Biochemical Pharmacology, Vol. 40, No. 3, pp. 425–429, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

THE ANTI-PROLIFERATIVE PROPERTIES OF 4-BENZYLPHENOXY ETHANAMINE DERIVATIVES ARE MEDIATED BY THE ANTI-ESTROGEN BINDING SITE (ABS), WHEREAS THE ANTI-ESTROGENIC EFFECTS OF TRIFLUOPROMAZINE ARE NOT

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(*Received* 19 *October* 1989; *accepted* 2 *February* 1990)

Abstract—We compared the anti-proliferative properties of 4-benzylphenoxy-*N* ethyl morpholine (morpho-BPE) and trifluopromazine (TFP) on both the human breast cancer cell lines, MCF_7, and its tamoxifen-resistant variant RTx_6. We found that the calmodulin antagonist trifluopromazine (TFP) which bound ABS weakly, inhibited MCF_7 cell growth but did not follow the relationship observed for diphenylmethane derivatives between MCF_7-inhibitory potencies and their K_i. Regarding the tamoxifen-resistant RTx_6 cells, TFP but not morpho-BPE induced inhibition of the proliferation. Using a tritiated derivative of morpho-BPE, two distinct binding sites could be demonstrated. Indeed, a low affinity binding site was present in both cell lines whereas a high affinity binding site was mainly found in MCF_7 cells although being in lower concentration (<10%) in RTx_6 cells. Both tamoxifen and TFP displaced morpho-BPE from the two binding sites. The uptake and efflux of the tritiated drug were similar in the two cell lines. The drug did not appear to be metabolized. We concluded that TFP and morpho-BPE belong to distinct classes of molecules and that ABS mediates the anti-proliferative action of diphenylmethane derivatives but not the inhibitory effect of the calmodulin antagonist TFP.

Triphenylethylene anti-estrogens have been reported to inhibit the proliferation of mammary cancer cells *in vitro*. Moreover, one of these compounds has been currently used in the treatment of more than a quarter of a million breast cancers per year over the past decade [1]. In addition, recent clinical investigations have shown that tamoxifen (NolvadexND) is valuable in post-menopausal patients irrespective of estradiol-receptor status [2]. The mechanism of action of triphenylethylene anti-estrogens is thus a matter of debate. Although these molecules are able to compete with estradiol (E_2) on its own receptor (ER§) [3–5] there is evidence to suggest that they may act through an estrogen-independent process also implicated in the regulation of cell proliferation. Specific intracellular anti-estrogen binding sites, distinct from ER, have been described [6]. They include calmodulin [7], and a specific high affinity binding site [8–10]: the anti-estrogen binding site (ABS). Diphenylmethane derivatives have no affinity for ER and compete with Tx on ABS; they have been found to inhibit the growth of the human breast cancer MCF_7 cell line [11, 12]. Their growth inhibitory potency was found to correlate well with their abilities to compete with [^{3}H]tamoxifen binding and was not reversed by estradiol [12]. ABS predominantly localized in microsomal membranes was previously solubilized and its molecular mass was estimated to be 110,000 [13]. In the present study, using tritiated morpho-BPE, we measured the affinity and the number of ABS binding sites on the post-mitochondrial subfraction of both MCF_7 cells and RTx_6, a tamoxifen resistant clone variant of MCF_7 cell line [10]. The uptake, efflux and metabolism of this drug were also determined. The growth inhibitory activity of morpho-BPE was compared to that of the calmodulin inhibitor trifluopromazine (TFP).

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§ Abbreviations: ER, estradiol receptor, ABS, anti-estrogen binding site; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; morpho-BPE, 4-benzylphenoxy-*N* ethyl morpholine; Tx, tamoxifen; TFP, trifluopromazine; PMF, post-mitochondrial fraction; K_d, equilibrium dissociation constant; K_i, inhibition constant of competing ligand.

MATERIALS AND METHODS

Chemicals

[^{3}H]Morpho-BPE (sp. act 2.2 Ci/mmol) was synthesized by the method of Poling *et al.* [14]; catalytic ^{3}H/H exchange was performed on the benzyl group (CEA, Saclay). The radiochemical and chemical purity of the labeled and unlabeled compounds was > 99% (HPLC and TLC). Trifluopromazine (TFP), reagent grade, was purchased from the Sigma Chemical Co. (Poole, U.K.).

