Pergamon

PII: S0960-0760(96)0003-9

J. Steroid Biochem. Molec. Biol. Vol. 58, No. 1, pp. 13–19, 1996

0960-0760/96 $15.00 + 0.00

Effects of Sex- and Glucocorticoid Steroids on Breast Cancer Cells Grown as either Multicellular Tumor Spheroids or Monolayers

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The effects of estradiol, medroxyprogesterone acetate (MPA) dexamethasone, dihydrotestosterone and the antihormones 4-OH tamoxifen and RU 38486 were studied in two established breast carcinoma cell lines, the estrogen-sensitive ZR-75-1 and the estrogen-independent BT 20 cells applying two different *in vitro* systems, spheroid and monolayer cell culture in steroid deprived medium. Growth of ZR-75-1 spheroids was dramatically stimulated by the addition of estradiol, an effect which was neutralized by the simultaneous addition of 4-OH tamoxifen. The antiestrogen alone as well as dihydrotestosterone and MPA reduced ZR-75-1 spheroid growth significantly. While growth of BT 20 spheroids was only transiently inhibited by tamoxifen and dihydrotestosterone, a persistant increase in BT 20 spheroid growth was observed under MPA treatment in a concentration of 1 μM. This effect, although statistically significant, was very moderate. With the exception of this finding, growth effects of the different test compounds were similar in both *in vitro* systems, tumor spheroids and monolayer cell cultures. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

The proliferative activity of estradiol (E2) together with the antagonistic effect of antiestrogens like tamoxifen (TAM) and its 4-OH metabolite (OHT) has been demonstrated widely using estrogen-sensitive breast cancer cells in various *in vivo* and *in vitro* experimental models [1–6]. Whereas there is a consensus about the role of estrogens in breast cancer cell growth promotion, experiments with progestins lead to conflicting results, i.e. either mitogenic, or antiproliferative [6–9] or no effect at all, depending on culture conditions [10] and type of cell lines (and variants) [9, 11] have been reported. Mainly progestins of the 19-nor-testosterone type in supraphysiological or pharmacological doses have been found to stimulate growth of a variety of breast cancer cell lines [12–15], whereas this was found only rarely for progestins of the progesterone type, e.g. medroxyprogesterone-acetate (MPA) and if so, then only in high concentrations (10^{-6} M) [13–15]. This growth stimulation seemed to occur via the estrogen receptor (ER), either directly or after aromatisation in the case of 19-nor-testosterone derivatives [16], as TAM in most cases was able to counteract this effect. Despite the intrinsic estrogenic potential of some progestins, when administered alone, they may display antiestrogenic activity in the presence of estrogens [11, 14]. In addition MPA was found to stimulate growth of mouse mammary carcinoma cells through the androgen receptor [17] and to inhibit proliferation of ZR-75-1 cells through the androgen- and glucocorticoid receptor [18].

Most of these studies were performed with cells grown as a monolayer, a system which does not allow an intense cell–cell contact as may occur during tumor growth *in vivo*. An interesting alternative to overcome this problem could be a three-dimensional growth in multicellular tumor spheroids (MCTS). The architecture of MCTS is similar to that of solid tumor nodules containing proliferative cells close to capillaries, quiescent cells next to these and necrotic areas at greater distances [19, 20]. Spheroids show a necrotic center surrounded by the so-called viable rim, which contains the inner mantle region with quiescent cells and the outer crust of proliferating cells [21]. Once the

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Received 6 Jul. 1995; accepted 17 Nov. 1995.

spheroids are initiated, they increase their size at a rate according to Gompertzian's equation, which accurately represents the growth of some tumor models and cancer *in vivo* [22, 23]. The MCTS model is intermediate in complexity between standard two-dimensional monolayer cultures *in vitro* and tumors *in vivo* [20]. Evidence of enhanced differentiation has been obtained in spheroids of a human colon adenocarcinoma cell line, which developed pseudoglandular structures mimicking the features of an adenocarcinoma *in vivo* [24].

