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Effects of Progestagens and Org OD14 in *In Vitro* and *In Vivo* Tumor Models

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Sex steroids, in particular estradiol (E2) and progesterone (P4), play, together with other hormones and growth factors, a role in the development of normal breast tissue. The effect of four progestagens (norethisterone, 3-ketodesogestrel, gestodene and P4) and Org OD14, a steroid with weak estrogenic, progestagenic and androgenic properties were studied on growth of breast tumor cells *in vitro* using two subclones of MCF-7 (H and A) and T47D (S and A) cells. In addition, we investigated the effects of 3-ketodesogestrel, gestodene and Org OD14 on the growth of 7,12-dimethyl-benz(*a*)anthracene(DMBA)-induced mammary tumors in rats. In the *in vitro* assays with MCF-7 cells norethisterone, 3-ketodesogestrel and gestodene stimulated growth only at high doses ($\geqslant 10^{-7}$ M), whereas P4 had no effect. Gestodene was more potent than 3-ketodesogestrel and norethisterone. Org OD14, stimulated cell growth at a dose of 10^{-8} M, while E2 is active at 10^{-10} M. In T47D-A cells similar effects were found, but the subclone S did not respond to the progestagens and Org OD14. The two T47D subclones also reacted differently to progestagens during growth stimulation with E2. In T47D-S the progestagens and Org OD14 inhibited, while in T47D-A these compounds did not modulate the effect of E2. In the DMBA model we found that gestodene and 3-ketodesogestrel were able to inhibit tumor growth to the same extent. Surprisingly, Org OD14 was even more effective in the DMBA model using the therapeutic approach. Using the prophylaxic approach tumor development was delayed and tumor growth was strongly suppressed. The inhibitory effects of Org OD14 on tumor growth in the DMBA model may be attributed to its mixed hormonal profile. From these studies we conclude that different cell lines and even subclones thereof respond quite differently to steroids. Both *in vitro* and *in vivo* studies are required to judge whether synthetic steroids might be involved in an increased risk for the development of breast tumors.

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INTRODUCTION

The role of both estrogens and progestagens in normal breast development has been discussed extensively (for review see [1]). Studies by Anderson *et al.* [2] with OC-users indicate that progestagens rather than estrogens induce cell proliferation of normal breast cells. Various breast cancer cell lines (MCF-7, T47D, ZR75-1) have also been used to study the role of both estrogens and progestagens on cellular proliferation in order to determine their potential tumor risk. These studies [3–7] clearly show a stimulating effect by estrogens. In contrast, the effects of progestagens alone or in combination with estradiol (E2) on proliferation of tumor cell lines are controversial. Both growth stimulation [8–10] and growth inhibition [11] have been found with progestagens derived from 19-nortestosterone. Progestagens of the pregnane series alone show no effect [9] or act stimulatory as well as inhibitory depending on the duration of the treatment [12, 13].

In combination with E2 the progestagens of the pregnane series counteract the proliferative effect of E2 [4, 6, 14–16] in T47D cells. The data of Coletta *et al.* [11] with respect to the effects of gestodene on the growth of MCF-7 cells are surprising. According to Coletta gestodene has, probably due to the presence of a gestodene binding protein [17], an inhibitory effect which is not seen with other progestagens like 3-ketodesogestrel, R5020 and medroxyprogesterone

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acetate (MPA). The results could not be confirmed by van den Burg *et al.* [9]. The above-mentioned differences may, at least partly, be due to the use of different cell lines (subclones, passage numbers), culture conditions (media, sera, presence of phenol red and insulin) and doses of the compounds used. Very often firm conclusions, based on *in vitro* studies, are drawn with respect to risk for pathology in the breast, but in the light of the conflicting data as well as the high concentrations at which effects are seen, some caution seems to be justified.

