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Ligands of the Antiestrogen-Binding Site Are Able to Inhibit Virion Production of Human Immunodeficiency Virus 1-Infected Lymphocytes

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SUMMARY

Since the discovery of human immunodeficiency retrovirus, the drug arsenal against retrovirus has rapidly increased. Concomitantly, new challenges in the therapy of acquired immune deficiency syndrome have arisen, including drug toxicities, drug resistance, and the development of various cancers as effective therapies prolong survival. Tamoxifen, a nonsteroidal antiestrogen with a low incidence of side effects, is widely used in cancer therapy; it is known to exert pleiotropic activities by binding essentially to the estrogen receptor and other unidentified proteins. In the present work, quantification of the p24 core protein of human immunodeficiency virus 1 produced by infected lymphocytes shows an inhibitory effect of tamoxifen on virion production. Moreover, we assume that this effect is not mediated by the estrogen receptor because antiestrogen ligands interacting with the antiestrogen-binding site exhibit efficacy related to their affinity for this site, although specific antiestrogens of the estrogen receptor are ineffective.

Although it is known that complex interactions exist between HIV and its host cells (1), CD4 of lymphocytes, ANR, and proteins of the viral genome remain the principal targets of retroviral research (for a review, see Ref. 2). Thus, a variety of small molecules have proved to be effective against HIV replication in several chronically infected cells *in vitro* (3, 4). Laurence *et al.* (4), surmising the very low toxicity of Tx, proposed that this widely used drug, due to its affinity with the estrogen receptor, should be able to inhibit HIV replication via interaction with its long terminal repeats. Tx is the second most widely used antitumoral drug, and the few clinically observed nocive side effects are generally mediated through its binding on the estrogen receptor (5, 6).

We have previously shown that the *in vitro* inhibitory effects of antiestrogens on the retroviral Moloney murine leukemia virus are clearly mediated via the ABS (7) in an estrogen-independent pathway. Specific ligands of ABS that were devoid of estrogenic activity and exhibiting less cytotoxicity than Tx *in vivo* (8)[1] could be useful for antiretroviral research on AIDS.

We investigated the antiviral activities of different antiestrogen species able to bind either to the estrogen receptor or to the ABS and to the Tx ligand of both. To evaluate the potential therapeutics of antiestrogens, we compared the effect of such drugs on virion production by infected human lymphocytes with the activities of AZT, which has been shown to reduce the replication of HIV both *in vitro* and *in vivo*, and of neomycin B, which is able to inhibit the *rev* function and viral production *in vitro* in chronically infected cells (3).

Experimental Procedures

Materials

We purchased lymphocyte separation medium (LSM Ficoll) from Organon; fetal calf serum and natural human IL-2 (10,000 units in 50 ml) from Boehringer Mannheim; penicillin, streptomycin, glutamine, PBS, and RPMI medium from BioWhittaker (Walkersville, MD); phytohemagglutinin from Difco (Detroit, MI); 1,5-dimethyl(1,5-diazodecamethylen)polymetho)bromide from Sigma (St. Louis, MO); Tx and [^{3}H]Tx (84 Ci/mmol) from Amersham (Arlington Heights, IL);

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[1] J.-C. Faye and F. Bayard, unpublished observations.

ABBREVIATIONS: HIV, human immunodeficiency virus; AZT, 3′-azido-2′,3′-dideoxythymidine; PBPE, pyrolidino-benzylphenoxyethanamine; PBMC, peripheral blood mononuclear cells; $TCID_{50}$, tissue culture 50% infective dose; Tx, tamoxifen; E2, estradiol; IL, interleukin; ABS, antiestrogen-binding site; AIDS, acquired immune deficiency syndrome; PBS, phosphate-buffered saline.

and AZT and neomycin B from Sigma. All other chemicals were obtained from Sigma. RU 58668 was a generous gift from Roussel Uclaf (Romainville, France). The specific ABS ligand PBPE was synthesized in our laboratory as described previously (9). The ligands were stored at −20° in 1000-fold concentrated ethanol solutions.