Based on the work of Olea [25], who described MCTS formation of MCF-7 breast cancer cells in estrogen-containing but not deprived media, we chose this model for further studies of endocrine susceptibility of breast cancer cells. Two established cell lines, namely the ZR-75-1 and the BT 20 cell lines, were selected for their well documented estrogen dependence and resistance, respectively. Both lines are derived from different patients and have no similarity with MCF-7 cells. As far as we know, no data have yet been published on MCTS formation of ZR-75-1 or BT 20 cells. Yuhas *et al.* postulated that the ability of breast cancer cell lines to grow as MCTS is dependent on the origin of the tumor cells [26]. They found that cells derived from pleural effusions are not able to grow in tight aggregates in contrast to the tumor cells from solid breast cancer samples. We therefore addressed the issue whether ZR-75-1 cells, derived from ascitic fluid [27] and BT 20 cells, derived from an infiltrating ductal carcinoma [28], could both form spheroids and examined how different classes of steroids influenced their growth and morphological structure.

MATERIAL AND METHODS

Monolayer cell culture

ZR-75-1, an estrogen-sensitive cell line [27] was generously supplied by Dr R. J. B. King, London, U.K. BT 20 is an established estrogen receptor (ER) negative cell line [29], kindly donated by Dr G. Buehring, Berkeley, CA. Cells were grown in Phenol Red-free Eagle's minimum essential medium (MEM) supplemented with 10% charcoal-treated fetal calf serum (FCS). Estradiol content of FCS was checked by radioimmunoassay and found to be below the detection limit (5 pg/ml). The cultures were maintained at 37°C in humid air containing 5% CO_2. The method for culturing the monolayers has been described in detail elsewhere [4].

Multicellular tumor spheroids

Cells for spheroid formation were obtained by trypsinization from exponentially growing monolayer cultures. The proportion of vital cells was evaluated after dilution in Trypan Blue. The culture method used to initiate spheroid growth was based on the liquid overlay technique [30]. The cell suspension was diluted adequately in charcoal-treated medium, and 4000 cells in 200 μl were seeded in each well of a 96-microwell test plate (Nunc, Falcon) coated with 1% agarose (Serva, Heidelberg, Germany). The cultures were maintained at 37°C in humid air containing 5% CO_2. MCTS were harvested on day 26 (BT 20) and day 32 (ZR-75-1), respectively.

Cell treatment

Monolayers as well as 30 spheroids of each cell line were treated by adding various concentrations of test-compounds either alone or in combination in ethanolic solution (final ethanol concentration was 0.1%). Control groups contained 0.1% of ethanol. Treatments were begun on day four. Medium was renewed every third day.

Test-compounds

17β-estradiol (E2), (Serva, Heidelberg, Germany), 4-OH tamoxifen (OHT), (Jeneca, Cheshire, U.K.), medroxyprogesterone-acetate (MPA), (Upjohn, Kalamazoo, Michigan, U.S.A.), RU 38486-17 (RU 486), (Roussel Uclaf, Paris, France), hydroxyflutamide (OHF), (Schering, Berlin, Germany), Dexamethasone (DEX), (Sigma Chemical Company, St Louis, MO, U.S.A.), and 5α-dihydro-testosterone (DHT), (Makor Chemicals Ltd, Jerusalem, Israel).

