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Additive Inhibitory Effects of Bromocryptine (CB-154) and Medroxyprogesterone Acetate (MPA) on Dimethylbenz[a]anthracene (DMBA)-induced Mammary Tumors in the Rat

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Abstract—*Treatment for* 18 *days of rats bearing dimethylbenz[a]anthracene-induced mammary tumors with the synthetic progestin medroxyprogesterone acetate (MPA) or the inhibitor of prolactin secretion* 2α-*bromocryptine (CB-*154*) inhibited total tumor area to* 30 ± 7% *of the original volume. Combination of the two drugs, on the other hand, caused further inhibition to* 10 ± 5% *of the pretreatment tumor area. The most striking effect of combination of the two drugs is a doubling of complete responses (no detectable tumor) from* 30% *when either drug was used alone to* 60% *in animals treated with the combination therapy. Both estradiol and progesterone receptors were further decreased when MPA was added to CB-*154*. The present data demonstrate that combination of the synthetic progestin MPA and the inhibitor of prolactin secretion CB-*154 *exerts maximal inhibitory effects on the growth of the DMBA-induced mammary tumor, the most widely used* in vivo *model of human breast cancer.*

INTRODUCTION

MAMMARY CARCINOMA induced in the rat by dimethylbenz[a]anthracene (DMBA) is the most widely used *in vivo* model for studies on the endocrine control of human breast cancer [1–6]. Processes which decrease the secretion of estrogens or prolactin are well known to inhibit the development and growth of this tumor [1–6].

Medroxyprogesterone acetate (MPA) is one of the most widely used compounds in the endocrine therapy of advanced breast cancer in women [7–11]. The overall response rate to this progestin averages 40% in unselected breast cancer patients [11], an efficiency comparable to that of the non-steroidal antiestrogen tamoxifen [12, 13]. Its more general use, however, is for breast cancer relapsing after other endocrine therapeutic modalities.

The mechanisms underlying the antitumor activity of MPA, however, are poorly understood. In order to better understand the action of MPA in breast cancer, we have studied the possibility of additive effects of inhibition of PRL secretion by bromocryptine (CB-154) and administration of MPA on the growth of DMBA-induced mammary carcinoma in the rat.

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MATERIALS AND METHODS

Animals

Mammary tumors were induced in female Sprague–Dawley (Crl:CD(SD)Br) rats (obtained from Charles River Canada Inc., St-Constant, Quebec) at 50–55 days of age by a single intragastric administration of 20 mg of DMBA (Sigma Chemical Co. St. Louis, MO) in 1 ml of corn oil. Animals were housed two per cage under a regimen of 14 h of light and 10 h of darkness (lights on between 05:00 and 19:00 h). Purina rat chow and water were given *ad libitum*.

Treatments

Three to four months after DMBA administration, animals bearing tumors having a diameter of 1 cm^2 or more were selected and tumor number and size were recorded. Tumors developed in 75–80% of animals. The animals were then divided into four groups: control, medroxyprogesterone acetate (MPA), bromocryptine (CB-154) and MPA + CB-154. At the start of the experiment, each group had similar average tumor size. The rats of the appropriate groups were treated twice daily for

18 days with either MPA (10 mg), CB-154 (0.5 mg) or both compounds injected subcutaneously (s.c.) in 0.5 ml of 1% gelatin–0.9% NaCl. Control animals received the vehicle alone.

Tumor measurements were performed on the first day of treatment and at 2–3-day intervals thereafter. The two largest perpendicular diameters of each tumor were measured with calipers and the product of these diameters were used to estimate tumor size. The size measured on the first day of the experiment was taken as 100%. Results are expressed as percentage of growth in relation with the size determined on the first day. At the end of the treatment period, the animals were killed by decapitation. Blood samples were collected individually. Tumors and uteri were immediately removed, freed from connective and adipose tissue, weighed, frozen in liquid nitrogen and stored at −80°C until assayed.

Radioimmunoassays

Plasma PRL and LH were measured by double-antibody radioimmunoassays using rat hormones: rat LH-I-6 and rat PRL-I-5 were used for iodination while rat LH-RP-2 and rat PRL-RP-3 were used as standards. These hormone preparations as well as the rabbit antisera anti-LH-S-8 and anti-PRL-S8 were generously supplied by the National Pituitary Program, Baltimore, U.S.A.

