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ANTITUMOR EFFECTS OF COMBINATION TOREMIFENE AND MEDROXYPROGESTERONE ACETATE (MPA) *IN VITRO* AND *IN VIVO*

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Summary—The estrogen (ER) and progesterone (PgR) receptor levels in various gynecological tumors were measured. The same tumors were exposed *in vitro* to toremifene, MPA or their combination and the growth of the tumors was followed by measuring the adenosine triphosphate (ATP) within the cells by a simple bioluminescence assay. Altogether 34 clinical samples were studied. DMBA-induced mammary tumors bearing rats were treated *in vivo* with toremifene, MPA and their combination.

About half of the ovarian cancers and 6 out of the 7 adenocarcinomas of uteri contained ER. The ovarian tumors were PgR rich in 25% and adenocarcinomas of uteri in 6 out of the 7 cases.

When compared to control toremifene (concentration 1 μmol/l) was able to decrease the number of living cells to 50% or less in 9/34 samples, MPA (concentration 10 μmol/l) in 17/34 samples, and the combination in 25/34 samples. In five cases the antitumor effect of the combination was synergistic. In two cases signs of weak antagonism were seen.

In vivo the antitumor effect of toremifene and MPA was clearly synergistic against DMBA-induced cancers. The effect was dose-dependent and at sufficiently high doses it was possible to eradicate the tumors and cure the animals.

INTRODUCTION

It is of clinical significance that the response of breast cancer patients to hormonal treatments can be predicted by determining the hormone receptor levels of the tumors [1], although some workers have been sceptical about the usefulness of receptors [2, 3].

Recently, predictive *in vitro* and *in vivo* tests have been the subject of intense study in selecting cytostatic treatment for each patient individually [4–6]. These predictive tests could be an alternative to receptor measurements in mammary and gynecological cancers [7]. In the present study, results of receptor measurements have been compared with *in vitro* sensitivity.

Toremifene, a new antiestrogenic antitumor compound, increases the progesterone receptor levels in the target tissue [8, 9] as a result of which the tumors might become more sensitive to progestins. The combination of antiestrogen + progestin is therefore theoretically interesting. Clinically, however the combination of tamoxifen and MPA has not given better results than either drug alone [10]. Therefore the effect of toremifene and MPA was evaluated both *in vitro* and *in vivo*. Several dose levels were used in *in vivo* studies.

MATERIALS AND METHODS

Reagents and instruments

ATP monitoring reagent (LKB-Wallac, Turku, Finland) containing purified firefly luciferase and Tris–HCl buffer (0.5 M, pH 7.75) as well as adenosine triphosphate (ATP) standard (LKB-Wallac) were needed for the bioluminescence reaction. Cell culture media, Medium 199 and RPMI 1640 as well as supplements, L-glutamine, fetal calf serum, penicillin and streptomycin were purchased from Flow Laboratories (Middlesex, England), Fluka (Buchs, Switzerland) or Sigma (St Louis, Mo., U.S.A.) chemical companies. Medium 199 was used in the transportation tubes from hospital to the laboratory. RPMI 1640 was used in the actual cell cultures. The radioactive ligands [^{3}H-2,4,6,7]estradiol, sp. act. 115 Ci/mmol, and (17-α-methyl[^{3}H]promegestone (= R-5020), sp. act. 87 Ci/mmol for ER and PgR determinations, respectively, were obtained from NEN (Dreieich, F.R.G.). The tested hormones were MPA and toremifene (both from Farmos Chemical Research Laboratory, Oulu, Finland). For cell cultures they were dissolved in ethanol and pipetted onto the vials. Ethanol concentration in the final growth medium never exceeded 0.07%. Cell suspension (100 μl) was added. The final concentrations of

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toremifene and MPA were 1 μmol/l and 10 μmol/l, respectively. These concentrations are related to IC_{50} values in MCF-7 cells in similar growth conditions. The hormones were considered effective if the amount of living cells was $\leq 50\%$ of that in control (no hormones) after 2 days cultivation. Synergism was defined according to Drewinko *et al.*[11].

