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ANDROGENIC ACTIVITY OF SYNTHETIC PROGESTINS AND SPIRONOLACTONE IN ANDROGEN-SENSITIVE MOUSE MAMMARY CARCINOMA (SHIONOGI) CELLS IN CULTURE

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Summary—A series of compounds designed to block the action of androgens in target tissues, and called antiandrogens, have been developed for the treatment of androgen-sensitive diseases, especially prostate cancer, hirsutism, precocious puberty and deviant sexual behavior. In order to further assess the androgenic activity of these compounds, we have studied their effect on the growth of an androgen-sensitive clone of the mouse mammary carcinoma Shionogi SC-115 cells in culture. Hydroxyflutamide did not affect the doubling time (7.40 ± 0.09 vs 7.20 ± 0.12 days) characteristic of these cells. However, all of the other compounds tested stimulated cell growth. Thus, in the presence of cyproterone acetate, cells had an accelerated growth rate and shorter generation time of 6.28 ± 0.06 days ($P < 0.01$). In the presence of 1 μM spironolactone, the generation time was 4.96 ± 0.04 days ($P < 0.01$). With chlormadinone acetate, the doubling time was reduced to 3.79 ± 0.08 days while for megestrol acetate, the doubling time was 3.63 ± 0.04 days ($P < 0.01$). The synthetic progestin Medroxyprogesterone acetate had the most potent androgenic effect reducing the doubling time to 1.85 ± 0.05 days ($P < 0.01$). For comparison, dihydrotestosterone gave a doubling time of 1.76 ± 0.07 days. When hydroxy-flutamide (5 μM) was added simultaneously with each "progestin", the ED_{50} value of action of all the compounds was increased in a competitive manner, thus indicating that the mitogenic effect on cell growth of all compounds is mediated by the androgen receptor. Of all the compounds used, only hydroxy-Flutamide was devoid of any androgenic activity and thus meets the criteria of a pure antiandrogen.

INTRODUCTION

Since the observation of Huggins and his colleagues in 1941 on the important role of testicular androgens in prostate cancer growth [1], the standard treatment of this disease has been orchiectomy and estrogens. However, orchiectomy is psychologically unacceptable for a large number of patients while treatment with estrogens in order to block gonadotropin secretion causes serious cardiovascular complications leading to death in 15% of patients during the first year of treatment [2]. A recently proposed alternative is the use of agonists of luteinizing hormone-releasing hormone (LHRH) which cause a complete blockade of testicular androgen secretion without side effects other than those related to hypoandrogenism [3]. However, since the effect of these peptides is limited to the blockade of testicular androgens, one cannot expect to improve the prognosis of prostate cancer beyond the results previously achieved with orchiectomy, namely a remission in 60–80% of cases for a limited time interval, thus leaving 20–40% of patients without any significant effect of treatment on the cancer [4, 5].

The well recognized sensitivity of prostate cancer to androgens has stimulated the development of compounds, called antiandrogens, which are aimed at blocking the action of androgens in target tissues [6, 7]. The interest in these compounds is strengthened by the recent observation that the blockade of androgens of both testicular and adrenal origins by combining a pure antiandrogen with castration at the start of treatment of advanced prostate cancer yields a higher response rate, a more prolonged disease-free period and an improved survival while maintaining a good quality of life [4, 8, 9]. The use of this combination therapy follows the recognition that adrenal steroids contribute approx 50% of all androgens present in the prostatic cancer tissue [4]. Antiandrogens thus offer an alternative to hypophysectomy or adrenalectomy without the complications of glucocorticoid replacement therapy.

Since the aim of endocrine therapy in prostate cancer is maximal blockade of androgen action, the ideal antiandrogen should be a compound having potent antiandrogenic activity while being devoid of any androgenic, glucocorticoid, progestational, estrogenic or any other hormonal or antihormonal

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action [10]). It thus becomes of major interest to investigate in detail the properties of available drugs in order to choose the best antiandrogen for clinical use.

So far, the androgenic/antiandrogenic activity of the various antiandrogenic compounds used has been measured in intact animal models [7, 11–14]. In the present study, we have taken advantage of the availability of a highly androgen-sensitive clone derived from the Shionogi carcinoma cell line in order to assess the androgenic activity of the compounds presently proposed for the treatment of prostate cancer and/or hirsutism, namely cyproterone acetate, medroxyprogesterone acetate, spironolactone, chlormadinone acetate, megestrol acetate and Flutamide. Specificity of the action of each drug on the androgen receptor has been tested using competition with the pure antiandrogen Flutamide [7, 11, 15].

