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The Effects of Retinoid Treatment and Antiestrogens on the Growth of T47D Human Breast Cancer Cells*

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Abstract—*The ability of all-*trans*-retinoic acid,* 13*-*cis*-retinoic acid, the free acid of etretinate (RO* 10*-*1670*), the 'arotinoid' RO* 13*-*6298 *and its free acid RO* 13*-*7410 *to affect the growth of T47D human breast cancer cells* in vitro *was investigated. The growth of T47D cells was inhibited by all of the retinoids tested, with the arotinoids being up to* 100 *times more effective than all-*trans*-retinoic acid. The presence of cellular retinoic acid binding protein (cRABP) was indicated by the cellular uptake of* [^{3}H]*all-*trans*-retinoic acid. Maximum binding was* 460 fmol/μg *DNA. All of the retinoids with a polar terminal free carboxyl group readily competed for the binding sites, but none of the retinoids competed for the estrogen or progesterone receptor. Co-treatment of the T47D cells with* 0.1 μM *all-*trans*-retinoic acid and either tamoxifen* (1 μM) *or hydroxytamoxifen* (10 nM *or* 0.1 μM) *produced an additive effect on growth inhibition. No such additive effect was observed when T47D cells were co-treated with arotinoids and antiestrogens. The results showed that the T47D cells can serve as a useful model* in vitro *to test the effects of the synthetic retinoids and antiestrogens on steroid receptor-positive human breast cancer.*

INTRODUCTION

RECENT results of controlled clinical trials on operable breast cancer indicated that hormonal therapy with antiestrogens, with or without adjuvant chemotherapy, prolonged the time to recurrence and overall survival [2, 3]. This benefit was most pronounced in women with estrogen receptor-rich [4] and/or progesterone receptor-rich [5] breast tumors. In addition, several animal models of endocrine-responsive mammary carcinoma are also available to study hormone manipulation, alone or in association with other therapeutic agents. Investigations using 7,12-dimethylbenz(a)anthracene (DMBA) or *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary carcinomas have demonstrated that ovariectomy significantly reduced tumor incidence and multiplicity. In other studies [6, 7] the combination of retinoid administration and ovariectomy appeared to act in a synergistic fashion to enhance the reduction of primary malignancies and preneoplastic lesions. This apparent synergistic inhibition of tumor growth is also observed when an antiestrogen (tamoxifen) and a prolatin suppressant (2-bromo-α-ergocryptine) are substituted for endocrine gland ablation in the DMBA-induced rat mammary carcinoma model [8].

While these investigations strongly suggested that the combination of hormone therapy and retinoid administration might be effective treatment for endocrine-responsive breast carcinoma, only limited studies have been performed on any human tumors using retinoids with acceptable therapeutic indices, i.e. 13-*cis*-retinoic acid and etretinate (RO 10–9359) [9, 10]. These two synthetic retinoids have achieved some limited therapeutic success in clinical trials [11]; however, a new series of synthetic aromatic retinoids with increased biological activity have been developed. These retinoidal benzoic acid derivatives [12] (frequently referred to as 'arotinoids' [13]) have recently demonstrated encouraging results in both *in vitro* systems [14] and in limited clinical studies [15]. These new compounds may be the most clinically useful synthetic retinoids for administration alone or in combination with hormonal therapy for some cancers. The study described in this paper was undertaken to assess the combined influence of antiestrogens and several synthetic retinoids on a hormone-responsive human breast carcinoma cell line *in vitro*.

MATERIALS AND METHODS

Retinoids

The retinoids used in this study were provided as a gift from Drs W. Bollag and J. Würsch of F.

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Hoffman–LaRoche & Co., Basel, Switzerland. The formulas are shown in Fig. 1. All-*trans*-retinoic acid (RA), 13-*cis*-retinoic acid and RO 13–6298 (ethyl-*p*-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoate) are soluble in ethanol; and the free acid of etretinate or RO 10–1670 (all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid) and the free acid of RO 13–6298 (RO 13–7410) are soluble in dimethyl sulfoxide. Stock solutions (10 mM) were stored in the dark at −20°C.