LKB Luminometer 1250 equipped with LKB 2210 potentiometric recorder (both from LKB-Wallac, Turku, Finland) was used for ATP-measurements.

Receptor determinations and cell cultures

Fresh human ovarian cancers ($n = 23$), adenocarcinomas of the uterus ($n = 8$) and vulvar cancers ($n = 3$; one of the patients was operated on twice) were studied. Tissue samples were divided into two pieces. One was used for ER and PgR receptor determinations, one for *in vitro* cell culture assays.

A fresh tumor fragment was immediately frozen at -70°C for receptor binding studies. Cytosol ER and PgR were determined with slightly modified DCC method of Korenman and Dukes[12]. Tumors with ER higher than 10 fmol/mg protein and PgR higher than 20 fmol/mg protein were considered ER-rich and PgR-rich, respectively.

A sample for cell culture assay was transferred immediately into a test tube containing medium 199. For the cultivation the sample was minced carefully with a scalpel. In several specimens a sufficient amount of cells was obtained by simple scraping. The red blood cells if present in large quantity were lysed with NH_4Cl. Living cells were collected by centrifugation and used for cultivation. If necessary, tumor cells were detached from the matrix by overnight incubation with collagenase after which the red blood cells were lysed. RPMI medium with 10% fetal calf serum, 292 mg/ml L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin was used as the growth medium. Cell cultivation conditions on microtiter plates or in plastic tubes were as follows: 5% CO_2, 37°C and growth time 1–5 days. The number of living cells were estimated by a simple bioluminescence assay which has been described earlier [13]. Shortly: intracellular ATP was released by TCA treatment and ATP was quantitated by adding ATP monitoring reagent which changes the chemical energy of ATP to light. Light was quantitated by luminometer. The method is technically very simple and rapid.

DMBA-induced tumors

Breast cancers were induced in 48–52 days old female Sprague–Dawley rats (Alab, Solluntuna, Sweden) by administering 12 mg of 7,12-dimethyl[a]-benzanthracene/animal orally with stainless steel cavage as a single dose in 1.0 ml of sesam oil. The induction was carried out in a special isolator (Metall & Plastik GmbH, Radolfzell, F.R.G.) wherefrom the animals were transferred to standard laboratory conditions after about 3 weeks. The animals had tap water and standardized laboratory chow (Anticimex, Stockholm, Sweden) available *ad libitum*. Treatment with hormones was started when the animals had palpable tumors, about 5–7 weeks after the induction. Treatment was continued for five weeks. Toremifene and MPA were given daily at the doses which appear in Fig. 1. Toremifene was administered orally and MPA intramuscularly. The treatment lasted for five weeks. Both compounds were suspended in vehicle containing polyethylene glycol 3000 28.8 g/l, NaCl 8.65 g/l, Tween 80 1.92 g/l, methyl-*p*-hydroxybenzoate 1.73 g/l and propyl-*p*-hydroxybenzoate 0.19 g/l in distilled water.

Tumors were detected individually by palpation once a week. The width (w) and length (l) of each tumor was measured by comparing them to the measuring scale in front of the observer. Tumor volume (V) was calculated by assuming the tumor half oval. Thus $V = \pi w^2 l/12$. The same equation is valid also if the tumor shape is half spherical. The number of tumors was recorded at every measurement. The tumors were divided into three groups according to the growth properties. Class I = actively growing tumors; the volume was increased more than 4-fold during the five weeks' treatment. Class II = stable tumors. Class III = regressing tumors; the size was decreased to less than one fourth of the start of the treatment, or tumor was disappeared completely.