Preparation of cytosol

All subsequent steps were performed at 0–4°C. Tissue was weighed before homogenization in 5 volumes (vol/wt) of buffer A (25 mM Tris–HCl, 1.5 mM EDTA, disodium salt, 10 mM α-monothioglycerol, 10% glycerol, and 1.5 mM sodium molybdate, pH 7.4) using a Polytron PT-10 homogenizer (Brinkman Instruments, Canada) at a setting of 5 for two or three periods of 10 s with intervals of 10 s for cooling. The homogenate was then centrifuged at 105,000 ***g*** for 60 min in a Beckman L5-65 centrifuge. Steroid binding assays were performed with freshly prepared cytosol fractions [5, 6]. Protein concentration was measured according to Lowry *et al.* [14] using bovine serum albumin as standard.

Progesterone and estrogen receptor assays

6,7[^{3}H]17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione (R5020) (87 Ci/mmole) and the corresponding unlabeled steroid were from New England Nuclear. [^{3}H]Estradiol (115 Ci/mmole) was also obtained from NEN. The unlabeled steroid diethylstilbestrol (DES) was from Sigma. [^{3}H]R5020 and [^{3}H]E_2 binding was measured using the dextran-coated charcoal adsorption technique; 0.2 ml aliquots of cytosol preparations were incubated with 0.1 ml 16 nM [^{3}H]R5020 (200,000 cpm) and 200 nM dexamethasone or 0.1 ml 8 nM [^{3}H]E_2 (90,000 cpm) in the presence or absence of a 100-fold excess of the unlabeled steroid (R5020 or DES) for 18–22 h at 0–4°C as described [5, 6]. Unbound steroid was then separated by incubation for 15 min at 0–4°C with 0.3 ml of 0.5% Norit A, 0.05% Dextran T-70 (DCC) in buffer B (1.5 mM EDTA disodium salt, 10 mM monothioglycerol and 10 mM Tris–HCl, pH 7.4) and centrifugation at 3000 ***g*** for 15 min. Aliquots of the supernatant (0.3 ml) were then removed for radioactivity measurement. After addition of 4 ml of Formula-963 scintillation liquid (New England Nuclear), the radioactivity was measured in a Beckman counter at a counting efficiency of 66%.

Calculations

Radioimmunoassay data were analyzed using a program based on model II of Rodbard and Lewald [15]. Statistical significance was calculated according to the multiple-range test of Duncan–Kramer [16]. All data are expressed as means ± S.E.M. of triplicate determinations of 17–20 animals per group.

RESULTS

Effect on tumor growth

As illustrated in Fig. 1, there was a rapid increase in total tumor area in the control animals to 250 ± 70% above pretreatment values after 18 days of observation. In the animals treated with MPA (10 mg, twice daily) or CB-154 (0.5 mg, twice daily), there was a similar reduction in total

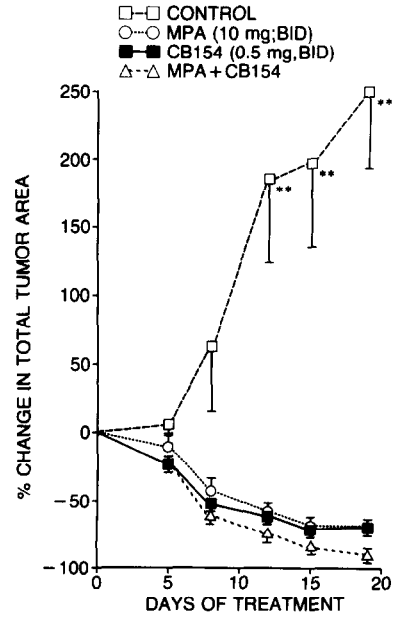

*Fig. 1. Effect of 18-day treatment with MPA (10 mg, twice daily), CB-154 (0.5 mg, twice daily) or the combination MPA + CB-154 on the average total DMBA-induced mammary tumor area (cm^2) in the rat. (**P < 0.01.) Data are expressed as a percentage of tumor area measured before starting therapy (day 0).*

tumor area to 30 ± 7% ($P < 0.01$) of pretreatment values. Moreover, it can be seen that treatment with the combination CB-154 + MPA caused further reduction in total tumor areas to 10 ± 5% of the pretreatment value ($P < 0.01$ vs. all groups).