Cells and culture conditions

MCF_7 cells were a gift from Dr Rich (Michigan Cancer Foundation, Detroit) and RTx_6 cells were a tamoxifen-resistant clone variant of MCF_7 obtained in our laboratory [10]. The cells were routinely grown

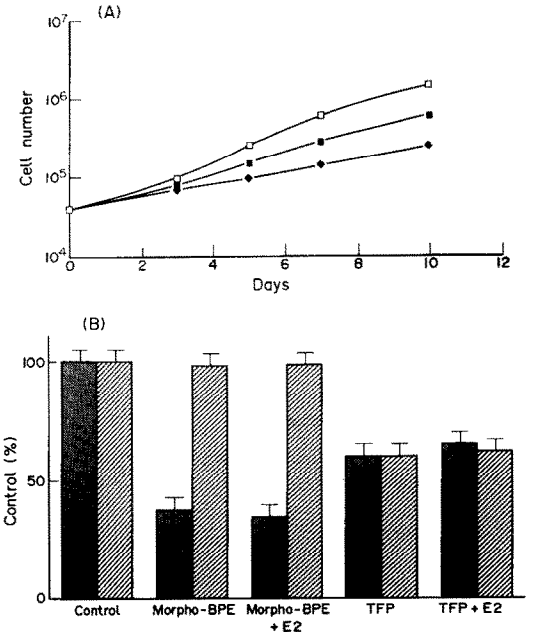

Fig. 1. (A) Time course of effect of (■) TFP (10^{-5} M) and (◆) morpho-BPE (10^{-5} M) on MCF_7 cell growth, (□) control is performed in absence of inhibitor. Each point is the mean of three individual determinations with <10% variation. (B) Inhibitory action of TFP (10^{-5} M) and morpho-BPE (10^{-5} M) on MCF7 (■) and RTx_6 (▨) in the absence or presence of E_2 (10^{-8} M). Results are expressed as the percentage difference between control and treated cells vs control cells (mean of nine determinations in three different experiments with a coefficient of variation < 10%).

at 37° in 5% CO_2 in 75 cm^2 Nunclon plastic flasks (Nunc) in RPMI 1640 medium supplemented with 2 g of sodium bicarbonate per liter, 2 mM glutamine, human insulin 0.08 I.U./mL (pH 7.4 at 23°) and 4% fetal calf serum stripped of endogenous hormones. The RTx_6 clone has remained resistant to tamoxifen for more than 10 years with the same karyotype as that of MCF_7 cells. For binding studies, tamoxifen was withdrawn from the culture medium of RTx_6 at least for 3 weeks before experiments started.

Cell proliferation studies

Cells in the logarithmic growth phase were plated in 35-mm Petri dishes (Nunclon) at a density of $\approx 4 \times 10^4$ cells/dish. On the following day, the medium was changed, and drugs stored in ethanol were added in the medium. Control cultures were grown in 0.1% ethanol. The medium was changed every 2 days. The inhibitory effects of the diphenylmethane derivatives have been previously analysed by detailed concentration studies [12]. A correlation between their inhibitory potencies and their K_i has been established at 10^{-5} M. Time course studies of growth inhibition were determined after a 5-day incubation period with 10^{-5} M morpho-BPE or 10^{-5} M TFP. Cell numbers were determined using a Coulter counter (Coultronics).

Uptake of [^{3}H]morpho-BPE by intact cells

Drug influx. Cells (3×10^6) were plated in 175 cm^2 Nunclon plastic flasks a week before the experiments. The incubation was started by adding [^{3}H]morpho-BPE (final concentration 7.5×10^{-9} M) to the 30 mL culture medium. The same experiment was performed by adjusting the drug concentration to 10^{-5} M with unlabeled morpho-BPE. Cellular and extracellular [^{3}H]morpho-BPE were separated by removing the culture medium; cells were washed twice with saline phosphate buffer, then lysed with 5 mL 1 N NaOH at the indicated times. An aliquot of the lysate was saved for radioactivity counting.

Drug efflux. Cells were incubated for 30 min in the presence of [^{3}H]morpho-BPE under the same conditions as for the drug influx studies. The culture medium was then removed and replaced by fresh medium. The released and intracellular radioactivities were determined after 0, 6, 18, 24 or 48 hr.