Growth measurements

MCTS. 10 spheroids from each treatment group were evaluated twice a week using a calibrated ocular micrometer on an inverted microscope. The volume of the spheroids was calculated by the equation

$$\text{volume} = a \times b^2 \times \pi/6,$$

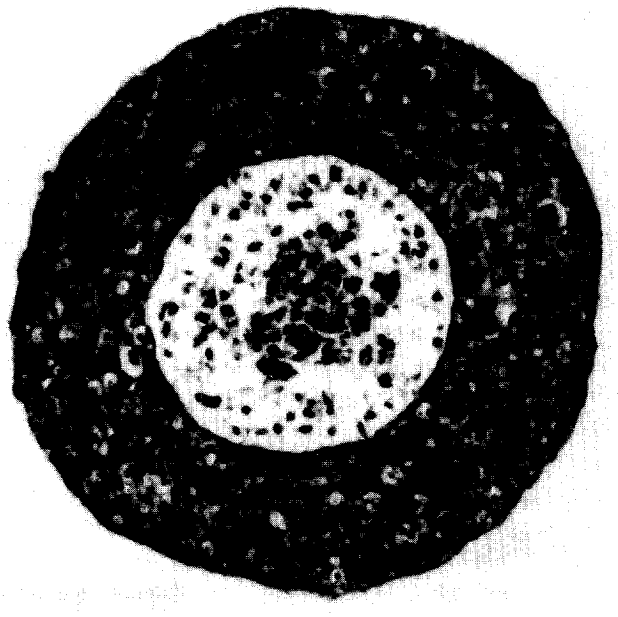

Fig. 1. Typical histological structure of a ZR-75-1 spheroid on day 35 with a necrotic center surrounded by viable tumor cells. H.-E. staining.

Table 1. Growth of ZR-75-1 multicellular spheroids under the different treatment regimens

	Day 29			Day 32		
ZR-75-1	% vol	CV (%)	Level of significance	% vol	CV (%)	Level of significance
Control	145	8	=	160	11	=
E2 10^{-9}	429	11	$P<0.001$	514	12	$P<0.001$
OHT 10^{-6}	87	10.5	$P<0.05$	89	9	$P<0.05$
E2 + OHT 10^{-9} 10^{-6}	77	9	$P<0.05$	79	11	$P<0.05$
MPA 10^{-6}	107	6	$P<0.05$	118	6	$P<0.03$
MPA 10^{-8}	90	3	$P<0.03$	97	2.5	$P<0.02$
RU 486 10^{-6}	135	10	NS	161	7	NS
MPA + RU 10^{-6} 10^{-6}	93	3	$P<0.03$	100	4	$P<0.03$
MPA + OHF 10^{-6} 10^{-6}	88	9.5	$P<0.05$	97	8	$P<0.03$
DEXA 10^{-8}	163	10	NS	183	8.5	NS
DHT 10^{-8}	117	8	$P<0.05$	122	6	$P<0.03$

Effects of different hormones and antihormones on ZR 75 1 spheroid growth as measured on days 29 and 32.
Results are indicated as percent of the spheroid volume on day 4 (100%).
Concentrations are indicated in mol/l.
CV = coefficient of variation.

where a and b are the longest and shortest diameters, respectively [25].

Monolayers. Cell number was evaluated using an electronic particle counter (Coulter Counter Electronics Ltd., Luton, U.K.). Cell numbers of treated groups were expressed as a percentage of untreated control groups. The coefficient of variation was always below 8%.

Histological staining of MCTS

The MCTS were fixed in 4% formaldehyde and paraffin embedded. Sections 4 μm thick were cut serially, and equatorial sections were stained with hematoxylin-eosin for evaluation of morphological structures.

Statistical analysis

Differences in median spheroid volume and cell number of monolayer cultures were evaluated with the non-parametric U-test according to Mann–Whitney.

RESULTS

Growth characteristics and morphology of MCTS

Both cell lines formed spheroids within 4 days. The mean diameter on day 4 was 290 μm in ZR-75-1 and 320 μm in BT 20 spheroids. No differences in morphological structure or in degenerative changes were found in the various treatment groups of one cell line. Histological structure was typical for a predominantly solid adenocarcinoma in both cell lines. ZR-75-1 spheroids began to develop a typical central necrotic region on day 32, BT 20 spheroids on day 26 (Fig. 1).

