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Structural Similitudes between Cytotoxic Antiestrogen-Binding Site (AEBS) Ligands and Cytotoxic Sigma Receptor Ligands. Evidence for a Relationship between Cytotoxicity and Affinity for AEBS or Sigma-2 Receptor but Not for Sigma-1 Receptor

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ABSTRACT. 1-Benzyl-4-(*N*-2-pyrrolidinylethoxy)benzene (PBPE) is a cytotoxic derivative of the antitumoral drug tamoxifen. PBPE binds with high affinity and specificity to the microsomal antiestrogen-binding site (AEBS). PBPE, as well as some other high-affinity AEBS ligands, shares structural features with high-affinity and selective sigma receptor ligands in the *N*-(arylethyl)-*N*-alkyl-2-(1-pyrrolidinyl)ethylamine class, such as BD1008, which are cytotoxic against tumoral cells. Based on these structural and pharmacological similitudes, we set out to examine whether AEBS and sigma receptors could be related binding sites. We showed that BD1008 had a high affinity for AEBS. However, prototypical sigma receptor ligands were very low-affinity competitors on AEBS. Surprisingly, AEBS ligands displayed a high affinity for sigma-1 and sigma-2 receptor subtypes, showing that AEBS and sigma receptor-binding sites were not mutually exchangeable. Moreover, phenytoin, which is an allosteric modulator of sigma-1 receptor, was a competitive inhibitor of [^{3}H]tamoxifen on AEBS. These results suggest that the tamoxifen-binding site on AEBS and the sigma ligand-binding site on sigma receptors were not identical but related entities. We also showed here that the high-affinity and specific AEBS ligands also bound sigma receptors with high affinity. Moreover, the compounds that were capable of displacing tamoxifen from AEBS were cytotoxic against tumoral cells but not against the AEBS-deficient cell line Rtx-6. These results confirm that AEBS and sigma receptors might belong to the same family of proteins, and that the tamoxifen-binding site might be involved in the cytotoxicity of AEBS ligands and some classes of sigma compounds. BIOCHEM PHARMACOL **58**;12:1927–1939, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. AEBS; sigma receptors; tamoxifen; diphenylmethane; BD1008; cytotoxicity

AEBS[‖] are high-affinity binding sites for tamoxifen, a well-known drug used in the treatment of breast cancer. AEBS are different from the estrogen receptors, and have no affinity for 17β-estradiol, other estrogens, or steroidal antiestrogens [1]. By developing specific AEBS-targeted ligands, we [2, 3] and others [4, 5] established that AEBS were associated with the cytotoxicity of these specific ligands on tumoral cells, thereby demonstrating that AEBS might provide targets in the development of new antitumoral drugs devoid of potential deleterious estrogen-like side effects. The AEBS specific ligand *N*,*N*-diethyl-2-[(4-phenylmethyl)phenoxy]ethanamine HCl (DPPE) potentiated the chemotherapeutic index of cytotoxic drugs *in vitro* [5, 6]. Recently reported clinical trials using DPPE, in association with various antineoplasic agents such as cyclophosphamide and doxorubicin, were efficient and of low toxicity for the treatment of metastatic prostate cancer [7] and metastatic breast cancer [8]. These compounds might provide an alternative means of developing new therapeutics. Moreover, our group has recently shown that tamox-

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‖ Abbreviations: AEBS, antiestrogen-binding sites; DTG, 1,3-di-o-tolylguanidine; MBPE, 1-benzyl-4-(*N*-2-morpholinylethoxy)benzene; MCPE, 1-(1-methyl-1phenylethyl)-4-(*N*-2-morpholinylethoxy)benzene; (+)-3-PPP, (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; PBPE, 1-benzyl-4-(*N*-2-pyrrolidinylethoxy)benzene; and PCPE, 1-(1-methyl-1phenylethyl)-4-(*N*-2-pyrrolidinylethoxy)benzene.

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ifen and AEBS-specific compound PBPE inhibit lymphocyte infectivity by HIV by an AEBS-related mechanism, underlining the importance of AEBS ligands both in cell growth control and antiretroviral effects [9]. AEBS have recently been characterized in rat liver microsomes as multiproteic complexes with microsomal epoxide hydrolase as one of the subunits [10]. Brandes proposed that AEBS was a cytochrome P450 associated with an intracellular histamine receptor [11]. Moebius reported the presence of a tamoxifen-binding site in the emopamil-binding protein expressed in yeast [12]. Altogether, these tentative assignments of structure to this binding site need more studies to clearly establish its identity.

Structure–affinity studies revealed that AEBS exhibit high affinity for two classes of compounds, notably the cationic amphiphilic aryl aminoethyl compounds, and the ketocholestanol-like oxysterols [1, 13]. The first class of AEBS ligands includes triarylethylene, triarylpropenone, diarylbenzopyran, diarylbenzofuran, and diphenylmethanes [14]. Although these compounds exhibit various affinities for AEBS, those compounds in each subclass bearing the *N*-morpholinylethyl or the *N*-pyrrolidinylethyl side chain show the highest affinity for AEBS [3, 13]. This structural feature is also common to selective ligands showing high affinity for sigma receptors [15–17]. Sigma receptors include a family of binding sites which can be divided into different subclasses based on their pharmacological profiles [18]. Sigma-1 receptors, which preferentially bind (+)-pentazocine and the dextrorotatory isomers of other benzomorphans. Sigma-2 receptors have low affinity for (+)-benzomorphans and preferentially bind (−)-pentazocine over the (+) isomer. Both subtypes bind haloperidol and DTG with high affinity. Recently, sigma-1 receptor genes have been cloned, expressed, and found to be related structurally to one of the enzymes of fungal sterol metabolism [19, 20]. Other sigma-like binding sites have been described which are distinguishable from sigma-1 or sigma-2 sites [21, 22]. Sigma-1 and sigma-2 receptors have been detected on a number of tumor cell lines from different tissue sources and have been linked to the cytotoxicity of their cognate ligands [23–25]. Specifically, the sigma-2 receptors have been recently implicated in the morphologic and cytotoxic action of sigma ligands [26].

