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Combined effects of estradiol, leuprorelin, tamoxifen and medroxyprogesterone acetate on cell growth and steroid hormone receptors in breast cancer cells

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Abstract. Both tamoxifen and medroxyprogesterone acetate have a direct antitumor effect and are widely used in breast cancer therapy. Luteinizing-hormone-releasing hormone analogs inhibit the growth of breast cancer cells and could represent an alternative treatment for patients affected by breast cancer. Our study was carried out to investigate the effect of leuprorelin (TAP-144) alone or combined with tamoxifen or medroxyprogesterone acetate in human breast cancer cells. Ineffective when used in the absence of estrogens, TAP-144 inhibited the estrogen-stimulated growth of MCF-7, CG-5 and ZR-75-1 cells cultured in medium supplemented with charcoal-treated serum. The growth of estrogen-unresponsive MDA-MB-231 cells was not affected by TAP-144. The combination of TAP-144 with tamoxifen in CG-5 cells did not determine any enhancement of inhibition of cell growth, whereas in both CG-5 and MCF-7 cells, when 1 μM TAP-144 was associated with 0.1 μM medroxyprogesterone acetate, cell growth inhibition was increased, resulting in a subadditive effect. Progesterone receptor levels of CG-5 cells were significantly increased by TAP-144 in the presence of 17β-estradiol with respect to those present in control and 17β-estradiol-treated cells.

Key words: Breast neoplasms – Leuprorelin – Tamoxifen – Medroxyprogesterone acetate – Estrogen and progesterone receptors

Introduction

Luteinizing-hormone-releasing hormone (LHRH) agonists have been shown to be effective in the treatment of advanced prostate cancer and other endocrine-related tumors (Schally et al. 1984; Eisenberger et al. 1986; Klijn and Foekens 1987). In breast cancer these compounds have been used both in pre- and in postmenopausal patients with metastatic disease (Plowman et al. 1986; Nicholson and Walker 1989; Crighton et al. 1989). A number of reports suggest that LHRH analogs may have a direct inhibitory effect on breast cancer cell proliferation "in vitro" (Blankenstein et al. 1985; Miller et al. 1985; Foekens et al. 1986; Eidne et al. 1987; Scambia et al. 1988). Nevertheless, the mechanism by which this effect is achieved is not completely clear. Different media and treatment schedules have been used by different authors to demonstrate the antiproliferative action of LHRH analogs in estrogen-sensitive breast cancer cells (Blankenstein et al. 1985; Miller et al. 1985; Foekens et al. 1986; Scambia et al. 1988). The role of specific binding sites for LHRH agonists, which have been detected both in estrogen-sensitive and -insensitive breast cancer cell lines, still needs clarifying (Eidne et al. 1987).

In the present paper we explored the effect of TAP-144 on the growth of a series of breast cancer cell lines, testing various culture conditions for each one. The action of TAP-144 combined with the antiestrogen tamoxifen or the synthetic progestin medroxyprogesterone acetate (MPA) was also investigated.

In order to clarify the mechanisms underlying some of the effects of TAP-144 in combination with these drugs, we also studied the variations induced by this drug on steroid hormone receptors.

Materials and methods

Cells. The MCF-7 and ZR-75-1, estrogen-sensitive and estrogen-receptor-positive (ER+) (Soule et al. 1973; Engel et al. 1978), and

Abbreviations: MPA, medroxyprogesterone acetate; LHRH, luteinizing-hormone-releasing hormone; TAP-144, leuprorelin; ER, estrogen receptor; PR, progesterone receptor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CH-FCS, charcoal-treated fetal calf serum

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MDA-MB-231 (estrogen-insensitive and ER −) (Cailleau et al. 1974) cell lines were a gift from Prof. Stefano Iacobelli (Clinical Oncology, University of Chieti, Italy).

CG-5 cells, estrogen-responsive and ER +, are a variant of the MCF-7 cell line and were obtained in 1982 in the Laboratory of Molecular Endocrinology of the Catholic University of the Sacred Heart, Rome, Italy (Natoli et al. 1983).

MCF-7 and CG-5 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Irvine, UK) supplemented with 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid: Flow Laboratories), 10% fetal calf serum (FCS; Flow Laboratories) and antibiotics.