Figure 2 illustrates the distribution of categories of responses achieved following the various treatments. While 63% of tumors progressed during the 18-day observation period in control rats, only 7, 20 and 7% of tumors progressed in the animals treated with MPA, CB-154 and MPA + CB-154, respectively. The number of stable tumors, on the other hand, was measured at 33, 26, 24 and 3% in control animals and those treated with MPA, CB-154 and MPA + CB-154, respectively. The number of tumors which regressed by more than 50% (partial response), on the other hand, increased from 4% in control rats to 37, 26 and 30%, respectively, in the above-mentioned groups of animals. A dramatic effect of treatment was observed on the number of complete responses (tumors undetectable at the end of treatment) which increased from 2% in control animals to 30, 30 and 60% in MPA, CB-154- and MPA + CB-154-treated animals, respectively.

Effect on tumor estrogen (ER) and progesterone (PR) receptor levels

In control animals, [^{3}H]estradiol binding was highest (59 ± 5.4 fmol/mg protein) in the tumors which progressed, the value being four times higher than in stable tumors. In the tumors which remained stable under treatment, lower values of [^{3}H]E_2 binding were found in the animals treated with MPA + CB-154, the value being 5.0 ± 2.0 fmol/mg protein compared to 26 ± 3.2 and 44 ± 4.2 fmol/mg protein in the animals treated with MPA and CB-154, respectively. In the regressing tumors still present at the end of the observation period, the lowest values of [^{3}H]estradiol binding were also found in the animals treated with both MPA and CB-154 (Fig. 3).

A marked effect of treatment on the level of progesterone receptors (PR) measured by [^{3}H]R5020 binding is illustrated in Fig. 4. In fact, PR decreased from 77 ± 4.2 fmol/mg protein in tumors progressing in intact control animals to 25 ± 1.8, 33 ± 2.2 and 2.6 ± 1.6 fmol/mg protein for tumors regressing in animals treated with MPA, CB-154 and MPA + CB-154, respectively. In stable tumors, PR levels were similar in control, MPA-treated and MPA + CB-154-treated animals while higher values were found in CB-154-treated animals (Fig. 4).

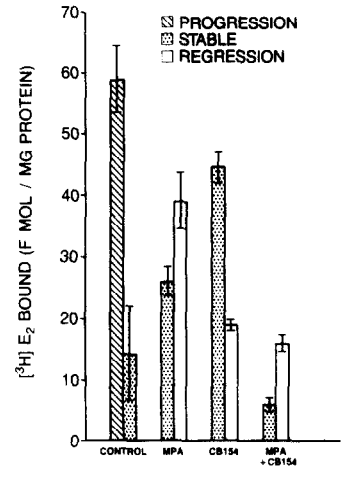

Fig. 3. Effect of 18-day treatment with MPA (10 mg, twice daily), CB-154 (0.5 mg, twice daily) or the combination MPA + CB-154 on the specific binding of [^{3}H] estradiol to DMBA-induced mammary tumor cytosol. Binding has been measured in progressing, stable and regressing tumors.

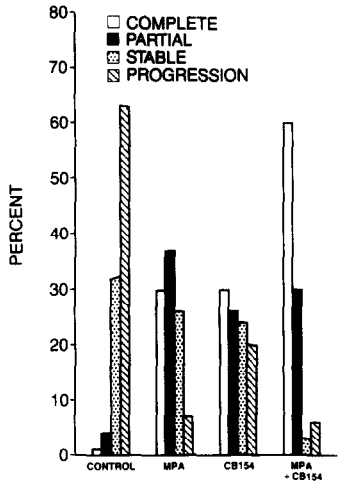

Fig. 2. Effect of 18-day treatment with MPA (10 mg, twice daily), CB-154 (0.5 mg, twice daily) or the combination MPA + CB-154 on the categories of responses (complete, partial, stable and progression) of DMBA-induced mammary carcinoma in the rat.

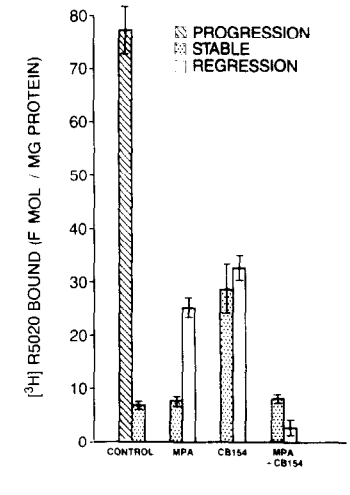

Fig. 4. Effect of 18-day treatment with MPA (10 mg, twice daily), CB-154 (0.5 mg, twice daily) or the combination MPA + CB-154 on the specific binding of [^{3}H]R5020 to DMBA-induced mammary tumor cytosol. Binding has been measured in progressing, stable and regressing tumors.

