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Serum Protects HeLa Cells from Antiestrogen Effects in Culture

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Abstract—*The effect of nafoxidine and tamoxifen on HeLa cells is reported in this study. The two antiestrogens showed no effect on HeLa cells when grown in the presence of fetal calf serum, the cytotoxic effects were observed only under serum-free conditions. BSA also protected the HeLa cells from antiestrogen effects. The effect of nafoxidine and tamoxifen on different established cell lines and a primary culture of rat skin fibroblasts has been studied.*

INTRODUCTION

THE MECHANISM of non-steroidal antiestrogens is not well understood yet. Different cell populations show varied responses to the antiestrogens [1, 2]. Antiestrogens inhibit the binding of estrogens to their receptors on the cell and the inhibition is reversible and competitive [3]. They also inhibit the synthesis of new estrogen receptors in the uterus [4]. Due to their competitive binding to the estrogen receptors, these compounds have been used as antitumor agents for different types of mammary cancer [5, 6]. Their effects on several other carcinomas like those of colon, rectum, stomach, kidney and bladder and a melanoma have been investigated [7]. Nafoxidine has also been shown to possess antitumor activity against an ascitic hepatoma [8]. Tamoxifen and its hydroxylated metabolite inhibited the proliferation of MCF-7 cells; this inhibition is mediated through the estrogen receptors [9]. At higher concentrations of tamoxifen, the inhibition was not reversed by estrogen.

Antiestrogens cause hypertrophy of luminal cells, the inhibiton of cell proliferation and an increased rate of cell death in rat uterus [10]. In this paper we have tested the effects of tamoxifen and nafoxidine on a human cervical carcinoma cell line, HeLa, *in vitro* under serum-free conditions.

MATERIALS AND METHODS

Materials

Nafoxidine and tamoxifen were gifts from Upjohn Co., U.S.A. and ICI Pharma, France respectively. DMEM was from Himedia, Bombay and FCS from Seralab, U.K. Fibronectin, insulin, transferrin and estradiol-17β were purchased from Sigma, U.S.A.

Cell lines

HeLa, Vero and CHO cells have been obtained from NIV, Pune. Meth A and L-929 cells are from NIH, U.S.A. and SV-40 transformed mouse fibroblast (MKSV) cells are from Villejuif, France. Primary cultures of rat skin fibroblasts (RSF) were prepared in our laboratory after the enzymatic dispersion of neonatal rat skin with collagenase and trypsin. All the above cell lines are regularly maintained in DMEM containing 5% fetal calf serum and penicillin (100 IU/ml) and streptomycin (50 μg/ml) under standard culture conditions.

HeLa cells were also grown under serum-free conditions and the medium contained DMEM, fibronectin (1 μg/ml), insulin (1 μg/ml) and transferrin (100 μg/ml). Under these conditions HeLa cells show the same growth pattern as in the presence of 5% FCS containing medium.

Incorporation of ^{3}H-thymidine

Cells, 5×10^4 cells/ml, were incubated in 24-well Linbro plates (Flow laboratories) in DMEM containing different concentrations of FCS. Cells were treated with ^{3}H-thymidine, 2 μCi/ml (BARC, Bombay) and incubated for 12 hr. After incubation the cells were harvested by trypsinization, washed 3–4 times with 5% TCA, dissolved in Soluene-350 (Packard) and counted in Bray's scintillation fluid.

Treatment with estradiol or antiestrogens

Nafoxidine, tamoxifen and estradiol were dis-

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*Table 1. Effect of antiestrogens on the viability of HeLa cells in culture**

Group	Concentration	Viability (%)†
Control	—	100
Nafoxidine	1 μM	0
Tamoxifen	1 μM	7
Estradiol	10 nM	98
Nafoxidine + Estradiol	1 μM + 10 nM	30
Tamoxifen + Estradiol	1 μM + 10 nM	40
Nafoxidine + FCS	1 μM + 100 μl	90
Nafoxidine + BSA	1 μM + 10 mg	0
Tamoxifen + FCS	1 μM + 100 μl	95
Tamoxifen + BSA	1 μM + 10 mg	97

*5×10^4 HeLa cells were plated in 1 ml of serum-free culture medium; and the cells were treated with antiestrogens for 24 hr. The results shown here are typical of 9 experiments performed with different cultures.
†Percentage viability was scored by trypan blue exclusion method using a hemocytometer.

solved in ethanol and then diluted in the culture medium. Equivalent amounts of ethanol were added to the control cultures as in the treated groups. The final concentration of ethanol was always below 1%. Cells were treated with antiestrogens either at the time of plating or after pretreatment with FCS or BSA. The effect of antiestrogens was studied after 24 hr treatment.

