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Tibolone and 5α-dihydrotestosterone alone or in combination with an antiandrogen in a rat breast tumour model

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Abstract

Tibolone was combined with the antiandrogen flutamide to determine whether the inhibition of tumour growth in the prophylactic 7,12-dimethylbenz(a)anthracene (DMBA) rat model could be attributed to androgenic properties of one of its metabolites. The mean tumour load after tibolone (0.25 or 1.0 mg/kg twice daily orally for 10 weeks) was 125 and 255 versus 718 mm^2 for placebo. The mean number of tumours were 1.2 and 2.0 versus 5.8, respectively. Combined with flutamide (10 mg/kg twice daily orally) both doses of tibolone did not result in an increase compared to placebo, but in significantly lower tumour loads (160 and 64 versus 718 mm^2, respectively) and smaller numbers of tumours (0.8 and 1.0 versus 5.8, respectively). The differences between tibolone monotherapy and the combination groups with flutamide were not statistically significant indicating that flutamide did not reverse tibolone's inhibition of tumour growth. The positive control, 5α-dihydrotestosterone (DHT), entirely suppressed tumour development and flutamide abolished the inhibitory effect of DHT. Thus, unlike DHT, tibolone does not exert its beneficial effect in DMBA-induced tumours via the androgen receptor, but acts via different mechanisms.

Keywords: Tibolone; Breast cancer; DMBA; Dihydrotestosterone; Flutamide

1. Introduction

Sex hormones, in particular oestradiol and progesterone, play a major role (together with other hormones and growth factors) in both the physiological development of breast tissue and the pathogenesis of breast cancer. Recent meta-analyses have shown prolonged use of oestrogen-containing oral contraceptive or hormone replacement therapies (HRT) to be associated with an increased risk of breast cancer [1,2]. Current opinion is that oestrogens may promote, and androgens reduce, breast cancer risk [3]. In particular, it has been suggested that the beneficial effects of medroxyprogesterone acetate (MPA) on breast tumours are attributable, next to the progestagenic activity, also to its androgenic activity [3]. In monkeys, testosterone has been reported to inhibit oestrogen-induced mammary epithelial proliferation [4]. Human breast cancer cells in *in vitro* systems have been used commonly to study the effects of hormones, but such studies have yielded inconsistent data. The well-established 7,12-dimethylbenz(a)anthracene (DMBA) model was therefore selected for the present study.

In this experimental breast cancer model, DMBA is used to induce mammary tumours in rats, the development of which have been shown to be under hormonal control. These tumours are dependent on oestrogens for their continued growth [5,6]; progestagens show either inhibitory or no effects [7–9], and androgens inhibit tumour growth in this system [10]. In the therapeutic DMBA model, in which treatment is started when tumours are already present, tibolone (Org OD14; 7α,17α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one) has been shown clearly to inhibit mammary tumour growth in intact rats [11]. In the prophylactic model, in which treatment with tibolone is started at same time as DMBA is administered, tumour development has been shown to be delayed, and tumour growth is strongly suppressed, over 10 weeks of treatment [11].

After oral administration, tibolone is rapidly converted to several metabolites which are present in the

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circulation predominantly as inactive sulphated compounds. The unsulphated 3α- and 3β-hydroxy metabolites display oestrogenic activity, whereas the parent compound and the Δ^4-isomer have progestagenic and androgenic activity [12–15]. The effects of tibolone on human breast tissue have not been firmly established, and the combination of oestrogenic, progestagenic and androgenic properties might be expected to result in either protection against or stimulation of tumour development. Tibolone nevertheless appears to inhibit tumour growth in both prophylactic and therapeutic DMBA models, an effect that might be explained by the localised conversion of tibolone and its 3β-hydroxy metabolite from the circulation to the progestagenic and androgenic Δ^4-isomer in the breast. In this study, therefore, the effects of a combination of tibolone and the antiandrogen flutamide in DMBA-induced mammary tumours were investigated, with 5β-dihydrotestosterone (DHT) and flutamide being used alone or in combination as positive controls.