Methods

Preparation of healthy human PBMC. Blood obtained from healthy donors was diluted with PBS (v/v) and loaded on LSM Ficoll (v/v). A ring of PBMC was recovered through centrifugation at 400 × *g* for 30 min and washed twice with PBS. Viable cells were counted with a hemocytometer.

Basic medium (RPMI, 20% fetal calf serum, 1% glutamine, 1% penicillin/streptomycin) was added to the lymphocyte pellet to obtain a concentration of ~2 million cells/ml.

Phytohemagglutinin activation. Lymphocyte stimulation was obtained by adding phytohemagglutinin at 5 μg/ml to the lymphocyte suspension for 48 hr at 37° in a 5% CO_2 atmosphere. PBMC were then washed with PBS and resuspended in a complete medium [basic medium with added IL-2, 10% (v/v)].

HIV infection of the lymphocytes. The infecting stock LAI, with a titer of 7000 $TCID_{50}$, was used as the source of infecting virus. Virus titers (expressed as $TCID_{50}$) were calculated according to the method of Reed and Muench (10). Briefly, samples of serial 10-fold dilutions (in quadruplicate) of the infecting stock LAI were used to infect healthy, previously stimulated lymphocytes at 1000, 100, and 10 $TCID_{50}$.

Infection was carried out for 2 hr at 37°. Infected cells were then pelleted (400 × *g*, 10 min) to remove the supernatant containing the nonabsorbed lymphocyte virions, washed with PBS, and resuspended in complete medium.

Treatment of infected lymphocytes. To treat the infected lymphocytes with different drugs, we plated 10^6 PBMC (0.5 ml)/well onto 96-well flat-bottomed plates (Falcon Plastics, Oxnard, CA). The supernatant was removed and substituted with 0.5 ml of complete medium supplemented with 1,5-dimethyl(1,5-diazodecamethylen)-polymetho)bromide at 2 μg/ml to injure the lymphocyte membranes.

Infected and noninfected cells were treated with Tx; PBPE at 1, 10, and 50 × 10^{-6} M; and E2 at 10^{-8} M, with neomycin B at 100 × 10^{-6} M and AZT at 1.5 × 10^{-6} M dissolved in complete medium with 20% IL-2 for 10 days. The culture media were removed on days 5, 7, and 10. The supernatants were conserved at −80° for enzyme immunoassay quantification of the p24 core protein. Uninfected lymphocytes were used as a negative control, as were lymphocytes treated with medium with ethanol at 1:1000. Drug cytotoxicity was determined with the use of Trypan blue staining. For each treatment and each measurement, live cells were concomitantly numbered after 10 days of treatment.

p24 assay. Viral capsid protein p24 production was quantified in 200 μl of the culture supernatant at an absorbance of 450 nm with