The MDA-MB-231 and ZR-75-1 cell lines were maintained in minimum essential medium Eagle (modified) (Flow Laboratories) supplemented with 10 mM HEPES buffer (Flow Laboratories), 10% FCS (Flow Laboratories) and antibiotics.

Drugs. TAP-144 {[D-Leu^6-(des-Gly^{10}-NH$_2$)]LHRH ethylamide} was kindly donated by Takeda Italia Farmaceutici S.p.A, Catania, Italy, and was dissolved in saline solution. Tamoxifen was donated by ICI-Pharma, Italy. MPA was provided by Upjohn S.p.A., Italy. 17β-Estradiol was purchased from Sigma, St. Louis, Mo., USA. All these compounds were dissolved in ethanol.

Cell proliferation experiments. Cells harvested by trypsinization were seeded into 60-mm plastic petri dishes at a density of 50 000 cells/ml in the culture medium indicated above for each cell line. In experiments performed to evaluate the effect of the analog alone or in the presence of the estrogen, the medium was replaced 24 h later with fresh medium containing 5% charcoal-treated FCS (CH-FCS), or 5% or 10% FCS and TAP-144 from 10 pM to 1 μM alone or in combination with estradiol. Estradiol was used at concentrations of 1 nM and 10 nM to stimulate the proliferation of CG-5 and MCF-7 cells respectively. In some experiments CG-5 cells were precultured in phenol-red-free medium for 12 or 22 days in the presence of 10% CH-FCS. Subsequently, the effect of 1 μM TAP-144 alone or combined with estradiol was tested in the presence of 5% CH-FCS. A 0.1 μM solution of tamoxifen or MPA was used in experiments in which the effect of TAP-144 combined with the antiestrogen or the progestin was studied. The final ethanol concentration never exceeded 0.2% in the control and treated cell medium.

The medium, supplemented with the hormones, was renewed every 48 h. Triplicate cultures were set up for each drug concentration. After 6 days of treatment, cells were collected by trypsinization and counted in a hemocytometer.

Cell viability was assessed by trypan blue dye exclusion.

Steroid hormone receptor assay. CG-5 cells were plated at an initial density of 50 000 cells/ml in 35-mm plastic petri dishes in DMEM supplemented with 10% FCS, which was renewed after 24 h with medium supplemented with 5% CH-FCS containing estradiol (1 nM) or TAP-144, at concentrations of 0.1 μM and 1 μM, alone or in combination. The medium, supplemented with the hormones, was changed every 2 days. Cell dishes were set up in triplicate and control dishes were cultured in parallel.

Steroid receptor determination was performed by a whole-cell assay after 6 days of treatment. Cells were washed three times with medium without FCS and left for 1 h in the same medium at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was then replaced with DMEM containing 5 nM [^{3}H]estradiol in the presence or absence of a 100-fold excess of diethylstilbestrol for the determination of ER and 5 nM [^{3}H]ORG-2058 in the presence or absence of a 200-fold excess of unlabelled ORG-2058 for the determination of progesterone receptor (PR)

After 1 h incubation with the ligands, cells were washed twice with Hanks' balanced salt solution at room temperature and radioactivity was then extracted with 1 ml 80% ethanol overnight.

Cell numbers were determined in a series of plates which underwent the same experimental procedure.

Radioactivity was measured by liquid scintillation counting. ER and PR contents were expressed as the number of sites/cell.

Statistical analysis. In cell growth experiments the significance of the difference between cell numbers in two groups was determined by the unpaired two-tailed Student's *t*-test.

To evaluate the effect of the combination of two drugs on cell growth we used the following definitions (Denz et al. 1985):

$SF_{A+B} > (SF_A) \times (SF_B)$	antagonistic
$SF_{A+B} > (SF_A) \times (SF_B)$ and $< SF_B$ when $SF_A > SF_B$	subadditive
$SF_{A+B} = (SF_A) \times (SF_B)$	additive
$SF_{A+B} < (SF_A) \times (SF_B)$	synergistic

where SF is the surviving fraction, and A and B indicate the agent used alone, while A + B refers to the drugs used in combination. The statistical analysis of the steroid receptor data was performed by Wilcoxon's signed-rank test.

Results

The LHRH analog TAP-144, used alone at concentrations ranging from 10 pM to 1 μM in medium supplemented with 5% CH-FCS, was ineffective on the growth of both estrogen-sensitive and estrogen-insensitive breast cancer cells (data not shown). Similarly, if the analog was tested on estrogen-sensitive cells cultured in medium supplemented with 5% or 10% unstripped serum (containing small amounts of estradiol) no variation of cell number was observed (data not shown).