The statistical analysis of the DMBA results was performed by Student's *t*-test (tumor numbers/

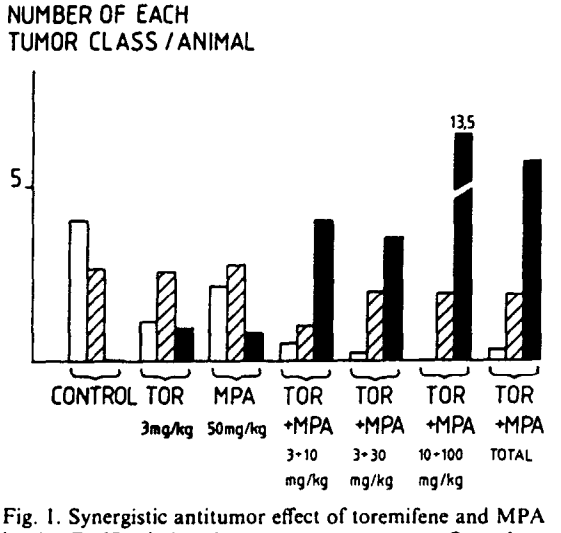

Fig. 1. Synergistic antitumor effect of toremifene and MPA in the DMBA-induced rat mammary cancer. Open bars illustrate the number of actively growing tumors/animal, shadowed bars the number of stable tumors/animal and filled bars the number of regressing or disappeared tumors/animal. The number of animals was 5 in each group.

Statistical significance by χ^2 test:

	P
Control vs TOR (3 mg/kg)	<0.05
Control vs TOR (50 mg/kg)	<0.05
Control vs TOR + MPA (3 + 10 mg/kg)	<0.001
Control vs TOR + MPA (3 + 30 mg/kg)	<0.001
Control vs TOR + MPA (10 + 100 mg/kg)	<0.001

animal) and by χ^2-test (number of tumors in each growth class).

RESULTS

ER and PgR levels as well as the effect of hormones on each clinical sample have been presented in Table 1. The table also includes comment on the interaction of toremifene and MPA. In ER+ ovarian cancers toremifene was effective (cell number <50% of control) in 5 out of 15 cases, in one of these cases cell number was <20% and in no case <10%. The respective figures for MPA were 10, 2 and 1. MPA therefore was more effective than toremifene. This may be due partly to high FCS concentration in the growth medium which diminishes the efficacy of antiestrogens (toremifene and tamoxifen). The combination of toremifene and MPA was effective in all ER+ ovarian cancer samples, in 7 cases the number of living cells was <20% and in 3 cases <10% of control. In the samples of adenocarcinoma of uteri toremifene alone was effective in 1 out of 7 samples, MPA in 4 out of 7 and the combination in all samples. All samples contained measurable amounts of ER. In vulvar cancer toremifene was effective in 2 out of 3 samples, MPA and combination in all samples. One patient (RE in Table 1) was clinically treated with MPA and determinations were carried out before and after the treatment. Before the treatment MPA was effective *in vitro* and after the treatment the sample was resistant to all assayed hormonal manipulations.

Effects of toremifene, MPA, and their combination on DMBA-induced rat mammary cancer have been presented in Table 2 and Fig. 1. The combination was, especially at high doses, very effective and eradicated all tumors in several animals. The efficacy was dependent on the dose of the compounds.

DISCUSSION

It has been demonstrated in the present study that many gynecological tumors contain ER and PgR. Therefore studies of their hormonal manipulations are well justified both on clinical and experimental grounds. The results in Table 1 demonstrate the

Table 1. Effect of toremifene and MPA on gynecological tumors *in vitro*. The given values (means of duplicates) represent the number of living cells as % of control