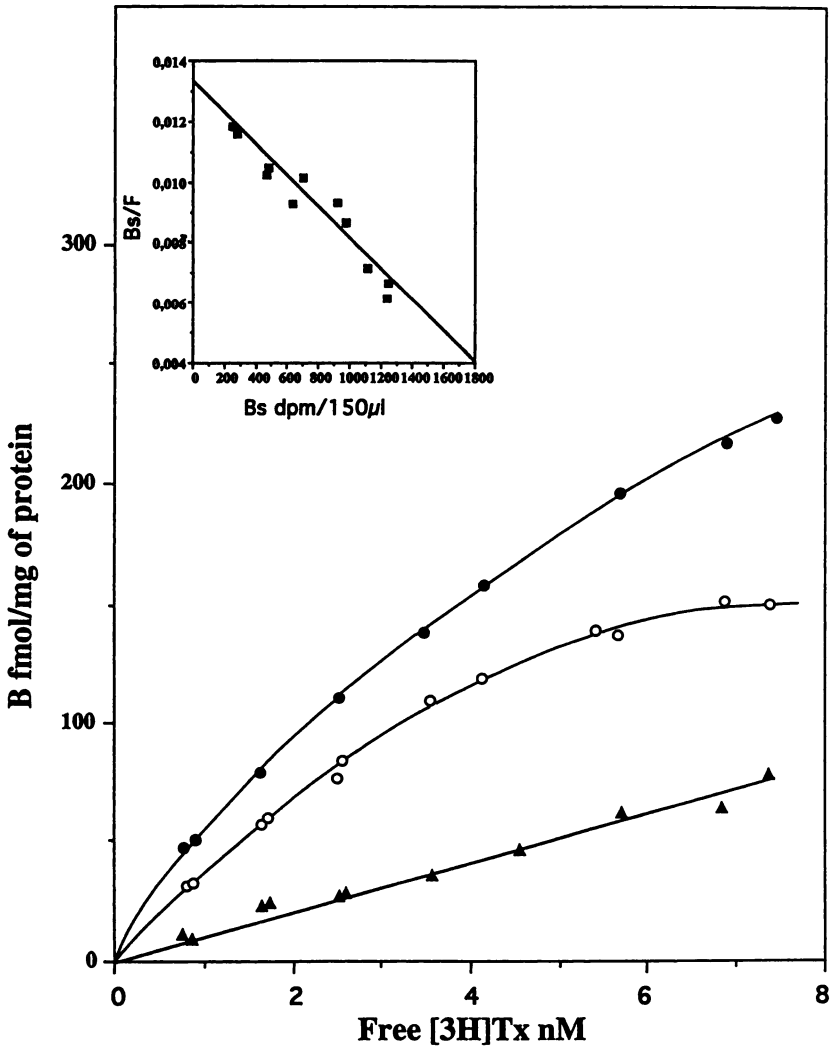

Fig. 1. Scatchard analysis of [^{3}H]Tx binding to membrane-bound ABS. Samples were incubated with increasing concentrations of [^{3}H]Tx in the absence (total binding) (●) or presence (nonspecific binding) (▲) of 10^{-6} M unlabeled Tx. Specific binding (○) was calculated from the difference between total and nonspecific binding. K_D was determined by computer analysis from the saturation curve. *Bs*, bound specific; *F*, free. The experiment was performed three times as described in Experimental Procedures, and data are expressed as the mean values of three experiments carried out in triplicate (standard error = <5%).

the use of Coulter HIV-1 p24 antigen assay (Coulter Products, Inc., Buffalo, NY).

ABS studies. All procedures were performed at 4°. PBMC (150 million) were pelleted through centrifugation at 1500 rpm for 15 min and washed with PBS. Homogenization was carried out through sonication in TTE buffer, pH 7.4 (50 mM Tris·HCl, 1.5 mM EDTA, 12 mM thioglycerol). The homogenate was centrifuged at 10,000 × *g* for 20 min, and ABS measurements were performed on the supernatant as described previously (11). Protein concentration was evaluated according to the method of Bradford (12).

Binding studies were carried out on 200-μl samples with 0.3 mg protein/ml incubated for 18 hr at 4° with increasing concentrations of [^{3}H]Tx (1–10 nM). Nonspecific bound ligand was evaluated through the addition of 100-fold excess of unlabeled Tx. Bound and free ligands were separated on a Sephadex LH20 column (Pharmacia, Piscataway, NJ). Experiments were performed in the presence of 1 μM E2 to saturate the estrogen receptor to which Tx could also bind. The equilibrium dissociation constant (K_D) was determined at saturation binding with the EBDA Ligand computer program (13).