Growth curves of MCTS

ZR-75-1 cells [Table 1 and Fig. 2(a, b)] were dramatically stimulated by the addition of estradiol: the mean spheroid volume on day 32 was three times that of the control group. This estrogen-induced proliferation was completely reversed by the addition of OHT. OHT, DHT and MPA alone significantly reduced spheroid growth. Effects observed by combination of MPA with either RU 486 or flutamide were not distinguishable from MPA alone. RU 486 alone, as well as dexamethasone, did not influence spheroid growth significantly.

Growth of BT 20 spheroids [Table 2 and Fig. 3(a, b)] was significantly stimulated by MPA in a concentration of 1 μM. This effect was neutralized by the addition of RU 486 but not by flutamide. Treatment with OHT and DHT alone resulted in a significant but transient growth inhibition between day 11 and day 22, whereas on day 26 this effect disappeared. Neither RU 486 nor dexamethasone nor E2 treatment influenced BT 20 spheroid growth significantly.

Growth of cells cultured as monolayers

In ZR 75 1 cells the addition of estradiol led to an increase of up to 550% in cells as compared to the control group (100%). This effect was reversed by the addition of OHT. OHT alone, MPA 1 μM, MPA 10 nM and DHT revealed a significant antiproliferative effect ($P<0.05$). The inhibitory influence of MPA was reduced but not neutralized by the addition of RU 486 or OHF. Treatment of ZR-75-1 cells with dexamethasone or RU 486 alone did not alter proliferation

