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Antiestrogens Inhibit the Replication of the Retroviral Moloney Murine Leukemia Virus *In Vitro*

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SUMMARY

Widely used in breast cancer therapy, tamoxifen exhibits *in vitro* and *in vivo* pleiotropic activities that are generally attributed to its binding to the estrogen receptor. However, several reports have shown that the antiestrogen binding site (ABS) is also an intracellular target of the drug. This dual affinity determines at least two modes of action for the triphenylethylenic antiestrogens; one would be estrogen reversible and the other irreversible. Here, tamoxifen is shown to inhibit the production of Moloney murine leukemia virus virions by fibroblastic A9 cells, in which estrogen receptor is not detectable either by binding or by radioimmunoassay. Moreover, a specific ligand of the ABS induces effects equivalent to those of tamoxifen, suggesting that tamoxifen inhibits Moloney murine leukemia virus replication through an estrogen-independent pathway involving the ABS.

The nonsteroidal antiestrogen tamoxifen (Nolvadex) is extensively used as primary or adjuvant therapy in breast cancer. Exhibiting few serious undesirable side effects, tamoxifen is accountable for a real survival benefit (1). Recent clinical investigations have shown that it is valuable in other diseases. It 1) modulates the immune system (2), 2) inhibits cholesterol biosynthesis (3), and 3) suppresses lymphocyte mitogenesis (4). Moreover, it has recently been proposed as an inhibitor of replication of human immunodeficiency virus in lymphocytes (5). Thus, although the mechanism of action of tamoxifen is still a matter of controversy, its potential for clinical application has been expanded. Among the multiple intracellular proteins that are able to bind triphenylethylenic antiestrogens, both the ER and the ABS do so with high affinity (nanomolar K_d). Diphenylmethane compounds (derivatives of *N,N*-diethylphenylmethylphenoxyethanamine) (6) bind to the ABS without interfering with the ER. Thus, they are essential tools for the differentiation of activities mediated by either the ER or the ABS.

In this study, the ability of tamoxifen and ABS-specific ligands to block the expression of M-MuLV in infected mouse A9 fibroblasts (7) was estimated by quantification of the reverse transcriptase activity and immunodetection of M-MuLV capsid protein CAp30 in the culture medium. Results indicate that tamoxifen is able to inhibit M-MuLV virion production by an estrogen-independent pathway.

Materials and Methods

Chemicals. All chemicals were from Sigma; [^{3}H]tamoxifen (87 Ci/mmol) was from Amersham. The ABS ligand was derived from BPE and was synthesized in our laboratory as described previously (8). It was stored at −20° in 1000-fold concentrated ethanol solutions. The compounds used were tamoxifen and pyrolidino-BPE.

Cells and culture conditions. A9 cells (kindly provided by Pr. P. MacIsaac (Medical Biology Institute, La Jolla, CA) and derived from M-MuLV-infected NIH-3T3 cells prepared by the infection method) (7) and NIH-3T3 (control) cells were routinely grown at 37° in 5% CO_2, in Dulbecco's modified Eagle's medium supplemented with 3.7 g of sodium bicarbonate (pH 6.8 at 20°) and 10% fetal calf serum stripped of endogenous hormones. For experiments, cells in logarithmic growth phase were plated in 35-mm Petri dishes (Nunclon Delta) at a density of 5×10^4 cells/dish. On the following day, the medium was changed and drugs stored in ethanol as 1000-fold concentrated solutions were added. Control cultures were grown in 0.1% ethanol. Cell numbers were determined using a Coulter counter (Coultronics).

Binding studies. All procedures were performed at 4°. Cells were harvested by scraping in phosphate-buffered saline and were pelleted at 800 × *g* for 10 min. Homogenization was carried out by sonication in TTE buffer (20 mM Tris·HCl, pH 7.4, 12 mM thioglycerol, 1.5 mM EDTA) for ABS binding and TTM buffer (20 mM Tris·HCl, pH 7.4, 12 mM thioglycerol, 20 mM sodium molybdate) for ER binding. Extraction of nuclear ER was performed for 30 min at 4° by adjusting TTM buffer to 0.4 M KCl.

The homogenate was centrifuged for 1 hr at 105,000 × *g*. Supernatant was kept for ER determination. The pellet was resuspended in TTE

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ABBREVIATIONS: ER, estrogen receptor; ABS, antiestrogen binding site; M-MuLV, Moloney murine leukemia virus; BPE, benzylphenoxyethanamine.

buffer for ABS measurement. Binding studies were performed with 200-μl aliquots (0.1 mg/ml) incubated for 16 hr at 4° with increasing concentrations of [^{3}H]tamoxifen (0.5–10 nM) or 17β-[^{3}H]estradiol (0.25–5 nM). Bound and free ligands were separated on Sephadex LH20 columns (9). Nonspecific binding was determined, as shown previously (6, 8, 9), in the presence of 1 μM concentrations of the corresponding unlabeled ligand. Because tamoxifen is able to bind the ER, the ABS binding experiment was performed in the presence of 1 μM 17β-estradiol. The equilibrium dissociation constant (K_d) was established by saturation binding and Scatchard representation (10).