When estrogen at 1–10 nM was added to the culture medium containing 5% CH-FCS, TAP-144, at the above-mentioned concentrations, significantly reduced the estradiol-induced stimulation of the growth of both MCF-7 and CG-5 cells (Fig. 1). This effect was more evident in CG-5 cells.

ZR-75-1, estrogen-sensitive and ER + cells, responded in a similar way to treatment with the analog, both in the presence and in the absence of estradiol (data not shown).

The CG-5 cell line was also cultured in phenol-red-free medium, in the presence of 10% CH-FCS, for a period of 12 or 22 days in order to address the question of how the presence of added estrogens could affect the proliferation and the response to TAP-144. CG-5 cells responded to the lack of estrogens by reaching confluence after a longer time than that usually employed. If precultured in the above conditions for 12 days, they responded to added estrogens with a marked stimulation of cell proliferation (about 160% over control). When estrogen withdrawal was prolonged (i.e. 22 days) the stimulation of cell growth observed after estrogen addition was lower (in agreement with data by Katzenellenbogen et al. 1987). On the other hand, the effect of the analog appeared to be reduced if compared to that observed in cells that were less estrogen-sensitive (−40% versus −70% of estradiol stimulation) (Fig. 2).

The effect of estradiol, tamoxifen and TAP-144 on the growth of CG-5 cells, cultured in the presence of 5% CH-FCS, is reported in Fig 3. Tamoxifen alone inhibited

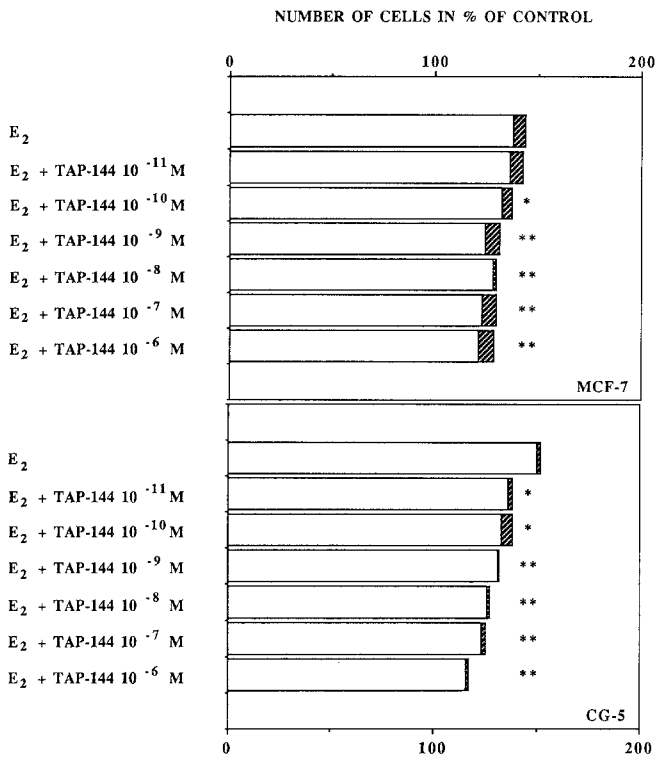

Fig. 1. Effects of leuprorelin (*TAP-144*) and 17β-estradiol (E_2) on proliferation of MCF-7 and CG-5 cells in the presence of 5% charcoal-treated fetal calf serum (*CH-FCS*). E_2 concentration was 10 nM for MCF-7 and 1 nM for CG-5 cells. *Bars* represent the mean of six observations from two different experiments + SE (*shaded area*). The cell number of the control groups ± SE was 2 322 500 ± 59 871 for MCF-7 and 2 186 000 ± 62 391 for CG-5 cells. $*P < 0.05$ versus estradiol-treated cells (Student's *t*-test); $**P < 0.01$ versus estradiol-treated cells (Student's *t*-test)