Viability of cells

Cells were treated with 0.1% trypan blue for 1–2 min and viability was scored by the dye exclusion method.

RESULTS AND DISCUSSION

Nafoxidine and tamoxifen did not show any effect on HeLa cells when grown in the presence of FCS. Antiestrogens were cytotoxic to HeLa cells only under serum-free conditions; therefore, conditions were set up to grow HeLa cells in the absence of FCS. Instead of FCS, the Dulbecco's modified essential medium was supplemented with fibronectin, insulin and transferrin, in which the HeLa cells showed the same growth pattern as in the case of 5% FCS.

Table 1 shows the viability of HeLa cells in the presence of nafoxidine and tamoxifen. Nafoxidine and tamoxifen (1 μM) significantly inhibited the viability of HeLa cells after 24 hr treatment. There was a total loss of viability, this could be due to the use of serum-free conditions for the growth of cells or may be due to the detergent effect of antiestrogens which is more pronounced under these conditions. Estradiol (10 nM) partially reversed the effect of antiestrogens when added simultaneously to the culture medium. Higher neutralizing effect by estradiol was observed for tamoxifen-treated cells than in the case of nafoxidine-treated cells. Fetal calf serum could also neutralize the effects of antiestrogens. BSA was ineffective against nafoxidine-treated cells but protected the cells from tamoxifen effects. This could be either due to the general protective effect of high concentrations of proteins present in FCS and BSA or due to the presence of certain bound estrogens or a combination of both the effects. Lack of effect of BSA on nafoxidine-treated cells may be due to the cytotoxic effects of this compound, in addition to its antiestrogenic activity [10]. Similar results were obtained when the HeLa cells were pretreated with FCS or BSA for 2 hr and then treated with the antiestrogens. Recently, tamoxifen has been shown to inhibit the elongation process in the synthesis of eukaryotic proteins [11]. Similar mechanism may be operative here where the action is not mediated through the specific receptors as has been shown in *Xenopus* oocytes or reticulocytes.

*Table 2. Effect of antiestrogens on the viability of different cell lines**

Cell line	% Viability Control	Nafoxidine†	Tamoxifen†
CHO	98	0	100
Vero	96	0	98
HeLa	100	0	0
L-929	100	0	0
Meth A	98	0	0
MKSV	100	0	0
RSF	95	95	96

*All the cell lines were plated in triplicate at a concentration of 10^5 cells/ml.
†Nafoxidine and tamoxifen were used at 1 μM concentration.

Table 2 shows the effect of nafoxidine and tamoxifen on the viability of different established cell lines and a primary culture of rat skin fibroblasts. Nafoxidine was cytotoxic to all the cell lines, whereas the skin fibroblasts were not affected. On the other hand tamoxifen was ineffective against CHO, Vero and the skin fibroblasts.

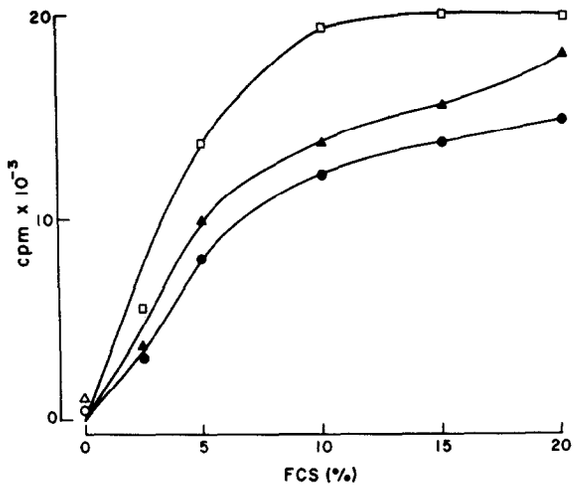

Fig. 1. Effect of different concentrations of FCS on the incorporation of ^{3}H-thymidine by HeLa cells. Control cells (□) grown in serum-free medium and the effect of nafoxidine (○) and tamoxifen (△) on this incorporation. Reversal of nafoxidine (●) and tamoxifen (▲) effects by FCS.