For showing the efficacy of anti-tumor agents testing compounds *in vivo* is essential. The 7,12-dimethylbenz(*a*)anthracene(DMBA)-induced tumor model in rats, developed by Huggins *et al.* [18] has been most widely used. The growth of the DMBA-induced tumors is dependent on E2 and prolactin [19]. Androgens display a tumor inhibiting activity [20]. The effect of progestagens varies with the dose, type of progestagen and the period of treatment after DMBA administration. At low doses progesterone (P4) stimulates while at high doses it has no effect [21]. Norethynodrel gave a protective effect whereas MPA shows no protection [22] when the compounds were administered at the same time as the tumor inducer. Surprisingly, when MPA treatment was started before DMBA administration a decrease in mammary carcinomas was found [23], whereas for norethynodrel an increase was seen [24]. In general the time of administration of the test compound in relation to the time of exposure of the carcinogen as well as the dose of the compound seem to be important. In the present study the compounds were tested in two different ways: according to a prophylaxic protocol, administrating DMBA and test compound at the same time and to a therapeutic protocol starting treatment several weeks after DMBA administration when tumors had been established.

In the investigations we compared the effects of norethisterone, 3-ketodesogestrel, gestodene and P4 on growth of tumor cell lines and on growth of DMBA-induced mammary tumors. For the *in vitro* studies with the progestagens we used two subclones of MCF-7 cells and two subclones of T47D cells applying standardized conditions in order to clearly determine cell type and subclone differences.

In addition Org OD14, a steroid with weak estrogenic, progestagenic and androgenic properties [25], was studied in the above-mentioned *in vivo* and *in vitro* models. This compound is used in humans for the treatment of climacteric complaints and it does not give endometrium stimulation. Moreover, it has beneficial effects on bone [26, 27].

MATERIALS AND METHODS

Animals

Sprague–Dawley rats were delivered by IFFA Credo-Broekman (Someren, The Netherlands) or by the Zentralinstitut für Versuchstierkunst (Hannover, Germany). The rats were housed in Macrolon cages in light- and temperature-controlled rooms. Tap water and pelleted food (RMH-B, Hope Farms, Linschoten, The Netherlands) were given *ad libitum*.

Cell lines

The MCF-7 cell line, subclone H, was kindly provided by Dr B. van der Burg (Hubrecht Laboratory, Utrecht, The Netherlands). The T47D cell line, subclone S, was obtained from Dr R. Sutherland (Garvan Institute of Medical Research, Darlinghurst, Australia). Both the MCF-7 and T47D cell line were also bought from the American Type Culture Collection (Rockville, MD, U.S.A.) and indicated by A in the text. The cells were maintained by culturing in a humidified atmosphere of air with 5% CO_2 at 37°C in polystyrene flasks in phenol-red free medium using Dulbecco's modified Eagles medium and Ham's F12 (Gibco, Paisley, Scotland) in a ratio of 1:1 with 5% fetal calf serum (Bocknec, Ontario, Canada). The cells were passaged once a week using trypsin and EDTA.

Compounds

All compounds were prepared by N.V. Organon (Department of Medicinal Chemistry, The Netherlands) except that 4-hydroxytamoxifen and ICI 164.384 were kindly provided by Dr A. E. Wakeling (Zeneka Pharmaceuticals, Cheshire, England). DMBA was purchased from Fluka (Basel, Switzerland) or Sigma Chemical Co. (St Louis, U.S.A.).

Methods

For both the MCF-7 and T47D cells the following protocol was used. The cells were seeded in 24 well plates and cultured as described above except that charcoal treated serum (CTS) was used.

After 2 days of adaptation the steroids were added in ethanol in such an amount that 0.2% ethanol was not exceeded. After 5 and 7 days the media were changed. After 9 days of culturing the media were removed and the cells were frozen at −70°C until the measurement of DNA content. The total DNA content per well was assessed by fluorescent staining with Hoechst 33342 and measuring the fluorescence with a Perkin–Elmer luminescence spectrometer model LS50.

The estrogen receptor competition studies were essentially carried out as described before for the steroid receptors [28] except that a larger dose range 10^{-10}–10^{-5} M was used for the competitors.

