as a function of time. Incubations were carried out at different periods of time, using 2 μM either $[^{14}\text{C}]$ -Gestodene or $[^{14}\text{C}]$ -Testosterone. (A) An early (15 min) bioconversion of Gestodene to 5 α -dihydrogestodene (5 α -GSD), and a subsequent large formation of 3 β ,5 α -tetrahydrogestodene (3 β ,5 α -GSD) and 3 α ,5 α -tetrahydrogestodene (3 α ,5 α -GSD), although in a lesser extent, were observed. (B) The major metabolic products formed in Testosterone incubations were 3 α ,5 α -androstenediol and 3 β ,5 α -androstenediol (Diols). Each point represents the mean \pm SD of three experiments in triplicate.

Effect of GSD and its 5 α -reduced derivatives on MCF-7 cell proliferation

Proliferation of MCF-7 cells was determined by quantitation of DNA content after exposure to GSD and its 5 α -reduced derivatives 5 α -GSD, 3 α ,5 α -GSD and 3 β ,5 α -GSD, at increasing concentrations (1, 10, 100 and 1000 nM) and for different periods of time (24, 48 and 72 h). The non-modified GSD molecule was the less effective compound to induce cell proliferation, as shown in Fig. 2(A). Indeed, highest doses of GSD, incubated for 72 h, induced a small, although significant ($p < 0.05$) increase of cell DNA, as compared with DNA baseline (49 ± 12.6 ng) obtained in cells incubated with vehicle alone, as can be seen in Fig. 2(A). Fig. 2(B) shows that 5 α -GSD induced a significant dose-time dependent effect ($p < 0.05$), on the increase of cell DNA, as compared with DNA baseline, noticeable at concentration of 10 nM and above and at 72 h of incubation.

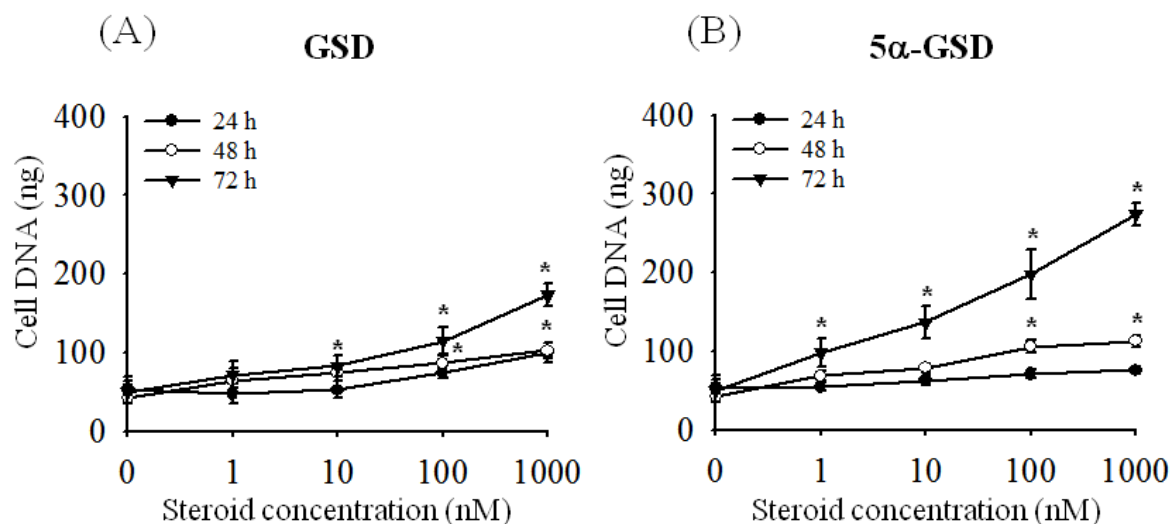


Fig. 2. Effect of Gestodene (GSD) and 5α-dihydrogestodene (5α-GSD) on MCF-7 cell proliferation. Cells were incubated for different periods of time with increasing concentrations of (A) GSD and (B) 5α-GSD. Proliferative cell response to steroid treatments was evaluated by quantitation of cell DNA using CyQUANT kit. Cell DNA baseline in incubations with vehicle alone was 49 ± 12.6 ng. Each point represents the mean \pm SD of three experiments in triplicate. * $p < 0.05$ vs. vehicle incubations.

