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SHORT COMMUNICATION

Modifications of Benzylphenoxy Ethanamine Antiestrogen Molecules: Influence Affinity for Antiestrogen Binding Site (AEBS) and Cell Cytotoxicity

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ABSTRACT. The antiestrogen binding site (AEBS) is a membranous protein complex that has been shown to be intimately linked with the antiproliferative and antiretroviral effects of certain antiestrogenic compounds such as tamoxifen (Tx). Various specific ligands of AEBS derived from benzylphenoxy ethanamine and a new benzoyl structure were synthesized either by modification of the aminoether side chain or by halogen substitution at the *meta-*, *ortho-*, and *para* position on the benzoyl group. Using the MCF-7 cellular strain and its RTx6 variant (a clone selected for its antigrowth resistance to tamoxifen), it was shown that under high drug concentrations the cytotoxicity of the ligands was directly correlated with their affinity for AEBS. In agreement with previous observations made on triphenylethylenic ligands, modification of the basic ethanamine side chain modulated the ligand affinities. Chloride in *meta* increased ligand efficacy, whereas chloride substitution in *ortho* and *para* decreased it. Effects on AEBS-positive MCF-7 cells were drug concentration- and time-dependent, whereas they were unspecific on the AEBS-negative RTx6 cell line. These cytotoxic effects were confirmed in the absence of estrogen receptor on human AEBS-positive uterine cervix cell carcinoma HeLa cells, but were non-specific on rat fibroblastic AEBS-negative (low concentration) NRK cells. The cytotoxicities of these ligands are related to their affinities for AEBS. BIOCHEM PHARMACOL **57**;6:657–661, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. AEBS; tamoxifen; diphenylmethane; benzophenone; cytotoxicity; cell

The antiestrogen tamoxifen has few known noxious side effects and has become the world's second most widely used antitumoral drug. Initially designed as an antagonist of the estradiol steroidal hormone on the ER,† the existence of alternate ER-independent mechanisms of action was first suggested by various clinical observations [1, 2]. Tamoxifen is known to affect many biological events more or less directly involved in its antiproliferative effects. Numerous biochemical parameters have subsequently been considered as molecular mediators of such antiestrogen activities. Tamoxifen is also suspected of producing adducts on DNA and proteins [3–5]. Its most well-known target proteins include protein kinase C [6], calmodulin [7], histamine [8], and prolactin receptors [9], their interactions with this particular ligand being of low affinity ($K_d > 10^{-6}$ M). Several groups [10, 11] including ours [12] noted the ubiquitous presence of an AEBS that specifically binds triphenylethylenic antiestrogens such as tamoxifen with high affinity ($K_d = 10^{-9}$ M) and focused attention on the potential existence of a receptor (different from ER) able to mediate the biological activities of tamoxifen [13, 14]. To differentiate the effects mediated via ER from those mediated via AEBS, and following the first publication on diphenylmethane derivative [15], we synthesized molecules which interacted with AEBS but were unable to bind ER [16, 17]. These molecules have recently been shown to synergize the antitumoral activity of cisplatin on human ovarian cancer [18] and melanoma [19]. One of these ligands had previously been used to identify AEBS [17, 20]. In the present work the affinities of these molecules were evaluated with regard to tritiated tamoxifen on the microsomal subfraction of mammary cancer MCF-7 cells. Verifications of their cytotoxic potentialities on the MCF-7 cell line and its variant, selected for its growth resistance to tamoxifen and named RTx6, was then carried out [16]. We have previously shown that this tamoxifen resistance could be associated with a low AEBS concentration in these cells [14], RTx6 cells remaining sensitive to pure estradiol antagonists [21]. The ER independence of this cytotoxicity

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† *Abbreviations:* ER, estrogen receptor; AEBS, antiestrogen binding site; PMF, post mitochondrial fraction; RBA, relative binding affinity; and Tx, tamoxifen.

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was confirmed on ER-negative cell lines exhibiting either high (HeLa) or negligible (NRK) AEBS concentrations.