The DMBA experiments were carried out in the Department of Endocrine Oncology (Dr Daniel Den Hoed Cancer Centre, Rotterdam, The Netherlands) according to the method described by Bakker *et al.* [29] when the therapeutic protocol was used. The prophylaxic experiments were performed in the Department of Endocrinology of N.V. Organon (Oss, The Netherlands) and the administration of the compounds begun

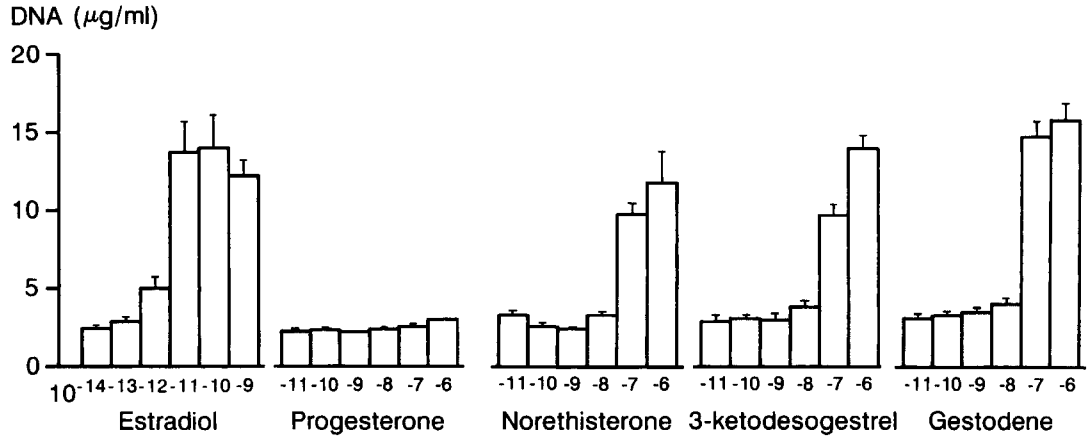

Fig. 1. Effect of E2, P4, norethisterone, 3-ketodesogestrel and gestodene on proliferation of MCF-7 (H) cells. Bars represent DNA concentration (μg/ml) ± SD in a well.

at the time the tumor inducer was given. Each treatment group consists of 8 animals. E2 was assayed with a kit obtained from EIR (Würlingen, Switzerland).

RESULTS AND DISCUSSION

Progestagens

The progestagens were tested *in vitro* under standardized conditions in the absence of estrogens (phenol-red free medium and CTS) and exogeneous insulin was not added. The cells were cultured for 7 days in the presence of steroids at various concentrations. The effects of the progestagens and E2 on proliferation of MCF-7 cells using the subclone H of the Hubrecht Laboratory are shown in Fig. 1. A clear dose–response curve is found with E2. A stimulation of proliferation is found with norethisterone, 3-ketodesogestrel and gestodene at concentrations of 10^{-7} and 10^{-6} M but no effect is seen with P4. Gestodene appears to be the most potent cell growth stimulator. Compared to E2 approx. 10,000 times higher concentrations of the progestagens are needed in order to give a similar response. With the other MCF-7 subclone, which has a somewhat higher proliferative basal activity, identical results were obtained (results not shown). Our results are in line with those of others [8–10]. However, in the present studies the difference in effects between gestodene and 3-ketodesogestrel as found by Coletta *et al.* [11, 17] could not be confirmed.

The stimulatory effect of the progestagens could not be blocked by anti-progestagens but the effects can be counteracted by anti-estrogens (Fig. 2). The progestagens may bind to the estrogen receptor or an estrogenic metabolite is formed during the incubation period of 7 days. The binding of the compounds to the estrogen receptor was determined by performing competition