The effect of 3α,5α-GSD and 3β,5α-GSD on MCF-7 cell proliferation is depicted in Fig. 3. Fig. 3(A) shows the capability of 3α,5α-GSD to induce an important and significant ($p < 0.05$) cell proliferation since the concentration of 1 nM, and particularly at 72 h of incubation, as compared with DNA baseline (53.6 ± 14 ng) obtained in cells incubated with vehicle alone. DNA induced increase observed, was dose- and time-dependent. As can be seen in Fig. 3(B), 3β,5α-GSD, induced a remarkable and significant ($p < 0.05$) increase on cell DNA content as compared with DNA baseline. However, 3β,5α-GSD highest concentration (1000 nM) at 72 h of treatment, showed a decrease in cell DNA content as compared to that induced with 100 nM concentration. Cell DNA content increase induced by E₂, used as control, was evident even at the lower dose employed (1 pM) and since the shortest time of incubation. A decrease in the DNA content also was observed at the highest concentration of E₂ (1000 pM) and at 72 h of incubation, as shown in Fig. 3(C).

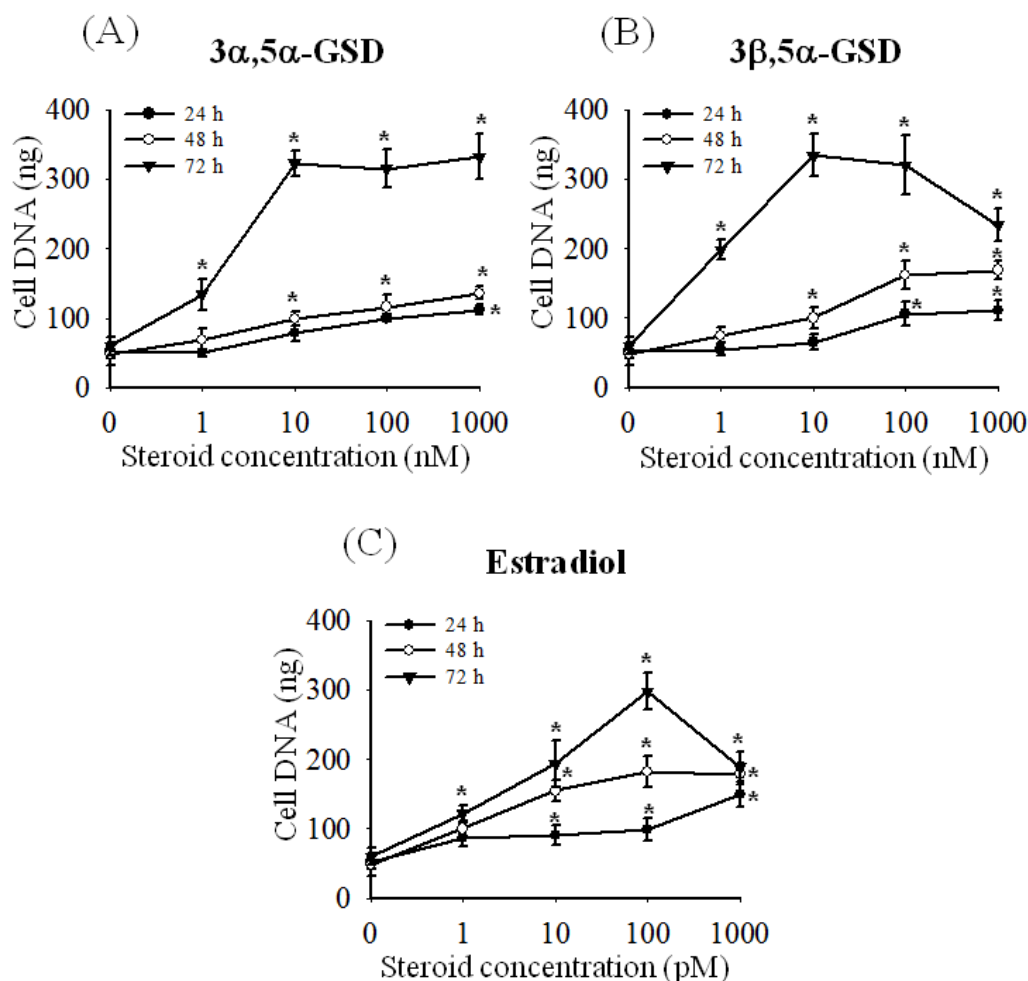


Fig. 3. Effect of 3 α ,5 α -tetrahydrogestodene (3 α ,5 α -GSD) and 3 β ,5 α -tetrahydrogestodene (3 β ,5 α -GSD) on MCF-7 cell proliferation. Cells were incubated for different periods of time with increasing concentrations of (A) 3 α ,5 α -GSD and (B) 3 β ,5 α -GSD. (C) Incubations with estradiol (E₂) were used as controls. Proliferative cell response to steroid treatments was evaluated by quantitation of cell DNA using CyQUANT kit. DNA baseline in the cells incubations with vehicle alone was 53.6 ± 14 ng. Each point represents the mean \pm SD of three experiments in triplicate. * $p < 0.05$ vs. vehicle incubations.

As shown in Fig. 4, incubations of MCF-7 cells with GSD and its reduced derivatives 5 α -GSD, 3 α ,5 α -GSD or 3 β ,5 α -GSD, in presence of the antiestrogen ICI, resulted in a significant decrease ($p < 0.05$) of the cell DNA content induced by the different steroids, in an identical manner as when ICI inhibited the effect of E₂. Only cells, cells in the presence of vehicle or ICI alone were used as controls. Neither vehicle nor ICI induced cell proliferation.