EXPERIMENTAL

Culture of Shionogi cells

Shionogi male mice bearing tumors of the androgen-sensitive mouse mammary carcinoma cell line SC-115 were kindly provided by Dr K. Matsumoto, Osaka, Japan. Cells were dispersed from finely minced tumors by a 2-h treatment at 37°C in 25 mM Hepes buffer (137 mM NaCl; 5 mM Na_2HPO_4; 10 mM glucose, pH 7.2) containing 510 U/ml collagenase (Clostridium, Boehringer), 550 U/ml hyaluronidase II (Sigma) and 4% bovine serum albumin (fraction V, Schwartz-Mann). Dispersed cells were collected by centrifugation (500 *g* for 10 min), washed twice by suspension in Minimal Essential Medium (MEM) containing 5% dextran-coated charcoal-treated fetal calf serum (DCC-FCS), 1% non-essential amino acids, 10 U/ml penicillin, 50 μg/ml streptomycin and 100 nM 5α-dihydrotestosterone (DHT) (Steraloids).

Cells were plated in the same medium at a density of 75,000 cells/ml in 75 cm^2 flasks under an atmosphere of 5% carbon dioxide in air at 37°C. The medium was changed weekly. 5α-dihydrotestosterone (DHT) was dissolved in ethanol and the final concentration of ethanol did not exceed 0.01% in the culture medium. Cells were subcultured at near-confluence by gentle digestion in a solution of 0.1% pancreatin (Flow Laboratories) in Hepes buffer containing 3 mM EDTA (pH 7.2). Cells were pelleted by centrifugation, resuspended in culture medium, counted in a Coulter counter and replated as described above.

Cloning of SC-115 cells

Soft agar cloning was performed as described [16, 17]. A single clone (SEM-1) showing DHT-sensitive growth was selected for cell growth experiments. The cloned cells were maintained in the MEM medium described above except that the DCC-FCS concentration was 2% and DHT was 100 nM. The medium was changed every third day and the cells subcultured as described above.

Doubling-time experiments

Cells were plated in 24-well plates at a density of 20,000 cells/well in MEM-2% DCC-FCS medium containing, when indicated, 1 μM of the following compounds: medroxyprogesterone acetate, megestrol acetate, chlormadinone acetate, spironolactone, cyproterone acetate and hydroxy-flutamide while the concentration of DHT was 100 nM. The control wells contained the same ethanol concentration (0.1%). Samples were taken daily for measurement of cell number. The medium was changed every second day and the cells were grown up to 40 days. At the end of the indicated incubation periods, cells were washed with Dulbecco's phosphate buffered saline (without calcium and magnesium). A pancreatin-EDTA incubation was then performed. The enzymatic treatment was stopped with MEM medium containing 10% FCS and the cells were counted with a Coulter counter.

Measurements of ED_{50} values of stimulation of cell proliferation

The cells were incubated with increasing concentrations of the indicated compounds in the presence or absence of 5 μM hydroxy-flutamide. DHT and medroxyprogesterone acetate were incubated during 8 days while chlormadinone acetate, spironolactone and megestrol acetate were incubated during 14 days and cyproterone acetate and hydroxy-flutamide during 25 days.

Calculations and statistical analyses

The potency of each compound as stimulator of cell proliferation was measured as ED_{50} value using an iterative least square method [18].

RESULTS

The stimulatory activity of the various "antiandrogens" on cell proliferation was first assessed by measurement of cell growth kinetics in the presence of each compound alone. As illustrated in Fig. 1, a wide range of stimulatory activities was observed when each compound was incubated with clone SEM-1 at a concentration of 1 μM (except for DHT which was used at a concentration of 100 nM). The cell number doubling-time for each compound was as follows: hydroxy-flutamide had no effect on cell growth, the doubling time being superimposable to that of cells incubated with control medium (7.40 ± 0.09 vs 7.20 ± 0.12 days) while all the other compounds stimulated cell growth: cyproterone acetate reduced the generation time to 6.28 ± 0.06 days ($P < 0.01$ vs control and Flutamide), spironolactone to 4.96 ± 0.04 days ($P < 0.01$), chlormadinone acetate to 3.79 ± 0.08 days ($P < 0.01$), megestrol acetate to 3.63 ± 0.04 days ($P < 0.01$) and medroxypro-