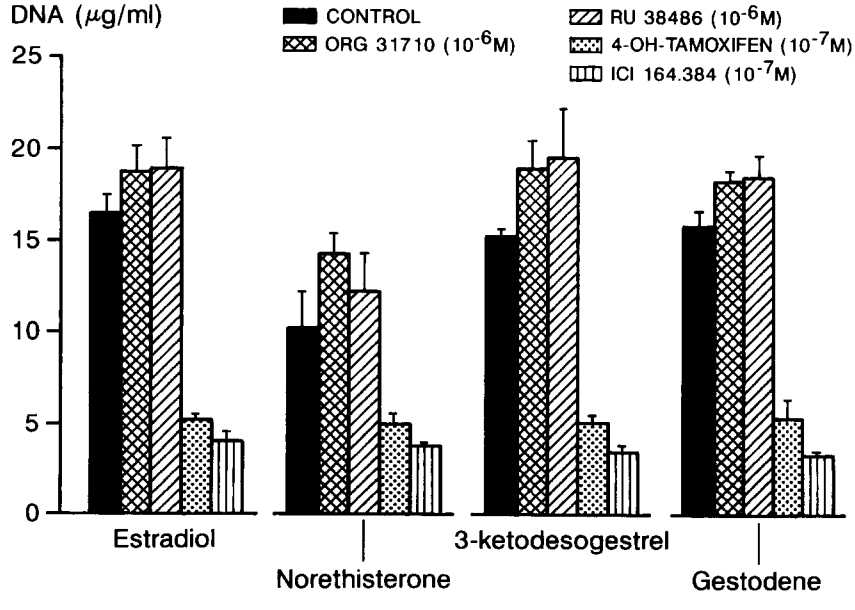

Fig. 2. Effects of anti-progestagens (Org 31710 and RU 38486) at a concentration of 10^{-6} M and anti-estrogens (4-hydroxy-tamoxifen and ICI 164.384) at a concentration of 10^{-7} M on E2 (10^{-10} M) and progestagen (10^{-6} M) stimulated growth. Bars ± SD represent DNA concentration (μg/ml) in a well.

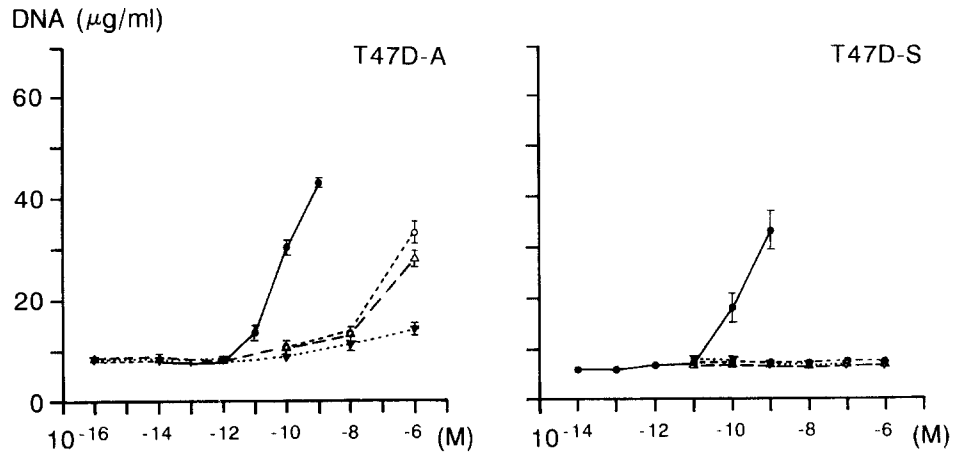

Fig. 3. Effects of E2 (●---●), gestodene (○---○), 3-ketodesogestrel (△---△) and P4 (▼---▼) on proliferation of T47D cells (subclone A left panel and subclone S right panel). Values ± SD for DNA concentration (μg/ml) in a well are given.

studies at higher concentrations because normally at 1 μmol/l no binding to the estrogen receptor is found. Gestodene competes for the estrogen binding site (RBA = 0.025% with E2 = 100%) whereas 3-ketodesogestrel and P4 do not compete. Norethisterone also competes weakly (RBA = 0.01). This indicates that both metabolic conversion into estrogenic compounds and intrinsic estrogenicity of the compounds may contribute to the estrogenic response. However, it is most likely that the effects are due to the intrinsic estrogenicity of the compounds because the conditions for metabolism are not optimal and the affinity is of such a magnitude that at μmol concentrations a full estrogenic response can be expected.