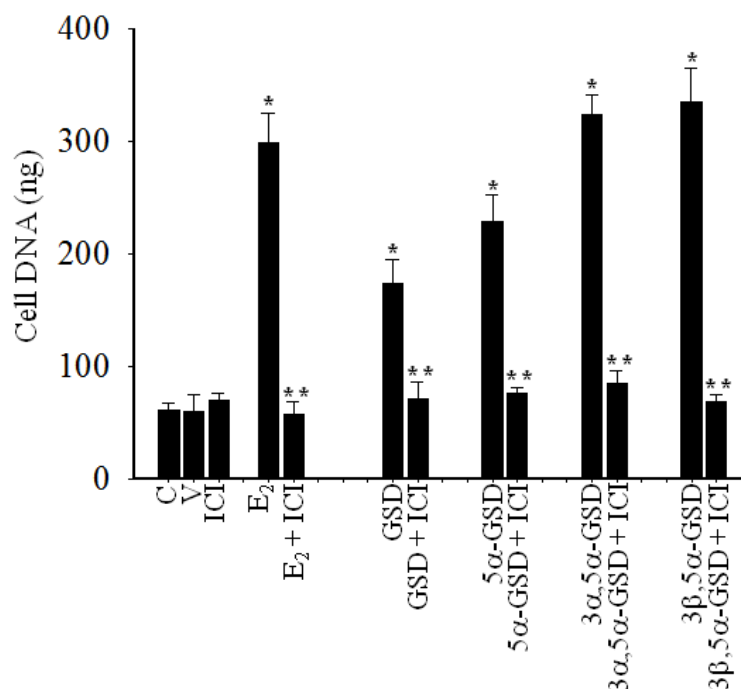


Fig. 4. Effect of the antiestrogen ICI 182,780 (ICI) on MCF-7 cell proliferation induced by Gestodene and its 5 α -reduced derivatives. Cells were incubated for 72 h with 1000 nM gestodene (GSD) and 5 α -dihydrogestodene (5 α -GSD) and 10 nM of 3 α ,5 α -tetrahydrogestodene (3 α ,5 α -GSD) and 3 β ,5 α -tetrahydrogestodene (3 β ,5 α -GSD), in the absence or presence of 50 nM ICI. Incubations with only cells (C), vehicle alone (V), 50 nM ICI and 100 pM estradiol (E₂) were used as experimental controls. Values represent the mean \pm SD of three experiments in triplicate. * p <0.05 vs. vehicle incubations; ** p <0.001 vs. incubations in absence of ICI.

As can be observed in Fig. 5, neither the presence of the progesterone receptor antagonist RU nor the non-steroidal antiandrogen FLU, inhibited the MCF-7 cell DNA content increase induced by GSD tetrahydro derivatives 3 α ,5 α and 3 β ,5 α . No effect on cell proliferation was observed in incubations with only cells and cells in the presence of vehicle, RU or FLU used as controls.

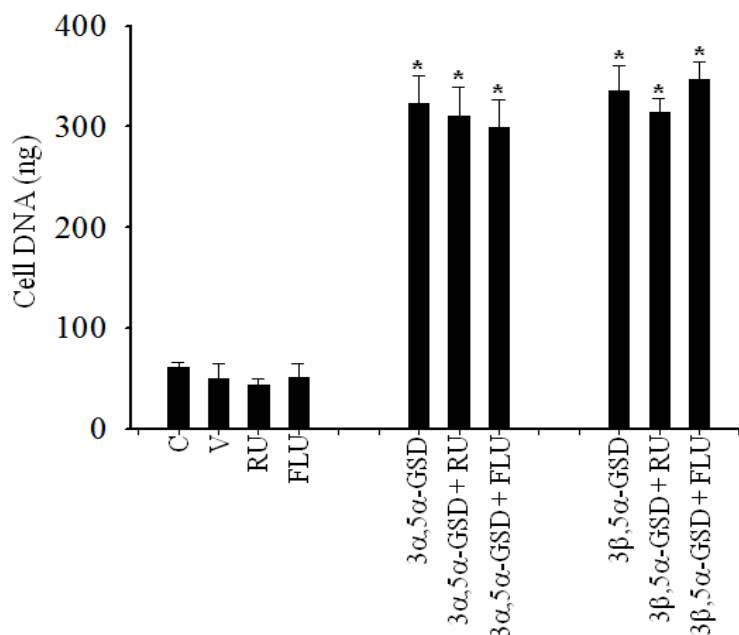


Fig. 5. Effects of the antiprogesterone Mifepristone (RU) and the antiandrogen Flutamide (FLU) on MCF-7 cell proliferation induced by 3α,5α-tetrahydrogestodene (3α,5α-GSD) and 3β,5α-tetrahydrogestodene (3β,5α-GSD). Cells were incubated for 72 