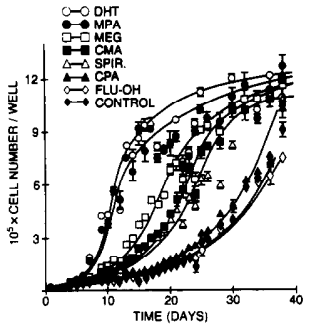

Fig. 1. Effect of a series of "antiandrogens" on the growth of an androgen-sensitive clone (SEM-1) of mouse mammary carcinoma Shionogi cells in culture. The cells were incubated up to 40 days in medium (MEM + 2% dextran-coated charcoal extracted fetal calf serum) containing the indicated compounds at a concentration of 1 μM (except 0.1 μM for DHT). Media were changed every second day. The compounds were: DHT: dihydrotestosterone; MPA: medroxyprogesterone acetate; MEG: megestrol acetate; CMA: chlormadinone acetate; SPIR: spironolactone; CPA: Cyproterone acetate; FLU-OH: hydroxy-flutamide. The control medium contained the same concentration of ethanol (0.1%).

gesterone acetate to 1.85 ± 0.05 days ($P < 0.01$). DHT included for comparison as the standard androgen showed an accelerated doubling-time of 1.76 ± 0.07 days ($P < 0.01$ vs all groups except medroxyprogesterone acetate).

Following this assessment of the relative stimulatory effect of each compound on cell growth at a concentration of 1 μM, we next examined the potency of each compound by measurements of ED_{50} values and have assessed the specificity of their action on the androgen receptor by simultaneous incubation

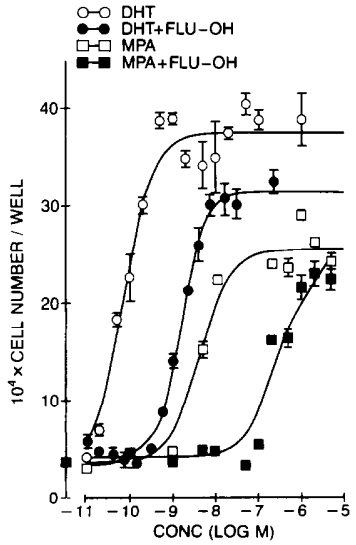

Fig. 2. Effect of increasing concentrations of dihydrotestosterone or medroxyprogesterone acetate in the presence or absence of hydroxy-Flutamide (5 μM) on the number of androgen-sensitive Shionogi SC-115 cells (clone SEM-1). The cells were incubated as described in Fig. 1, except for the concentrations of the indicated compounds and an incubation time of 8 days.

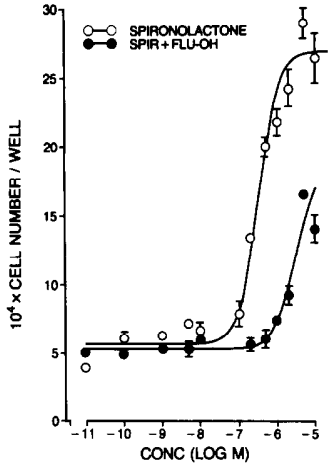

Fig. 3. Effect of increasing concentrations of spironolactone (SPIR) in the presence or absence of hydroxy-flutamide (5 μM) on the growth of androgen-sensitive Shionogi SC-115 cells in culture (clone SEM-1). The conditions were those specified in Fig. 1. The incubation was performed during 14 days.

with the pure antiandrogen hydroxy-flutamide. Figure 2 compares the ED_{50} values of DHT and medroxyprogesterone acetate action measured after 8 days of incubation with increasing concentrations of the two compounds. The ED_{50} value of DHT action was calculated at 0.073 nM while that of medroxyprogesterone acetate is 42.5 nM. When hydroxy-flutamide (5 μM) was added to the incubation medium, the ED_{50} values of DHT and MPA action increased to 5.9 and 222 nM, respectively.

It can be seen in Fig. 3 that incubation with increasing concentrations of spironolactone for 14 days showed an ED_{50} value of action of the compound at 344 nM while, in the presence of hydroxy-flutamide, it was increased in a competitive manner

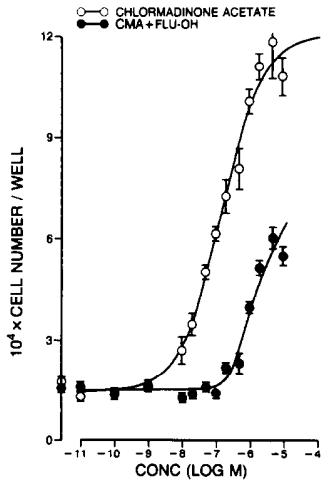

Fig. 4. Effect of increasing concentrations of chlormadinone acetate (CMA) in the presence or absence of hydroxy-flutamide (5 μM) on androgen-sensitive Shionogi (clone SEM-1) cell growth. The conditions were as described in Fig. 1. The experiment was performed for 14 days.