MATERIALS AND METHODS

Chemicals

17β-Estradiol was from Steraloid, [^{3}H]tamoxifen (81Ci/mmol) from Amersham, and unlabeled tamoxifen was a gift from Imperial Chemical Industries; AEBS ligands were synthesized in our laboratory and their synthesis will be published elsewhere. Their chemical purity was higher than 95%, assessed by elementary analysis, and routinely checked by TLC. Stock solutions were prepared in ethanol at concentrations 1000 times the working concentration, and stored at −20°.

Cell Culture

Cells were routinely grown in Falcon plastic flasks (75 cm^2) in a humidified incubator at 37° in 5% CO_2-enriched atmosphere. The human breast cancer cell line, MCF-7, and its variant RTx6 were cultured in RPMI 1640 medium (Seromed) as previously described [14]. The human cervical cancer cell line HeLa and the fibroblastic normal rat kidney NRK were cultured in Dulbecco's Modified Eagle's medium (Seromed) supplemented with 5% fetal bovine serum.

AEBS Quantitation

AEBS were labeled as previously described using cell microsomal membrane and [^{3}H] tamoxifen in the presence of 1 μM of 17β-estradiol to mask residual estrogen receptors. Assays were performed in 50 mM Tris–HCl, 1 mM EDTA, 12 mM thioglycerol pH 7.4 for 18 hr at 4° in a volume of 200 μL with 80 μg microsomal proteins and 3 nM radioligand. Non-specific binding was carried out with 2 μM tamoxifen. Assays were terminated by loading 150 μL of the incubate through a 3.5-mL Sephadex LH-20 column equilibrated with 50 mM Tris–HCl, 1 mM EDTA, 12 mM thioglycerol pH 7.4. Elution was performed by adding 1 mL of 50 mM Tris–HCl, 1 mM EDTA, 12 mM thioglycerol pH 7.4. The eluates were collected and counted in a Ready Safe cocktail (Beckman).

Competitive Binding Studies

The cells were harvested by scraping in PBS and then pelleted at 800 *g* for 10 min. All procedures were performed at 4° as previously described [16]. The cells were then homogenized 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 supernatant PMF was kept for the binding studies. As previously described [22], a subcellular fractionation using CHAPS (3-[(3-cholamidopropyl) dimethylammonio] propane-1-sulphonic acid) detergent, was carried out to obtain solubilized microsomal protein. Competitive binding assays were conducted in the presence of 1 μM estradiol to eliminate the [^{3}H]tamoxifen interaction with the ER. The RBAs were calculated by using : RBA = $100 \times IC_{[tam]50}/IC_{[comp]50}$ where $IC_{[tam]50}$ is the concentration of radioinert tamoxifen and $IC_{[comp]50}$ the concentration of competitor inhibiting 50% of the [^{3}H]Tx binding.

Cytotoxicity Assay

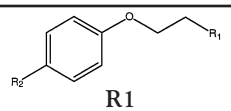
Cells growing in exponential phase were plated at a density of 10^5 into triplicate Petri dishes (9.6 cm^2). The medium was changed the following day and increasing concentrations of drugs added for increasing times of treatment. At the indicated times, the cells were washed three times with PBS to remove dead cells and the number of surviving cells determined with a Coulter counter (Coultronics) hemocytometer.

RESULTS AND DISCUSSION

We first quantitated AEBS in different tumoral cell lines. In MCF-7 cells, we measured 1.5 ± 0.1 pmol/mg of proteins, and a k_D = 5.4 ± 1.7 nM, while in Hela cells the B_{max} (maximum binding) was 1.1 ± 0.1 pmol/mg of proteins and the k_D = 5.2 nM. Hela cells expressed 27% fewer AEBS than the MCF-7 cells, and Hela cells did not express estrogen receptors, unlike MCF-7 cells. In RTx-6, the B_{max} = 0.1 ± 0.1 pmol/protein with a k_D = 4.7 ± 2.0. In NRK, the B_{max} was 0.1 ± 0.1 pmol/mg of protein and the k_D = 110 ± 23 nM. Because of its low affinity for tamoxifen, this binding site might be different from AEBS.