Cells

T47D is a human breast cancer cell line derived from the pleural effusion of a 54-yr-old Caucasian woman [16]. This cell line has been characterized as having an estrogen receptor which is in a permanently activated state in the nucleus, inducing high levels of progesterone receptor [17]. Although little or no cytoplasmic estrogen receptor is found, the cells are still responsive to antiestrogens [18, 19]. The cell line was obtained from the Human Cell Culture Bank, National Cancer Institute, Bethesda, MD, and were routinely cultured in Eagle's minimal essential medium supplemented with L-glutamine (2mM), non-essential amino acids, gentamicin (50 μg/ml), insulin (200 ng/ml; Sigma Chemical, Co., St. Louis, MO) and 10% (v/v) fetal calf serum and incubated in a humidified atmosphere with 5% CO_2 at 37°C. Tissue culture media, additives, and serum were purchased from Seromed GmbH, Munich, F.R.G.

Growth assays

Dose–response curves of retinoid treatment were determined using the method of Taylor *et al.* [18]. Triplicate determinations for each treatment were performed in 24-well dishes obtained from Falcon (Becton Dickinson and Co., Oxnard, CA), Lux (Lux Scientific Corp., Newburg Park, CA) or Costar (Cambridge, MA). Each well was seeded with 30,000 cells and incubated overnight in the media and incubation conditions as described for routine cell culture. The following day, the retinoid stock solution was serially diluted in supplemented Eagle's minimal essential medium with 2% charcoal-stripped serum to yield final concentrations of 10μM to 1 nM. Control cultures received 0.1% solvent (v/v) in media. All procedures involving retinoids were carried out in subdued light. For dose–response curves, the cells were exposed to retinoids for a total period of 7 days, with changes of freshly diluted retinoid in media with 2% charcoal-stripped serum every 2 days. For the time course study, cells were treated with fresh 0.1 μm retinoid for 2, 5 or 7 days. Co-treatment of cells with tamoxifen, 4-hydroxytamoxifen (ICI Ltd, Macclesfield, U.K.) or 17-β-estradiol (E_2); Sigma Chemical Co., St. Louis, MO) and the retinoids was performed by using dilutions of the drugs as described by Taylor *et al.* [18]. An appropriate aliquot was added to each well to yield the desired final concentration in combination with 10 nM retinoid. After the incubation period each experiment was terminated by removal of the medium, and the total cellular DNA was measured directly in the multi-well plates [20].

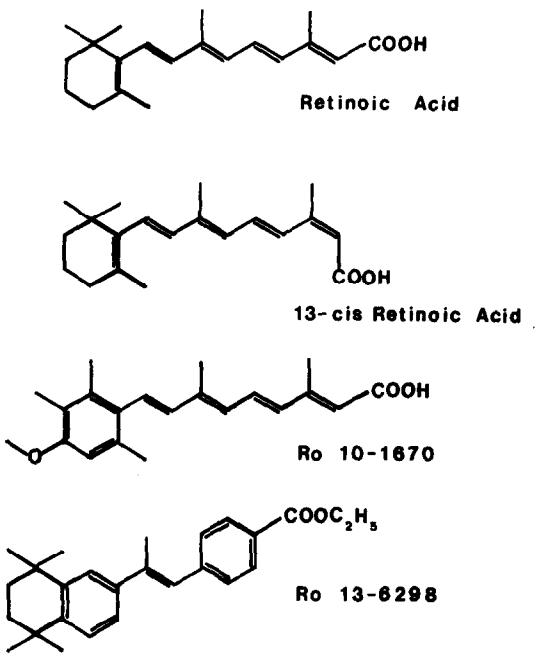

Fig. 1. Chemical structure of the retinoids

Whole cell uptake of radiolabelled retinoic acid

[^{3}H] All-*trans*-retinoic acid (32 Ci/mmol) was provided by Dr S. Würsch of F. Hoffman-LaRoche & Co, Basel, Switzerland. Whole cell binding of all-*trans*-retinoic acid was demonstrated by the method of Takenawa *et al.* [21]. Triplicate cultures adjusted to 5 × 10^5 T47D cells were suspended in phosphate-buffered saline and incubated for 1 hr at 37°C with various concentrations of [^{3}H] all-*trans*-retinoic acid plus or minus a 100-fold molar excess of unlabeled all-*trans*-retinoic acid. After incubation, the cells were collected by centrifugation and the supernatant was removed. The pellet was washed twice with cold phosphate-buffered saline. Extraction with ethanol of the [^{3}H]-all-*trans*-retinoic acid taken up by the cells gave the total uptake of all-*trans*-retinoic acid. The ethanol solution was transferred to a scintillation vial (Bio Vials, Beckman, Inc.). Three milliliters of Beckman HPb scintillator was added to each vial and radioactivity was counted using a Beckman

LS1800 scintillation counter. Specific binding was assessed by subtraction of the dpm counted in the presence of cold ligand from those counted in its absence. Scatchard analysis [22] of the data obtained in this manner gave the total number of all-*trans*-retinoic acid binding sites.