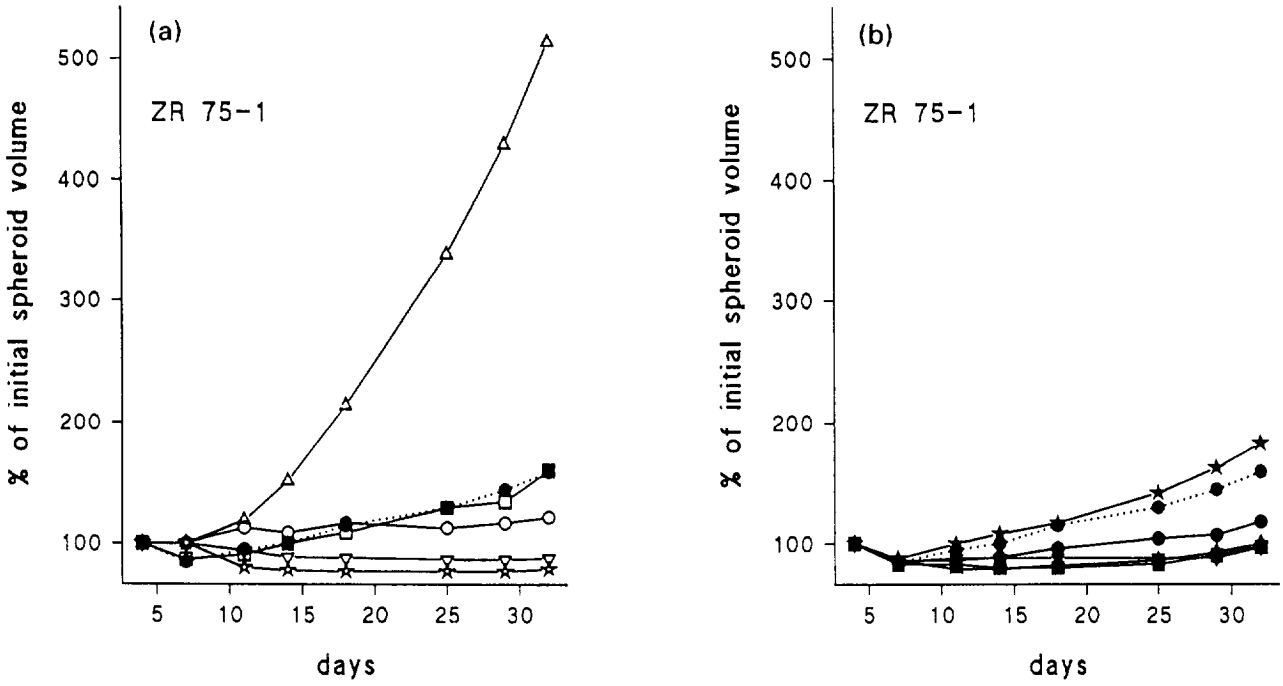

Fig. 2. Growth curves of ZR-75-1 spheroids cultivated in estrogen-deprived medium and with the addition of the following substances to the medium: (a) without (● - - - ● control), ○——○ DHT, □——□ RU 486, △——△ E2, ▽——▽ 4-OHT, ☆——☆ E2 + 4-OHT. (b) without (● - - - ● control), ●——● MPA (1 μM), ■——■ MPA (10 nM), ▲——▲ MPA + RU 486, ▼——▼ MPA + OHF, ★——★ DEX. Each point represents the mean volume of 10 spheroids measured in relation to initial control spheroid volume. The corresponding coefficients of variation as well as concentrations of test compounds used are indicated in Table 1.

significantly. BT 20 cells cultured as monolayers did not respond either to high dose MPA or to any other hormonal treatment performed in this study (data not shown).

DISCUSSION

The data presented above demonstrate that BT 20 as well as ZR-75-1 breast carcinoma cells are able to grow as MCTS. This fact was unexpected for ZR-75-1 cells

Table 2. Growth of BT 20 multicellular spheroids under the different treatment regimens

	Day 22			Day 26		
BT 20	% vol	CV (%)	Level of significance	% vol	CV (%)	Level of significance
Control	182	3	=	183	5	=
E2 10^{-9}	172	8	NS	166	12	NS
OHT 10^{-6}	140	8	$P<0.05$	161	11	NS
E2 + OHT 10^{-9} 10^{-6}	164	7	NS	175	7	NS
MPA 10^{-6}	212	4	$P<0.05$	219	5	$P<0.05$
MPA 10^{-8}	190	7	NS	184	9	NS
RU 486 10^{-6}	179	9	NS	182	8	NS
MPA + RU 10^{-6} 10^{-6}	190	4	NS	190	7	NS
MPA + OHF 10^{-6} 10^{-6}	196	7	NS	202	4	$P<0.05$
DEXA 10^{-8}	179	9	NS	181	7	NS
DHT 10^{-8}	156	8.5	$P<0.05$	174	9	NS

Effects of different hormones and antihormones on BT 20 spheroid growth as measured on days 22 and 26.
Results are indicated as percent of the spheroid volume on day 4 (100%).
Concentrations are indicated in mol/l.
CV = coefficient of variation.