2. Materials and methods

2.1. Animals

Spf-bred Sprague–Dawley rats aged 54–61 days were obtained from Iffa-Credo (Someren, The Netherlands). These animals weighed 182–223 g at the start of the study. The rats were individually housed in type III Macrolon cages (Komeco, Alkmaar, The Netherlands) on wood shavings in light- and temperature-controlled rooms (14 h of light alternating with 10 h of darkness at 21–23 °C). Animals had free access to standard pelleted food (RMH-B, Hope Farms, Linschoten, The Netherlands) and tap water. The study complied with the Dutch Animal Experimentation Act (1997) and the Royal Decree on Animal Experimentation (1985).

2.2. Compounds

Tibolone, 5α-dihydrotestosterone (DHT) and flutamide were obtained from the Department of Pharmaceutics at NV Organon, Oss, The Netherlands. DMBA was obtained from the Sigma Chemical Co, St Louis, MO, USA.

2.3. Pharmaceutical formulations

Suspensions of tibolone (5 mg/ml) and flutamide (50 mg/ml) were prepared in aqueous solutions of gelatine and mannitol. DHT was dissolved in arachis oil. An oily solution of DMBA was prepared by dissolving 1 g in 100 ml of olive oil and stirring constantly in the dark for 36 h before use.

2.4. DMBA model

The DMBA model used was a modification of the therapeutic method described by Bakker and colleagues [16]. Briefly, mammary tumours were induced in each rat with 2 oral 1 ml doses of a 10 mg/ml DMBA solution, given over a 1-week interval (first dose at week 0 and second at week 1). Thus, each rat received a total dose of approximately 40 mg/kg. In contrast to the method used by Bakker and colleagues [16], treatment with tibolone, DHT or flutamide, alone or in combination, or inert vehicle, was started at the same time as the first dose of DMBA and continued for 10 weeks. All compounds were administered twice daily at volumes of 1.0 ml/kg to animals in all 10 groups ($n=9$) (1 flutamide, 2 tibolone, 2 DHT, 2 tibolone + flutamide and 2 DHT + flutamide dosage groups, and 1 placebo (vehicle) group; dosages are shown in Table 1). All agents except DHT (which was given subcutaneously (s.c.)) were administered orally by gavage.

The rats were weighed and palpated once weekly to detect tumours. Numbers of tumours were counted and tumour loads determined by measurement of perpendicular diameters once weekly from week 7 onwards. The tumour load was expressed as the product of the two perpendicular diameters in mm^2. Rats were killed and tumour weights determined at week 10. Animals were sacrificed earlier than planned if they were in poor condition or were impaired by their tumours, which accounted for the variation in the number of rats per group at autopsy.

2.5. Design and statistical analyses

A randomised block experimental design, starting with nine blocks of 10 rats, was used. The mean tumour load and mean number of tumours per group were calculated with data from all of the animals in each group, with the last measured tumour load of each sacrificed animal being used for the calculation of the mean tumour load at subsequent time points. Tumour loads and tumour numbers in animals without tumours were counted as zero. The statistical significance of the differences between groups in tumour load and number of tumours was analysed with the Wilcoxon test for randomised experiments. For statistical evaluation of tumour weights, Student's *t*-test was used. Results are presented as group arithmetic means ± the standard error of the mean (S.E.M.).

3. Results

Tumour load results at weeks 7, 8, 9 and 10 are presented in Fig. 1 and mean numbers and weights of tumours at autopsy are shown in Table 1.

Table 1
Effect of 10 weeks of treatment with tibolone or 5α-dihydrotestosterone (DHT) and/or the antiandrogen flutamide on tumour induction, mean number of tumours and mean tumour weight at autopsy in female rats treated with 7,12-dimethylbenz(a)anthracene (DMBA)[a]

Treatment	Dosage (mg/kg) twice daily	No. of rats with tumours (total evaluable)	Mean no. of tumours per rat[b]	Mean tumour weight (mg)[b]
Placebo		9 (9)	5.8±1.8	5907±2001
Flutamide	10	8 (9)	5.3±1.9	3894±1892
Tibolone	0.25	5 (9)	1.2±0.5	733±408*
Tibolone	1.0	6 (8)	2.0±0.7	1838±791
DHT	0.25	0 (8)	0.0*	0.0*
DHT	1.0	0 (8)	0.0*	0.0*
Tibolone + flutamide	0.25 + 10	4 (9)	0.8±0.4*	1392±1351*
Tibolone + flutamide	1.0 + 10	4 (7)	1.0±0.4*	617±316*
DHT + flutamide	0.25 + 10	7 (8)	2.6±0.8	1453±784*
DHT + flutamide	1.0 + 10	5 (8)	3.6±1.2	2450±838

*Statistically significantly different from controls ($P<0.05$) according to the Wilcoxon test for number of tumours and Student's t-test for tumour weight.