In the present study, we questioned the possible existence of a functional relationship between AEBS and sigma receptors. We first evaluated the binding potency of sigma ligands on AEBS by comparison with tamoxifen, diphenylmethane compounds, and a new class of AEBS ligands, i.e. the cumyl derivatives (for chemical structures, see Fig. 1). We then measured the affinity of AEBS ligands for sigma-1 and sigma-2 receptors. We then determined whether phenytoin, an allosteric modulator of sigma-1 receptor, affected the binding of [^{3}H]tamoxifen on AEBS. Finally, since AEBS ligands and some sigma compounds displayed cytotoxicities against various tumoral cell lines, we then tested the cytotoxicity of these ligands on human MCF-7 cells (a mammary epithelial cell line sensitive to tamoxifen), on Rtx-6 (an MCF-7 variant resistant to tamoxifen and AEBS ligands which expresses a low level of AEBS), on human neuroblastoma (SK-N-SH), and rat C6 glioma, which were first used to show the cytotoxic potency of sigma ligands [27].

MATERIALS AND METHODS

Molecular Structure Analysis

Computational chemical calculations were performed on a Silicon Graphics Indigo workstation using Insight II version 97.0 (Biosim/MSI). Minimum energy conformations were calculated using the Discover module (2.9.7./95.0/3.0.0) with CVFF forcefield. Van der Waals volumes and van der Waals volume intersections were determined using the Search-Compare module version 95.0 (Biosim/MSI).

Membrane Preparation

Male Sprague–Dawley rats (150–200 g) were killed by decapitation and their livers removed to ice-cold 10 mM Tris–HCl, pH 7.4, containing 0.9% saline or 0.32 M sucrose. The livers were homogenized (after mincing with scissors) using a Potter–Elvehjem Teflon–glass homogenizer in 10 mL/g tissue wet weight of ice-cold 10 mM Tris–HCl/0.32 M sucrose, pH 7.4. The crude homogenate was centrifuged for 10 min at 1000 *g* and the pellet discarded. The resulting supernatant was centrifuged at 31,000 *g* for 15 min. The pellet was resuspended in 3 mL/g 10 mM Tris–HCl, at pH 7.4 by vortexing, and the suspension allowed to incubate at 25° for 15 min. The pellet was then resuspended to 1.53 mL/g in 10 mM Tris–HCl, pH 7.4 by gentle Potter–Elvehjem homogenization and aliquots stored at −80° until use. Protein concentration was determined by the method of Bradford [28] using BSA as standard. Guinea-pig brain membranes were prepared from frozen guinea-pig brains (Harlan Sprague–Dawley), without cerebellum. Cerebella were removed from frozen brains (stored at −80° prior to use) and the brains allowed to thaw slowly on ice. After weighing and mincing with scissors, the tissue was treated as described above for rat liver.

Ligand-Binding Assay (AEBS)

AEBS were labeled as previously described using rat liver membranes, a rich source of AEBS, and [^{3}H]tamoxifen in the presence of 1 μM of 17β-estradiol to mask residual estrogen receptors [29]. Assays were performed in 50 mM Tris–HCl, 1 mM EDTA, 12 mM thioglycerol at pH 7.4 for 18 hr at 4° in a volume of 200 μL with 80 μg of 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, and 12 mM thioglycerol at pH 7.4. Elutions were performed by adding 1 mL of 50 mM Tris–HCl, 1 mM EDTA, and 12 mM thioglycerol at pH