The effects of the progestagens on two T47D cell lines were tested under the same conditions as those used for the MCF-7 cell lines. Figure 3 shows that with the subclone A similar stimulating effects are found for 3-ketodesogestrel and gestodene at high, unphysiological concentrations. P4 again has no effect. In the subclone S no stimulation was found with any of these progestagens. Both cell lines respond to E2 but the T47D-A subclone already gives a growth response at 10^{-11} M whereas the S subclone starts to respond at a concentration of 10^{-10} M. Apparently the estrogen receptor concentration in the latter cell line is lower under these culture conditions.

The experiments show that the responses of progestagens in an estrogenic environment are quite different from that in an estrogen deficient milieu. Figure 4 shows that in the T47D-A subclone no modulation of the estrogenic effect is seen but in the other clone the progestagens 3-ketodesogestrel and gestodene inhibit the estrogen-induced proliferation. P4 shows a weak inhibiting effect. Similar inhibiting effects were found by Sutherland *et al.* [12] using MPA. It is suggested that both the progestagen and estrogen receptors are involved in growth regulation of the T47D-S cell line [30].

The *in vitro* data clearly show that the various cell lines respond differently. The estrogenic effect of progestagens is not observed in the cell line of Sutherland which may be due to a difference in metabolism, the receptor population present in the cells

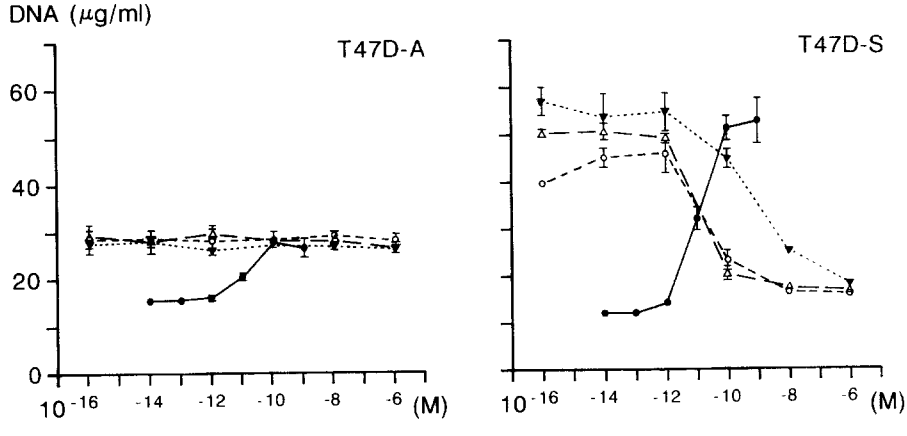

Fig. 4. Effect of gestodene (○---○), 3-ketodesogestrel (△---△) and P4 (▼---▼) on E2 (10^{-10} M) stimulated growth in T47D cells. Left panel subclone A and right panel subclone S. The growth curve with E2 alone (●---●) is also given. Values ± SD represent DNA concentration (μg/ml) in a well.

