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INHIBITION OF THE ESTRADIOL-INDUCED GROWTH OF CULTURED HUMAN BREAST CANCER CELLS BY THE ANTI-ESTROGENS TAMOXIFEN, DESMETHYL-TAMOXIFEN, 4-HYDROXY-TAMOXIFEN AND ENCLOMIPHENE

CHRISTIAN MARTH,* GÜNTHER DAXENBICHLER*, GERTRUDE C. BUEHRING†, FERDINAND HOFSTÄDTER‡ and OTTO DAPUNT*

*Department of Gynecology and Obstetrics, University of Innsbruck, Austria, †School of Public Health, University of California, Berkeley, CA, U.S.A., ‡Department of Pathology, University of Innsbruck, Austria

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Abstract—The growth effects of tamoxifen (T), desmethyl-tamoxifen (dMeT), 4-hydroxy-tamoxifen (OHT) and enclomiphene (Clo) on cultured human breast cancer cell lines have been related to published binding affinities for the estrogen receptor. Only in cells which were stimulated by estrogens did these anti-estrogens markedly inhibit growth. In both estrogen sensitive cell lines tested, 734 B and ZR 75.1, the anti-estrogen activity showed the identical rank: OHT ≫ Clo ≅ T = dMeT; this anti-proliferative potency agrees with reported affinities of these compounds for the estrogen receptor. In culture media containing defined amounts of estradiol we observed that a 10,000-fold molar excess of OHT was required to inhibit the estradiol-induced growth, but the estradiol-independent proliferation was not affected.

The endocrine treatment of early and metastatic breast cancer with anti-estrogens is now widely established [1–3]. Tamoxifen treatment is a commonly used strategy and much research has been conducted to investigate its mechanism of action; however, many questions remain unanswered.

Anti-estrogens bind to the cytoplasmic estrogen receptor (ER)§ in competition with estradiol (E_2). This complex is able to translocate into the nucleus but is unable to induce the same cell response as the ER conjugated with E_2 [4]. The growth inhibitory action of anti-estrogens on breast cancer cells is thought to be related to its binding to the ER [5]. In humans, tamoxifen (T) is mainly metabolized into desmethyl-tamoxifen (dMeT [6]), whereas 4-hydroxy-tamoxifen (OHT), which is the major metabolite in rats, is found only in low levels in the plasma or is not detectable at all [6–9]. Enclomiphene (Clo) is another triphenylethylene derivative with anti-estrogenic action [10] and is metabolized in rat liver microsomes in a manner similar to T [11]. T, dMeT, and Clo bind with a comparable affinity to ER. OHT, however, shows a more pronounced ability to compete with estradiol for the ER (Table 1). Coezy *et al.* have recently shown that the growth

§ Abbreviations: T, tamoxifen; OHT, 4-hydroxy-tamoxifen; dMeT, desmethyl-tamoxifen; Clo, enclomiphene; E_2, estradiol; ER, estrogen receptor; MEM, Minimum Essential Medium; FBS, fetal bovine serum; Strip, charcoal treatment.

Table 1. Comparison of the relative binding affinity of the anti-estorgens as reported in literature (selected cases)

Relative binding affinity (E_2 = 100)				Incubation			
T	dMeT	OHT	Clo	Temp. (°C)	Time	Cytosol from	Reference
6	4	280	—	30	30 min	MCF 7	12
1	1	400	—	0	24 hr	MCF 7	13
—	—	—	2	4	16 hr	MCF 7	10
2	—	167	—	4	16 hr	MCF 7	14
0.3	0.2	34	—	4	24 hr	Pooled breast carcinomata	15
—	—	—	0.71	4	2 hr	Rat uterus	11
—	—	—	5.3	25	2 hr	Rat uterus	11
38	21	110	—	0	2 hr	Rat uterus	6
2	1	188	—	25	2 hr	Rat uterus	6
2–6	—	278–300	8	30	30 min	Rat uterus	16
5.6	—	—	4.4	4	18 hr	Chicken oviduct	17

inhibitory effects of the anti-estrogens on MCF 7 cells correlate well with their affinity for the ER [13]. Murphy *et al.* have also found that the growth inhibitory potency of Clo and its analogs on MCF 7 cells is associated with their binding affinity to the ER [10]. The aim of our investigation was to repeat these studies with other hormone sensitive breast cancer cells, since we have found a divergence in Hs578T cells between the affinity for the ER and growth inhibitory action of dMeT [15]. This anti-estrogen binds very weakly to the ER (Table 1) but showed the same effects on proliferation as OHT. In addition to growth stimulation, estradiol induces the secretion of a 46 kD protein in cultured breast cancer cells [18, 19]. OHT is able to inhibit estradiol-induced production of this protein; however, nearly a 1000-fold excess is required in comparison to estradiol, which is difficult to explain in the light of the high binding affinity of OHT. We have tried to determine if this enormous excess is also necessary to inhibit the estradiol-induced growth in cultured breast cancer cells.