[a] Data are given as arithmetic means±standard error of the mean (S.E.M.). All drugs were given orally, with the exception of DHT, which was given by subcutaneous (s.c.) injection.

[b] Calculated over the total number of rats per group.

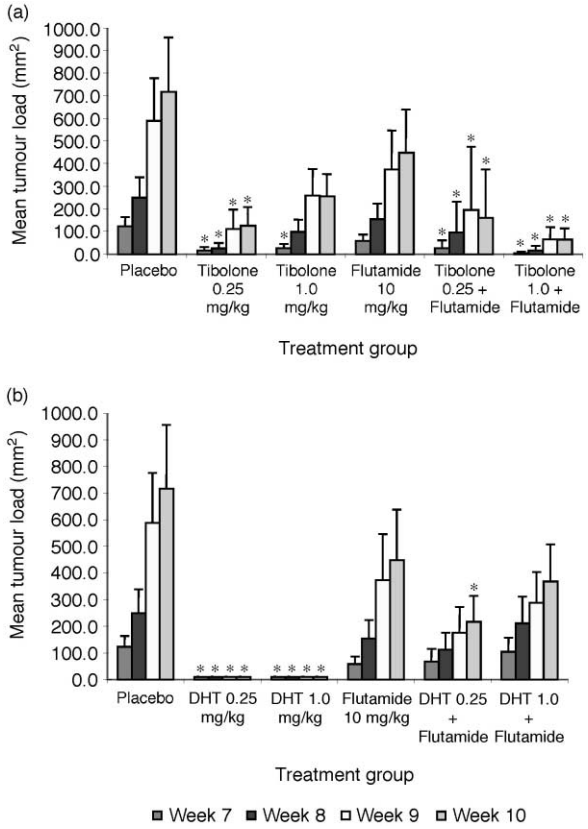

Fig. 1. Effect of tibolone (a) or dihydrotestosterone (DHT) (b) with and without the antiandrogen flutamide on mean tumour loads in female Sprague–Dawley rats treated with 7,12-dimethylbenz(a)anthracene (DMBA). All drugs shown were given twice daily for 10 weeks. To facilitate comparisons, placebo and flutamide monotherapy results are reproduced in both charts. Data are shown as arithmetic means±standard error of the mean (S.E.M.). *Statistically significantly different from placebo ($P<0.05$) according to the Wilcoxon test.

3.1. Placebo group

At week 7, tumours were found in 7 of 9 evaluable vehicle-treated rats; at weeks 8 and 9, tumours were present in 8 of 9 rats. Two rats had to be sacrificed at week 9 because of the size of the tumours. The mean tumour load and the number of tumours increased over time up to and including week 10. At autopsy, mean tumour load, weight and number per rat were 718±240 mm^2, 5907±2001 mg and 5.8±1.8, respectively.

3.2. Tibolone groups

At weeks 7 and 10, 2 and 5 of 9 rats, respectively, in the 0.25 mg/kg tibolone group had tumours. At all time points, the mean tumour load was significantly lower than that in the placebo group (Fig. 1). At autopsy, the mean number of tumours was 1.2±0.5, which was not significantly lower than in the placebo group (5.8±1.8; Table 1). Mean tumour load (125±83 mm^2) and weight (733±408 mg) at autopsy were significantly lower than those in the placebo group.

In the 1.0 mg/kg tibolone group, 3 of 8 rats showed tumours at week 7; this increased gradually to 6 of 8 animals at week 10. Although the mean tumour load and number of tumours during the measurement period were lower than those in the placebo group, the differences between groups were not significant (except for tumour load at week 7) (Table 1 and Fig. 1). At autopsy, the number of tumours (2.0±0.7), tumour load (255±98 mm^2) and tumour weight (1838±791 mg) did not differ significantly from those in the vehicle treated rats.

No statistically significant differences in mean numbers of tumours, tumour loads and tumour weights were apparent between the two tibolone groups.