	ER (fmol/mg prot)	PgR	TOR (1 μmol/l)	MPA (10 μmol/l)	TOR + MPA	Comment on combination
Ovarian cancers (n = 23)						
ER-poor						
HH	0	0	86	115	85	=TOR
AU	0	0	55	38	21	=MPA
HS	0	5	93	104	76	Addit
HL	0	8	34	28	3	Synerg
EK	1	0	93	76	39	Synerg
SL	1	6	14	2	1	=MPA
HA	6	0	50	16	11	=MPA
ER-rich						
AS	12	70	45	21	20	=MPA
MU	13	16	45	21	20	=MPA
LA	28	10	78	51	35	=MPA
VG	31	16	72	58	35	Addit (MPA)
HJ	43	13	73	78	51	Addit
TI	50	16	51	55	19	Synerg
NS	60	222	62	85	58	=TOR
LH	93	42	70	68	54	=MPA
KM	216	54	74	94	80	>TOR
ER-unknown						
SK	?	?	62	38	5	Synerg
KE	?	?	54	78	13	Synerg
SS	?	?	79	48	16	Synerg
HV	?	?	71	42	47	>MPA
HaH	?	?	42	65	29	=TOR
SN	?	?	55	38	21	=MPA
KI	?	?	85	164	90	>TOR
	ER + 9/16	PR + 4/16				
Adenoca. uteri (n = 8)						
SA-L	6	4	71	51	40	=MPA
EH	11	45	72	60	45	=MPA
SA	12	70	45	21	20	=MPA
AS	12	70	83	58	58	=MPA
KE	34	47	93	76	39	Synerg
LM	86	73	58	49	2	Synerg
MH	127	1141	63	47	40	=MPA
NS	?	?	55	38	34	=MPA
	ER + 6/7	PR + 6/7				
Vulvar cancers (n = 3)						
NS	?	?	25	10	4	=MPA
NK	?	?	35	10	13	>MPA
RE, before MPA	?	?	50	5	2	=MPA
RE, after MPA	?	?	95	61	62	=MPA

Table 2. Effect of toremifene (TOR) and MPA on DMBA-induced breast cancer in rats. The animals were treated daily for five weeks with toremifene (p.o.), MPA (i.m.) or their combinations. The number of animals in each treatment group was 5. The statistical significance of change in the tumor number when compared to control has been calculated by Student's t-test

Group	Number of tumors in the beginning	Number of disappeared tumors per animal	Change of tumor number per animal	Statistical significance (P)
Control	63	0	4.4 ± 3.3	
TOR (3 mg/kg)	61	0.54	0.5 ± 1.1	<0.05
MPA (50 mg/kg)	10	0	0.5 ± 0.7	<0.05
TOR + MPA (3 + 10 mg/kg)	19	3.3	−2.5 ± 1.3	<0.001
TOR + MPA (3 + 30 mg/kg)	22	2.0	−1.8 ± 1.5	<0.001
TOR + MPA (10 + 100 mg/kg)	34	8.0	−5.3 ± 3.1	<0.001

varying sensitivity of the tumors to toremifene and MPA—from sensitivity to complete resistance. ER and PgR alone could not predict the response to these agents. These results are in agreement to Goldenberg and Froese[14] with respect to the ER levels and effect of tamoxifen in breast cancer samples *in vitro*.

The predictive testing for breast cancer treatment is generally based on ER and PgR levels. The value of these tests has been criticized as only 50–60% of ER positive tumors and 10% of ER negative tumors respond to tamoxifen treatment [8, 10]. Direct exposure of the tumor cells *in vitro* to antiestrogens and/or progestins could therefore be more valuable predictive assay. Predictive subrenal capsule assay *in vivo*, which presumably predicts effectively the response to cytotoxic treatments, seems not to be suited—at least in the standard version—for testing of less aggressive hormonal therapies [15]. The present study refers to the importance of predictive assays: if the synergistic effect which was seen in a few samples *in vitro* and very clearly *in vivo* is true in humans, the chosen individual patients (albeit few) would greatly benefit from the combination.