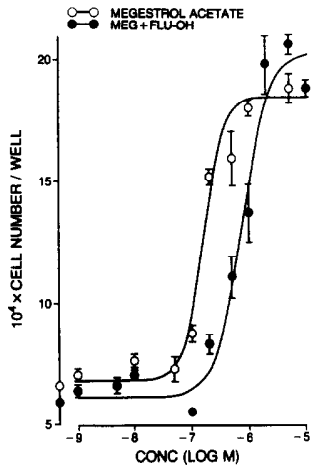

Fig. 5. Effect of increasing concentrations of megestrol acetate (MEG) in the presence or absence of hydroxy-flutamide (5 μM) on androgen-sensitive Shionogi (clone SEM-1) cell growth. The conditions are as described in Fig. 1. The experiment lasted 11 days.

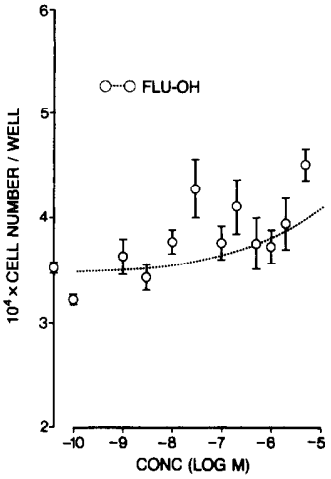

Fig. 7. Effect of increasing concentrations of hydroxy-flutamide on androgen-sensitive Shionogi cell growth of the androgen-sensitive clone SEM-1 of Shionogi cells. The conditions are as described in Fig. 1. The experiment was performed for 25 days.

to 1.88 μM. Figure 4 shows that the effect of chlormadinone acetate on cell growth during the same incubation period of 14 days was exerted at ED_{50} values of 151 and 896 nM in the absence and presence of 5 μM hydroxy-flutamide, respectively. Megestrol acetate (Fig. 5) had an ED_{50} value of action of 161 nM, this value being shifted to 746 nM in the presence of hydroxy-flutamide (5 μM). It can be seen in Fig. 6 that after 25 days of incubation, cyproterone acetate stimulated cell growth at an ED_{50} value of 65 nM, this value being increased to 739 nM in the presence of 5 μM hydroxy-flutamide. Hydroxy-flutamide alone, on the other hand, showed no significant stimulation of cell growth during a 25-day incubation period (Fig. 7).

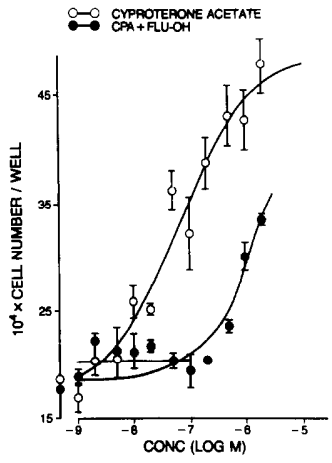

Fig. 6. Effect of increasing concentrations of cyproterone acetate (CPA) in the presence or absence of hydroxy-flutamide (5 μM) on androgen-sensitive Shionogi cell growth. The experiment was performed as described in Fig. 1 and lasted 25 days.

DISCUSSION

Antiandrogens must be strictly defined as compounds which inhibit androgen action at the target tissue level and do not act through inhibition of gonadotropin secretion [10]). To be maximally effective, it is clear that an antiandrogen should counteract the effect of androgens at the receptor level without exerting any androgenic activity by itself. Compounds having mixed agonistic–antagonistic activity (such as cyproterone acetate and spironolactone, 11, 12, 19) cannot reach the complete inhibition achieved with pure antiandrogens [11, 19]. Any intrinsic agonistic activity stimulates the androgen receptor, such as stimulatory androgenic activity being more important in cancer cells where the stimulatory action is amplified by rapid cell division. The present data clearly show that all the compounds tested, except hydroxy-flutamide, exert significant and sometimes marked agonistic androgenic activity as assessed by stimulation of proliferation of an androgen-sensitive clone of cancer cells. Although an androgenic activity has been previously suggested for most of these compounds using androgen-sensitive parameters of response in normal tissues, it is likely that the present data obtained with cancer cells are even more relevant to human cancer.