Competitive binding experiments

Cells were grown in four similarly treated multiwell plates for 8 days as described for growth assays. Competitive binding studies were determined [20] between 100 nM [^{3}H]-all-*trans*-retinoic acid and increasing concentrations of all-*trans*-retinoic acid, 13-*cis*-retinoic acid, RO 10-1670, RO 13-6289 and RO 13-7410 (1 nM to 10 μM). Medium was removed from the cells and replaced with medium containing 0.1% BSA plus [^{3}H] all-*trans*-retinoic acid and the appropriate concentration of unlabeled competitor. After incubation at 37°C for 2 hr the cells were washed, extracted with ethanol and ^{3}H counted as described above. 17-β-[2,4,6,7-^{3}H]estradiol (104 Ci/mmol) and 17,21-dimethyl-19-nor-4,-9-pregnadiene,3,20dione[6,7-^{3}H] (87 Ci/mmol, R5020) were purchased from New England Nuclear (Boston, MA). The relative binding affinities of [^{3}H]E_2 (2nM) for estrogen receptor and [^{3}H]R5020 for progesterone receptor in intact T47D cells were compared to the binding affinities of increasing concentrations of all-*trans*-retinoic acid, RO 13-6298 or RO 13-7410 (1 nM to 10μM) for the same binding sites.

RESULTS

Effects of the retinoids on the growth of T47D cells

Figure 2 shows the antiproliferative effects of all-*trans*-retinoic acid, 13-*cis*-retinoic acid, RO 10-1670, RO 13-6298 and RO 13-7410 on T47D cells. The effect on growth was proportional to the dose of retinoid tested. The T47D cells were not affected by all retinoids to the same degree. 13-*cis*-retinoic acid and RO 10-1670 were not as potent as all-*trans*-retinoic acid, but RO 13-6298 and RO 13-7410 had a marked antiproliferative effect on the cells, even at the lowest concentration (1 nM). RO 13-7410 appeared to exhibit cytotoxic effects at the highest concentration (10 μM) since only non-viable cells were found in any treated culture at the experiment's end. Figure 3 demonstrates that the growth inhibition of 1 μM retinoic acid, RO 13-6298 or RO 13-7410 occurred early and to similar degrees. Cell proliferation differed little between days 1 and 8.

Uptake of [^{3}H] *all-*trans*-retinoic acid by T47D cells and retinoid competition for the binding sites*

The specific binding and Scatchard analysis are

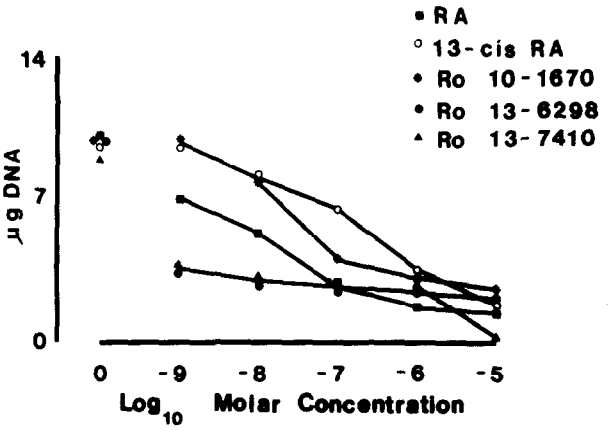

Fig. 2. Effect of retinoids on the growth of T47D cells. Cells were grown as described in Materials and Methods and treated for 7 days *with a retinoid, and cell growth was assessed by measurement of total cell DNA. Points are the means of triplicate determinations, the standard error of the mean (S.E.M.) of each point being* 5% *or less* (S.E.M. = S.D./n).