h with 10 nM 3α,5α-GSD or 3β,5α-GSD, in the absence or presence of 50 nM RU or FLU. Incubations with only cells (C), vehicle alone (V), RU and FLU were used as controls. Each point represents the mean \pm SD of three experiments in triplicate. * $p < 0.05$ vs. vehicle incubations.

DISCUSSION

The results of this study clearly demonstrate that estrogen-dependent MCF-7 breast cancer cells efficiently biotransformed GSD into non-phenolic A-ring tetrahydro reduced metabolites. Indeed, MCF-7 cells incubated with radiolabelled GSD showed a great capacity for biotransform the progestin molecule to 5α-GSD, 3α,5α-GSD and 3β,5α-GSD, indicating a great activity of 5α-steroid reductase and two aldo-keto reductases, the 3α-hydroxysteroid dehydrogenase and the 3β-hydroxysteroid dehydrogenase. The major metabolic conversion products were 3β,5α-GSD and 3α,5α-GSD, representing a bioconversion of 12.1 % of the incubated substrate GSD. These findings are in line with previous reports from our group, demonstrating that other sex steroid hormone sensitive tissues, such as hypothalamus, pituitary and ventral prostate, incubated with the isotopically labelled 19-norprogestins norethisterone and levonorgestrel, were able to bioconvert progestin molecules into their corresponding A-ring reduced metabolites 5α-dihydro and 3α- and 3β-tetrahydro metabolites.^[26, 27, 32, 33]