MATERIAL AND METHODS

Materials

T (Imperial Chemical Industries, Cheshire, U.K. ICI 46,474, Nolvadex_), OHT (ICI 79,280), and dMeT (ICI 55,548) were a gift from Dr. John Patterson, ICI (Macclesfield, England). Clo was obtained from Arcana (Austria). E_2 was supplied by Serva (Heidelberg, F.R.G.). For all experiments 10^{-3} M ethanolic solutions were prepared and tested monthly by thin layer chromatography on silica-gel plates with fluorescent indicator (Machery-Nagel, Germany) developed in benzene:triethylamine (9:1 v/v). The compounds were considered pure if a single band was observed. Three human breast cancer cell lines were used in this study. BT 20, which originated from an infiltrating ductal carcinoma [20], is an ER negative cell line [21]. ZR 75.1, which originated from ascites fluid [22], is well characterized as an estrogen sensitive cell line and contains significant amounts of ER [22, 23]. This cell line was generously supplied by Dr. R. J. B. King, Imperial Cancer Research Fund, London. 734 B was established from a pleural effusion and is also an ER positive cell line [24]. The cells were cultured in Minimum Essential Medium (MEM) (Eurobio, Paris, France), supplemented with 10% fetal bovine serum (FBS) (Eurobio), glutamine (2 mM) (Eurobio) and gentamicin (50 μg/ml) (Serva). For the stock culture, cells were grown as a monolayer in T 75 plastic flasks (Falcon Plastics Company, Oxnard, CA) under a humid atmosphere and 5% CO_2 in air at 37°. Cells were harvested with trypsin (0.05%)–EDTA (0.02%) in saline.

Estrogens in the FBS were removed by stripping with charcoal (50 mg charcoal/ml FBS for 3 hr at 20°). This procedure removed more than 99% of E_2 as shown by adding tritiated E_2 as a tracer, and by radioimmunoassay against E_2. The charcoal was removed by centrifugation and the FBS-Strip was sterilized by filtration.

Methods

Growth effects. Cells from stock flasks were harvested and then seeded in Nunc 24 well tissue culture plates (Nunc 146485, Roskilde, Dk) as 1 ml suspensions in MEM 10% FBS or 10% FBS-Strip. The cells were allowed to attach for 24 hr, and then the medium was changed to the appropriate concentration of anti-estrogens and/or E_2. The culture medium was changed every 2–3 days. To construct growth curves, the cell number was determined using an electronic particle counter (Coulter Electronics Ltd., Dunstable, U.K.) at the beginning of each experiment (day 0, 12 wells) and at suitable successive time intervals (4 wells per group per day). Measurement was not disturbed by clusters. Less than 3% doublets or triplets were observed. The recovery of the cells after preparing the single cell suspension was always higher than 98%. The values obtained by electronic particle counter and by haemocytometer were not statistically distinguishable and correlated highly significantly. Precision of the automatized procedure, however, was markedly better. To demonstrate dose dependence of the anti-estrogen effect, 4 concentrations (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) with 4 wells per concentration were treated as described above and counted after 10–14 days of treatment. In a similar approach the anti-estrogenic activity was evaluated by culturing the cells in MEM 10% FBS-Strip containing defined concentrations of E_2 and increasing amounts of OHT or T. Control groups were cultured either in unsupplemented medium or in medium containing E_2 (10^{-9} or 10^{-10} M). The difference in cell number after 10–14 days of culture was defined as E_2 induced growth.

Statistical evaluation. Most of the data were analyzed using nonparametric tests. Growth curves were evaluated by the two-way analysis of variance by ranks according to Wilcoxon and Wilcox or by the Wilcoxon matched pairs signed rank test. Multiple comparisons were performed by the Kruskal–Wallis one way analysis of variance by ranks or by the Link and Wallace test [25].