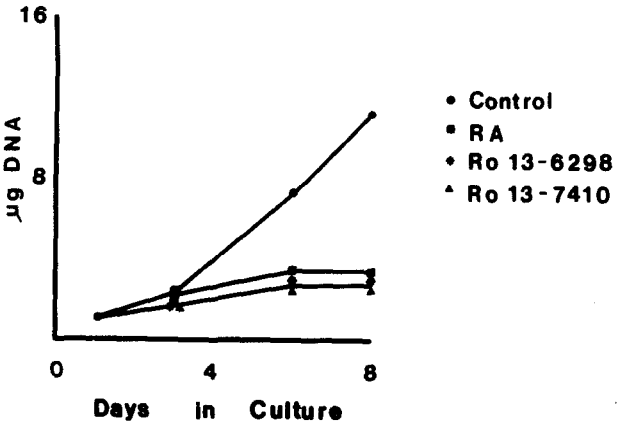

Fig. 3. Time course of effect of retinoids (0.1 μM). *T47D cells were treated for* 2, 5 *or* 7 days *with* 0.1 μM *retinoid. Other conditions and statistics were as described in the legend to Fig.* 2.

shown in Fig. 4. Specific uptake of [^{3}H]all-*trans*-retinoic acid showed a dissociation constant (K_d) of 50 nM with a maximal binding of 460 fmol/μg DNA. The synthetic retinoids with a free acid, RO 10-1670 and RO 13-7410 were effective competitors for the all-*trans*-retinoic acid binding sites (Fig. 5). The ethyl ester of RO 13-7410 (RO 13-6298) and 13-*cis*-retinoic acid were less effective competitors. None of the retinoids tested competed for either the estrogen (Fig. 6A) or progesterone receptor (Fig. 6B).

Co-treatment of T47D cells with retinoids, estrogen or antiestrogen

The growth of T47D cells was readily inhibited by tamoxifen and hydroxytamoxifen in a dose-responsive manner (Fig. 7). Estradiol at low concentrations was also shown to have no significant effect upon the growth of T47D cells. However, 10

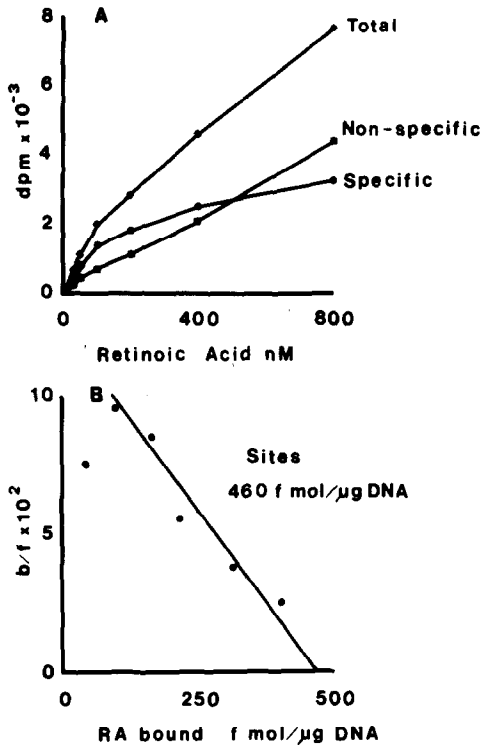

Fig. 4. (A) Binding of [^{3}H] *all–*trans*–retinoic acid by intact T47D cells. Whole cells were incubated for* 1 hr *at* 37°C *with various concentrations of* [^{3}H] *all–*trans*–retinoic acid plus or minus a* 100*-fold molar excess of unlabelled all–*trans*–retinoic acid to assess non-specific binding. Cells were treated as described in Materials and Methods. Specific binding: total binding minus non-specific binding. (B) Scatchard analysis of specific binding expressed as* fmol *all–*trans*–retinoic acid/µg DNA. The correlation coefficient (r) for the regression analysis of five data points is* 0.97.

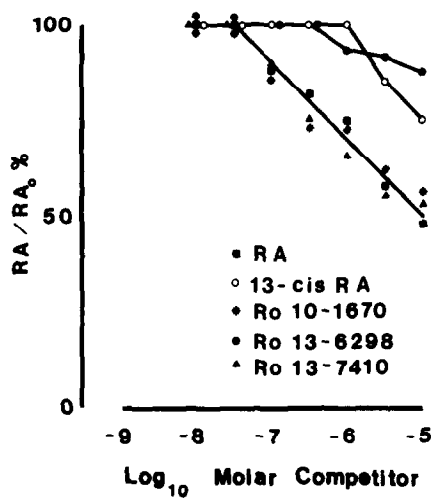

Fig. 5. Competition of retinoids for the binding of [^{3}H]–all–trans*–retinoic acid in whole T47D cells. Cells were grown as described for Fig. 2 and in Materials and Methods. Results are expressed as percentage maximal* [^{3}H]*all–*trans*–retinoic acid bound.*

nM all–*trans*–retinoic acid has been shown to be as effective as hydroxytamoxifen in inhibiting the growth of T47D cells; the combination of these two agents results in an additional near-two-fold growth inhibitory effect. At 10 nM concentrations the two synthetic retinoids, RO 13–6298 and RO 13–7410 are as effective as the combination of hydroxytamoxifen and retinoic acid, but no additive growth inhibitory effect is demonstrated between these retinoids and the antiestrogens.