Previous studies using androgen-sensitive parameters in normal tissues have demonstrated the androgenic activity of cyproterone acetate [7, 11, 13, 19–22]. In fact, when cyproterone acetate was administered to pregnant guinea pigs [13], and rabbits [21], all female fetuses showed signs of virilization of the secondary sex organs. Recently, a growth-promoting effect of cyproterone acetate has been described on the growth of Shionogi tumors in castrated mice [22]. The present data, using a simpler system, namely a clone of the same androgen-

Table 1. Effect of synthetic progestins, spironolactone and hydroxy-Flutamide on the doubling time (expressed in days) and the ED_{50} value of stimulation of growth (expressed in nM in the absence or presence of 5 μM hydroxy-Flutamide) of Shionogi carcinoma cells

Compound	Doubling time	ED_{50} value of growth response	
		No hydroxy-Flutamide	+hydroxy-Flutamide
	(days)	(nM)	
Control	7.20 ± 0.12	—	—
Hydroxy-flutamide	7.40 ± 0.09	—	—
Cyproterone acetate	6.28 ± 0.06*	65	739
Spironolactone	4.96 ± 0.04*	344	1880
Chlormadinone acetate	3.79 ± 0.08*	151	896
Megestrol acetate	3.63 ± 0.04*	161	746
Medroxyprogesterone	1.85 ± 0.05*	42.5	222
DHT	1.76 ± 0.07*	0.073	5.9

*$P < 0.01$.
For the doubling time, all compounds were present at a concentration of 1 μM except DHT (0.10 μM).

sensitive Shionogi cancer cells maintained *in vitro*, show that cyproterone acetate exerts a direct stimulatory effect on cell proliferation. The androgenic nature of the stimulatory effect of cyproterone acetate is well demonstrated by the inhibition of its effect by the pure antiandrogen hydroxy-flutamide [15], the active metabolite of flutamide [23].

The presence of megestrol acetate on the prostatic androgen receptor following *in vivo* administration was incorrectly interpreted as an antiandrogenic action [24]. In the light of the present and previous [11, 14, 19] results, binding of megestrol acetate to the androgen receptor induces an activation rather than an inactivation of the receptor. Since a major argument put forward to support the use of megestrol acetate for the treatment of prostate cancer is the potential blockade of intracellular androgen action in the prostatic cancer tissue, the discovery of an androgenic action of the compound in normal [11, 14, 19] and cancer (present study) tissues should seriously limit the potential usefulness of this compound for the treatment of prostate cancer.

Spironolactone, in addition to being an aldosterone antagonist [25], has been shown to have some antiandrogenic activity [26, 27]. Based on these antiandrogenic properties, spironolactone has been used for the treatment of hirsutism [26–30]. The androgenic activity of spironolactone has recently been described on rat ventral prostate growth and prostatic ornithine decarboxylase activity in the rat [19] and is clearly confirmed in the present study. In fact, spironolactone is a weak and only partial androgen antagonist possessing a relatively important intrinsic androgenic activity.

The androgenic activity of medroxyprogesterone acetate has previously been reported in normal tissues. These data pertain to the masculinization of female fetuses when the compound was administered to pregnant animals [31, 32]. In addition to its masculinizing effects on the external genitalia and reproductive tract of female fetuses, this synthetic progestin has been shown to stimulate the weight of the preputial glands [12, 33]. Moreover, when injected into female mice, medroxyprogesterone acetate, as well as megestrol acetate, induced a marked stimulation of kidney β-glucuronidase activity [12]. When the two 17-acetoxyprogesterone derivatives were injected in the androgen-insensitive (tfm/y) mouse, there was no increase in kidney β-glucuronidase activity, thus further indicating that medroxyprogesterone acetate and megestrol acetate exert their action through interaction with the androgen receptor.

In agreement with the findings of a potent androgenic activity of medroxyprogesterone acetate, [^{3}H]medroxyprogesterone acetate has been found to bind to the mouse kidney androgen receptor directly without transformation [34]. In fact, medroxyprogesterone acetate binds to the androgen receptor with high affinity, its K_d value of interaction being comparable to that of DHT itself [34]. As illustrated in Fig. 2, medroxyprogesterone is also a potent stimulator of androgen-sensitive cell growth, a half-maximal effect being exerted at 43 nM.

In previous studies performed with rat anterior pituitary cells in culture, medroxyprogesterone acetate was found to inhibit LHRH-induced LH release to 20% of control at an ED_{50} value of 4.6 ± 0.7 nM [35]. The potency of this effect was similar to that obtained with testosterone or DHT [36], thus indicating that MPA also exerts full androgenic activity on LH release. Under the same conditions, progesterone had no effect. In agreement with the present data on androgen-sensitive cell growth, megestrol acetate and chlormadinone acetate had higher ED_{50} values on LHRH-induced LH release measured at 200 and 100 nM, respectively.

Another important finding reported with medroxyprogesterone acetate, megestrol acetate and cyproterone acetate is their potentiating effect on the stimulatory action of androgens on kidney β-glucuronidase activity. This marked enhancement of the

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