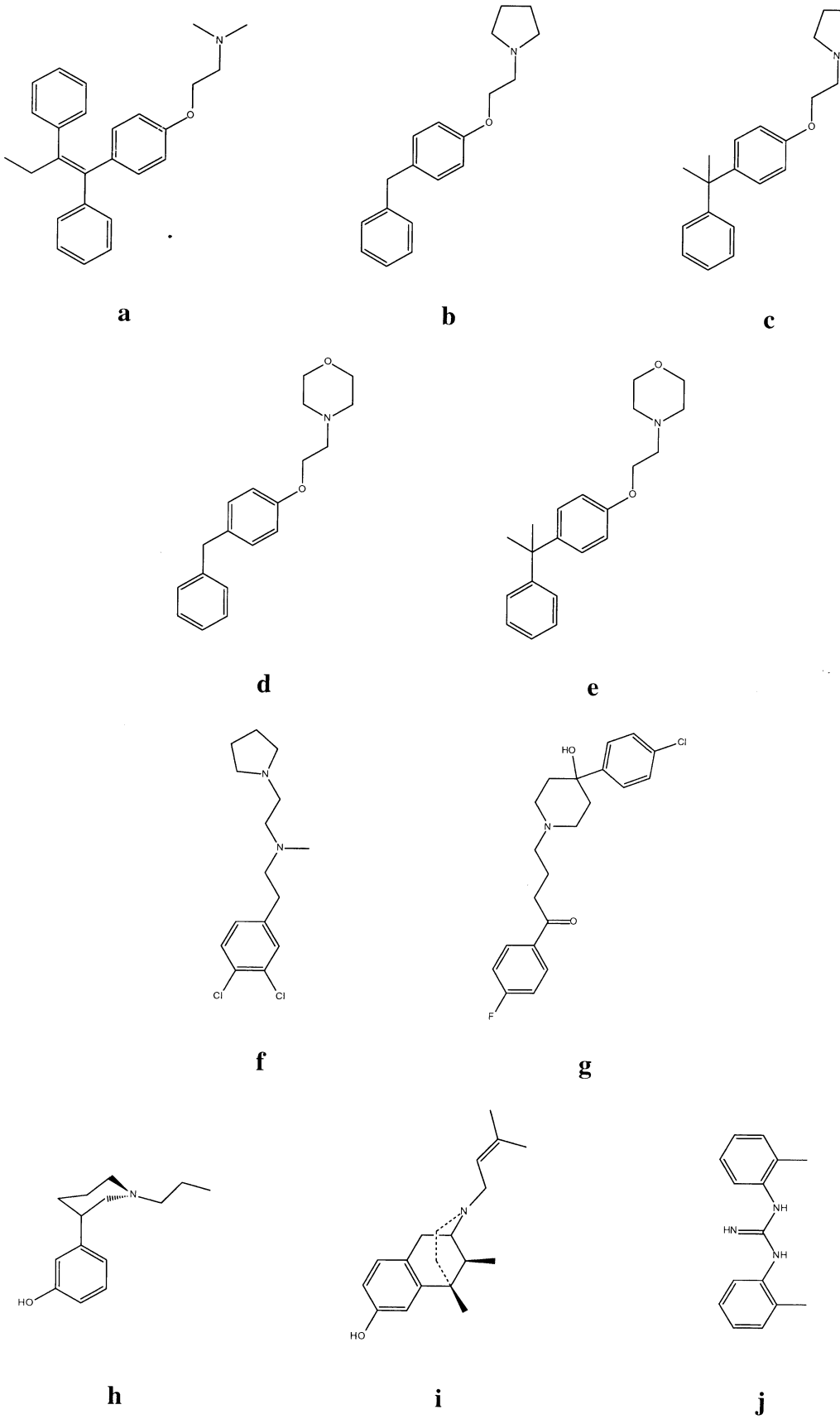
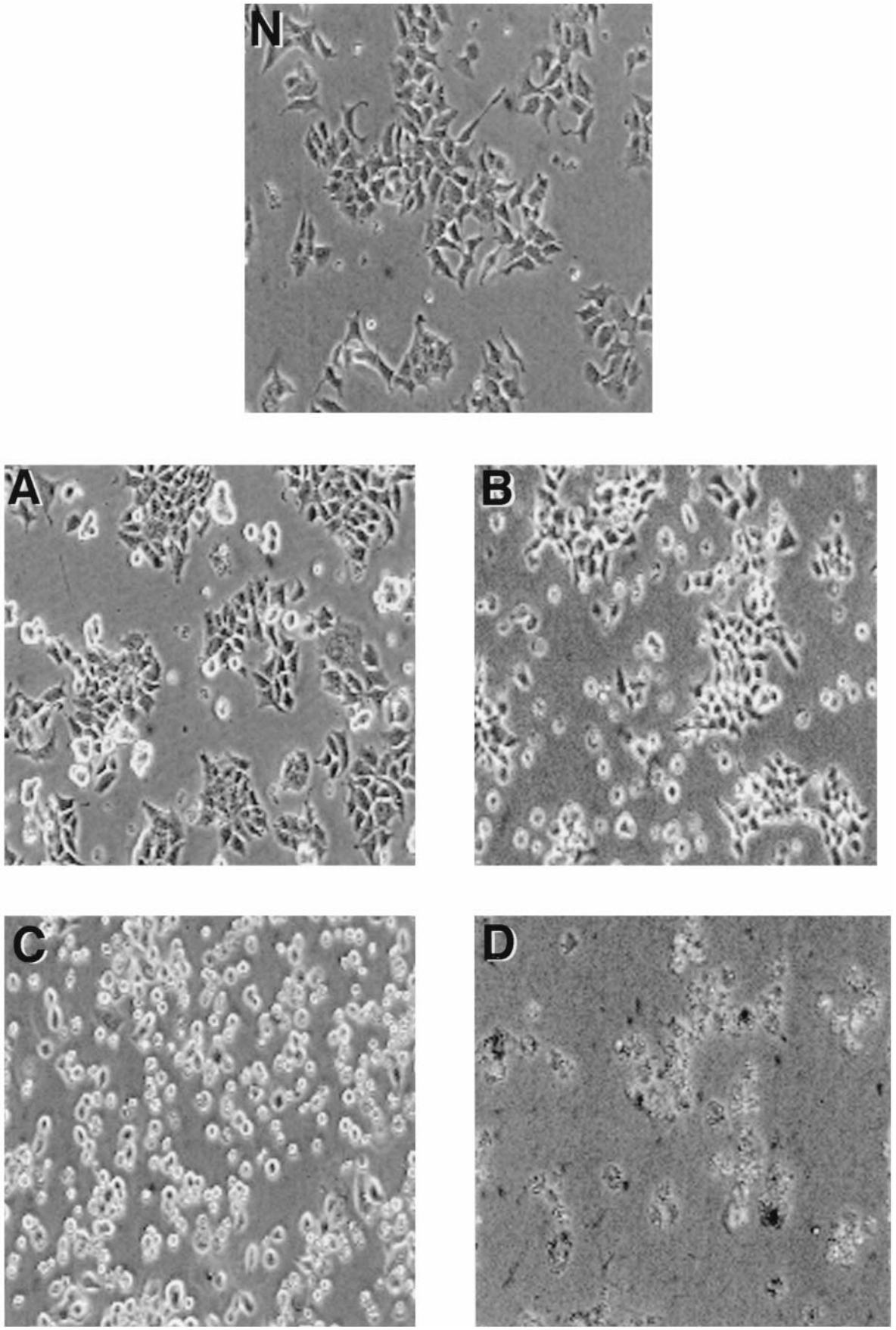


FIG. 1. Chemical structures of compounds: a) tamoxifen; b) PBPE; c) PCPE; d) MBPE; e) MCPE; f) BD1008; g) haloperidol; h) (+)-3-PPP; i) (+)-pentazocine; j) DTG.


N
A
B
C
D

7.4. The eluates were collected and counted in a Ready Safe cocktail (Beckman).

Ligand-Binding Assay (Sigma-1 Receptors)

Sigma-1 receptors were labeled as previously described, using the sigma-1-selective probe [^{3}H](+)pentazocine and guinea-pig brain membranes [30]. Guinea-pig brain membranes (300–500 μg of membrane protein) were incubated with 3 nM [^{3}H](+)pentazocine in a total volume of 0.5 mL of 50 mM Tris–HCl, at pH 8.0. The incubations were carried out for 120 min at 25°. Non-specific binding was determined in the presence of 10 μM unlabeled haloperidol. The assays were terminated by dilution with 5 mL of ice-cold 10 mM Tris–HCl, at pH 8.0 and vacuum filtration through glass fibre filters using a Brandel cell harvester. Filters were soaked in 0.5% polyethyleneimine for at least 30 min at 25° prior to use and then washed twice with 5 mL of ice-cold 10 mM Tris–HCl, at pH 8.0 and counted in a CytoScynt cocktail (ICN) after an overnight extraction. Membranes were prepared from frozen guinea-pig brains (minus cerebella) as described above [30].

