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ADDITIVE AND SYNERGISTIC ANTITUMOR EFFECTS WITH TOREMIFENE AND INTERFERONS

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Summary—MFC-7 cells were exposed to toremifene, human alpha and gamma interferons and combinations of them *in vitro*. Growth of the cells was followed by ATP bioluminescence method. Rats bearing DMBA-induced tumors were treated with toremifene, rat gamma interferon and their combination daily for five weeks. The growth of the tumors was followed by palpation weekly.

Toremifene and interferons inhibited the growth of MCF-7 cells. Interferons alpha and gamma were additive; toremifene and interferons were additive or at the best synergistic.

Toremifene inhibited the growth of DMBA-induced tumors. Rat gamma interferon alone had no clear effect on the tumor growth. Combination of toremifene and gamma interferone was the most effective treatment and did not show any detectable toxicity.

Toremifene and interferons have interesting interactions. Clinical studies using the combination might be warranted.

INTRODUCTION

Interferons belong to the biological response modifiers used in anticancer therapy. They are structurally polypeptides and are produced by vertebrate cells in response to a variety of stimuli. They have complex effects, both direct and immuno-mediated, on tumor growth [1]. In the treatment of breast cancer interferons have interesting indirect effects: they change the steroid metabolism [2] and increase the level of estrogen receptors (ER) in the estrogen target tissue [3, 4]. Tumors could therefore become more responsive to events which are mediated through ER under the influence of interferons. Additive and synergistic antitumor effects of toremifene, a new antiestrogen, and human alpha and gamma interferons *in vitro* against MCF-7 cells have been described by us earlier [5]. The aim of the present study was to extend the earlier studies using both *in vitro* and *in vivo* tests.

MATERIAL AND METHODS

Cell cultures

Drugs. Toremifene was synthesized by Chemical Research Laboratory of Farmos Group (Oulu, Finland). The natural human leukocyte alpha interferon was produced and purified as described earlier [6, 7]. The purification procedure yields two fractions (P-IFA and P-IFB) which have different compositions of alpha interferon subtypes. The fractions were pooled for the preparation used in this study. The preparation contained 6×10^6 IU/ml and had a sp. act. of 3.3×10^6 IU/mg of protein. The natural human gamma interferon was produced and purified by monoclonal antibodies as described previously [8]. It contained 1×10^6 IU/ml and had a sp. act. of 2×10^7 IU/mg of protein. The human interferon preparations did not contain detectable amounts of tumor necrosis factors or interleukin-2 [8, 9].

Rat gamma interferon was prepared from a CHO DHRF$^+$ transformant in which the genomic rat gamma interferon gene was introduced and which constitutively produces this interferon [10]. The interferon was purified by monoclonal antibody affinity chromatography to >99% purity of 4×10^6 units/mg protein.

Cell culture experiments. The MCF-7 cells were kindly provided by Dr Charles McGrath, Michigan Cancer Foundation. The cultivations were carried out in Eagle's MEM (Gibco Europe Ltd, Renfrewshire, Scotland) which was supplemented with 1% of unstripped FCS (KC Biological Inc., Lenexa, U.S.A.), L-glutamine (292 mg/l; Fluka AG, Buchs, Switzerland), gentamycin (10 μg/ml) and insulin (0.6 μg/ml); both from Collaborative Research Inc., Lexington, Mass, U.S.A., sodium pyruvate (111 mg/ml; Merck, Darmstadt, F.R.G.), non-essential amino acids (Gibco), Hepes buffer 25 mM; Sigma, St Louis, Mo., U.S.A.). The cells were repeatedly shown to be free of mycoplasma contamination during the course of the study [11]. The cells were cultured at 37°C and 5% CO_2 in the humidified incubator for 1–11 days.

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Table 1. Effect of toremifene and human interferons (IFN) alpha and gamma on MCF-7 cells *in vitro*. Number of living cells, expressed as ATP-bioluminescence (mV) has been given. Each value is mean ± SD of 4 replicates

		Cultivation time (days) 1	3	7
Control		1.11 ± 0.13	1.45 ± 0.25	11.7 ± 0.86
IFN alpha	500	1.03 ± 0.06	1.50 ± 0.18	8.65 ± 1.16
(IU ml)	1000	1.05 ± 0.17	1.28 ± 0.18	7.58 ± 1.56
	2000	1.15 ± 0.11	1.00 ± 0.12	4.83 ± 0.54
IFN gamma	100	0.96 ± 0.17	1.24 ± 0.19	3.63 ± 1.30
(IU ml)	150	0.84 ± 0.05	1.36 ± 0.24	3.45 ± 0.34
	300	1.00 ± 0.06	1.14 ± 0.09	3.65 ± 0.73
Toremifene	0.1	1.08 ± 0.06	1.43 ± 0.06	5.73 ± 0.39
(μmol/l)	1.0	0.95 ± 0.13	1.35 ± 0.10	1.03 ± 0.10