Metabolism. Cells were incubated for various times in the presence of [^{3}H]morpho-BPE, washed and lysed as described above. Ten mL of the culture medium and 3 mL of the cell lysate were treated with 40 and 15 mL of ethanol, respectively, vortexed and cooled for 2 hr at −20°. The precipitated proteins were pelleted at 12,000 *g* and the supernatant removed and evaporated under nitrogen. The dried pellet was then solubilized in 50 mM ammonium carbonate buffer (pH 9.5), and extracted twice with ethyl ether. At this pH, at least 98% of the radioactive material is in the amino form and is recovered in the ether phase. The solvent was dried under nitrogen, and the products were solubilized in acetonitrile/ammonium acetate 50 mM pH 5 (50/50). Using this same buffer for elution, the extracts were analysed by HPLC on a C_{18} reverse phase system at a flow rate of 1.5 mL/min. From each fraction collected (900 μL), 500-μL aliquots were saved for radioactivity counting.

Binding studies

Cells were harvested by scraping in PBS and then pelleted at 800 *g* for 10 min. Homogenization was carried out by sonication in a buffer containing 1 mM EDTA, 20 mM Tris–HCl, pH 7.4. The homogenate was centrifuged at 12,000 *g* for 20 min and the remaining supernatant, the so called post-mitochondrial fraction (PMF), was kept for binding studies. Bound (B) and unbound (U) morpho-BPE or tamoxifen were measured after incubation of 200 μL of PMF (1 mg protein/mL) with appropriate dried ligand for 16–20 hr at 4°. B and U were separated by the charcoal-dextran procedure [15]. Measurements on microsomal CHAPS-extracts were carried out as described elsewhere [13]. Because tamoxifen is able to bind ER, all binding experiments were performed in presence of 5 μM 17β-estradiol.

Scatchard analysis

PMF from MCF_7 and RTx_6 cells were incubated with increasing concentrations of [^{3}H]morpho-BPE (0.5 nM–0.5 μM) in the absence or presence of 10 μM of either tamoxifen or unlabeled morpho-BPE. Saturable components of the binding were obtained from the difference between total and non-specific binding corrected by the Rosenthal method [16].

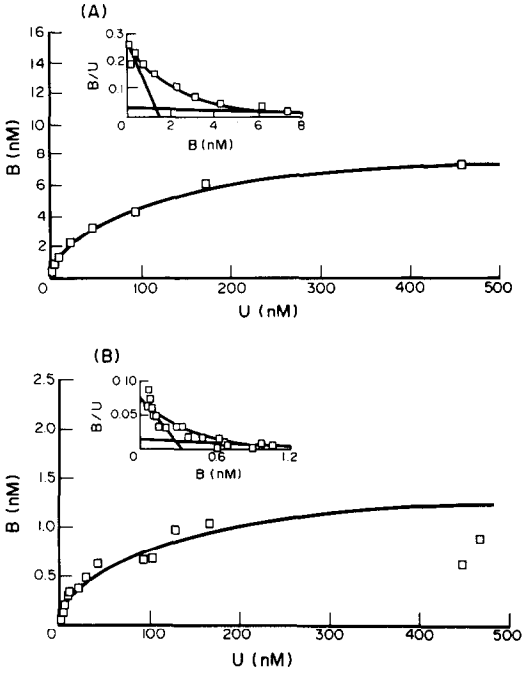

Fig. 2. Scatchard analysis of [^{3}H]morpho-BPE. (A) MCF_7. (B) RTx_6. Results are the mean of four individual experiments carried out in triplicate with a coefficient of variation < 5%.

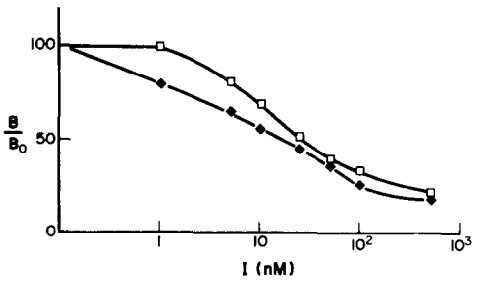

Fig. 3. Competition of tamoxifen (◆) and morpho-BPE (□) for high affinity binding site of [^{3}H]morpho-BPE (10 nM). Results are the mean of two individual experiments carried out in triplicate with a coefficient of variation < 5%.