3.3. Flutamide group

Mean tumour load, tumour weight and number of tumours in the flutamide group did not differ significantly from the placebo group values (Fig. 1 and Table 1).

3.4. Tibolone + flutamide groups

The mean tumour loads at weeks 7, 8, 9 and 10 in the tibolone 0.25 mg/kg twice daily monotherapy group were lower than the corresponding loads in the group treated with a combination of tibolone 0.25 mg/kg and flutamide 10 mg/kg. The differences between the tibolone monotherapy and combination group were not statistically significant. No significant differences were found between these groups at autopsy in mean number of tumours, tumour load or tumour weight (Fig. 1 and Table 1). The differences between the combination group and the placebo group were statistically significant.

In rats receiving the combination of 1.0 mg/kg tibolone twice daily and flutamide, the mean tumour load at weeks 7, 8, 9 and 10 was lower than those in the tibolone 1.0 mg/kg group, although differences between monotherapy and combination group were not significant. Furthermore, at autopsy, the mean tumour load, tumour weight and mean number of tumours were lower in the combination than in the monotherapy group; these differences were not significant (Fig. 1 and Table 1). As with the earlier results, the differences with the placebo group were statistically significant.

3.5. DHT groups

No tumours were found in either DHT group (2×0.25 or 1.0 mg/kg/s.c.). This indicates the complete suppression of tumour induction or growth by DHT.

3.6. DHT + flutamide groups

In both groups treated with the combinations of DHT and flutamide, tumours were found from week 7 onwards. Mean tumour loads, weights and numbers of tumours per group were not significantly different from those in the placebo animals except for the 0.25 mg dose at week 10.

4. Discussion

The results presented show that tibolone and DHT reduce loads, numbers and weights of mammary tumours in DMBA-treated rats. This confirms results previously obtained with this model for DHT [10] and tibolone [11]. This suppression of tumour development by tibolone suggests that the oestrogenic 3α- and 3β-hydroxy metabolites, which are formed rapidly in the circulation from tibolone after oral administration, do not exert significant effects on tumorigenesis. Oestrogenic activity would be expected to increase (not decrease) tumour loads, especially as breast tissue in women is able to concentrate, produce and metabolise oestrogens and thus generate localised oestradiol concentrations 10–40 times higher than those in the circulation [17]. In contrast, in this prophylactic DMBA model, tibolone reduced tumour growth. Since the androgen DHT also suppressed tumour growth in this model, the tumour-inhibiting effect of tibolone may be linked to the localised conversion of tibolone and its 3β-hydroxy metabolite from the circulation to the progestagenic and androgenic Δ^4-isomer by the enzyme 3β-hydroxysteroid dehydrogenase/isomerase. The presence of this enzyme has been demonstrated in human endometrial tissue [18,19] and in human breast tissue [20]. It is not known whether this enzyme is also present in the female rat.

The results presented also show that the suppression of tumour development observed after administration of DHT can be reversed by the antiandrogen flutamide, via its blockade of the androgen receptor. This has already been described for DHT [10] and dehydroepiandrosterone (DHEA) which has both androgenic and oestrogenic activities [21–23]. This suggests that DHT and DHEA exert their antitumour effects in the DMBA model via the androgen receptor. In contrast to these and the present results with DHT, the results of this study show that inhibition of the formation and development of DMBA-induced tumours by tibolone (2×0.25 and 2×1.0 mg/kg/orally (p.o.)) were not reversed by the antiandrogen flutamide. This shows that the inhibitory effects of tibolone on these tumours are apparently not mediated via androgens and androgen receptor. This implies that either the local conversion of tibolone and of its 3β-hydroxy metabolite from the circulation to the progestagenic and androgenic Δ^4-isomer does not occur or that the expression of the androgen receptor in the breast tissue of the DMBA treated rats is reduced. However, the enzyme responsible for the conversion, 3β-hydroxysteroid dehydrogenase/isomerase, has been demonstrated to be present in human breast tissue [20]. Therefore, it is highly unlikely that the progestagenic and androgenic Δ^4-isomer is not formed in rat breast tissue in the DMBA model. Its capacity to bind to the androgen receptor would have resulted in the observed reduction of the tumours by tibolone, but it would also have resulted in the impairment of this reduction by the anti-androgen flutamide as observed with DHT. To explain the absence of androgen-medi-