Figure 1 shows the effect of different concentrations of FCS on the incorporation of ^{3}H-thymidine by antiestrogen-treated or untreated HeLa cells. In the absence of FCS, there was significant inhibition of the incorporation of labelled thymidine by nafoxidine and tamoxifen. This inhibition was reversed with the addition of higher concentrations of FCS in the medium.

Antiestrogens have been shown to bind the estrogen receptors and thus compete with the binding of estrogens [12]. They have also been shown to possess specific binding sites on the cell surface [13]. Previous studies have suggested the binding of nafoxidine and tamoxifen to the same site where estradiol binds. In addition to their competition with the estrogen for binding to the receptor, they have also been shown to have cytotoxic effects *in vivo* and *in vitro* which are not reversed by estrogens [9, 10] and are not mediated through the receptors. Similar observations are reported here when the cell viability is partially restored in the presence of 10 nM estradiol (Table 1). Higher concentrations of estradiol did not increase significantly the viability of antiestrogen-treated HeLa cells. These observations suggest the use of serum-free conditions for any studies on the action of antiestrogens on cultured cells.

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REFERENCES

1. Terenius L, Ljungkvist I. Aspects on the mode of action of antiestrogens and antiprogestagens. *Gynec Invest* 1972, **3**, 96–107.
2. Clark JH, Hardin JW, Padykula HA, Cardasis CA. Role of estrogen receptor binding and transcriptional activity in the stimulation of hyperestrogenism and nuclear bodies. *Proc Natl Acad Sci USA* 1978, **75**, 2781–2784.
3. Terenius L. Structure–activity relationships of antiestrogens with regard to interaction with 17-β-estradiol in the mouse uterus and vagina. *Acta Endocr Copenh* 1971, **66**, 431–447.
4. Jordan VC. Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. *Cancer Treat Rep* 1976, **60**, 1409–1419.
5. McGuire WL, Chamness GC, Horwitz KB, Zava DT. Hormones and receptors in breast cancer. In: O'Malley BW, Birnbaumer L, eds. *Receptors and Hormone Action*. New York, Academic Press, 1978, Vol. 2, 401–441.
6. Bodwin JS, Hirayama PH, Rego JA, Cho-Chung YS. Regression of hormone-dependent mammary tumors in Sprague–Dawley rats as a result of tamoxifen and pharmacologic dose of 17-β-estradiol: cyclic adenosine 3′,5′-mono-phosphate-mediated events. *J Natl Cancer Inst* 1981, **66**, 321–326.
7. Leake RE, Laing L, Calman KC, Macbeth FR. Estrogen receptors and antiestrogen therapy in selected human solid tumors. *Cancer Treat Rep* 1980, **64**, 797–799.
8. Khar A. Nafoxidine possesses antitumor activity against an ascitic hepatoma. *Eur J Cancer Clin Oncol* 1986, **22**, 1475–1478.
9. Bardon S, Vignon F, Derocq D, Rochefort H. The antiproliferative effect of tamoxifen in breast cancer cells: mediation by the estrogen receptor. *Mol Cell Endocr* 1984, **35**, 89–96.
10. Martin L. Effects of antiestrogens on cell proliferation in the rodent reproductive tract. In: Sutherland RL, Jordan VC, eds. *Non-steroidal Antiestrogens*. Sydney, Academic Press, 1981, 143–163.
11. Guille MJ, Arnstein HRV. The antioestrogen drug tamoxifen is an elongation inhibitor of eukaryotic protein biosynthesis. *FEBS Lett* 1986, **207**, 121–126.
12. Sutherland RL, Murphy LC. Mechanisms of estrogen antagonism by non-steroidal antiestrogens. *Mol Cell Endocr* 1982, **25**, 5–23.
13. Sutherland RL, Foo NS. Differential binding of antiestrogens by rat uterine and chick oviduct cytosol. *Biochem Biophys Res Commun* 1979, **91**, 183–191.