Virion reverse transcriptase assay. Viral medium was clarified (10,000 × *g* supernatant) and used according to the method of Goff *et al.* (11). Samples were incubated for 2 hr at 37°, in 50 mM Tris·HCl, pH 8.3, 20 mM dithiothreitol, 60 mM NaCl, 0.05% Nonidet P-40, 0.6 mM $MnCl_2$, 0.01 mM dTTP, 10 μg/ml poly(A)-oligo(dT), with 0.5–1 μCi of [^{32}P]dTTP. They were then filtered on 0.45-μm DEAE membranes. After being rinsed twice with 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0, the filters were dried and autoradiographed. Control experiments were performed under the same conditions with 10 units of purified M-MuLV reverse transcriptase (Boehringer Mannheim).

Immunodetection of the CAp30 viral protein. The viral medium was diluted in sample buffer (50 mM Tris·HCl, pH 6.8, 8% glycerol, 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, 6 M urea) and loaded on a 10% tricine slab gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and immunodetected with rabbit anti-p30 serum (12).

Results

Binding studies. As shown in Fig. 1, [^{3}H]tamoxifen bound specifically, with high affinity and in a saturable manner, to microsomal ABS in A9 cells. Scatchard analysis (Fig. 1, *inset*) revealed high affinity (K_d = 0.96 nM) and a binding capacity (B_{max}) of 0.62 pmol/mg of protein for ABS studies. The inhibition constant (K_i = 2.7 nM) for pyrolidino-BPE was determined as described previously (8). Neither specific binding of 17β-[^{3}H]estradiol nor immunoreactive ER (Abbott Laboratories ER-EIA assay) was detected (data not shown). These results suggest that, in A9 cells, tamoxifen preferentially binds to the ABS.

Reverse transcriptase activity in A9 cells. Tamoxifen and pyrolidino-BPE inhibited A9 cell proliferation only after a 48-hr treatment (Fig. 2). This inhibition was reversible up to a drug concentration of 10 μM; withdrawal of either tamoxifen or pyrolidino-BPE after a 48-hr treatment allowed cell proliferation recovery, demonstrating the nontoxic effect of the treatment.

M-MuLV reverse transcriptase activity (standardized to the cell number) was tested in the culture medium of cells treated for different times with 10 μM antiestrogen. Inhibition of M-MuLV replication by tamoxifen and, to a lesser extent, by pyrolidino-BPE could be observed after a 48-hr treatment (Fig. 3). All reverse transcriptase measurements were done at this time because antiestrogens produced a slight reduction in cell number.

Although no detectable estrogen binding was found in A9 cells, to eliminate the possibility of the presence of a low level of ER the ability of estradiol to reverse antiestrogen inhibition of M-MuLV replication was nonetheless determined in cells treated with tamoxifen or pyrolidino-BPE in the presence of 17β-estradiol (20 and 200 nM). As shown in Fig. 4, estradiol was unable to prevent the antiestrogenic effect. The kinetics of the antiestrogen effect on reverse transcriptase activity suggest that these drugs do not interact directly with the enzyme, because they produced inhibitory action only after 24 hr of cell treatment (Fig. 3). At the same concentrations, tamoxifen and pyrolidino-BPE were inactive with commercial reverse transcriptase and with conditioned medium from untreated A9 cells (data not shown).

Western immunoblotting. To confirm that antiestrogen blocks M-MuLV virion release, we analyzed by Western immunoblotting the amount of viral capsid protein present in the medium. Typical results are shown in Fig. 5 for CAp30 protein immunodetection. For an equal number of cells, the percentage