Effect on plasma prolactin (PRL) and luteinizing hormone (LH) levels

Treatment with MPA alone caused a decrease of 60% ($P < 0.01$) in plasma PRL levels while plasma LH was decreased by 45%, $P < 0.01$ (Fig. 5). CB-154, on the other hand, decreased plasma PRL by 80% ($P < 0.01$) but had no effect on plasma LH levels. While MPA + CB-154 caused a further decrease of plasma PRL to 2.0 ± 0.2 ng/ml, plasma LH levels were not further decreased by adding CB-154 to the MPA treatment (Fig. 5).

Effect on uterine weight

The slight 9% decrease in uterine weight (from 425 ± 45 to 385 ± 45 mg) following 18 days of treatment with MPA was not significant while CB-154 had no effect on this parameter (data not shown).

DISCUSSION

The present data demonstrate that while treatment with MPA or CB-154 independently inhibits the growth of DMBA-induced mammary carcinoma, combined administration of the two drugs causes further inhibition of tumor growth. The mechanism of action of the dopaminergic agonist CB-154 is well documented [17–21]. The importance of prolactin has been demonstrated for both the induction [22] and growth [2, 3, 5] of DMBA-induced mammary tumors. Although precise correlation between plasma PRL levels and DMBA-induced tumor growth is not available, an increase in plasma PRL [23–25] is clearly associated with accelerated tumor growth while a reduction in plasma PRL inhibits tumor growth [26, 27].

The role of prolactin in human breast cancer is still unclear. Hyperprolactinemia has been reported as a poor prognosis indicator in metastatic breast cancer [28]. Moreover, plasma PRL levels have been found to be higher in post-menopausal women with breast cancer than in normal post-menopausal women [29, 30]. Although it is generally agreed that inhibition of PRL secretion inhibits the growth of DMBA-induced mammary carcinoma in the rat, the situation is still debatable in the human. The first clinical trials with bromocryptine and L-DOPA have yielded non convincing results [31, 32].

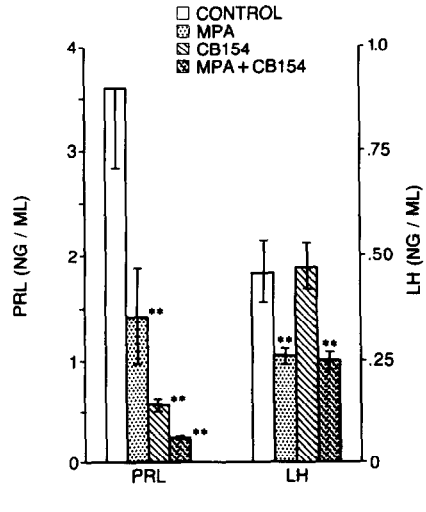

*Fig. 5. Effect of 18-day treatment with MPA (10 mg, twice daily), CB-154 (0.5 mg, twice daily) or the combination MPA + CB-154 on plasma prolactin (PRL) and LH levels in rats bearing DMBA-induced mammary tumor. (**P < 0.01 vs. control.)*

The mechanism of action of MPA, on the other hand, is more complex. In fact, MPA is a synthetic progestin having progestin, androgenic and glucocorticoid activities [33]. It is somewhat unlikely that the progestin-like properties of MPA play a major role in the inhibition of tumor gowth induced by the drug. In fact, treatment with progesterone has given variable effects on the growth of DMBA-induced mammary tumors. Progesterone stimulated tumor growth when given after DMBA administration [34–37]. On the other hand, Terenius [38] has observed that daily treatment for 13 days with approximately 4 mg progesterone per rat started at the time of DMBA administration had no effect on the number or size of tumors. It has also been observed that when administered early enough, progesterone can have an inhibitory effect on tumor growth [39, 40]. Thus, injection of 4 mg of progesterone 20 days before and after DMBA administration reduced the percentage of rats with tumors as well as the number and size of tumors.