Results

Binding studies. The first step of our study was to verify the presence in the lymphocytes of the two proteins (estrogen receptor and ABS) known for their high affinity for Tx. Although we were not able to determine the concentration of the estrogen receptor through either binding or immunoreactivity analyses, the mRNA coding for this protein was detected with the use of reverse-transcription polymerase chain reaction studies (data not shown). On the other hand, the only available method of determining the presence of ABS in a given cell type is to carry out binding studies with [^{3}H]Tx. In the presence of E2 (10^{-6} M) to saturate the potential estrogen receptor, Tx binding (Fig. 1) analyzed with the EBDA Ligand computer program showed a high affinity ($K_D = 8 \times 10^{-9}$ M) for a microsomal protein classified as ABS based on its pharmacological profile [i.e., binding of PBPE ($K_i = 8 \times 10^{-9}$ M), no affinity for E2, or no affinity for RU 58668, a specific antagonist of the estrogen receptor (14)].

Cell proliferation. After phytohemagglutinin activation of 10^6 lymphocytes and treatment with IL-2, viable cells were counted with a hemocytometer at different times. Variations in lymphocyte proliferation were observed depending on the batch of PBMC used in the experiment. However, in all experiments with $\leq 10^{-5}$ M, none of the antiestrogens exhibited an inhibitory effect at 10 days, whereas at 5×10^{-5} M Tx, a dramatic cytotoxic activity was demonstrated from 5 days on (Fig. 2), even though PBPE exerted a inhibitory effect at the same concentration as the PBMC proliferated slowly. In further experiments, live cells were always numbered after 10 days of treatments. Each antiestrogen checked showed significant inhibition of the proliferation compared with untreated PBMC at $\leq 10^{-5}$ M. The viral load did not produce any effect on cell proliferation (data not shown).

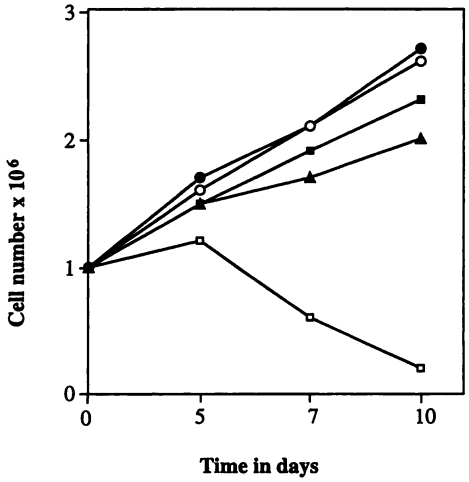

Fig. 2. Time course of the effect of Tx and PBPE on PBMC. After phytohemagglutinin activation, cells were seeded at 10^6 cells/dish and treated for various times with control medium containing ethanol (1:1000) (●), 10^{-5} M Tx (■), 5×10^{-5} M Tx (□), 10^{-5} M PBPE (○), or 5×10^{-5} M PBPE (▲). Data are from one standard experiment carried out in quadruplicate.