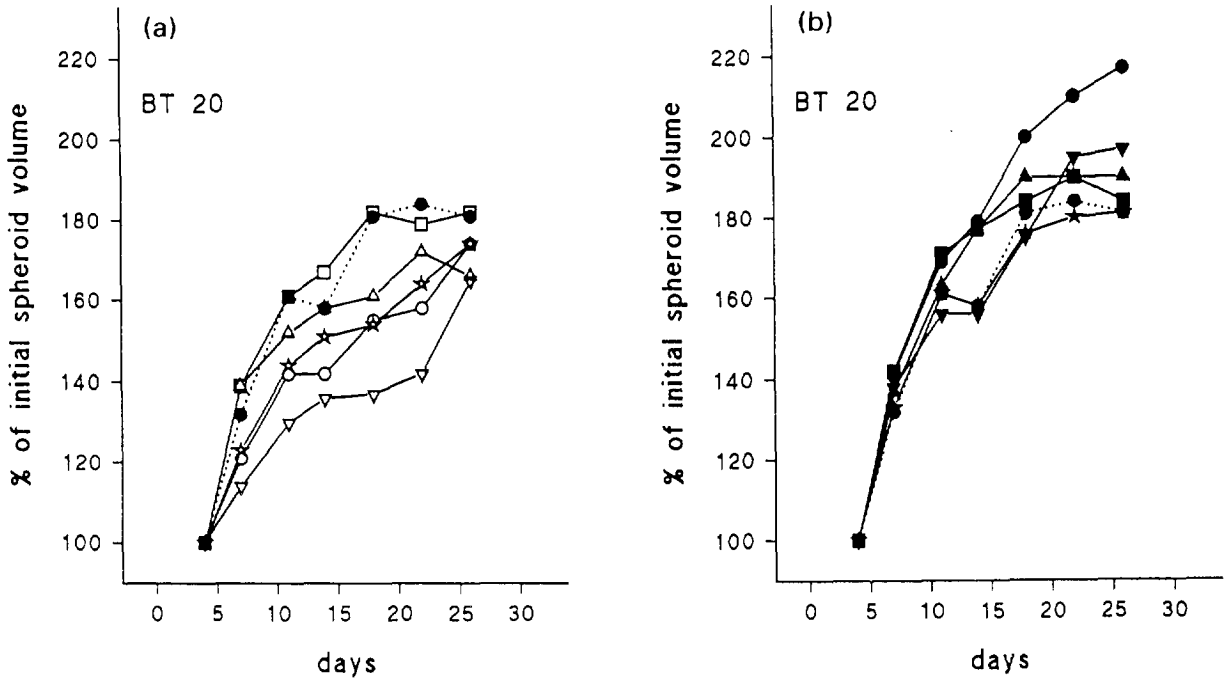

Fig. 3. Growth curves of BT 20 spheroids cultivated in estrogen-deprived medium and with addition of the following substances to the medium: (a) without (●- - -● control), ○——○ DHT, □——□ RU 486, △——△ E2, ▽——▽ OHT, ☆——☆ E2 + OHT. (b) without (●- - -● control), ●——● MPA (1 μM), ■——■ MPA (10 nM), ▲——▲ MPA + RU 486, ▼——▼ MPA + OHF, ★——★ DEX. Each point represents the mean volume of 10 spheroids measured in relation to initial control spheroid volume. The corresponding coefficients of variation as well as concentrations of test compounds used are indicated in Table 2.

as they are derived from ascitic fluid and therefore should, according to Yuhas *et al.* [26] not be able to grow as spheroids. Moreover, Olea *et al.* [25] reported that the estrogen-dependent breast cancer cell line MCF-7 requires estrogens in order to grow as MCTS. However, in order to establish spheroids as a model for studying the influence of estrogens, MPA and their antagonists without falsification by hormone-containing culture medium, we had to use estrogen deprived and Phenol Red-free medium [31]. Under these conditions we did not succeed in culturing MCF-7 cells as MCTS. ZR-75-1 spheroids grown in estrogen-free medium were smaller than those grown in estrogen-containing medium, but nevertheless developed the typical structure of an MCTS with the central necrotic area and the outer viable rim. Although spheroids of all treatment groups continued to grow after the occurrence of the central necrotic areas, growth measurements were performed when necrosis occurred, as necrotic material is known to induce growth-inhibitory effects on the proliferating population of cells [20].

There were no differences in the morphological structure of spheroids in the various treatment groups as evaluted by light microscopy. Some spheroids displayed glandular lumina like structures, but there was no association with the different substances added to the medium. In general, histological appearance was similar to that of a biopsy specimen from a human breast carcinoma *in vivo*.

Cell lines used differed with respect to their steroid receptor status: the ZR-75-1 cell line possesses functional estrogen (ER), progesterone (PR), androgen (AR) and glucocorticoid receptors (GR) [27], whereas BT 20 cells are negative for ER, PR and AR, but positive for GR [29].