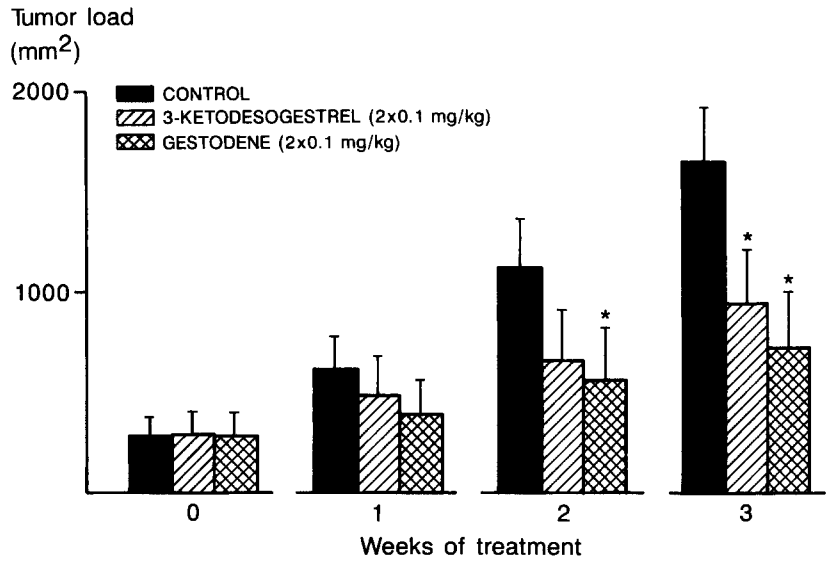

Fig. 5. Effect of 3-ketodesogestrel (2 × 0.1 mg/kg) and gestodene (2 × 0.1 mg/kg) after subcutaneous administration (therapeutic model) on DMBA-induced mammary tumors in rats. Bars represent tumor load (mm^2) ± SEM measured *in vivo*. Statistical analysis was carried out with the Sign test ($P < 0.05$). Tumor load at week "0" is just before treatment starts.

and/or the history of the cell line. Both estrogen and progestagen receptors are present in the two MCF-7 cell lines in low amounts but changes may occur during hormonal treatment.

Because both gestodene and 3-ketodesogestrel show some estrogenic effect in the cancer cell lines the two compounds were tested in the DMBA model. In this tumor model the induced tumors are estrogen-dependent. The compounds were tested subcutaneously at a dose 10 times higher than required for 100% ovulation inhibition in the therapeutic model. Figure 5 shows the effects of the two compounds in this model. Both compounds inhibit tumor growth. The difference in effect of 3-ketodesogestrel and gestodene is not statistically significant.

Org OD14

Org OD14 is a compound used for the treatment of climacteric complaints which possess weak estrogenic, progestagenic and androgenic properties [25].

In the two subclones of MCF-7 cells Org OD14 stimulates proliferation at a concentration of 10^{-9} M or higher as depicted in Fig. 6. The compound is approx. 100 times less active than E2. Similar effects are seen with subclone A of T47D but as with the progestagens no stimulation is found in the Sutherland cell line (data not shown).

Identical results as with the progestagens (see above: Fig. 4) in the presence of E2 are found in the T47D cells and also in MCF-7 cells (data not shown). From the *in vitro* results we can conclude that Org OD14 acts like an estrogen by stimulating proliferation. The estrogenic effect is higher in MCF-7 cells of subclone A (1% of the activity of E2) than in MCF-7 (H) cells and T47D (A) cells (0.2–0.35% of E2). The progestagenic activity of Org OD14 is most likely responsible for the

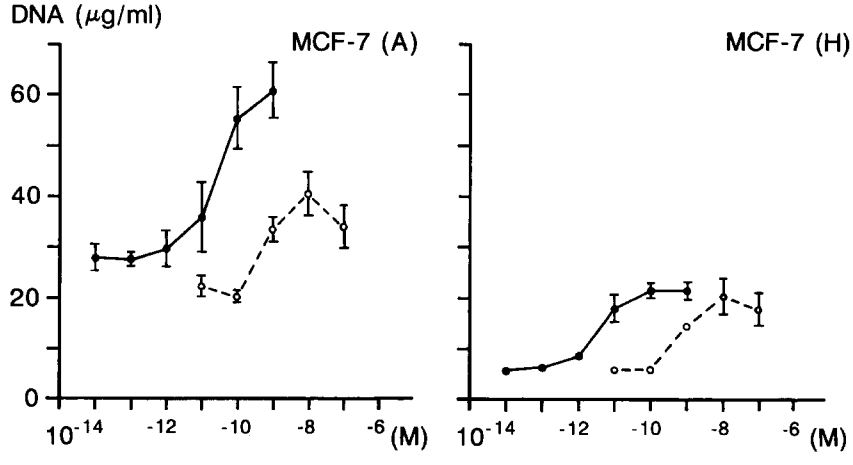

Fig. 6. Growth stimulation curves of MCF-7 cells (left subclone A and right subclone H) with E2 (●---●) and Org OD14 (○---○). Values ± SD represent DNA concentration (μg/ml) in a well.