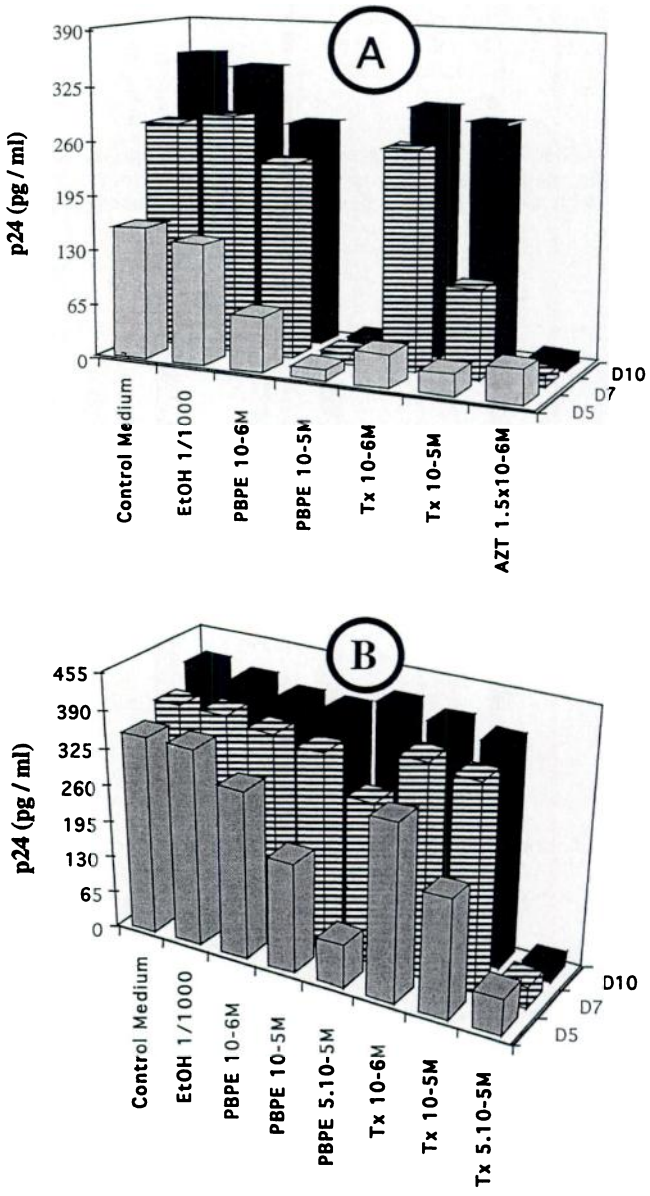

Fig. 3. Time course variation in p24 capsid protein production with dose-dependent treatment with antiestrogens. A, $TCID_{50}$ = 10. B, $TCID_{50}$ = 100. p24 concentrations were determined with the use of enzyme-linked immunosorbent assays. Data are from one of seven experiments carried out in quadruplicate. Intra-assay variations are <5%. *EtOH*, ethyl alcohol; *D5*, *D7*, and *D10*, days 5, 7, and 10, respectively.

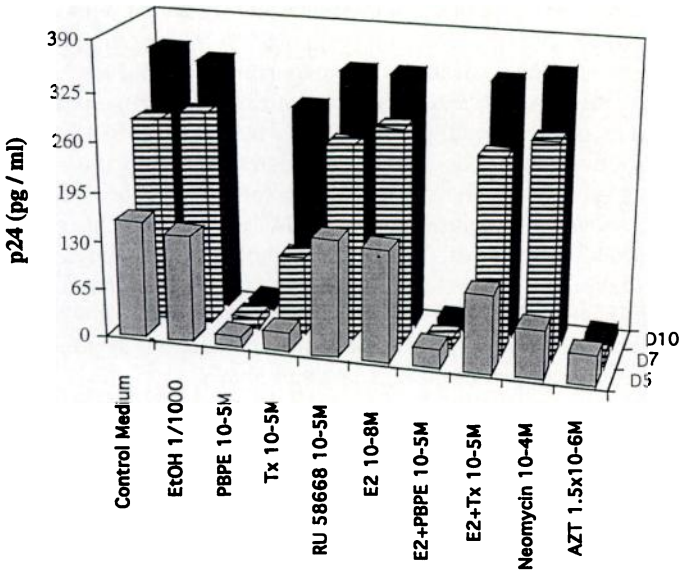

Fig. 4. Effect of various drugs on p24 capsid protein production. Data are from one of seven experiments carried out in quadruplicate. *EtOH*, ethyl alcohol; *D5*, *D7*, and *D10*, days 5, 7, and 10, respectively.