Ligand-Binding Assay (Sigma-2 Receptors)

The sigma-2 receptors were labeled as previously described using rat liver membranes, a rich source of sigma-2 sites, and [^{3}H]DTG in the presence of dextrallorphan [31]. Assays were performed in 50 mM Tris–HCl, pH 8.0 for 120 min at 25° in a volume of 0.5 mL with 160 μg membrane protein and 5 nM radioligand. Assays included 1 μM dextrallorphan to mask sigma-1 binding. Non-specific binding was determined in the presence of 10 μM haloperidol. All other manipulations were as described above for the sigma-1 receptor assay. The rat liver membranes were prepared from the liver of male Sprague–Dawley rats as described above [31].

Cell Culture

Rat C6 glioma cells and SK-N-SH human neuroblastoma cells (American Type Culture Collection) were cultured in Dubelcco's modified Eagle's medium (DMEM) supplemented with 44 mM of sodium bicarbonate and 10% fetal bovine serum (FBS) (Advanced Biotechnologies) in 24-well plastic plates (Costar) in a humidified atmosphere of 5% CO_2/95% air (normal conditions, pH 7.2–7.4). For all the drug additions, the FBS was reduced from 10% to 1%. The sigma receptor assay was performed exactly as described before [23]. The MCF-7 cells were initially from M. Rich (Michigan Cancer Fondation) and the Rtx-6 cells were from our laboratory [32]. These cells were adapted to grow in a chemically defined medium without serum [33]. They were cultured at 37° in 5% CO_2 in RPMI 1640 supplemented with 2 g/L of sodium bicarbonate, 1.2 mM glutamine (pH 7.4 at 23°), 0.08 unit/mL human recombinant insulin, and 0.1 mg/mL of human apo-transferin.

Ligand-Binding Assay on Cell Lines

The AEBS-binding assay was performed exactly as described before [3]. Fifteen concentrations were used over a range of 0.1 to 25 nM for [^{3}H]tamoxifen, over a range of 0.1 to 500 nM for [^{3}H](+)pentazocine, and over a range of 0.1 to 400 nM (in the presence of 1 μM of dextrallorphan) for [^{3}H]DTG. Data were analyzed using the iterative curve-fitting program BDATA (EMF Software) or GraphPad Prism (version 2.0). Values are the average 2 to 3 experiments ± SEM, each carried out in duplicate. Values for [^{3}H](+)pentazocine and [^{3}H]DTG in C6 glioma cells and neuroblastoma SK-N-SH cells were taken from Bowen and colleagues [23] and are the average of 3–5 experiments ± SEM, each carried out in duplicate. K_d values are in nM and B_{max} values are in fmol/mg protein.

Scoring Morphological Effects

After exchanging the culture medium for medium containing the test compound (30 μM unless otherwise specified), the cells were monitored at various times up to 72 hr by phase contrast microscopy at 10× or 20×. The effect of each compound was scored in relation to a control that only received the solvent vehicle. The cells were observed every 2 hr for the first 6 hr and then once every 24 hr after drug addition. Scoring was done with respect to the time after addition of the test compound (usually 6, 24, and 72 hr) and the extent of cellular change (N, A, B, C, D) as follows (see Fig. 2): N, no observable effect on cells; A, a significant proportion of cells exhibit loss of fine processes; B, all cells had lost fine processes and some cells remained polygonal with coarse processes, while others became spindle-shaped or round; C, most or all cells had become round; D, cell death with the presence of cell debris. These different stages are presented in Fig 2. In some cases, cell viability was determined by trypan blue exclusion to confirm the visual inspection.

FIG. 2. Morphological changes in MCF-7 cells produced by AEBS ligands. Phase contrast microscopy of mammary adenocarcinoma cells (MCF-7) cultured in the absence or presence of a test compound. Cells were monitored at various times up to 72 hr by phase contrast microscopy at 10× or 20×. The panels represent various stages of the effect of test compound as described in detail in the text. The score assigned (N, A, B, C, and D) corresponds to the letter of the panel. N, normal cells as they appear before addition of any test compound. Cells were given this score when there was no effect relative to an untreated control. A, significant proportion of cells exhibit loss of fine processes. B, all cells have lost fine processes and some cells become spindle-shaped, or round. C, most or all cells become round. D, cell death with presence of cell debris. Photographs are from representative experiments.

Data Analysis

For the Scatchard analysis and competition curves, the data were analyzed using Graphpad Prism (version 2.0) from Graph Pad Software Inc.

Chemicals

[^{3}H]Tamoxifen (specific activity 86 Ci/mmol), [^{3}H]DTG (specific activity 35 Ci/mmol), and [^{3}H](+)-pentazocine (specific activity 31.4 Ci/mmol) were from DuPont New England Nuclear. Tris–HCl, KCl, NaN_3, glycerol, EDTA, propanediol, and *N*,*N*′-dimethylformamide were from Merck. Tamoxifen ([Z]-1-[*p*-Dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene), haloperidol (4-[4-(*p*-chlorophenyl)-4-hydroxy-piperidino]-4′-fluorobutyrophenone), diphenyl hydantoin (5,5-diphenyl-2,4-imidazolidinedione), and biochemical and tissue culture reagents were from Sigma. (+)-3-PPP was from RBI and DTG was from Aldrich Chemicals. (+)-Pentazocine was synthesized by Dr. Ivy Carroll (Research Triangle Institute). BD1008, *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)ethylamine was synthesized as described [16], as were PBPE and MBPE [2]. The synthesis of PCPE and MCPE will be described elsewhere.* All compounds were checked for purity either by TLC or by HPLC and were more than 99% pure.