The ability of various benzylphenoxy ethanamines to compete with [^{3}H]Tx for binding to PMF AEBS is shown in Table 1. As for the triphenylethylene derivatives, the structure of the side chain terminal amino group seems to play an important role in determining competition efficiency [23]. The order of binding affinity as determined by the terminal amino group structure was -c-NC_4H_8 (pyrrolidino) > -c-$N(CH_2CH_2)O$ (morpholino) > -c-$N(CH_2CH_2)CH_2$ (piperidino) > $N(CH_3)_2$ > $N(C_2H_5)_2$. Only the RBA of the morpholino and pyrolidino derivatives was inverted when compared with the order found in triphenylethylene derivatives [23]. The most efficient ethanamine side chain was in subsequent structural modification. When the RBAs were measured on solubilized proteins in CHAPS buffer, their order was not modified and the values found were not significatively different from those of PMF samples (Table 1). Replacement of the benzylic group by a benzoyl induced the lost of affinity for AEBS observed in the PMF samples (Table 1). However, these molecules were still able to compete with the [^{3}H]Tx on the protein solubilized fraction (Table 1). In this series, the ethanamine basic side chain had the same effect on RBA as in the benzylic series (data not shown). As these molecules are potential photoprobes for solubilized AEBS, halogens were introduced into the benzoyl group to modify

TABLE 1. RBA of various diphenylmethane and benzophenonic compounds on AEBS



Compounds	R1	R2	RBA/Tx (%) PMF	RBA/Tx (%) Solubilized
Tamoxifen			100	100
DeBPE	$-N(C_2H_5)_2$	$C_6H_5CH_2$	10	16
DmBPE	$-N(CH_3)_2$	$C_6H_5CH_2$	18	23
PipBPE	$-N(C_2H_4)_2CH_2$	$C_6H_5CH_2$	46	45
MBPE	$-N(C_2H_4)_2O$	$C_6H_5CH_2$	100	95
PBPE	$-N(C_2H_4)_2$	$C_6H_5CH_2$	300	370
PBoPE	$-N(C_2H_4)_2$	C_6H_5CO	0	20
4-ClPBoPE	$-N(C_2H_4)_2$	$4Cl\text{-}C_6H_4CO$	0	0
2-ClPBoPE	$-N(C_2H_4)_2$	$2Cl\text{-}C_6H_4CO$	0	1
3-ClPBoPE	$-N(C_2H_4)_2$	$3Cl\text{-}C_6H_4CO$	0	28
3,5-ClPBoPE	$-N(C_2H_4)_2$	$3,5Cl\text{-}C_6H_3CO$	0	20
4-BrPBoPE	$-N(C_2H_4)_2$	$4Br\text{-}C_6H_4CO$	0	0
2-BrPBoPE	$-N(C_2H_4)_2$	$2Br\text{-}C_6H_4CO$	0	3
3-BrPBoPE	$-N(C_2H_4)_2$	$3Br\text{-}C_6H_4CO$	0	7
4-IPBoPE	$-N(C_2H_4)_2$	$4I\text{-}C_6H_4CO$	0	0

Solubilized: detergent extract of PMF. Tamoxifen: [Z]-1-[dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene; DeBPE: diethyl{2-[4-benzylphenoxy]ethyl}amine; DmBPE: dimethyl{2-[4-benzylphenoxy]ethyl}amine; PipBPE: 1-benzyl-4(2-piperidinylethoxy)benzene; MBPE: 4(2-morpholin-4yl-ethoxy)-1-benzylbenzene; PBPE: 1-benzyl-4(2-pyrrolidinylethoxy)benzene; PBoPE: phenyl 4-(2-pyrrolidinylethoxy)phenyl ketone; 4-ClPBoPE: 4chlorophenyl 4-(2-pyrrolidinylethoxy)phenylketone; 2-ClPBoPE: 2chlorophenyl 4-(2-pyrrolidinylethoxy)phenylketone; 3-ClPBoPE: 3phenyl 4-(2-pyrrolidinyl ethoxy)phenylketone; 3,5-ClPBoPE: 3,5dichlorophenyl 4-(2-pyrrolidinylethoxy)phenylketone; 4-BrPBoPE: 4bromophenyl 4-(2-pyrrolidinylethoxy)phenylketone; 2-BrPBoPE: 2bromophenyl 4-(2-pyrrolidinylethoxy)phenylketone; 3-BrPBoPE: 3bromophenyl 4-(2-pyrrolidinylethoxy) phenylketone; 4-IPBoPE: 4iodophenyl 4-(2-pyrrolidinylethoxy)phenylketone.