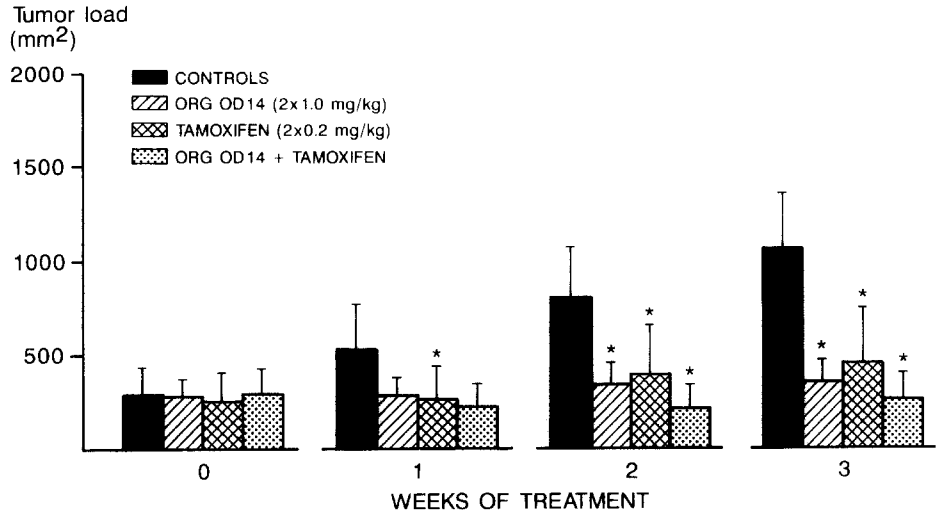

Fig. 7. Effect of Org OD14 (orally), tamoxifen (subcutaneously) and combined treatment on DMBA-induced mammary tumors using the therapeutic model. See Fig. 5 for further explanation.

inhibition of estrogen-induced proliferation in the T47D-S cells. Markiewicz and Gurpide [31] found that also in human endometrial tissue the progestagenic activity of Org OD14 prevails over the estrogenic activity.

Due to the properties of Org OD14 we also tested this compound in the DMBA model applying two modalities. In the therapeutic model the effect of Org OD14 was compared with that of tamoxifen and surprisingly Org OD14 at a daily dose of 2 × 1.0 mg/kg has a similar effectiveness as tamoxifen at a dose of 2 × 0.2 mg/kg. The effect of Org OD14 may be due to an inhibitory effect on gonadotropin release and thus on E2 levels. Indeed a significant reduction in E2 levels was found (data not shown). Therefore Org OD14 was also tested at a lower dose at which E2 levels were not influenced e.g. 2 × 0.25 mg/kg. Figure 8 shows the effects of three different doses (2 × 0.25, 2 × 0.5 and 2 × 1.0 mg/kg) of Org OD14 on the tumor load after treatment for 3 weeks. Org OD14 gives significant inhibition of tumor growth at all three doses after 1 (except the highest dose), 2 and 3 weeks of treatment.

The compound was also tested in the prophylaxic model in which the tumor inducer and Org OD14 are administered at the same time. Almost complete abolishment of tumor development is found with 2 × 0.25 and 2 × 1.0 mg/kg Org OD14 in this treatment regimen (Fig. 9). The lowest dose of Org OD14 gives an even better response than the highest dose. The estrogenic activity of the compound may be responsible for the higher tumor load at the high dose. At the dose of 2 × 0.25 mg/kg the E2 levels were not significantly decreased. In another experiment a four times lower dose than 2 × 0.25 mg/kg also gave tumor reduction but this effect was not significant (results not shown).