RESULTS

Structural Comparison of Compounds

We first compared the structure and physico-chemical properties of two prototypical compounds: PBPE, a high-affinity and selective AEBS ligand, and BD1008, a high-affinity and specific sigma receptor ligand. Both compounds were cationic and amphiphilic, containing the protonated hydrophilic tertiary amine *N*-pyrrolidine ethyl. Their calculated hydrophobicities (ClogP) were in the same range (4.92 and 4.21, respectively). Three-dimensional analyses were performed on BD1008 and PBPE: calculations of the minimal conformational energies showed that both compounds were flexible, did not contain rotational energy barriers, and consequently may adopt a great variety of conformations, although both are extended in their lowest energy conformation (Fig. 3, A and B). Their van der Waals volumes were 242.27 Å^3 for PBPE and 244.65 Å^3 for BD1008. After their superimposition in their lowest energy conformation, we measured the intersection of their Van der Waals volumes, which was 148.91 Å^3. This represented 61% of the molecules they had in common (Fig. 3, A and B), including the high-affinity determinant *N*-pyrrolidine ethyl. Two- and three-dimensional structural analyses showed that PBPE and BD1008, although initially belonging to different pharmacological classes of compounds, shared physico-chemical properties. We then compared their structure with tamoxifen.

The three-dimensional structure of tamoxifen having been determined previously, a stick model constructed from the atomic coordinate is shown in Fig. 3C [34]. Tamoxifen exhibited a propeller structure which is characteristic of triphenylethylenic compounds. The amine-aryl ether chain of tamoxifen was extended as in PBPE and BD1008. However, unlike PBPE and BD1008, the aromatic part of tamoxifen was not flexible. Phenyl groups were conformationally restricted with a high energy barrier of rotation. The van der Waals volume of tamoxifen was 321.95 Å^3. We then did a superimposition of the van der Waals volumes of the minimum energy conformation of PBPE and the van der Waals volumes of the crystallographic structure of tamoxifen (Fig. 3, A and C). The minimum energy conformation of PBPE matched that of the diphenylmethane and *N*,*N*-dimethylaminoethoxy moieties of tamoxifen. They had a common van der Waals volume of 191.62 Å^3, which was 36.64 Å^3 more than the intersection between the van der Waals volume of PBPE and BD1008 (see above), and represents 80% of the van der Waals volume of PBPE. When we compared the conformation of BD1008 with tamoxifen, we found that they shared a volume of 138.16 Å^3 (Fig. 3, B and C), including the amine side chain and part of the hydrophobic moiety of tamoxifen. This common shared volume was 43.46 Å^3 less than the intersection of PBPE with tamoxifen which might be predictive of a lower affinity of BD1008 than PBPE for AEBS. We then performed competition analysis in order to measure the affinities of these compounds to AEBS.

Binding to AEBS

Competition analyses of [^{3}H]tamoxifen on AEBS are reported in Table 1. The diphenylmethane compounds PBPE and MBPE bound AEBS with K_i of 8.79 ± 0.91 and 17.57 ± 0.42 nM, respectively which, in the liver, were lower than the K_i of tamoxifen (2.5 ± 0.52 nM). PBPE and MBPE differ in their basic side chain, which is a pyrrolidine in the case of PBPE and a morpholine in the case of MBPE. The cumyl derivatives PCPE and MCPE bound with high affinity to AEBS, and, as in the diphenylmethane series, the pyrrolidinic derivative PCPE showed a higher affinity than the morpholinic compound MCPE. The effect of the presence of the dimethyl group at the benzyl position was to decrease the affinity for AEBS by a factor of 1.14 for the pyrrolidinic derivatives and by a factor of 2.73 in the morpholinic series, the effect thus being more pronounced in the latter. As expected, the sigma ligand BD1008 bound with high affinity to AEBS with a K_i of 83.49 ± 5.62 nM. This compound contains the pyrrolidine ethyl moiety, which is a high-affinity determinant for AEBS. Its affinity was 9.5 times less than that of PBPE, so that replacement of the diphenylmethane group by 2-(3,4-dichloro-phenyl) ethyl produced only a weak decrease in this affinity towards AEBS. This difference in affinity parallels the shared van

* Poirot M, Delarue F, Klaebe A, Perie J and Faye JC, manuscript in preparation.