RESULTS

The proliferation of BT 20, the ER negative cell line, was neither stimulated by E_2 nor inhibited by any of the anti-estrogens tested (Table 2). 734 B as well as ZR 75.1, both ER positive cell lines, showed weak growth stimulation in MEM 10%) FBS by E_2

Table 2. Growth effects of E_2, T, OHT, dMeT, and Clo on BT 20 cells cultured in MEM 10% FBS

Treatment	Number of cells × 10^{-5} median (range) N = 6
Control	2.12 (2–2.28)
E_2 (10^{-9} M)	2.26 (1.96–2.34)
T (10^{-6} M)	2.14 (2.06–2.31)
OHT (10^{-6} M)	2.08 (1.96–2.4)
dMeT (10^{-6} M)	2.30 (1.92–2.48)
Clo (10^{-6} M)	2.18 (1.87–2.42)

3×10^4 cells were seeded and harvested after 14 days of treatment.

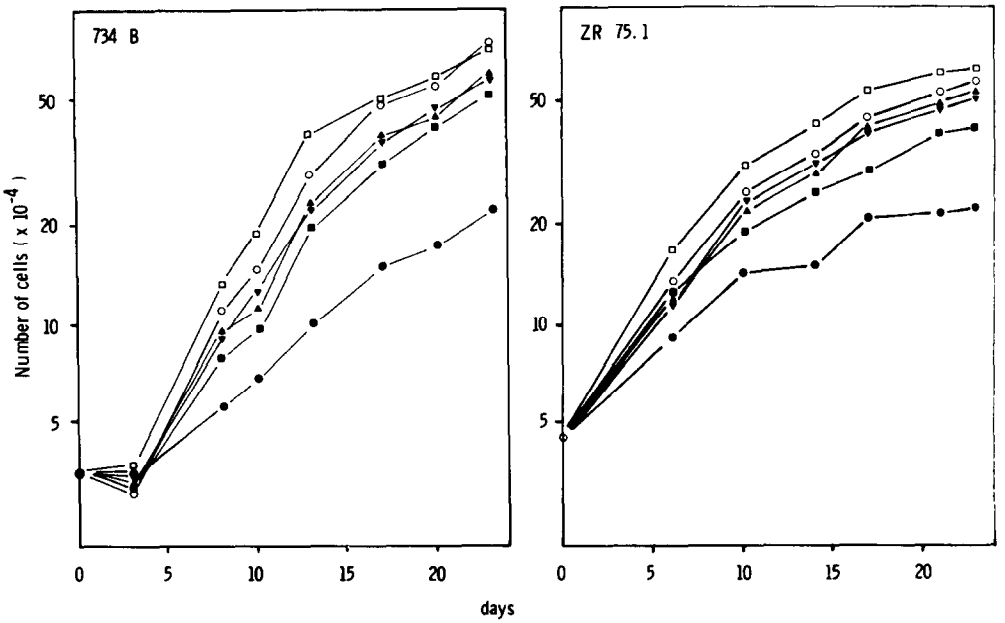

Fig. 1. Growth curves of 734 B and ZR 75.1 cells cultured in MEM 10% FBS. The cells were incubated with the vehicle alone as control (○), E_2 (10^{-9} M; □), T (10^{-7} M; ▲), OHT (10^{-7} M; ●), dMeT (10^{-7} M; ▼), and Clo (10^{-7} M; ■). The results are plotted as the mean of 4 wells counted. The variation coefficient was always lower than 10% and is not shown in this figure to maintain clarity.

(10^{-9} M) prior to the plateau phase of the growth curve (Fig. 1). T and dMeT both showed a similar and significant ($P < 0.01$) growth inhibitory action vs the control group.

Clo is statistically distinguishable from the control group as well as from T and dMeT ($P < 0.01$ and $P < 0.05$ respectively). These effects were similar for both cell lines tested. The action of OHT was much more pronounced and a strong growth inhibition was seen for both cell lines. In contrast, when MEM supplemented with 10% stripped FBS was used, E_2 showed a highly significant stimulation of growth in both ER positive cell lines (Fig. 2). The population doubling time in the E_2 treated cells in MEM 10% FBS-Strip and MEM 10% FBS are 102 and 44 hr respectively for 734 B, and 106 and 68 hr respectively for ZR 75.1. Among the anti-estrogens tested only OHT showed a significant ($P < 0.05$) but weak growth inhibitory action with similar effects on each cell line. Using untreated MEM 10% FBS all anti-estrogens tested over a concentration range from 10^{-9} to 10^{-6} M showed a significant growth inhibitory action, but the agents T, dMeT, and Clo were not very potent (Fig. 3). OHT, however, markedly reduced the proliferation in an obviously dose-related manner. To determine the excess of anti-estrogen needed to inhibit the estrogen-induced growth the cell lines 734 B and ZR 75.1 were incubated with 10^{-9} M E_2 and increasing amounts of OHT in medium with charcoal treated FBS (Fig. 4). For each cell line 10^5 cells/well were seeded. After 10 days the 734 B cells in the control group (without E_2 and without OHT) had nearly doubled (1.8×10^5 cells/well) whereas ZR 75.1 cells showed