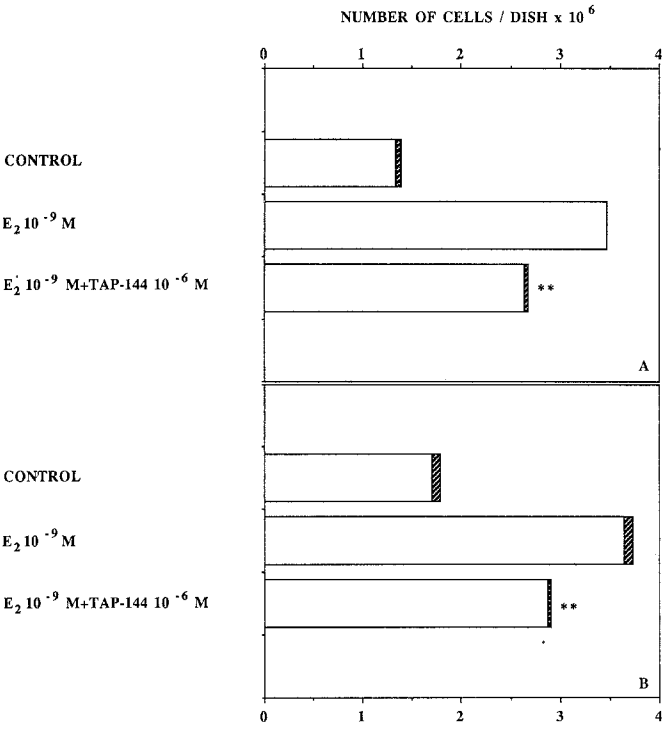

Fig. 2A, B. Effects of TAP-144 and E_2 on proliferation of CG-5 cells cultured for 12 (**A**) and 22 (**B**) days in the absence of estradiol (see Materials and methods). The assay was performed using phenol-red-free medium supplemented with 5% CH-FCS. *Bars* represent the mean of three observations from a representative experiment + SE (*shaded area*). $**P < 0.01$ versus estradiol-treated cells (Student's *t*-test)

cell growth by 40%. In the presence of estradiol, the anti estrogen reduced estrogenic stimulation by 69%.

When tamoxifen was combined with TAP-144 (0.1 μM and 1 μM) in the presence of estradiol, no statistically significant increase in the reduction of cell proliferation was observed (62% and 66% of cell growth inhibition, respectively) compared to that obtained by the antiestrogen alone in estradiol-treated cells, suggesting negative interference between the two drugs.

Figure 4 shows the effect of estradiol, MPA and TAP-144 on the growth of CG-5 cells. MPA alone caused an inhibition of 23%. It was able to suppress the estrogen-induced cell proliferation completely. The inhibition of estrogen-induced cell growth was not enhanced by the association of 0.1 μM TAP-144 with MPA. By contrast, when MPA was combined with 1 μM TAP-144, the cell number was below that of controls (−14%). This inhibition, although not considerable, is statistically significant with regard to estradiol/MPA-treated cells ($P < 0.01$) and the effect is subadditive.

The same increase of the effect of MPA induced by 1 μM TAP-144 was observed in MCF-7 cells ($P < 0.01$) (Fig. 5).

When TAP-144 (0.1 μM and 1 μM) and MPA (0.1 μM) were tested in combination in the absence of estradiol on

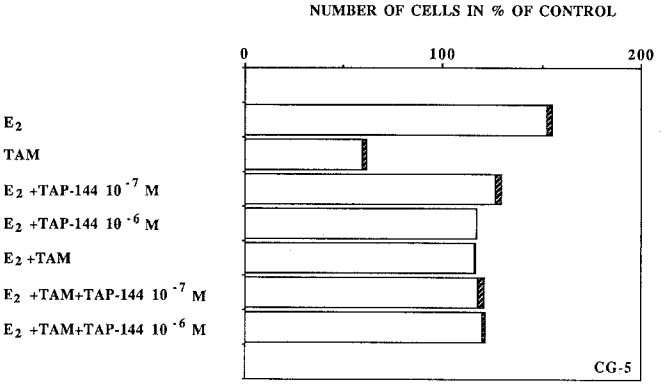

Fig. 3. Effects of TAP-144, 1 nM E_2 and 0.1 μM tamoxifen (*TAM*) on proliferation of CG-5 cells in the presence of 5% CH-FCS. *Bars* represent the mean of six observations from two different experiments + SE (*shaded area*). The cell number of the control group ± SE, set as 100%, was 2 325 666 ± 22 371

CG-5 cells no increase in the inhibition obtained by MPA alone was observed (data not shown).