The antitumor effect of antiestrogens and progestins is generally assumed to be mediated through specific receptors, although many details are obscure [1, 16, 17]. The measurable cytosolic ER level is strongly decreased by tamoxifen [18, 19] and toremifene [9]. PgR complex is thought to be bound to the sites in chromatin already occupied by estrogen or antiestrogen receptor [20]. MPA down regulates the ER and PgR levels in the cytoplasm [21]. Therefore, in ER-rich tumors which respond to antiestrogens by increasing PgR levels, the combination of antiestrogen and progestin might allow better control of hormone-dependent cancers. Synergistic antitumor effect of estrogen and MPA in DMBA-induced rat mammary cancer has been demonstrated by Huggins *et al.*[22]. It is more difficult to explain in pharmacological terms why all ER- and PgR-rich tumors do not respond to the combination of antiestrogen and progestin. Lack of favorable interaction is however in good agreement with the clinical findings of Mouridsen *et al.*[10] according to which tamoxifen alone is as effective as tamoxifen + MPA in the treatment of breast cancer. Thus it is evident that receptors alone cannot explain all antitumor effects and interactions of antiestrogens and progestins. One reason could be a difference of the structure of ER in malignant and normal breast tissue which has been described by Iqbal *et al.*[23]. Other possible pharmacological mechanisms have also been described such as cAMP linked phosphorylation-dephosphorylation of ER [2, 24]. These phosphorylation reactions could be connected to several hormonal mechanisms, e.g. to alpha adrenergic receptors (as shown in rabbit myometrium [25]), and prostaglandin synthesis [26]. There is no evidence, however, that these mechanisms could have direct effect on cell growth. The relation of oncogene expression, growth factors and hormonal growth regulation [27, 28] is an interesting possibility to further explain the antitumor effects of antiestrogens and progestins. Thus far the interpretation of results has been very difficult.

REFERENCES

1. Clark J. H., Peck, E. J. Jr, and Anderson J. N.: Oestrogen receptors and antagonism of steroid hormone action. *Nature* **251** (1974) 446–448.
2. Bodwin J. S., Hirayama P. H., Rego J. A. and Cho-Chung Y. S.: Regression of hormone dependent mammary tumors in Sprague–Dawley rats as a result of tamoxifen or pharmacological doses of 17-beta-estradiol: cyclic adenosine 3′,5′-monophosphate-mediated events. *J. Natn. Cancer Inst.* **66**, (1981) 321–326.
3. Steward J. F., Hayward J. L., Rubens R. D. and King R. J. B.: Estrogen receptor status of advanced breast cancer immediately before chemotherapy does not predict for response. *Cancer Chemother. Pharmac.* **9** (1982) 124–125.
4. Hamburger A. W. and Salmon S. E.: Primary bioassay of human tumor stem cells. *Science* **197** (1977) 461–463.
5. KSST; Group for sensitivity testing of tumors (KSST): *In vitro* short-term test to determine the resistance of human tumors to chemotherapy. *Cancer* **48** (1977) 2127–2135.
6. Bogden A. E., Costanza M. E., Reich S. D., Griffin T. W. and Cobb W. R.: Chemotherapy responsiveness of human breast tumors in the 6-day subrenal capsule (SRC) assay: an update. *Breast Cancer Res. Treat.* **3** (1983) 33–38.
7. Mäenpää J.: The subrenal capsule assay in predicting responsiveness of gynecological cancers to drug therapy. Academic Dissertation, Ann. Univ. Turkuensis. (Ser D) Medica-Odontologica (1985).
8. Namer M., Lalanne C. and Baulieu E. E.: Increase of progesterone receptor by tamoxifen as a hormonal challenge test in breast cancer. *Cancer Res.* **40** (1980) 1750–1752.