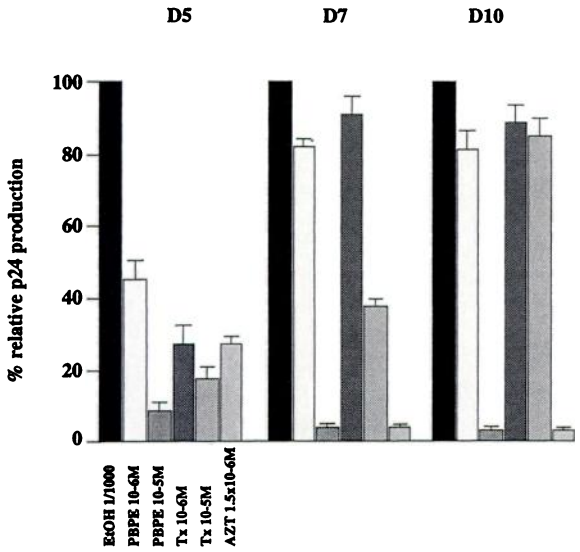

Fig. 5. Activities of various molecules on p24 production as a percentage of the p24 concentrations exhibited with ethyl alcohol (*EtOH*) (1:1000) control, 10^{-6} M PBPE, 10^{-5} M PBPE, 10^{-6} M Tx, 10^{-5} M Tx, and 1.5×10^{-6} M AZT at 5-, 7-, and 10-day treatments. Data are representative of >10 independent experiments. *D5*, *D7*, and *D10*, days 5, 7, and 10, respectively.

Effect of antiestrogens on production of the capsid p24 protein. Up to 5 days of treatment, 10^{-6} M was the lowest ligand concentration for which the Tx and the PBPE showed a significant inhibitory effect with 10 $TCID_{50}$ infection. This activity was, however, very low at 7 days and not visible at 10 days, and no effect could be measured at a higher infection titer (Fig. 3). In the absence of an antiproliferative effect at 10^{-5} M, Tx and PBPE are inhibitors of p24 protein production, and PBPE is more effective after 5, 7, and 10 days of treatment than Tx for both 10 and 100 $TCID_{50}$ (Fig. 3).

Mediators of the antiviral effect of antiestrogens. Another series of experiments was carried out to determine the respective roles of the estrogen receptor and ABS on Tx activity. RU 58668, an antiestrogen in the steroid family that interacts with the estrogen receptor, appeared ineffective in controlling p24 protein production (Fig. 4) regardless of the time and viral load. E2, which is not itself active, remained inactive on PBPE but was able to partially reverse the inhibitory activity of Tx. The reversing effect of E2 could account for the lower efficiency of Tx compared with PBPE because Tx can exhibit estrogenic effects (15) to counteract its inhibitory activity.

Control cells with AZT showed higher activity, and neomycin B, which was active on transfected cells (3), was much less active than were the two ligands of ABS.

Discussion

Although Tx is the second most widely used anticancer drug in the world, the elucidation of its mechanism of action remains a matter of considerable debate. Its clinical use, which initially increased because of the few nocive side effects, is now sustained by its broad-spectrum tumoral activity. As early as 1990, Laurence *et al.* (4), working with cells chronically infected with HIV-1, proposed that Tx be evaluated as a possible adjunct in the therapy of chronic, asymptomatic HIV infection. We used normal infected human lymphocytes to show that although Tx is able to inhibit virion production, PBPE, another molecule of the ABS ligand family, exhibits at least the same viral down-regulation, and such activity is not reversed by E2. The fact that RU 58668 has a potent effect on estrogen-dependent mammary tumors but no effect on down-regulation of the virus confirms that Tx activity is mediated by proteins other than by the estrogen receptor. In this regard, it may be of interest that mifepristone (RU 486), a chemical analogue of RU 58668, is able to inhibit HIV production by interfering with the activity of the *vpr* gene via a glucocorticoid receptor (16). Although we have not defined its precise molecular mechanism of action, we have shown that such activity is not due to direct inhibition of reverse transcriptase. PBPE is a member of a family of drugs used clinically to produce hypocholesterolemia. Its toxicity is extremely low, and, unlike Tx, it has no undesirable estrogenic effect. Under our experimental conditions, PBPE was completely effective if the viral load was 10 $TCID_{50}$, whereas at 100 $TCID_{50}$, its effect on the p24 protein production depended on the PBMC sources. In all cases, however, its relative effect with regard to untreated cells was significant (Fig. 5). This means that it cannot be used alone for the clinical treatment of AIDS but might have a use in the treatment of asymptomatic seropositive individuals and/or as an adjunct with other antiretroviral drugs.