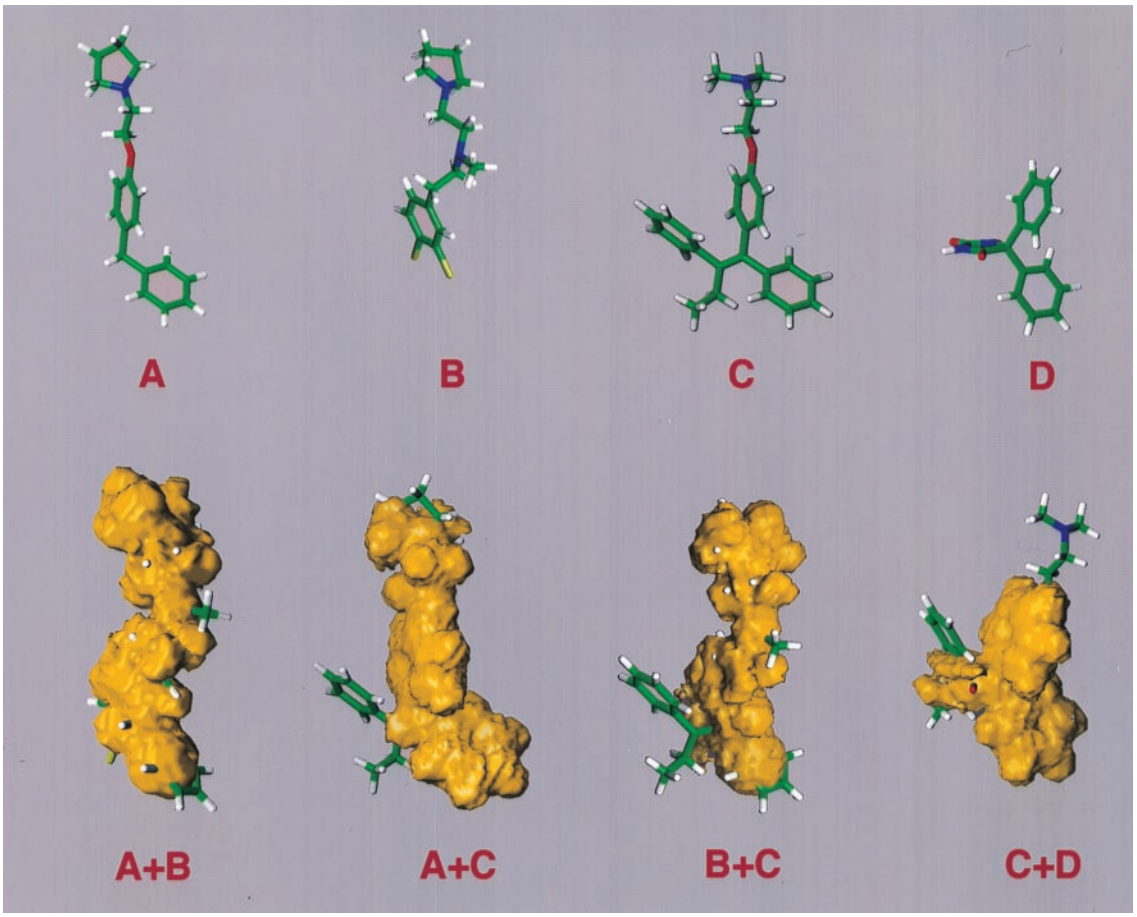


FIG. 3. Molecular structure of tamoxifen, PBPE, BD1008, and phenytoin. Superimposition and intersection of their van der Waals volumes. Upper level: minimum energy conformation of PBPE (A), BD1008 (B), and phenytoin (D). Three-dimensional structure of tamoxifen (C) was depicted using coordinates from x-ray crystallographic analysis by Précigoux and colleagues [32]. Lower level superimposition of molecules A, B, C, and D; the intersection of their van der Waals volumes is drawn in yellow. Superimposition and intersection of A and B: A + B, of A and C: A + C, of B and C: B + C, of C and D: C + D.

der Waals volumes as described in the above section. Prototypical sigma receptor ligands were also tested on AEBS and displayed a weak capacity to displace [^{3}H]tamoxifen on AEBS: the K_i of haloperidol was 64.03 ± 0.143 μM, that of (+)-3-PPP 107.80 ± 5.20 μM, that of (+)-pentazocine 149.70 ± 5.20 μM, and that of DTG 346.70 ± 1.07 μM. Pentazocine and (+)-3-PPP are phenolic compounds, and the presence of a phenolic group at

TABLE 1. Affinity of AEBS and sigma ligands for AEBS, sigma-1, and sigma-2 receptors

	AEBS	sigma-1	sigma-2
Compound	K_i (nM) [^{3}H]tamoxifen	K_i (nM) [^{3}H](+)pentazocine	K_i (nM) [^{3}H]DTG
Tamoxifen	2.52 ± 0.52	268 ± 15	135 ± 17
PBPE	8.79 ± 0.91	0.24 ± 0.004	121 ± 14
PCPE	10.13 ± 1.18	1.04 ± 0.06	120 ± 1.9
MBPE	17.57 ± 0.42	1.74 ± 0.04	1853 ± 51
MCPE	48.06 ± 2.45	14.74 ± 2.26	1347 ± 33
BD1008	83.49 ± 5.62	2.10 ± 0.81	8.1 ± 2.2
Haloperidol	64,030 ± 143	1.9 ± 0.3	79.8 ± 20.6
(+)-3-PPP	107,800 ± 107	79.3 ± 2.8	120 ± 24
(+)-Pentazocine	149,700 ± 5200	6.7 ± 1.2	1361 ± 134
DTG	346,700 ± 1072	74.3 ± 14.9	61.2 ± 13.4

Rat liver membranes were incubated with 3 nM [^{3}H]tamoxifen or 30 nM [^{3}H]DTG and 12 concentrations of unlabeled test ligand ranging from 0.1 to 10,000 nM or 1 to 10,000 μM. Guinea-pig brain membranes were incubated with 3 nM [^{3}H](+)-pentazocine and 12 concentrations of unlabeled test ligand ranging from 0.1 to 10,000 nM. Assays with [^{3}H]DTG included 1 μM dextrallorphan and assays with [^{3}H]tamoxifen 1 μM 17β-estradiol. IC_{50} values were determined using the iterative curve-fitting program GraphPad Prism. IC_{50} values were converted into the apparent K_i using the Cheng–Prusoff equation [41], and the K_d values.

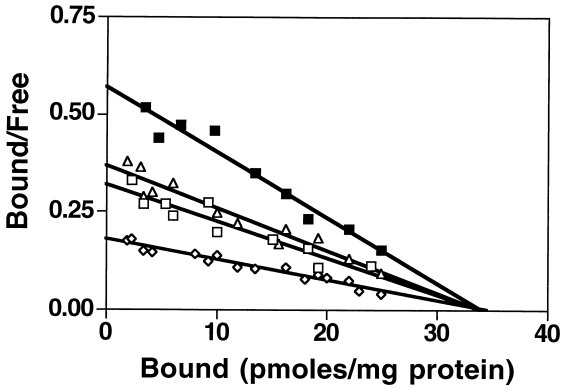


FIG. 4. Scatchard plots of [^{3}H]tamoxifen binding to microsomal AEBS without or with sigma ligands. Rat liver microsomal proteins (40 μg/assay) were incubated with increasing concentrations of [^{3}H]tamoxifen (0.3–30 nM) in the absence (■) or the presence of 300 μM DTG (△), 100 μM pentazocine (□), and 50 nM BD1008 (◇). Experiments were performed as described in Materials and Methods. Each value is represented as the mean of triplicate determinations.

the opposite position to the ammonium side chain is known to drastically decrease the affinity for AEBS [13]; DTG is a hydrophilic guanidinium derivative which is different from prototypical AEBS ligands. We then did experiments in order to characterize the kind of competition produced by sigma ligands. Scatchard analyses on AEBS with [^{3}H]tamoxifen were carried out in the absence or presence of BD1008, (+)-pentazocine, and DTG. The concentration of inhibitors chosen for these studies were at IC_{50} or higher concentrations. Figure 4 shows that, in the absence of inhibitors, [^{3}H]tamoxifen bound AEBS with a K_d of 2.42 ± 0.24 nM and a B_{max} of 34.13 ± 3.4 pmol/mg protein. In the presence of 50 nM BD1008, the K_d was 7.65 ± 1.27 nM and the B_{max} 34.54 ± 5.3 pmol/mg protein. In the presence of 100 μM (+)-pentazocine, the K_d was 4.25 ± 1.57 nM and the B_{max} 34.10 ± 5.2 pmol/mg protein. Finally, in the presence of 300 μM DTG, the K_d was 3.68 ± 2.34 nM and the B_{max} 33.88 ± 1.1 pmol/mg protein. These sigma ligands increased the K_d without affecting B_{max}. We did the same experiments with different concentrations of inhibitors which produced, with increasing concentrations, an increase in the K_d without affecting the B_{max} (not shown). These experiments showed that prototypical sigma compounds and BD1008 might be competitive inhibitors of tamoxifen on AEBS.