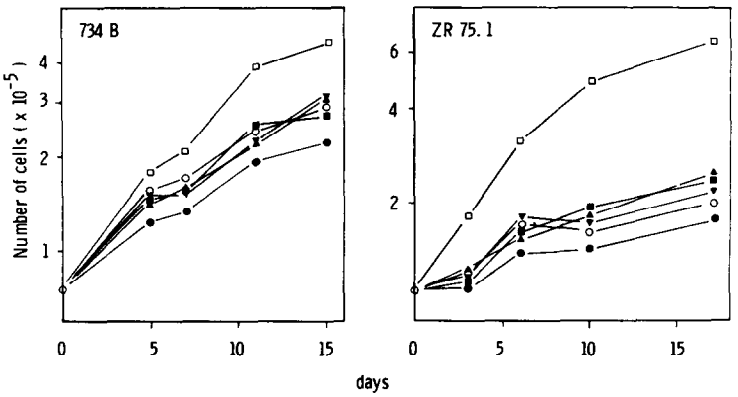

Fig. 2. Growth curves of 734 B and ZR 75.1 cells cultured in MEM 10% FBS Strip. All other procedures were performed in the same way as described in Fig. 1.

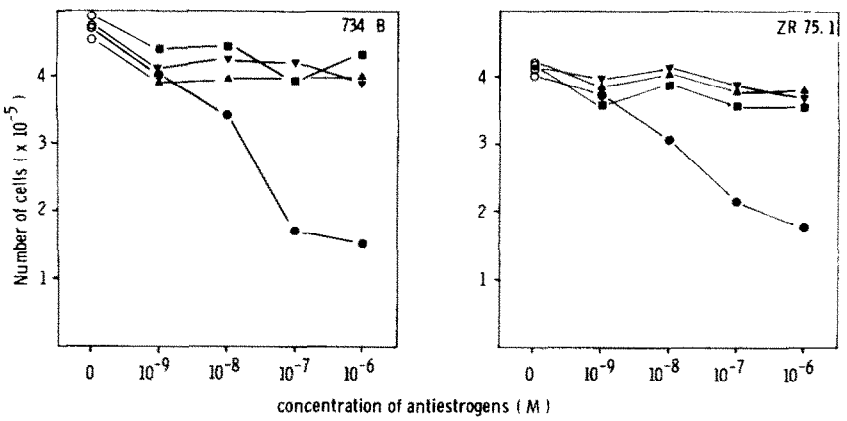

Fig. 3. Dose dependence of the anti-estrogenic action on 734 B and ZR 75.1 cells cultured in MEM 10% FBS. 3×10^4 cells were seeded and then treated with the vehicle alone (○) or with T (▲), OHT (●), dMeT (▼), and Clo (■) in the indicated concentrations for 14 days and thereafter harvested and enumerated. Results are expressed as mean of 4 wells counted.

little increase (1.04×10^5 cells/well). E_2 stimulated the proliferation of ZR 75.1 cells (3.9×10^5 cells/well) more than that of 734 B cells (2.5×10^5 cells/well). OHT inhibited the E_2 stimulated growth of both 734 B and ZR 75.1 cells, but even a 1000-fold molar excess of OHT was not sufficient to reverse completely the E_2 effect. To compare the efficiency of T and OHT in inhibiting E_2 induced proliferation ZR 75.1 cells were seeded at a density of 1.35×10^5 cells. MEM 10% FBS Strip was supplemented with 10^{-10} M E_2 and increasing concentrations of either T or OHT (Fig. 5). Again, even a 10,000-fold excess of OHT was unable to inhibit totally E_2 stimulated proliferation. The ratio of the OHT and T concentrations which led to a 50% growth inhibition was about 100:1.