Estrogen induced growth of ZR-75-1 cells grown as MCTS as well as in the monolayer culture, an effect which was completely reversed by the addition of OHT in both systems. DHT led to a significant growth inhibition in ZR-75-1 spheroids and monolayers. As experiments were performed under estrogen-free conditions, this growth inhibitory effect seems more likely to be mediated directly through the AR than through a DHT induced down-regulation of estrogen receptors, as has been described by Poulin *et al.* [32].

In addition to its progestin-like action, MPA was assumed to exhibit androgen- and glucocorticoid-like activities (18). In our study MPA displayed growth inhibitory potential in ZR-75-1 cells grown as MCTS and as monolayer, whereas DEX did not. Thus one can exclude that MPA acts via the GR. The ineffectivity of RU 486 and flutamide to overcome MPA-induced growth inhibition does not allow one to conclude that PR and AR are not involved in MPA action as, to our knowledge, it has up to now not been demonstrated that

growth inhibitory action of a steroidal compound could be overcome by an antihormone. The fact that MPA displays a maximal effect at 10^{-8} M (or lower) indicates, however, that the MPA effect is PR-mediated. Tamoxifen initiated growth inhibition of ZR-75-1 cells either as MCTS or monolayer under estrogen-deprived and Phenol Red-free conditions. This might lead to the assumption that estrogen receptor-independent mechanisms are involved as already suggested in earlier studies [33–37].

A different, probably more plausible interpretation of our results obtained in ZR-75-1 cells would occur if one assumed that after charcoal treatament of fetal calf serum (FCS) small concentrations of E2, below the detection limit of our radio-immunoassay (5 pg/ml = 18 pM) are still present. As E2 levels as low as 10^{-12} M can already stimulate growth [11] the probability of a moderate estrogenic stimulus in the controls cannot fully be excluded in our study (and probably in other studies too, when using stripped FCS). In this case the effects of OHT and MPA would reflect the well known antiestrogenic action of these compounds and the moderate antiproliferative effect of DHT could be attributed to its ability to decrease ER levels as mentioned above [32].

For BT 20 cells, slide discrepancies between results obtained in MCTS and monolayers were observed which is in agreement with earlier reports [26, 30]. Whereas growth of BT 20 cells in monolayer culture was not influenced by any of the hormones tested, BT 20 spheroids showed a transient but significant growth inhibition by OHT and DHT between day 11 and 22 but not any more on day 26. As effects were moderate and only transient, speculations on this phenomenon do not seem to be meaningful. Treatment of BT 20 spheroids with MPA in a concentration of 1 μM led to a continuous growth stimulation which was neutralized by RU 486 but not by flutamide. Again this effect, although statistically significant, is very moderate. This inhibition by RU 486 indicates that MPA acts via the PR.

High dose MPA administration is widely used in the clinical therapy of advanced breast cancer and it induces remission rates of 30–40% [38], which are comparable to those obtained with other types of endocrine therapies (e.g. tamoxifen) [39–40]. Our results with MCTS of BT 20 cells show that MPA may exert weak growth stimulation in certain breast cancer cells at a concentration of 1 μM, which can nearly be achieved in clinical application ($2–3 \times 10^{-7}$ M). Further studies should illuminate whether this phenomenon is found in other breast cancer cell lines as well.

With respect to the comparison of the two *in vitro* models, MCTS versus monolayer culture, for the evaluation of growth effects of steroid hormones on two breast cancer cell lines we conclude that, with the exception of the very limited stimulatory effect of MPA at high doses in BT 20-spheroids, no significant differences could be demonstrated in this study. This answer, however, should not be generalized.

Nevertheless, MCTS have been proved to be an excellent tool for *in vitro* studies, where cell-to-cell interaction, production of extracellular matrix, morphologic and functional differentiation, etc. are the main subject of interest, as this model is certainly more comparable to the *in vivo* situation than are monolayer cultures [41–47].

Acknowledgements—This work was supported in part by the Austrian "Fonds zur Förderung der Wissenschaftlichen Forschung", project number P 5655.

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