Org OD14 is a synthetic steroid which possesses weak estrogenic, progestagenic and androgenic activities [25]. The tumor growth inhibiting activity of

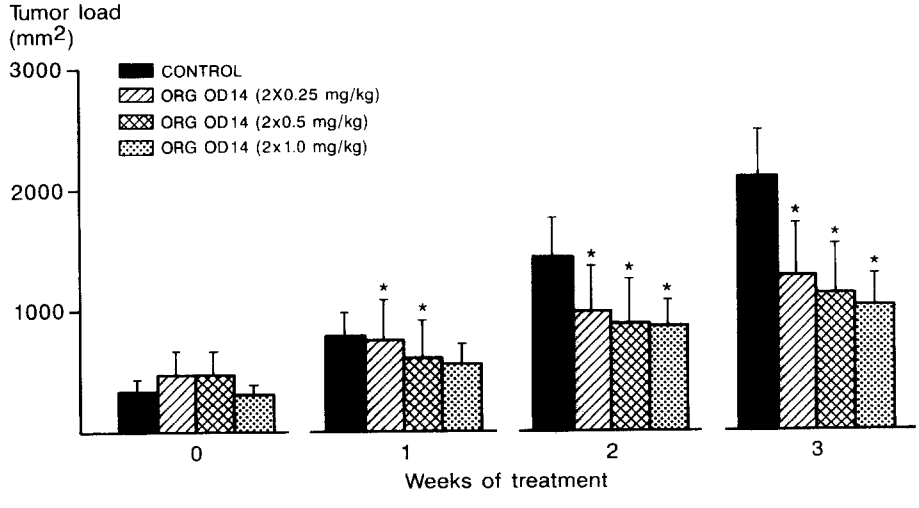

Fig. 8. Effects of various doses of Org OD14 on DMBA-induced mammary tumors using the therapeutic model. See Fig. 5 for further explanation.

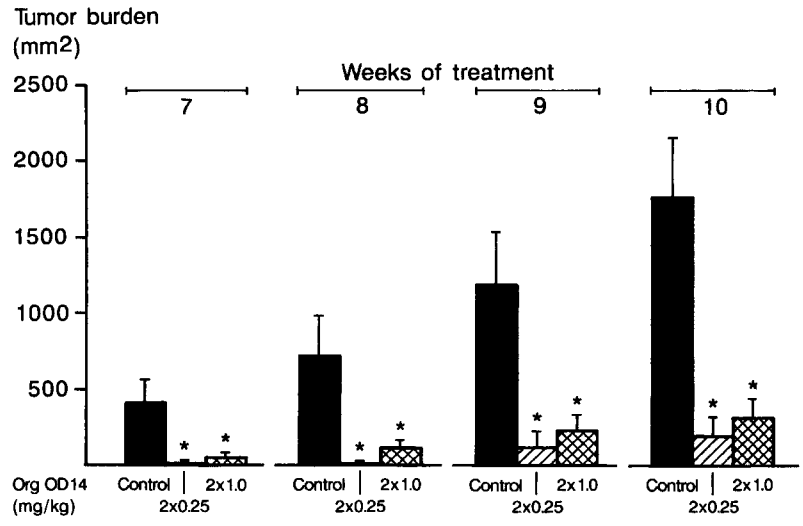

Fig. 9. Effect of Org OD14 on the development of DMBA-induced mammary tumors using the prophylaxic model. Statistical evaluation was done with the Wilcoxon test. See Fig. 5 for further explanation.

Org OD14 may be attributed to its androgenic and progestagenic activities because both progestagens [21, 23] and androgens [20] can diminish tumor growth in the DMBA model.

It can therefore be concluded that Org OD14 has due to its mixed hormonal profile most likely no tumor promoting activity. A direct effect of Org OD14 on the tumor can not be excluded completely but needs further investigations.

CONCLUSIONS

At high non-physiological concentrations estrogenic activity of 19-nortestosterone derived progestagens like gestodene, norethisterone and 3-ketodesogestrel becomes apparent by stimulating the proliferation of tumor cell lines but *in vivo* this does not lead to a stimulation of estrogen-sensitive tumors as in the DMBA model. Org OD14 also shows a stimulating effect on the proliferation of the tumor cell lines but the potency is far less than that of E2. This weak estrogenic activity does also not give rise to a tumor promoting effect in DMBA-treated rats. Surprisingly Org OD14 gave a strong inhibitory effect which can be attributed to the progestagenic and/or androgenic activity of the compound.

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