DISCUSSION

Our data in general support the prevailing hypothesis that anti-estrogens exert their growth inhibitory effect via the estrogen receptor (ER). We observed an effect by E_2 and the anti-estrogens only on the ER positive breast cancer cell lines 734 B and ZR 75.1, and not on the ER negative line BT 20. In accordance with Coezy [13] and Murphy [10] we found that for the anti-estrogens tested the reported binding affinities to the ER agree with the observed

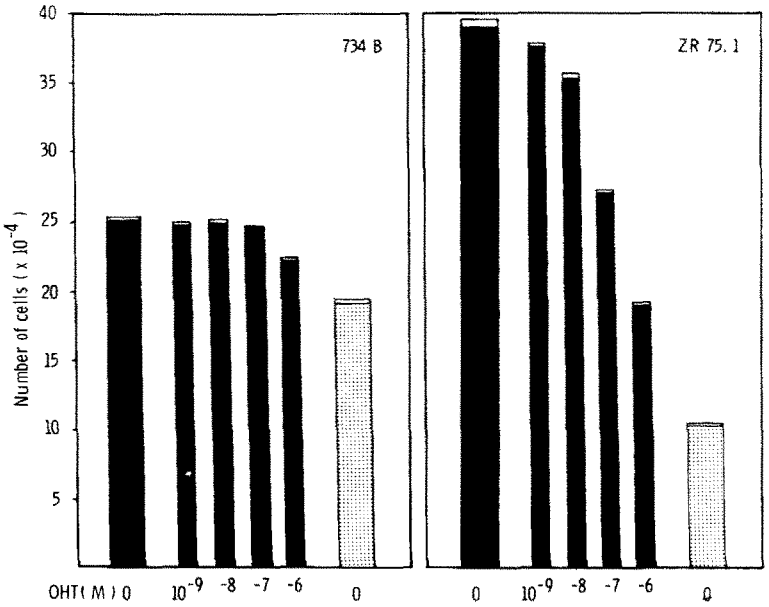

Fig. 4. Anti-estrogenic activity of OHT on the E_2-induced growth. The 734 B as well as ZR 75.1 cells were incubated in MEM 10% FBS Strip in the absence (dotted bar) or presence of 10^{-9} M E_2 (black bars). The black bars represent the number of cells grown in MEM 10% FBS Strip supplemented with E_2 (10^{-9} M) and increasing concentrations of OHT (10^{-9}–10^{-6} M). Results are presented as mean of 4 wells enumerated + S.E.M.

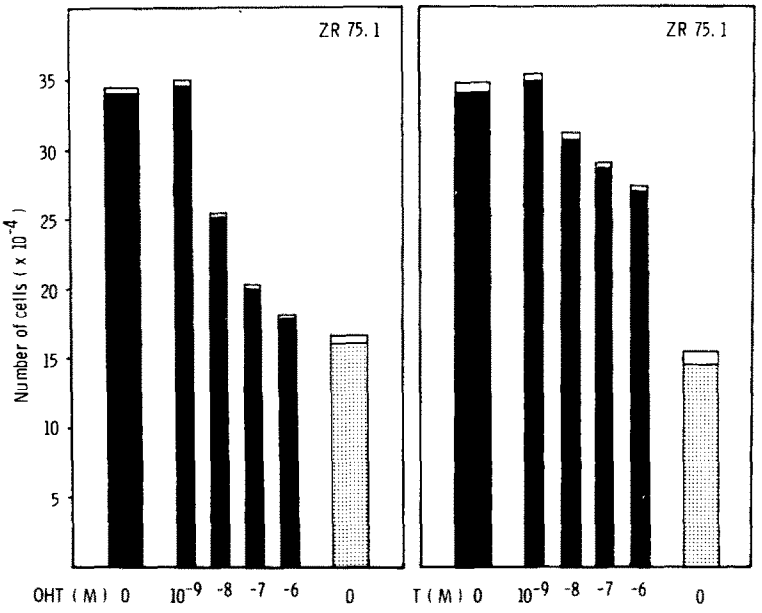

Fig. 5. Anti-estrogenic efficacy of OHT (left) and T (right) in competing the E_2 induced growth of ZR 75.1 cells. The culture medium of this experiment consisted of MEM 10% FBS Strip which was supplemented with 10^{-10} M E_2 and increasing amounts of anti-estrogen (black bars). The group represented by the dotted bar was cultured in MEM 10% FBS-Strip supplemented with the vehicle alone. Results are expressed as mean of 4 wells counted + S.E.M.