Since progesterone added to 17β-estradiol (E_2) in ovariectomized or hypophysectomized–ovariectomized animals has been shown to cause greater stimulation of tumor growth than E_2 alone [5, 6], it is likely that the androgenic component of MPA [33] is, at least to a large extent, responsible for the marked inhibitory effect on tumor growth. This is well supported by our recent data showing that the main action of MPA on ZR-75-1 human breast cancer cell growth is due to its androgen receptor-mediated inhibitory action [41].

In intact animals, the androgenic activity of MPA on tumor growth can be exerted through two mechanisms: (a) inhibition of gonadotropin secretion and (b) a direct inhibitory effect at the tumor level. A direct inhibitory effect of DHT on the growth of mammary fibroadenomas is suggested by data showing an additional decrease of carcinoma growth in animals already ovariectomized [42]. In addition, androgens have been found to inhibit the growth of DMBA-induced mammary tumors in intact animals [43, 44]. However, in intact animals, one cannot differentiate between an effect of androgens exerted through inhibition of gonadotropin secretion or a direct inhibitory effect on tumor growth. The first demonstration of a direct antiproliferative effect of androgens has recently been

obtained in the ZR-75-1 human breast cancer cells [45]. In addition to exerting their inhibitory effect on ZR-75-1 cell growth when present alone, androgens counteract the stimulatory effect of estrogens on the same parameter [45].

MPA has recently been shown to be a highly potent androgen having a potency comparable to that of the most potent natural androgen, namely 5α-dihydrotestosterone (DHT) itself [33]. The androgenic activity of MPA had previously been reported. These data pertain to the masculinization of female fetuses when the compound was administered to pregnant animals [46, 47]. This synthetic progestin has also been shown to stimulate the weight of the preputial glands [48]. Moreover, when injected into female mice, MPA, as well as megestrol acetate, induced a marked stimulation of kidney β-glucuronidase activity [48]. In addition, when the two 17-acetoxy progesterone derivatives were injected in the androgen-insensitive (tfm/y) mouse, there was no increase in kidney β-glucuronidase activity, thus indicating that MPA and megestrol acetate exert their action through interaction with the androgen receptor. In agreement with the findings of a potent androgenic activity of MPA, [^{3}H]MPA has been found to bind to the mouse kidney androgen receptor directly without transformation [49]. In fact, MPA binds to the androgen receptor with high affinity, its K_D value of interaction being comparable to that of DHT itself [49].

Since PRL stimulates DMBA-induced tumor growth, the inhibition of plasma PRL induced by MPA (probably secondary to inhibition of gonadotropin and estradiol secretion) is also likely to play a significant role in mediating the inhibitory effects observed following treatment with MPA. It is also possible that the further inhibition of plasma PRL levels observed in animals receiving both CB-154 and MPA could explain at least in part, the additional benefits of the combination therapy. However, the higher levels of plasma PRL observed after treatment with MPA alone compared to CB-154 alone and the superimposable effects observed on tumor growth indicate that MPA, in addition to its effect on plasma PRL, decreases tumor growth by mechanism(s) independent from changes in PRL secretion.

The decrease in tumoral ER levels observed following treatment with CB-154 is in agreement with previous data showing a stimulatory effect of exogenous treatment of OVX animals with PRL on ER levels in DMBA-induced tumors [5]. Since PR depends upon ER levels, it is logical to find a parallel decrease in the PR concentration in the animals treated with the inhibitor of PRL secretion. Although other mechanisms can be involved, the decrease in ER and PR levels observed in tumors of the animals treated with MPA, could result, at least in part, from the inhibitory effects of MPA on gonadotropin secretion resulting in lower plasma E_2 and PRL concentrations. It can also be seen that both ER and PR levels are decreased to a greater extent when MPA and CB-154 are administered together, thus suggesting the possibility of a direct inhibitory effect of MPA on ER and PR levels.

DMBA-induced mammary tumors in the rat have different sensitivities to PRL. In fact, some tumors require PRL, some require PRL + estrogens and some (a small number) are apparently growing without the influence of PRL or estrogens [4]. The present data clearly indicate the heterogeneity of growth rate of different tumors in the four groups of animals. Although the origin of tumors is believed to be monoclonal [50], it is clear that most, if not all, advanced tumors are composed of mixed populations of cells having a wide range of phenotypes [51, 52]. This well demonstrated heterogeneity of the sensitivity of tumors to hormones can well explain the additive beneficial effects of drugs acting through different mechanisms, thus increasing the chance of maximal inhibition of cell growth as shown by the present data using MPA and CB-154 in the DMBA-induced mammary carcinoma model.

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