In all our experiments cell viability was higher than 90%. As far as CG-5 steroid hormone receptors are concerned, TAP-144 at 0.1 μM and 1 μM did not affect either the ER or PR level when compared to controls. In agreement with the literature, estradiol treatment determined a decrease of ER and an enhancement of PR. When 1 μM

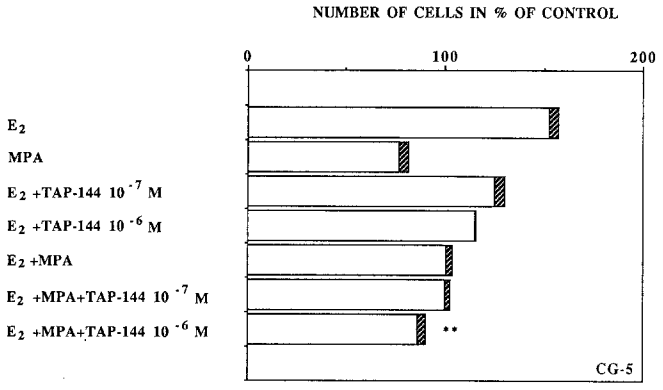

Fig. 4. Effects of TAP-144, 1 nM E_2 and 0.1 μM medroxyprogesterone acetate (*MPA*) on proliferation of CG-5 cells in the presence of 5% CH-FCS. *Bars* represent the mean of six observations from two different experiments + SE (*shaded area*). The cell number of the control group ± SE was 1985833 ± 24947. $^{**}P < 0.01$ versus estradiol + MPA-treated cells (Student's *t*-test)

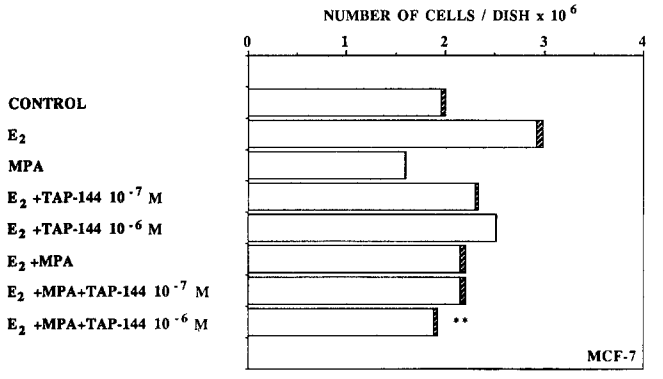

Fig. 5. Effects of TAP-144, 10 nM E_2 and 0.1 μM MPA on proliferation of MCF-7 cells in the presence of 5% CH-FCS. *Bars* represent the mean of three observations from a representative experiment + SE (*shaded area*). $^{**}P < 0.01$ versus estradiol + MPA-treated cells (Student's *t*-test)

TAP-144 was associated with 1 nM estradiol, a trend in favour of ER enhancement compared to estradiol-treated cells was observed while PR increased significantly compared to both control and estradiol-treated cells ($P \leqslant 0.05$) (Tables 1, 2).

Discussion

Our data relating to the direct effect of TAP-144 on breast cancer cells are in accordance with previous reports concerning the effects of various LHRH agonists (Blankenstein et al. 1985; Foekens et al. 1986; Scambia et al. 1988; Neri et al. 1990). In estrogen-insensitive cells such as MDA-MB-231, which represent a good model for testing the antiproliferative activity of LHRH analogs apart from the influence of estrogens, no inhibition of cell growth due to the analog was observed, as was already demonstrated in the same model by Eidne et al. (1987) and Sharoni et al. (1989) with buserelin. On the other hand, when tested on

Table 1. Effect of leuprorelin (TAP-144) and 17β-estradiol (E_2) on estrogen receptor levels in CG-5 cells

Conditions	Sites/cell	
	Median	Range
Control	29662	15838–35475
E_2 1 nM	16610*	10539–25266
TAP-144 0.1 μM	29666**	17663–33486
TAP-144 1 μM	28782**	15790–57655
E_2^a + TAP-144 0.1 μM	23151***	14198–32149
E_2^a + TAP-144 1 μM	24154***	12896–46361

Values derived from six different experiments
* $P \leqslant 0.05$, versus control cells, Wilcoxon's signed-rank test
** Not significantly different from control cells
*** Not significantly different from estradiol-treated cells
[a] 1 nM 17β-estradiol