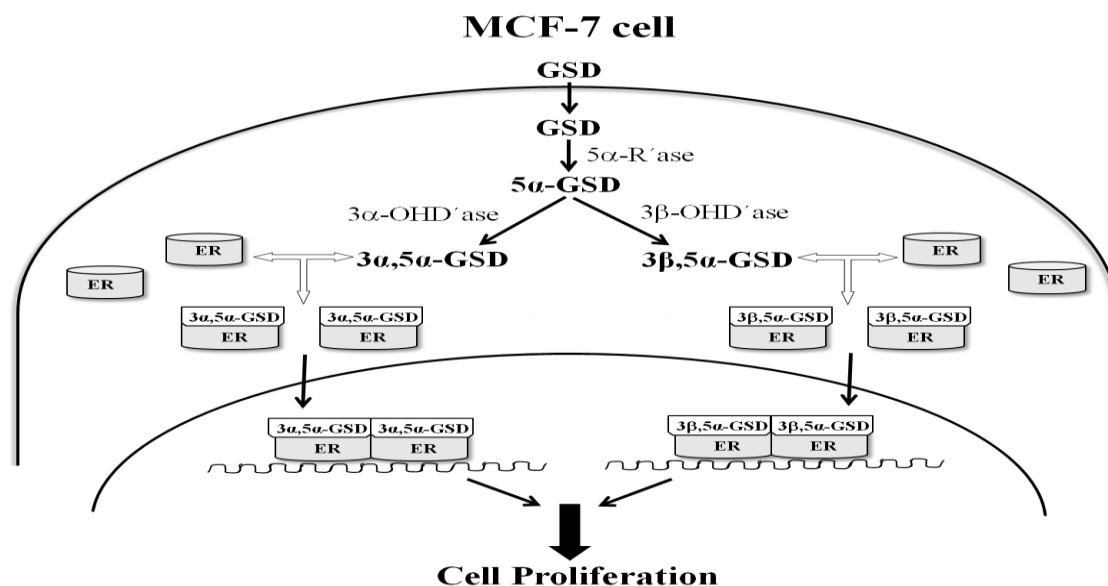


Fig. 6. An intracrine model of the effect of 19-norprogesterin Gestodene (GSD) in MCF-7 cell proliferation. The bioconversion of GSD into two tetrahydro reduced metabolites 3α,5α-GSD and 3β,5α-GSD, as demonstrated in this study, which bind to estrogen receptor (ER), ^[24, 31] represents an efficient intracrine system that allows GSD, a progesterone receptor agonist, to exert in MCF-7 cells, potent estrogen-like effects mediated by ER. The switch molecules that turn on the MCF-7 cell system are: the 5α-steroid reductase (5α-R'ase) and two members of the aldo-keto reductases family, the 3α-hydroxysteroid dehydrogenase (3α-OHD'ase) and the 3β-hydroxysteroid dehydrogenase (3β-OHD'ase). The GSD tetrahydro reduced metabolites 3β,5α-GSD and 3α,5α-GSD, although in a minor proportion, enhance MCF-7 activities involved in cell proliferation, whereas GSD molecule was almost ineffective.