The cells were plated in 24 well dishes (Nunc, Roskilde, Denmark) in 1.0 ml of the medium. Toremifene (concentrations are given in Table 1 and Fig. 1) was added to the medium in a small volume of ethanol. The concentration of ethanol never exceeded 0.07%. Interferons were diluted in growth medium and added to the wells in concentrations indicated in the tables. At intended times the number of living cells was determined in each well by a simple bioluminescence assay of adenosine triphosphate (ATP) as described by Kangas *et al.*[12]. Shortly: ATP is the basic energy source of the living cells and is in good correlation with the number of living cells. ATP is released from the cells by trichloric acetic acid (TCA). An aliquot of the TCA containing medium is taken to a measuring cuvette which contains ATP Monitoring Reagent (LKB-Wallac, Turku, Finland). The principal component of this is firefly luciferase which changes the chemical energy of ATP into light. The released light is quantitated by luminometer (Model 1250, LKB-Wallac, Turku, Finland).

In vivo experiments

Mammary adenocarcinomas were induced in 48–52-day-old female rats by a single 12 mg p.o. dose of 7,12-dimethyl-[a]-benzanthracene (DMBA). The induction was carried out in a specific isolator (Metall und Plastic GmbH, Radolfzell, F.R.G.). Palpable tumors appeared after about 6 weeks. After this the treatment of the animals was started and continued daily for five weeks. Toremifene at the dose of 3 mg/kg was given per os by stainless steel gavage, rat gamma interferon at the daily dose of 10000 U/rat was administered subcutaneously. The tumors were palpated individually once a week and the tumors were classified into growing, stable, and regressing ones as described in this issue [13]. After the study samples were taken from several tumors for histological and electron microscopical analyses. The results of these analyses will be described elsewhere.

The statistical analysis of additive or synergistic effect was carried out according to Drewinko *et al.*[14]. Shortly; the calculation is based on the surviving fractions (SF) in single and multiple drug treated cultures. In two drug combinations drugs A and B are additive if $SF(A) \times SF(B) = SF(AB)$. Deviation of additivity (D) is obtained from the equation $D = SF(AB) - SF(A) \times SF(B)$. The t-value analogous with Student's t-value at d.f. $= \infty$ is obtained from the following equation:

$$t = \frac{D}{\sqrt{\text{Var } SF(AB) + \text{Var } SF(A) + SF(B)}}$$

In the *in vivo* studies the statistical analysis of the efficacy of the treatments was carried out by χ^2 test using the numbers of tumors in each growth class.

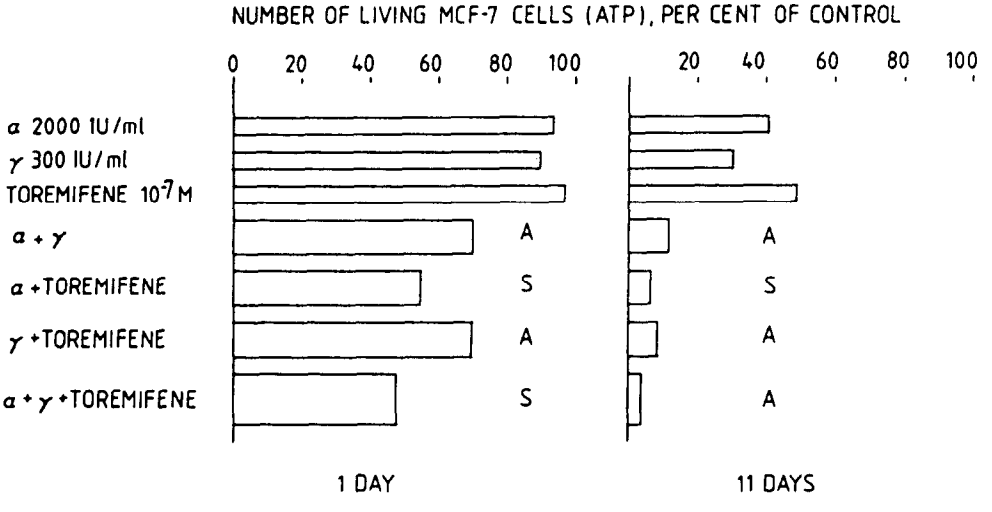

Fig. 1. Effect of toremifene and interferons alpha and gamma on the number of living MCF-7 cells *in vitro* as single agents and in combinations. The cultivation times of the cells were 1 and 11 days. Same concentrations of the agents were used both in single and in combination cultures. A = additive effect, S = synergistic effect. The number of living cells was determined by bioluminometric ATP method, and has been expressed as % of control cells grown without toremifene and interferons. An improved antitumor effect was evident with all combinations.