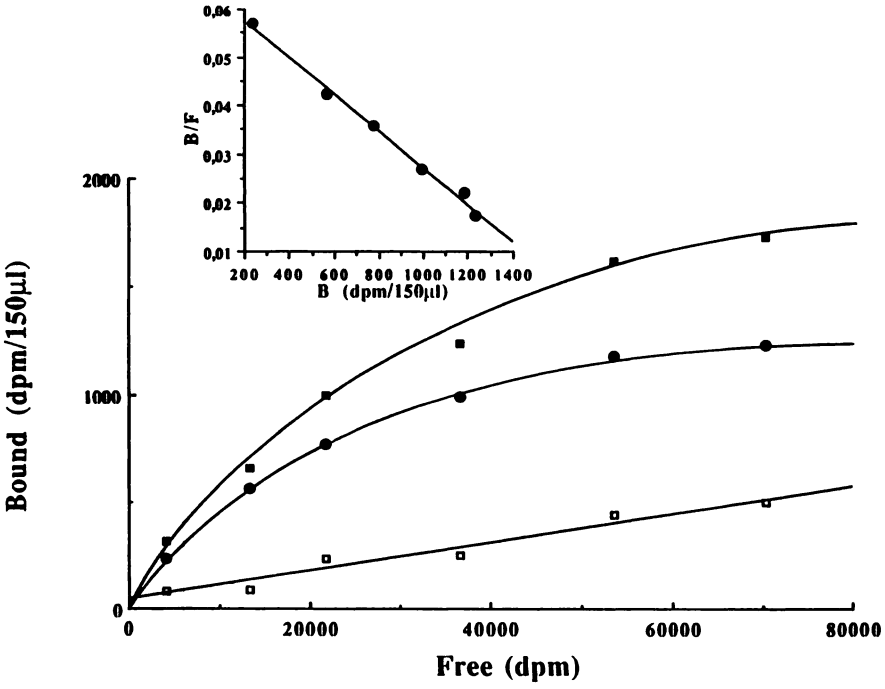

Fig. 1. Scatchard analysis of [^{3}H]tamoxifen binding to membrane-bound ABS. Samples (150 μl) were incubated with increasing concentrations of [^{3}H]tamoxifen in the absence (total binding) (■) or in the presence (nonspecific binding) (□) of 1 μM tamoxifen. Specific binding (●) was estimated from the difference between total and nonspecific binding. *Inset*, Scatchard analysis of specific binding. B, bound; F, Free.

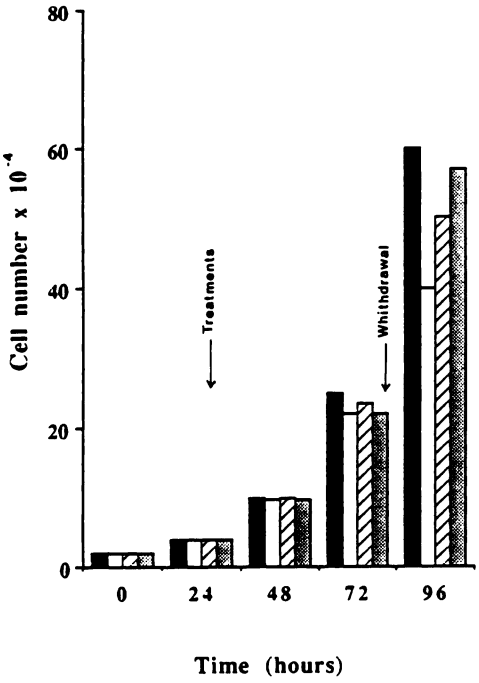

Fig. 2. Time course of the effect of tamoxifen and its reversal on A9 cell growth. Cells were seeded as described in Materials and Methods and treatments were started at 24 hr. ■, A9 cell proliferation under normal conditions; □, tamoxifen treatment; ▨, pyrolidino-BPE treatment; □, growth rescue of cells after drug withdrawal at 72 hr and refeeding with normal culture medium. Results are expressed as mean of three experiments (standard error, <5%).

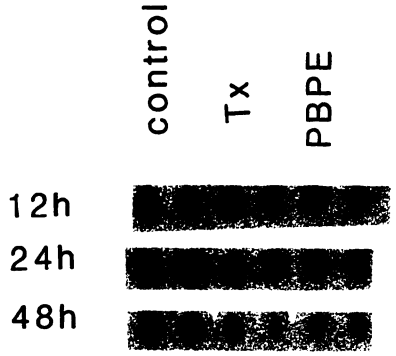

Fig. 3. Time course of the effect of tamoxifen (*Tx*) and pyrolidino-BPE (*PBPE*) on reverse transcriptase activity. Slot blots were prepared in duplicate as described in Materials and Methods, and times after treatments are indicated.

of CAp30 in the culture medium decreased dose-dependently after tamoxifen or pyrolidino-BPE treatment. This activity was detectable from 0.1 μM, a concentration that was apparently without effect on cell proliferation. Moreover, antiestrogens of the steroid family (RU 39411 and ICI 164384), which are known to interact preferentially with the ER, were ineffective on reverse transcriptase and CAp30 protein production (data not shown). These results supported the hypothesis of an inhibition by ABS ligands of viral particle release in the medium.

Discussion

The *in vitro* pleiotropic activities of tamoxifen and its low propensity to generate undesirable side effects *in vivo* led to evaluation of its therapeutic effect against various diseases.