ated anti-tumour effects in the presence of the androgenic Δ^4-isomer it should be concluded that the expression of androgen receptor in the DMBA breast tumours of rats is greatly reduced after tibolone treatment and not after DHT treatment. A possible mechanism for the tumour reduction in tibolone treated rats could then be the progestagenic effects of the Δ^4-isomer of tibolone, since it has been demonstrated that progestagens are able to reduce tumour growth [7–9]. Unfortunately, this cannot be tested, since an antiprogestagen by itself already reduces tumour growth. Apart from this mechanism, other mechanisms not mediated by androgen and androgen receptor may be operational for the tibolone-induced inhibition of DMBA-induced tumours. The surprising findings that the androgenic activity of tibolone is not involved in the DMBA model in the rat prompted us to evaluate alternative mechanisms in tissues of humans, since this is the species in which tibolone is being used.

Observations in breast cancer cell lines MCF-7 and T47-D, in which tibolone and its metabolites have been shown to interfere with the oestrogen metabolism by inhibiting the enzyme sulphatase [24–26], provide evidence of an alternative potential mechanism. Since oestradiol is one of the most important agents contributing to the development and growth of breast tumours, inhibition of formation of oestradiol in the breast would be expected to be beneficial. Three main enzymatic mechanisms are involved in the local formation of oestradiol: the aromatase pathway, which converts androgens into oestrogens; the sulphatase pathway, which converts oestrone sulphate into oestrone, and the 17β-hydroxysteroid dehydrogenase (17β-HSD) pathway, which transforms oestrone into oestradiol [27]. Co-incubation studies using 10-fold excess concentrations of tibolone and its metabolites have shown that the aromatisation of 1 μM androstenedione by human placental aromatase is not impaired (data not shown). This suggests that tibolone has no effects on aromatase. However, in addition to the effects on the sulphatase enzyme, tibolone and its two 3-hydroxy metabolites also decrease the rate of conversion of oestrone to oestradiol by inhibiting the enzyme 17β-HSD [27]. Since tibolone and its metabolites interfere with two of three pathways leading to the formation of biologically active oestradiol, the favourable effect of tibolone on mammary tumours may be partly mediated by inhibition of the local biosynthesis of oestradiol in the breast. The inhibition of the sulphatase enzyme may also interfere with the generation of the active oestrogenic metabolites of tibolone from their sulphated forms, thus further reducing local levels of oestrogenic activity. In addition, it has been found that tibolone and its metabolites stimulate sulphotransferases, an enzyme system that keeps sulphated oestrogenic compounds in the inactive form [28].

Anti-oestrogens, progestagens and androgens can promote apoptosis in breast cancer cells [29]. Tibolone and its Δ^4-isomer increase the number of apoptotic cells in T47-D and ZR75-1 cells, which contain progestagen and androgen receptors [29,30]. This indicates that the beneficial effect of tibolone may be mediated, at least in part, by its pro-apoptotic action. Data from numerous laboratories suggest 'cross-talk' between progesterone receptors and growth factor and cytokine signalling pathways at multiple levels in the breast [31]. It is possible to speculate that tibolone and its Δ^4-isomer may interfere with this 'cross-talk' resulting in changes in proliferation, differentiation or programmed cell death. In order to establish the mechanism of action of tibolone in breast cancer, further studies are required.

Clinical studies show that tibolone is tissue-specific in its action: the drug has a oestrogenic effects in climacteric conditions, and prevents postmenopausal osteoporosis in bone, whereas no oestrogenic stimulation has been observed in the endometrium or the breast [32,33]. Compared with oestrogen-containing HRT preparations, tibolone causes less breast tenderness and pain and does not increase breast density in humans [34–36]. These clinical data also indicate that tibolone acts on the breast in a manner that differs from conventional oestrogen-containing HRT preparations.

It is therefore concluded that the inhibition of induction and growth of DMBA-induced mammary tumours by tibolone is not reversed by the anti-androgen flutamide. The above data also show the reversal by flutamide of inhibition by DHT of tumour induction. In contrast to DHT, therefore, tibolone does not exert its beneficial effect on DMBA-induced tumours via the androgen receptor, but by other mechanisms such as interference with the local biosynthesis of oestradiol or induction of apoptosis.

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