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References

1. Mitsuya, H., R. Yarchoan, and S. Broder. Molecular targets for AIDS therapy. *Science (Washington D. C.)* **249**:1533–1544 (1990).
2. Mitsuya, H., R. Yarchoan, S. Kageyama, and S. Broder. Targeted therapy of human immunodeficiency virus-related disease. *FASEB J.* **5**:2369–2981 (1991).
3. Zapp, M. L., S. Stern, and M. R. Green. Small molecules that selectively

block RNA binding of HIV-1 Rev protein inhibit Rev function and viral production. *Cell* **74**:969–978 (1993).
4. Laurence, J., H. Cooke, and S. K. Sikder. Effect of tamoxifen on regulation of viral replication and human immunodeficiency virus (HIV) long terminal repeat directed transcription in cells chronically infected with HIV-1. *Blood* **75**:696–703 (1990).
5. Marshall, E. The politics of breast cancer. *Science (Washington D. C.)* **264**:1524–1527 (1994).
6. Fornander, T., L. E. Rutqvist, B. Cedermark, U. Glas, A. Mattson, C. Silfverswärd, L. Skoog, A. Somell, T. Theve, N. Wilking, J. Askergren, and M.-L. Hjarlmar. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* **1**:117–120 (1989).
7. Chailleux, C., F. Mesange, F. Bayard, A.-C. Prats, and J.-C. Faye. Antiestrogens inhibit the replication of the retroviral Moloney murine leukemia virus *in vitro*. *Mol. Pharmacol.* **44**:324–327 (1993).
8. Brandes, L. J., K. J. Simons, S. P. Bracken, and R. C. Warrington. Results of a clinical trial in humans with refractory cancer of the intracellular histamine antagonist, N,N-diethyl-2-[4-(phenylmethyl) phenoxy] ethanamine-HCl, in combination with various single antineoplastic agents. *J. Clin. Oncol.* **12**:1281–1290 (1994).
9. Fargin, A., F. Bayard, J. C. Faye, M. Traore, M. Poirot, A. Klaebe, and J. J. Perie. Further evidence for a biological role of antiestrogen binding sites in mediating the growth inhibitory action of diphenylmethane derivatives. *Chem. Biol. Interact.* **66**:101–109 (1988).
10. Reed, L. J., and H. Muench. A simple method of estimation fifty percent endpoints. *Am. J. Hyg.* **27**:493–497 (1938).
11. Poirot, M., M. Garnier, F. Bayard, I. Riviere, M. Traore, M. Wilson, A. Fargin, and J.-C. Faye. The antiproliferative properties of 4-benzyl phenoxy ethanamine derivatives are mediated by the antiestrogen-binding site (ABS), whereas the anti-estrogenic effects of trifluopromazine are not. *Biochem. Pharmacol.* **40**:425–429 (1990).
12. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**:248–254 (1976).
13. McPherson, G. A. Analysis of binding experiments: a collection of computer programs for the IBM PC. *J. Pharmacol. Methods* **14**:213–228 (1985).
14. Van de Velde, P., F. Nique, F. Bouchoux, J. Bremaud, M. C. Hameau, D. Lucas, C. Moratille, S. Viet, D. Philibert, and G. Teutsh. RU 58 668, a new pure antiestrogen inducing a regression of human mammary carcinoma implanted in nude mice. *J. Steroid. Biochem. Mol. Biol.* **48**:187–196 (1994).
15. Legros, N., G. Leclerc, and R. McCague. Effect of estrogenic and antiestrogenic triphenylethylene derivatives and progesterone and estrogen receptors levels of MCF-7 cells. *Biochem. Pharmacol.* **42**:1837–1841 (1991).
16. Refaeli, G., D. N. Levy, and D. B. Weiner. The glucocorticoid receptor type II complex is a target of HIV-1 *vpr* gene product. *Proc. Natl. Acad. Sci. USA* **92**:3621–3625 (1995).

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