9. Kallio S., Kangas L., Blanco G., Johansson R., Karjalainen A., Perilä M., Piippo I., Sundquist H., Södervall M. and Toivola R.: A new triphenylethylene compound, Fc-1157a. Hormonal effects. *Cancer Chemother. Pharmac.* **17** (1986) 103–108.
10. Mouridsen H. T., Palshof T. and Rose C.: Therapeutic effect of tamoxifen alone versus tamoxifen in combination with gestagen and estrogen in advanced breast cancer. *Rec. Res. Cancer Res.* **71** (1980) 169–177.
11. Drewinko B., Loo T. L., Brown B., Gottlieb J. A. and Freireich E. J.: Combination chemotherapy *in vitro* with adriamycin. Observations of additive, antagonistic, and synergistic effects when used in two-drug combinations on cultured human lymphoma cells. *Cancer Biochem. Biophys.* **1** (1976) 187–195.
12. Korenman S. G. and Dukes B. A.: Specific estrogen binding by the cytoplasm of human breast cancer. *J. Clin. Endocr. Metab.* **30** (1980) 639–645.
13. Kangas L., Nieminen A.-L. and Grönroos M.: Bioluminescence of cellular ATP: a new method for evaluation of cytotoxic agents *in vitro*. *Med. Biol.* **62** (1984) 338–343.
14. Goldenberg G. J. and Froese E. K.: Drug and hormone sensitivity of estrogen receptor-positive and negative human breast cancer cells *in vitro*. *Cancer Res.* **42** (1982) 5147–5151.
15. Mäenpää J., Grönroos M., Leppänen M. and Kangas L.: The subrenal capsule assay in mice: prediction of rat tumor sensitivity to chemotherapy. *Cancer* **54** (1984) 1530–1534.
16. Horwitz K. B. and McGuire W. L.: Nuclear mechanisms of estrogen action: Effects of estradiol and antiestrogens on estrogen receptors and nuclear receptor processing. *J. Biol. Chem.* **253** (1978) 8185–8191.
17. Binart N., Mester J., Baulieu E. E. and Catelli M. G.: Combined effects of progesterone and tamoxifen in the chick oviduct. *Endocrinology* **111** (1982) 7–16.
18. Lippman M. E., Bolan G. and Huff K.: Interactions of antiestrogens with human breast cancers in long-term tissue culture. *Cancer Treat. Rep.* **60** (1976) 1421–1428.
19. Jordan V. C., Dix C. J., Naylor K. E., Prestwich G. and Rowsby L.: Nonsteroidal antiestrogens: their biological effects and potential mechanisms of action. *J. Toxicol. Environ. Hlth* **4** (1978) 363–390.
20. Okulicz W. C., Evans R. W. and Leavitt W. W.: Progesterone regulation of the occupied form of nuclear estrogen receptor. *Science* **213** (1981) 1503–1505.
21. Leavitt W. W., Evans R. W., Okulicz W. C., MacDonald R. G., Hendry W. J. III and Robidoux W. R. Jr: Progesterone inhibition of nuclear estrogen receptor retention. In *Hormone Antagonists* (Edited by M. K. Agarwal), de Gruyter, Berlin (1982) pp. 213–232.
22. Huggins C., Moon R. C. and Morii S.: Extinction of experimental mammary cancer I. Estradiol-17-beta and progesterone. *Proc. Natn. Acad. Sci. U.S.A.* **48** (1962) 379–386.
23. Iqbal M. J., Colletta A. A., Houmayoun-Valayani S. D. and Baum M.: Differences in oestrogen receptors in malignant and normal breast tissue as identified by the binding of a new synthetic progestogen. *Br. J. Cancer* **54** (1986) 447–452.
24. Migliaccio A. and Auricchio F.: Hormone binding of estradiol-17-beta receptor: evidence for its regulation by cytoplasmic phosphorylation and nuclear dephosphorylation. Prevention of dephosphorylation by antiestrogens. *J. Steroid Biochem.* **15** (1981) 369–373.
25. Williams L. T. and Lefkowitz R. J.: Regulation of rabbit myometrial alpha adrenergic receptors by estrogen and progesterone. *J. Clin. Invest.* **60** (1977) 815–818.
26. Fenwick L., Jones R. L., Naylor B., Poyser N. L. and Wilson N. H.: Production of prostaglandins by the pseudopregnant rat uterus, *in vitro*, and the effect of tamoxifen with the identification of 6-keto-prostaglandin F1 as a major product. *Br. J. Pharmac.* **59** (1977) 191–199.
27. Rozengurt E., Sinnett-Smith J. and Taylor-Papadimitriou J.: Production of PDGF-like growth factor by breast cancer cell lines. *Br. J. Cancer* **36** (1985) 247–252,
28. Dickson R. B., McManaway M. E. and Lippman M. E.: Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. *Science* **232** (1986) 1540–1543.