the RBAs. Chloride was the most effective, either increasing the RBA in *meta* or decreasing it in *ortho*, while substitutions in *para* inhibited the competitive effect irrespective of whether Cl, Br, or I was used.

AEBS has been shown to be involved in cellular growth and cell cytoxicity [19, 24, 25]. To define the cytotoxic effect more specifically, we evaluated the effectiveness of various AEBS ligands on 4 different cell lines containing high or low AEBS concentrations. A time-course study of the exposure of cells to high concentrations of ligand was conducted up to 24 hr. MCF-7 and RTx6 were compared using compounds listed in Table 2. As shown in this table, MCF-7 cell death occurs rapidly. With PBPE after 2 hr, 30% of the cells died at a concentration of 10^{-4} M and more than 90% at 1.5×10^{-4} M. The lethal doses giving 50% cell kill (LD_{50}) were determined at different times of treatment and are reported in Table 2. Obviously, the cytotoxic effect of AEBS ligands on MCF-7 was time- and drug concentration-dependent; conversely, in RTx6 this effect occurred independently of the treatment time over a narrow range between 10^{-4} M (no toxicity) and 1.5×10^{-4}M (more than 80% of dead cells), and under these conditions no reliable LD_{50} could be determined.

Because the PBoPE are distinguished by their differences in RBA between microsomal and solubilized AEBS, the PBoPE molecules exibited little or no cytotoxicity at the concentrations checked. The carbonyl function, by decreasing the hydrophobicity of these compounds, counteracted their amphiphilic properties as well as their membranous and cellular captation, whereas the bindings on solubilized AEBS remained and were modulated by halogen substitution. The cytotoxicity of these products obviously depended mainly on their amphiphilic structure.

Another series of experiments on ER-negative cell lines was carried out after only 24 hr of treatment using AEBS-positive HeLa cells and AEBS-negative NRK cells. Cyto-

TABLE 2. Cytotoxic activity of diphenylmethane derivatives against tumoral cell lines

	LD_{50} (μM)						
	MCF-7 (RE+, AEBS+)				Hela (RE−, AEBS+)	RTx-6 (RE+, AEBS−)	NRK (RE−, AEBS−)
Compounds	2 hr	5 hr	10 hr	24 hr	24 hr	24 hr	24 hr
Tamoxifen	150	100	25	10	180	NM	>500
DeBPE	300	270	210	160	240	NM	>500
DmBPE	300	260	200	140	200	NM	>500
PipBPE	260	220	180	97	210	NM	>500
MBPE	300	230	172	88	170	NM	>500
PBPE	250	180	92	72	130	NM	>500

LD_{50} values are means of 3 determinations.

NM = not measurable. See the legend to Table 1 for chemical structures of compounds.