growth inhibitory action on cultured breast cancer cells. In 734 B as well as in ZR 75.1 cells the anti-estrogen activity showed the identical rank: OHT ⩾ Clo > T ≅ dMeT. The difference between T or dMeT and Clo is in our study statistically significant but probably not relevant, whereas the effect of OHT was much more pronounced. The anti-estrogenic potency of OHT expressed as the concentration needed to inhibit 50% of E_2-induced growth was in the same order of magnitude for both cell lines. Since MCF 7, 734 B and ZR 75.1 agree concerning the above mentioned findings we conclude that there is a common and specific mechanism of action and speculate that these results are valid for all estrogen dependent mammary carcinomata. Our earlier findings in the breast carcinosarcoma cell line Hs578T differed considerably from those in neoplastic cells of epithelial origin. This may be due to peculiar metabolic pathways in this very rare type of mammary carcinoma [26]. Several aspects of our data indicate that while the ER may play a role in anti-estrogen action, the mechanism may not be one of straightforward competition with E_2 for the ER. Like Westley *et al.* [18], who found a high molar excess of OHT was required to inhibit E_2-stimulated production of a 46 kD protein, we found a high excess of anti-estrogens was needed to achieve marked inhibition of E_2 stimulated growth. For example, OHT has about the same binding affinity as E_2 for the ER, yet even a 1000–10,000-fold molar excess compared to E_2 could not nullify the E_2 induced proliferation of the two breast cancer cell lines tested. Among the possible explanations for this, high affinity anti-estrogen binding sites are improbable for the following reasons: (1) the kD of the anti-estrogen protein complex is about 10^{-9} M [14], thus 90% binding and nearly maximal effect should occur at 10^{-8} M of the anti-estrogenic compound. (2) According to the binding specificity [14], T should have at least equal anti-estrogenic potency as OHT. Preferential adsorption of anti-estrogens to the plastic wall of the microwell test plates, another possible explanation, could be excluded by measuring the uptake of tritiated T and E_2 by the plastic wall (data not shown). Metabolic inactivation of a great proportion of T and OHT could not be observed by thin layer chromatography analysis of an ether extract of the culture medium. However further studies are needed to define the fate of OHT in cell culture in order to explain the above mentioned discrepancies between binding affinity and active concentrations of anti-estrogens. Charcoal treatment of fetal bovine serum depletes steroids as well as other growth factors. Indeed, proliferation in E_2-supplemented FBS-Strip is slower than in untreated FBS. 734 B cells grew better in MEM 10% FBS-Strip than did ZR 75.1 cells. On the other hand, estrogen stimulus was more pronounced for ZR 75.1 cells and we therefore conclude that this cell line is more E_2 sensitive. The pure anti-estrogenic nature of OHT was displayed by the fact that it only inhibited E_2 induced growth and barely influenced proliferation in estrogen deprived media. The weak growth inhibition of OHT on cells cultured in MEM 10% FBS-Strip (Fig. 2) may be explained by residual estrogens or estrogen sulfates after charcoal treatment [27]. These results do not fully agree with those of Sutherland *et al.* [28] who have published that not all the growth-inhibitory and cell cycle effects of anti-estrogens could be completely reversed by E_2. This

was interpreted as evidence for effects of anti-estrogens on cell proliferation in addition to those influenced by estrogen and mediated by ER [29]. However, the experiments which led to this conclusion were performed at anti-estrogenic concentrations $> 1\ \mu M$. These concentrations were found by Taylor *et al.* [20] to be effective on every cell line tested, independent of its origin (e.g. melanoma) and ER content. They suggest that these high levels of the drug are cytotoxic by a non-specific mechanism [30]. Since T and dMeT have a similar antiestrogenic activity the metabolic desmethylation of T is not likely to cause a loss of effectivity of the endocrine treatment of breast cancer. The role of OHT in human breast cancer therapy should be further elucidated as contradictory results about OHT plasma levels in T treated patients have been published [6, 7, 9]. *In vitro* studies indicate that low concentrations of OHT resulting from metabolism of T might contribute more to the therapeutic effects than do dMeT and T. Considering the pure anti-estrogenic nature of T and metabolite we suggest that patients with significant endogenous estrogen levels should benefit preferentially from anti-estrogens provided the tumour is purely hormone sensitive. This agrees with the findings of Margreiter *et al.* [3] that only premenopausal breast cancer patients benefit from an adjuvant T therapy.

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