Table 2. Effect of TAP-144 and 17β-estradiol (E_2) on progesterone receptor levels in CG-5 cells

Conditions	Sites/cell	
	Median	Range
Control	57035	36667–73195
E_2 1 nM	85050*[1]	46748–116454
TAP-144 0.1 μM	56559*[3]	32015–80406
TAP-144 1 μM	61434*[3]	31997–85812
E_2^a + TAP-144 0.1 μM	95800*[4]	32939–158203
E_2^a + TAP-144 1 μM	106065*[2]	54561–149054

Values derived from six different experiments
*[1] $P \leqslant 0.05$, versus control cells, Wilcoxon's signed-rank test
*[2] $P \leqslant 0.05$, versus E_2-treated cells, Wilcoxon's signed-rank test
*[3] Not significantly different from control cells
*[4] Not significantly different from E_2-treated cells
[a] 1 nM 17β-estradiol

estrogen-sensitive cells, TAP-144 showed an inhibitory effect when 1–10 nM estradiol was added to medium supplemented with 5% CH-FCS. Our findings represent the first evidence of the efficacy of TAP-144 in inhibiting estrogen-stimulated cell growth. In fact, Wilding et al. (1987), using the same culture conditions, observed that MCF-7 cell proliferation was not affected by TAP-144.

In experiments designed to verify whether the effect of TAP-144 on estrogen-induced cell proliferation could occur when minimal amounts of estradiol are present, such as those contained in unstripped serum, we could not find any inhibition of cell growth. These last observations appear to be different from those of Miller et al. (1985), who found that MCF-7 cells cultured in the presence of 10% heat-inactivated unstripped serum were sensitive to the antiproliferative action of buserelin. Notably, the same authors report, in two more recent publications, that different batches of serum influenced the degree of inhibition of cell growth caused by buserelin and that their MCF-7 clone became LHRH-agonist-insensitive when cultured in another laboratory with media supplemented with a different source of FCS (Scott et al. 1991; Mullen et al. 1991).

When CG-5 cells are cultured in phenol-red-free medium and acquire an enhanced sensitivity to estrogens,

TAP-144 is still active, although the activity is reduced. These data document that culture conditions may greatly influence cell responsiveness to LHRH agonists and that estrogen deprivation induces a series of events probably involved in the mechanism of action of these drugs which, at present, are not completely known. On the other hand, the action of LHRH analogs on estrogen-stimulated cell proliferation per se requires a better understanding. It has been speculated that these little peptides may behave like growth factors (Scambia et al. 1988) or interfere with the estrogen-induced production of growth factors (Scott et al. 1991).

When TAP-144 is associated with tamoxifen no vari ation of the inhibition of cell growth was observed, in agreement with published data on other agonists (Foekens et al. 1986; Scambia et al. 1988). The slight increase of the antiproliferative effect of MPA, both in CG-5 and in MCF-7 cells, by TAP-144 merits further investigation. In fact, Scambia et al. (1988), testing a combination of LHRH agonists with MPA on CG-5 cell growth, could not demonstrate any increased activity of the drugs. Whether such a discrepancy is attributable to the molecular structure of the compounds, drug concentration or cell line sensitivity remains to be determined. Moreover, it is worth noting that, in our CG-5 cells, TAP-144 produced, in the presence of estradiol, a significant enhancement of PR content in six separate experiments.

This receptor increase was not found by Scambia et al. (1988) or Neri et al. (1990), who explored the effect of other LHRH analogs on ER and PR levels in CG-5 and MCF-7 cells respectively. Nevertheless, when their data are analyzed, a trend in favor of PR up-regulation in the presence of estradiol seems to occur. Data from Foekens et al. (1986) disagree with our and the above-mentioned observations. In fact, these authors reported a down-regulation of nuclear PR when MCF-7 cells were treated with estradiol and buserelin.

In any case, the subadditive effect of TAP-144 and MPA on the inhibition of the growth of estrogen-sensitive cells could be of interest in view of the treatment of patients with metastatic breast cancer. Indeed, the combination of buserelin with high doses of progestins (megestrol acetate) has already been used with encouraging results by Klijn et al. (1985) to treat a very small number of patients affected by advanced breast cancer. From the endocrine point of view this combination appeared more suitable than the association with tamoxifen, which was shown to overcome the suppressive effect of the drug on hypothalamo-pituitary function (Klijn et al. 1985).

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