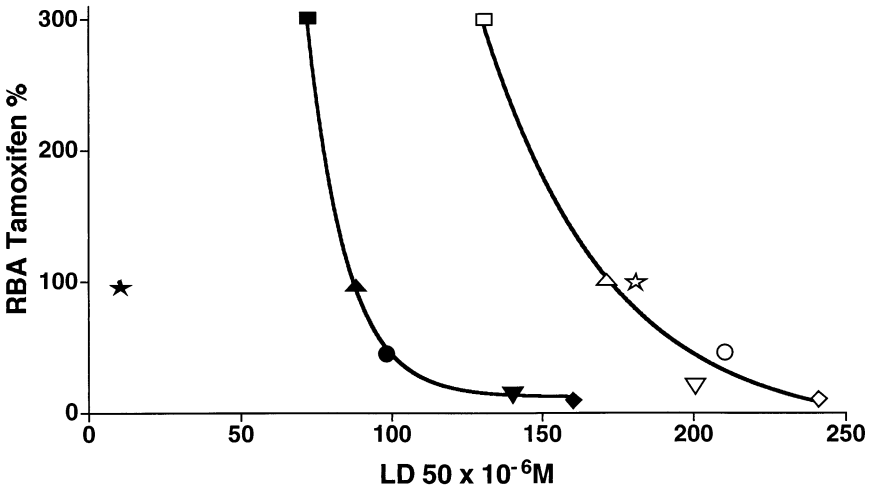


FIG. 1. Correlation between AEBS binding affinities with cytotoxic effect in MCF-7 cells (plain symbols) and Hela cells (open symbols). Cells were treated with tamoxifen (★), PBPE: 1-benzyl-'(2-pyrrolidinylethoxy)phenylketone (■), MBPE: 4(2-morpholin-4yl-ethoxy)-1-benzylbenzene (▲), PipBPE: 1-benzyl-4(2-piperidinylethoxy-)benzene (●), DmBPE: dimethyl{2-[4-benzylphenoxy]ethyl}amine (▼), or DeBPE: diethyl{2-[4-benzylphenoxy]ethyl}amine (◆).

toxic concentrations were higher in NRK than in RTx6 cells, and this effect was not dose- and time-dependent as opposed to what occurred in MCF-7 and Hela cells (Table 2). Hela cells were less sensitive to AEBS ligands than MCF-7 cells, one explanation being that there is 27% less AEBS found in Hela cells than in MCF-7 cells. The higher cytotoxic potency of tamoxifen in MCF-7 cells, which have a lower affinity for AEBS than PBPE and 4(2-morpholin-4yl-ethoxy)-1-benzylbenzene, might mainly account for tamoxifen interaction with ER and calmodulin.

Although the antiestrogen tamoxifen has become the second most used anticancer drug, its mechanism of action remains unclear. Obviously, part of its effect is mediated by the ER as has been reported in many studies [26]; however, experimental and clinical data have suggested the involvement of other proteins and, in particular, the AEBS in its cytotoxic activities [24, 25, 27]. In this work, tamoxifen was more active on MCF-7 than on the other cell lines, suggesting that ER plays a role in its cytotoxic effect. In contrast, this activity was low on RTx6, where the ER has been shown to be identical to that of MCF-7 [14]. On ER-negative cells Tx exhibited a cytotoxic effect corresponding to its AEBS affinity. The microsomal concentration of this binding site could be associated with the effectiveness of the different AEBS ligands tested in this study. Moreover, the affinity of these ligands for microsomal AEBS was correlated with their cytotoxic efficacies (Fig. 1). Tamoxifen, the sole ligand that bound ER, was also the only one to exhibit a different behavior on ER-positive MCF-7 cells, but not on the other cell lines. It appears from this study, in agreement with previous reports [24, 25, 27], that such high concentrations of AEBS ligands were highly cytotoxic and that these effects would be considered non-specific if they were not associated with the AEBS intracellular concentrations and their differential affinities for AEBS. Cells died by lisis in the RTx6 and NRK cell lines. In the MCF-7 and Hela cell lines, the toxicity of ligands was associated with a change in their morphology and will be published elsewhere.* This work provides new evidence for the indispensability of high affinity for AEBS in cytotoxic activity. Mechanisms involved in cytotoxity are currently under investigation and will be published elsewhere (see footnote below). Whether the AEBS is directly involved in mediating these effects remains unclear. It may be that high affinity for a ligand will facilitate cellular uptake and increased intracellular drug concentration, resulting in an enhanced potency through mechanisms that are unrelated to AEBS. However, we ruled out this hypothesis during previous work on MCF-7 and RTx6 [16]. On the other hand, the interaction of tamoxifen with several other intracellular sites has been reported and although the evidence is generally against any of these proteins being AEBS, they may mediate the high concentrations of AEBS ligands. Whatever the proteins involved in AEBS, the efficacy of its ligands in clinical trials can be used satifactorily to define the drug structure conferring the best AEBS affinity.

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