DISCUSSION

Evidence has been accumulating which indicates that the retinoids have a profound effect upon the growth of human steroid hormone receptor-positive and receptor-negative breast cancer cell lines [23, 24]. The results of this study (Fig. 2) agree with the previous work using other breast epithelial cell lines, which indicate that the naturally occurring retinoid, all–*trans*–retinoic acid, exhibits an antiproliferative effect at concentrations of 0.1–1 µM. The new class of synthetically prepared analogs, RO 13–6298 and RO 13–7410, are capable of achieving a similar degree of antiproliferative effect, but at 10- to 100-fold lower concentrations. This effectiveness at low concentrations suggests that they may be more useful therapeutic agents than retinoic acid, 13–*cis*–retinoic acid or etretinate.

While the specific mechanism for the biological actions of retinoic acid or the synthetic derivatives has not been elucidated, several investigations strongly implicate the binding of retinoids with a free carboxyl group to cellular retinoic acid-binding protein (cRABP) as an important step in the mediation of their biological activity. Investigations using all–*trans*–retinoic acid, 13–*cis*–retinoic acid and RO 10–1670 have demonstrated an association of retinoid binding to cRABP with the differentiation of embryonal carcinoma cells [25] and other biological activities *in vivo* and *in vitro* [26]. RO 13–7410, which binds to cRABP, has recently been shown in low concentrations to stimulate differentation in Nulli-SCC1 embryonal carcinoma cells [27] and to reverse the keratinization in hamster trachea organ cultures [28]. Previous studies using human mammary cell lines show a good correlation between the growth inhibition of these cells and either the specific binding of [^{3}H]all–*trans*–retinoic acid or the presence of cRABP [29, 30], although this correlation may not be true of all breast cancer cells or cells derived from other malignancies [31, 32]. All–*trans*–retinoic acid readily binds to T47D cells (Fig. 4), and all retinoids tested with a polar terminal free carboxyl group readily compete for the all–*trans*–retinoic acid binding sites. RO 13–6298 is not as effective a competitor (Fig. 5), but is biologically as active as RO 13–7410. If the assumption is made that cRABP is involved in the function of the retinoids, this fact indirectly suggests that the ethyl ester is metabolized by the T47D cells, converting the ethyl ester to the free acid form. While the precise

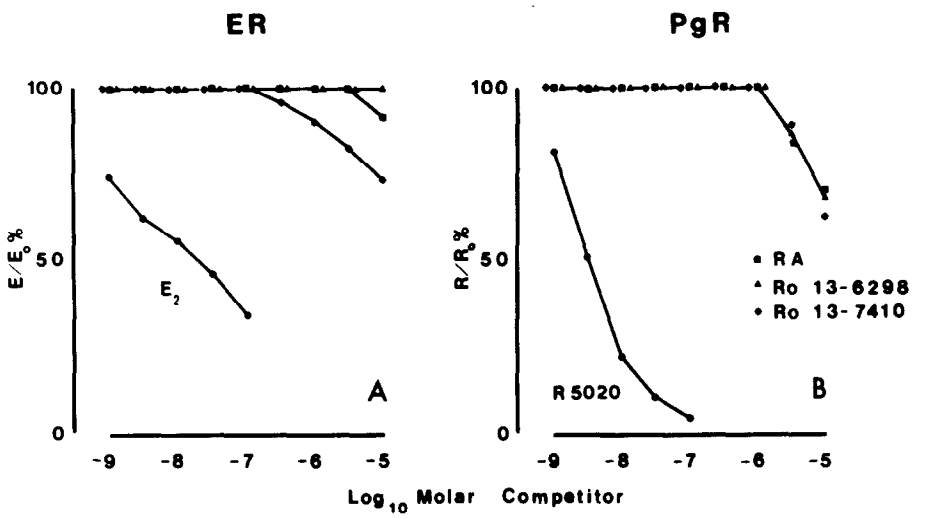

Fig. 6. Competition of the retinoid for estrogen and progesterone receptors in whole T47D cells. Cells were grown as described for Fig. 2. Conditions were described in Materials and Methods. Results are expressed as per cent maximal [^{3}H] E_2 (E/E_0%) *or,* [^{3}H]*R5020* (R/R_0%) *bound.*