RESULTS

Effects of human interferons and toremifene as single agents against MCF-7 cells have been shown as a function of cultivation time in Table 1. MCF-7 cells were more susceptible to gamma interferon than to alpha interferon. The efficacy of the agents was also clearly time-dependent: longer exposure increases the efficacy. The antitumor effect of the combinations of toremifene and interferons has been illustrated in Fig. 1 after 1 and 11 days cultivation. Interferons alpha and gamma were additive with each other and addition of toremifene further increased the efficacy.

As shown in Table 2, RIF gamma alone had no significant antitumor effect in DMBA-induced mammary carcinoma. Toremifene had a statistically significant antitumor effect. The combination of RIF gamma and toremifene had even stronger effect, although the difference against toremifene alone did not reach statistical significance. The histological analysis of the tumors after the 5 weeks treatment will be published elsewhere.

The weight gain of all interferon-treated animals, including single and combination treatments, was comparable to control animals receiving saline only. Toremifene slightly decreased the weight gain. In gross necropsies there were no signs of toxic effects with toremifene or interferons.

DISCUSSION

Clinically human interferons have been included in several clinical trials as investigative antitumor compounds. Their activity in breast cancer has been poor [15]. In our experimental breast cancer models both human alpha and gamma interferons were moderately active against MCF-cells *in vitro*, but the rat gamma interferon had no clear effect against DMBA-induced rat mammary cancer *in vivo*. This might refer to direct antitumor effect of interferons *in vitro* at concentrations which perhaps cannot be reached *in vivo* in the tumor tissue. Breast cancer as an indication of interferon treatment is however interesting, because interferons have been shown to increase the levels of estrogen receptors in the estrogen target tissues [3, 4] and to have anti-estrogenic action in breast cancer [16]. The tumors could therefore become more responsive to anti-estrogen therapy. The present study gives a strong support to this suggestion.

Table 2. Effect of toremifene, rat gamma interferon (RIF) and their combination in DMBA-induced rat mammary cancer

Group	Change of tumor number per animal	Classification I	II	III	Ratio growing/ others
Control	2.4 ± 1.8	36	22	8	1.50
RIF (10 000 U/rat)	2.4 ± 1.3	29	26	12	0.76
Toremifene (3 mg/kg)	1.6 ± 1.1	18	21	6	0.67
RIF + Toremifene	0.6 ± 1.0	9	22	8	0.30

Classification: I = number of growing tumors (tumor size increased at least 4-fold during the 5 weeks treatment), II = number of stable tumors, III = number of regressing tumors (disappear or final size less than 1/4 of the initial). Number of animals in each group = 5.

Statistical significance by χ^2 test in the classification:
Control vs RIF N.S.
Control vs Tor $\chi^2 = 4.12$, $v = 1$, $P < 0.05$.
Control vs. RIF + Tor $\chi^2 = 13.0$, $v = 1$, $P < 0.05$.
RIF vs Tor $\chi^2 = 0.12$, $v = 1$, N.S.
RIF vs RIF + Tor $\chi^2 = 4.38$, $v = 1$, $P < 0.05$.
Tor vs RIF + Tor $\chi^2 = 2.74$, $v = 1$, N.S.

We have shown earlier that the combinations of toremifene and human alpha and gamma interferons exert additive or synergistic antitumor effects in MCF-7 cells *in vitro* [5]. The preparation of gamma interferon used in that study contained other lymphokines [9]. The virtually pure gamma interferon in the present study appeared to be somewhat less effective than the impure preparation. However, the pure gamma interferon had also definite growth inhibitory activity in MCF-7 cells and the activity was evidently increased by alpha interferon or toremifene.

Interferons increase the natural killer (NK) cell activity *in vivo*. It has been shown that estrogens suppress the NK cell activity in mice [17]. Almost all known antiestrogens have intrinsic estrogenic effects *in vivo*. One can therefore speculate that antiestrogens may inhibit the indirect effects of interferons. According to preliminary results [18] toremifene does not affect the basal or interferon stimulated NK cell activities in mice. The risk of antagonism in the antitumor effect with toremifene and interferons *in vivo* is therefore minimal.

Fleischmann [19] has shown a synergistic antitumor effect with interferons alpha and gamma *in vitro*. The present study is in accordance with his finding, although our results show only additive antitumor effect. As the side effect of different interferons are slightly different it might be possible to diminish the doses and thereby the side effects of single interferons without loss of antitumor efficacy by combining interferons. Such combinations might therefore be valuable clinically.

The treatment of rats with combination of toremifene and interferon gamma did not cause any signs of toxicity in the present work. Toremifene has not caused serious side effects in clinical trials. It is therefore reasonable to expect that toremifene and interferons could be used safely in combination.

CONCLUSION

Interferons may increase the estrogen receptor levels in breast cancer tissue and thereby change the tumor responsiveness to antiestrogens. As there were no signs of toxicities with the combination of interferon and toremifene, clinical trials with the combination might be warranted.

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