The finding in the present study, that a higher formation of 5α-GSD occurred as early as 15 min, followed by a marked diminution and a concomitant increase in the formation of both 3α,5α-GSD and 3β,5α-GSD, agree with the concept that 5α-reduction of synthetic 19-norprogestins is an obligatory metabolic step for the formation of 3α,5α- and 3β,5α-tetrahydro metabolites ^[32-35, 37] and is in line with several reports demonstrating an enhanced 5α-steroid reductase activity and an over-expression of the 5α-steroid reductase gene in MCF-7 cells. ^[34, 38-41] Control incubations with [¹⁴C]-T, the natural substrate for 5α-reductase, showed an efficient bioconversion of radiolabelled T into 5α-DHT and Diols (3α,5α-diol and 3β,5α-diol), thus validating our experimental conditions. The formation of Δ⁴A, although in a

small amount, indicated the presence of 17 β -hydroxysteroid dehydrogenase activity in MCF-7 cells.

The effect of GSD and its dihydro and tetrahydro reduced derivatives on MCF-7 cell proliferation, was evident as demonstrated by the changes on cell DNA content, as compared with DNA baseline in cells incubated only with vehicle. The most relevant finding of this study was that the major metabolic conversions products of GSD in MCF-7 cells, 3 β ,5 α -GSD and 3 α ,5 α -GSD, were also the most active compounds to induce increase on MCF-7 cell proliferation, in a similar manner to that of estradiol, yet with lower capacity, and that this effect was completely inhibited by an antiestrogen (ICI), but not by an antiprogestin (RU) nor by an antiandrogen (FLU). These results strongly suggest that GSD tetrahydro derivatives are exerting estrogen-like effects mediated throughout their interaction with intracellular estrogen receptors, observation that correlates well with previous reports from our group which have demonstrated that GSD tetrahydro derivatives induced specific activation of an estrogen receptor α -dependent reporter gene in a co-transfected HeLa cells expression system. [29, 30] The observation that 3 β ,5 α -GSD and estradiol presented a sensible diminution of MCF-7 cell proliferation at highest concentrations and at the longest time of treatment, is in line with the characteristic dual effect described for estrogens, that at low doses have a stimulatory effect, while higher doses can exert inhibitory effects. [42-45]

The 5 α -dihydro GSD derivative was less effective than tetrahydro GSD compounds to increase cell proliferation which was significant only at 72 h of treatment. GSD was the less effective compound to induce MCF-7 cell proliferation which was observed at the highest concentrations used, and only in cells that were treated for 72 h. The small increase of cell proliferation induced by 5 α -GSD and GSD, particularly observed at highest doses and time of treatment, and that was efficiently inhibited by ICI, could be due to a biotransformation of these compounds into 3 α - and 3 β -tetrahydro GSD metabolites. Thus, our results can offer an explanation to the question derived from several reports, [13-15, 17-19] which show that high doses of GSD induced proliferation in estrogen-dependent MCF-7 cells, particularly because it has been well documented that GSD molecule neither, is able to be aromatized, because of its chemical structure, [13, 22-23] nor interacts with intracellular estrogen receptors. [20-21] However, as it was demonstrated in the present study, GSD was efficiently metabolized to non-phenolic 3 α ,5 α - and 3 β ,5 α -tetrahydro metabolites, that were also the most efficient compounds for inducing MCF-7 cell proliferation.

In summary, our studies show that MCF-7 breast cancer cells possess the capability to biotransform GSD, a potent progesterone receptor agonist, into potent estrogen receptor agonists 3 α ,5 α -GSD and 3 β ,5 α -GSD. The complete intracrine cycle offers a plausible explanation for the effects of GSD on MCF-7 cell proliferation (Figure 6).

CONCLUSION

The substantive outcome of our studies was the finding that MCF-7 breast cancer cells exhibited an important enzymatic capacity to biotransform isotopically labelled GSD molecule into non-phenolic metabolites 5 α -dihydro, 3 α ,5 α -tetrahydro and 3 β ,5 α -tetrahydro, presumably through 5 α -steroid reductase and 3 α - and 3 β -hydroxysteroid dehydrogenases. Estrogen-like effect of tetrahydro GSD derivatives on MCF-7 cell proliferation increase was also demonstrated. The overall data underline the important role that metabolic fate of GSD can play in the modulation and expression of its estrogen-like effects.

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