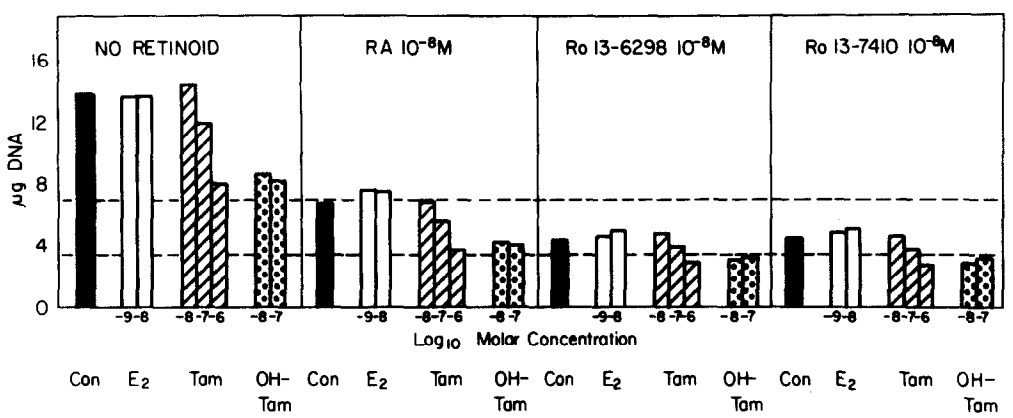

Fig. 7. Effect of retinoic acid or arotinoids on the growth of T47D cells co-treated with various concentrations of control 0.1% *solvent* (CON), *estradiol* (E_2), *tamoxifen* (*TAM*) *or hydroxytamoxifen* (OH–*Tam*). *Conditions and statistics were as described in the legend to Fig.* 2. *Upper and lower dashed lines indicate* 50 *and* 25% *growth of controls respectively.*

mechanism of retinoid action is still not known, it is clear through the studies presented here that the retinoids do not act through competition for the steroid hormone receptor binding sites (Fig. 6) in a manner similar to that of the antiestrogens [18].

The following evidence indicates that the retinoids effect T47D cell proliferation directly, and are not merely cytotoxic: (a) Fig. 3 demonstrates that although the T47D cells were growth inhibited compared to the untreated controls, the population still divides and increases in number; (b) there was a lack of karyorrhexis in the attached treated cells as observed with the phase contrast microscope; (c) the concentrations of the arotinoid RO–6298 which demonstrated an antiproliferative effect on T47D cells have been used by other investigators to induce terminal differentiation in HL–60 cells [14].

Since the retinoids exhibit an antiproliferative effect on a known steroid hormone-responsive cell line, we anticipated a synergy in growth inhibition between the retinoids and the antiestrogens. The growth of T47D cells was inhibited in almost 50% of the controls by low doses of either tamoxifen or its *in vivo* metabolite 4-hydroxytamoxifen. All-*trans*-retinoic acid alone was as effective as the antiestrogen; the combination of the two resulted in an overall 75% inhibition of growth (Fig. 7). No such additive effect was observed when the cells were co-treated with RO 13–6298 or RO 13–7410 and an antiestrogen. The apparent maximum growth inhibition of T47D by non-toxic antiproliferative agents was approximately 75% of the controls. Ten nanomolar all-*trans*-retinoic acid was used for the co-treatment studies; potentially, a concentration of 0.1 µM retinoic acid would not have demonstrated the observed additive effect. This suggests that the concentration and type of retinoid utilized for cancer therapy or prevention studies should be taken into consideration when growth inhibitory effects of both retinoids and antiestrogens are to be tested.

Our findings indicate that the T47D cells offer an *in vitro* model to study the effects of retinoids and antiestrogens on steroid receptor-positive human breast cancer. Cellular retinoic acid binding protein has been detected in many, but not all, breast lesions of varied histopathological types [33–35] and steroid hormone receptor content [36]. While the binding of all-*trans*-retinoic acid to extracts of human mammary tumors does not suggest that cRABP functions by a mechanism similar to the steroid hormone receptors, the association of cRABP in some breast cancers together with the results presented here suggest that the new generation of synthetic retinoids alone, or in conjunction with antiestrogens, merit further investigations into the treatment of chemoprevention of human breast cancer.

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