Competition studies

The ability of ligands to compete with [^{3}H]tamoxifen binding to ABS was determined by incubating two concentrations (1–3 nM) of [^{3}H]tamoxifen for 16 hr at 4° with increasing concentrations of unlabeled ligand ranging from 2.5 to 1000 nM. The K_i values were determined from Dixon plots [17]. Similarly the ability of tamoxifen to compete with [^{3}H]morpho-BPE was also determined.

RESULTS

Although both morpho-BPE and TFP inhibited the growth of MCF_7 cells, only TFP was active on RTX_6 cell proliferation (Fig. 1). Addition of 17β-estradiol did not modify any of these inhibitory activities. TFP at 10^{-5} M induced a 42% growth inhibition for a K_i of 120 nM. From a previous study [12], a 10% inhibition would have been expected for such a K_i if the anti-proliferative effect was ABS-mediated. These results indicate that the actions of morpho-BPE and TFP are probably not mediated through the same mechanism. They suggest that TFP belongs to a different class of molecules that do not act through an ABS interaction for their MCF_7-inhibitory properties.

Scatchard analysis

[^{3}H]Morpho-BPE displays affinity for at least two distinct types of binding sites (Fig. 2), being displaced by the unlabeled ligands, tamoxifen and TFP (data not shown). The high affinity binding site is about 10 times more abundant in MCF_7 than in RTx_6 cells. Its binding parameters $K_d = 5.4 \pm 1.7$ nM, $B_{max} = 1.5 \pm 0.6$ pmol/mg protein for MCF_7 cells (Fig. 2A); $K_d = 4.7 \pm 2.0$ nM, $B_{max} = 0.15 \pm 0.1$ pmol/mg protein for RTx_6 cells (Fig. 2B), correspond to those previously determined using [^{3}H]tamoxifen. Moreover, the high affinity (Fig. 3) and low affinity (data not shown) binding sites are both competable by tamoxifen whereas they are not by 17β-estradiol, progesterone, diethylstilbestrol, dihydrotestosterone and dexamethasone, up to a concentration of 10^{-5} M. Consequently, these results suggest the identify between this newly described site and ABS.

The low affinity binding site ($K_d = 450 \pm 75$ nM, $B_{max} = 10 \pm 3$ pmol/mg protein) is present to an equal extent in the two cell lines and is still to be characterized.

[^{3}H]Morpho-BPE uptake

Using 7.5×10^{-9} M of tritiated morpho-BPE maximum uptake was reached after 30 min and remained constant for 48 hr (Fig. 4A). Addition of increasing concentration (up to 10^{-5} M) of unlabeled ligand did not modify the uptake kinetic. This process appeared non-saturable and [^{3}H]morpho-BPE uptake at 30 min was identical for both cell lines (Fig. 4B).

Drug efflux

MCF_7 and RTx_6 cells were preincubated in the presence of 7.5×10^{-9} M [^{3}H]morpho-BPE for 30 min and the intracellular radioactivity was determined for the following 48 hr. Figure 5 shows that there was a similar decrease in [^{3}H]morpho-BPE concentration in the two cell populations, demonstrating that the RTx_6 resistance was not attributable to a more rapid loss of the drug in these cells.

Drug metabolism

After incubation of MCF_7 and RTx_6 cells in presence of [^{3}H]morpho-BPE for 0, 30 min, 6, 24 and 48 hr, cell and medium radioactivities were extracted as described in Materials and Methods, and were then analysed by HPLC. A single peak of radioactivity was observed, corresponding to non metabolized morpho-BPE with a retention time of 14.5 min. The same elution profile of radioactivity