Binding to Sigma Receptors

Binding to sigma receptors was done following established methodology that allowed pharmacological characterizations of sigma-1 and sigma-2 receptors [30, 31] by using prototypical sigma ligands. BD1008 does not exist in a radiolabeled form and consequently we were not able to focus on this compound. AEBS ligands were tested against sigma receptors. Table 1 summarizes the binding constants for the inhibition of [^{3}H]pentazocine binding to sigma-1 receptor and [^{3}H]DTG to sigma-2 receptor.

BINDING TO SIGMA-1. Prototypic AEBS ligands bind sigma-1 receptors in guinea-pig brain with high affinity. PBPE had a K_i of 0.24 ± 0.004 nM, MBPE a K_i of 1.74 ± 0.04 nM, and tamoxifen a K_i of 268 ± 15 nM. The sigma ligand BD1008 had a K_i of 2.1 ± 0.8 nM. Compared to other sigma ligands, PBPE displayed the highest affinity for sigma-1 receptor. The affinity of PBPE was 10 times higher than BD1008. The affinity of MBPE was similar to that of BD1008 and haloperidol for sigma-1. Tamoxifen displayed a moderate affinity for sigma-1 with a K_i of 268 ± 15 nM, which was 558 times higher than that of pentazocine for AEBS.

BINDING TO SIGMA-2. PBPE, with a K_i of 121 ± 14 nM, was the most potent of the AEBS ligands in inhibiting DTG binding. Tamoxifen had a K_i of 135 ± 17 nM, MBPE a K_i of 1853 ± 51 nM, and BD1008 a K_i of 8.1 ± 2.2 nM. Tamoxifen displayed a 2568-fold higher affinity for sigma-2 than DTG for AEBS. MBPE and PBPE both had a lower affinity for sigma-2 receptor than for sigma-1, with sigma-1/sigma-2 ratios of 1050 and 600, respectively, thereby demonstrating the good selectivity of these compounds for sigma-1 receptor. Tamoxifen displayed a 2-fold higher affinity for sigma-2 receptor than for sigma-1 receptor.

Effect of the Sigma-1 Receptor Allosteric Regulator Diphenylhydantoine (Phenytoin) on [^{3}H]Tamoxifen Binding on AEBS

Phenytoin is reported to be an allosteric modulator of sigma compounds by increasing their affinity with sigma-1 receptors [18]. Phenytoin (Fig. 3D) contains the diphenylmethane backbone present in several classes of AEBS ligands, including tamoxifen. We did conformational studies on this compound and showed that phenytoin in its lower energy conformation (Fig. 3D) was superimposable to the diphenylmethane moiety of the crystallographic structure of tamoxifen (Fig. 3, C and D). This suggests that phenytoin might compete with tamoxifen. We then performed Scatchard analyses with [^{3}H]tamoxifen in rat liver microsomes in the absence or presence of 100 and 300 μM phenytoin. These concentrations were chosen because they correspond to the concentrations required to monitor the allosteric effect on sigma-1 receptor [18]. Figure 5 shows that [^{3}H]tamoxifen bound AEBS with a K_d of 2.42 ± 0.24 nM and a B_{max} of 34.13 ± 3.4 pmol/mg protein. In the presence of 100 μM phenytoin, the K_d was 3.29 ± 0.34 nM and the B_{max} was 33.58 ± 2.3 pmol/mg protein, whereas in the presence of 300 μM phenytoin the K_d was 4.17 ± 0.36 nM and the B_{max} 32.34 ± 4.5 pmol/mg protein. Phenytoin produced a diminution in the affinity of tamoxifen for AEBS of 1.37 and 1.73 with 100 and 300 μM, respectively, without affecting the B_{max} (Fig. 5). This shows that phenytoin is not an allosteric regulator of AEBS as de-

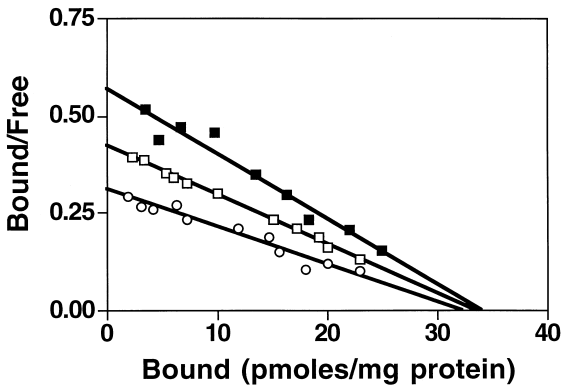


FIG. 5. Scatchard plots of [^{3}H]tamoxifen binding to microsomal AEBS without or with phenytoin. Rat liver microsomal proteins (40 μg/assay) were incubated with increasing concentrations of [^{3}H]tamoxifen (0.3–30 nM) in the absence (■) or the presence of 100 μM phenytoin (□) and 300 μM phenytoin (○). Experiments were performed as described in Materials and Methods. Each value is represented as the mean of triplicate determinations.

scribed for sigma-1 receptor, but instead might be a competitive inhibitor of tamoxifen on AEBS.