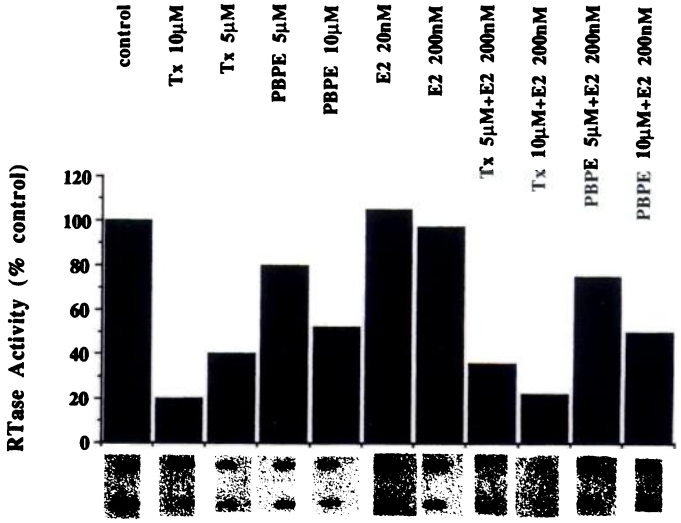

Fig. 4. Effects of increasing concentrations of tamoxifen (*Tx*) and pyrolidino-BPE (*PBPE*) on reverse transcriptase (*RTase*) activity. Estradiol (E2) effect was determined. Treatments were performed for 48 hr; percentages of reverse transcriptase/control (untreated cells) were determined by scanner analysis of slot blots on 0.45 μm DEAE-membranes. Experiments were performed three times in duplicate (two separate cell dishes).

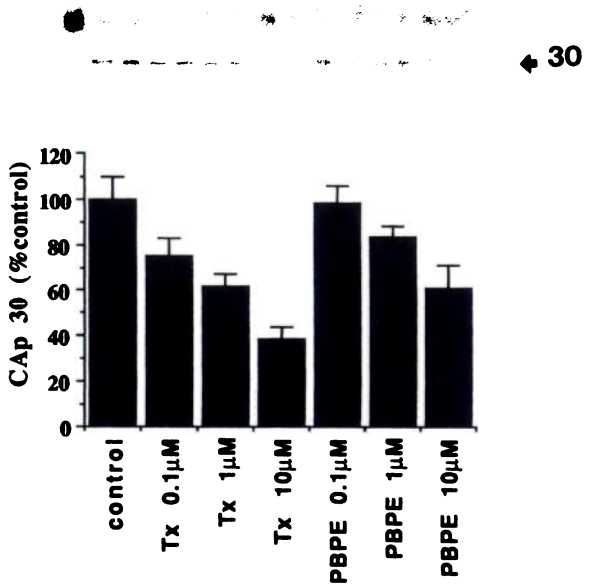

Fig. 5. CAp30 capsid protein variations under antiestrogenic treatments. Scanner analyses of immunodetection of the CAp30 protein were performed on autoradiograms prepared as described in Materials and Methods. *control*, negative control with 3T3 cell culture medium. Each dosage was in duplicate (two separate cell dishes) and the experiment was performed three times. *Tx*, tamoxifen; *PBPE*, pyrolidino-BPE.

Many proteins apart from the ER were also predicted to mediate part of the activity of antiestrogens (6). Thus, we have worked for several years on the ABS primarily demonstrated by Sutherland *et al.* (13).

Laurence *et al.* (5) have reported that interactions of tamoxifen-ER complexes with the estrogen-responsive element carried by the long terminal repeat of the human immunodeficiency virus probably account for the negative regulation of viral replication. However, in other cell lines high tamoxifen concentrations (>10 μM) are known to act via an estrogen-

irreversible pathway (14), and those authors showed neither the presence of the ER nor the reversibility of tamoxifen activity by estrogen. Thus, the hypothesis of another mode of action for tamoxifen cannot be ruled out. To distinguish between ER and ABS pathways, we examined the effect of tamoxifen on the expression of the M-MuLV retrovirus, which has never been reported to contain a canonical estrogen-responsive element in its long terminal repeat, in ER-devoid fibroblastic A9 cells. In agreement with this model, we show that tamoxifen inhibits the secretion of M-MuLV virions and that these effects are not reversed by estradiol. Moreover, pyrolidino-BPE, a specific ligand for ABS without affinity for ER, exhibits the same activity as tamoxifen, demonstrating the involvement of ABS in the antiviral action of antiestrogens. These results suggest that at least some of the effects reported by Laurence *et al.* (5) could be mediated via ABS and that the antiviral effect of tamoxifen might be estrogen independent.

Acknowledgments

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