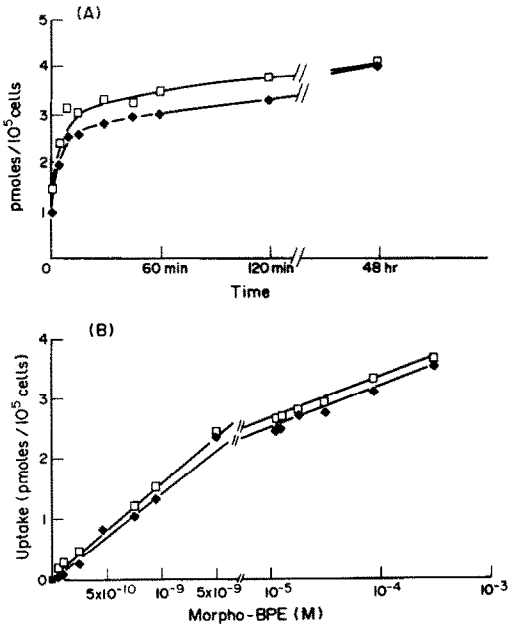

Fig. 4. MCF_7 (□) and RTx_6 (◆) uptake of [^{3}H]morpho-BPE. (A) The tritiated ligand is 7.5×10^{-9} M in the culture medium of both cell lines, the uptake of radioactivity is estimated at various times. (B) Cells are incubated in the presence of increasing concentrations of the tritiated molecules for 30 min as in (A). The radioactivity incorporated in cells is determined after washing as described in Materials and Methods.

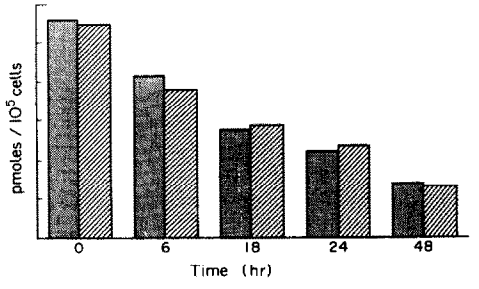

Fig. 5. Drug efflux; the two cell lines, MCF_7 (■) and RTx_6 (▨) are incubated in the presence of [^{3}H]morpho-BPE 7.5×10^{-9} M for 30 min, then the culture media are replaced by non-radioactive fresh media, and the radioactivity remaining in cells is estimated after various times.

was obtained at each treatment time for both cell and medium extracts of the two cell lines. The recovery determined in each condition was 85 ± 10% for cell extracts and 75 ± 10% for medium. The incubation time had no influence on these recoveries, moreover, the same recoveries were obtained when measurements were performed with medium alone incubated with [^{3}H]morpho-BPE. Non specific complexation of the ligand with proteins, peptides and lipids could account for the loss of 25 ± 10% of the radioactivity. This study clearly shows that the drug metabolism was the same for both cell lines and cannot account for the resistance of RTx_6.

DISCUSSION

Although the anti-estrogen tamoxifen is a competitive inhibitor of estrogens on their own receptor, part of its action could also be mediated by another protein with high affinity for this drug which has been designated as ABS. This protein has been identified as a protein of the calmodulin family by various laboratories [7, 18]. We show here that the growth inhibition induced by the calmodulin inhibitor TFP is not mainly mediated through ABS. This was demonstrated by its inhibitory action on RTx_6 cell proliferation and the discrepancy between its affinity for ABS and its growth inhibitory effect. Using [^{3}H]morpho-BPE which displays no affinity for the estrogen receptor, we have shown that the uptake, efflux and metabolism of this drug are identical for the MCF_7 and RTx_6 cell lines. These results strongly suggest that, in our conditions, ABS does not substantially interfere with the pharmacokinetic of the drug in MCF_7, and thus do not support that ABS could behave as a storage protein [19]. Performing Scatchard analysis, with either [^{3}H]morpho-BPE or [^{3}H]tamoxifen, we confirmed our previous results relating that ABS is present at different concentrations in the two cell lines [10]. Moreover, the two ligands are able to fully displace each other on their binding site. However, a second binding site demonstrating affinity for both morpho-BPE and Tx was observed in equal amounts in both cell lines. On the other hand, we have observed in a preliminary study that morpho-BPE treatment, decreases the cellular concentration of estrogen receptor and induces cell morphological changes. Both of these modifications may be relevant to an advanced step in cellular differentiation. We report in this paper that the interaction of morpho-BPE only with ABS is strongly correlated to its growth-inhibitory action on MCF_7 cell growth. On the contrary, TFP exerts its anti-proliferative activity through a mechanism independent of its interaction with ABS. This result favors the contribution of ABS in the mechanism of action of tamoxifen, a compound widely used for the endocrine treatment of breast cancer.

Acknowledgements—We thank Mrs F. Delassus and G. Tatinian for technical assistance. This work was supported by INSERM and a grant from U.P.S. M.P. is supported by a fellowship from the Ligue Française contre le Cancer.

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