Cytotoxicity

The morphological effects associated with the cytotoxicity produced by the compounds were described in the Materials and Methods section and illustrated in Fig 1. Table 2 shows that the AEBS ligands were potent inducers of cytotoxicity on MCF-7 cells, as previously reported [3]. Here, we report that the effect of MCF-7 on proliferation is associated with morphological changes. Tamoxifen was the most potent compound of the series: its effect was rapid even at low doses (from 0.5 μM), the cells first being arrested and then dying by cytolysis. This effect was not reversed by adding up to 10 nM 17β-estradiol, illustrating an estrogen receptor-

TABLE 2. Effect of AEBS and sigma ligands on MCF-7 breast adenocarcinoma cells

	Effect (30 μM)			
Compound	6 hr	48 hr	72 hr	Notes
Tamoxifen	C-D	C-D	D	
PBPE	A	C	C-D	C (100 μM) 6 hr
PCPE	A	C	C	
MBPE	N	B	B	D (100 μM) 48 hr
MCPE	A	B	B	C (100 μM) 48 hr
BD1008	N	B>C	B-C	B-C (100 μM) 48 hr
Haloperidol	N	A	A	B (100 μM) 48 hr
(+)-3-PPP	N	N	N	A (100 μM) 72 hr
(+)-Pentazocine	N	N	N	A (100 μM) 72 hr
DTG	N	N	N	A (100 μM) 72 hr

Compounds were incubated with MCF-7 cells at a concentration of 30 μM. The effects were observed over time and scored at 6 hr (or less), 48 hr, and 72 hr using the letter code as described in Materials and Methods and text and shown in Fig. 6. Where indicated under notes, cells were also monitored at other concentrations of test ligand. All experiments were repeated at least three times, with similar results. Duplicate wells were used for each test compound in each experiment.

TABLE 3. Effect of AEBS and sigma ligands on C6 glioma cells

	Effect (30 μM)			
Compound	6 hr	48 hr	72 hr	Notes
Tamoxifen	N	C-D	D	
PBPE	N	B<C	C>D	
PCPE	N	C	D	
MBPE	N	N	B>C	B<C>D (100 μM) 72 hr
MCPE	N	N	N	B-C (100 μM) 48 hr
BD1008	N	B>C	B-C	D (100 μM) 48 hr
Haloperidol	N	N	B-C	B (100 μM) 48 hr
(+)-3-PPP	N	N	N	A (100 μM) 72 hr
(+)-Pentazocine	N	N	N	A (100 μM) 72 hr
DTG	N	N	N	A (100 μM) 72 hr

Experiments were carried out as described in Table 2 caption.

independent cytotoxic effect (not shown). The diphenylmethane compound PBPE was efficient, producing an effect which was time- and dose-dependent, beginning by a loss of fine processes (Fig. 2, stage A) followed by cell rounding, after which 50% of the cells became round (Fig. 2, stage B). The totality of the cells then became round (Fig. 2, stage C), associated with a reorganization of the plasma membrane followed by cytolysis, leading to the appearance of cell debris (Fig. 2, stage D). Lower concentrations of PBPE incubated for longer times led to the gradual appearance of the same phenomenon. For example, the incubation of 0.5 μM PBPE for 5 days induced rounding in 50% of the cells (Fig. 2, stage B) (not shown), the point being that increasing the dose accelerated the kinetics of the cytotoxicity. Six hours of incubation with 30 μM produced a loss of the fine processes (Fig. 2, stage A) (Table 2). After 48 hr of incubation, 100% of the cells became round. Higher doses such as 100 μM produced a more robust response (stage C after 6 hr of incubation) (Table 2). With PBPE the cytotoxicity appeared more gradually than with tamoxifen. Other targets might be implicated in this latter mechanism, such as estrogen receptors, protein kinase C, and calmodulin [35]. The cumyl derivative PCPE induced the same gradual effect as PBPE, but at higher doses (Table 2). The morpholinic derivatives MBPE and MCPE demonstrated a lower potency than their pyrrolidinic homologues (48 hr at 100 μM induced stage D for MBPE and stage C for MCPE) (Table 2). Sigma ligand BD1008 induced morphological changes on MCF-7 as with the diphenylmethane and cumyl derivatives (Table 2). This efficiency of BD1008 placed it between PCPE and MBPE in the range of potency. Haloperidol induced a loss of fine processes that was apparent after 48 hr of incubation. The same time of incubation with 100 μM haloperidol induced stage B. (+)-Pentazocine and DTG up to 30 μM did not produce any morphological changes or induce cytolysis at 72 hr, and at 100 μM these compounds produced only stage A at 72 hr. Table 3 shows that these compounds produced the same qualitative changes in C6 glioma cell morphology as in MCF-7 cells. Table 4 shows that these compounds produced the same

TABLE 4. Effect of AEBS and sigma ligands on SK-N-SH neuroblastoma cells

Compound	Effect (30 μM) 6 hr	48 hr	72 hr	Notes
Tamoxifen	N	D	D	
PBPE	N	C-D	C<D	
PCPE	A	C	D	
MBPE	N	N	N	C>D (100 μM) 72 hr
MCPE	N	N	N	A-B (100 μM) 72 hr
BD1008	N	B>C	B-C	D (100 μM) 72 hr
Haloperidol	N	N	B	C (100 μM) 48 hr
(+)-3-PPP	N	N	N	A (100 μM) 72 hr
(+)-Pentazocine	N	N	N	A (100 μM) 72 hr
DTG	N	N	N	A (100 μM) 72 hr

Experiments were carried out as described in Table 2 caption.

qualitative changes in human neuroblastomas SK-N-SH. Interestingly, with the exception of tamoxifen, none of the compounds (up to 30 μM) produced strong morphological changes on Rtx-6 cells in this time–course study (Table 5). Tamoxifen did produce cell rounding, but was much less potent than in other cell lines (Table 5). However, as described for AEBS compounds [3], cytolysis occurred without morphological changes at doses over 300 μM in the case of AEBS ligands and BD1008. The low cytotoxic potency for tamoxifen and AEBS-specific ligands has been related to the low content of AEBS [2, 3, 32], which suggests that the cytotoxic potency of BD1008 on MCF-7 cells might be related to its affinity for AEBS.

Quantitation of AEBS in the Different Cell Lines

Binding parameters for [^{3}H]tamoxifen were measured on the different cell lines that were used for cytotoxic tests. In MCF-7 cells, [^{3}H]tamoxifen bound AEBS with a K_d of 5.3 ± 1.9 nM and a B_{max} of 1534 ± 69 fmol/mg of protein. In C6 cells, [^{3}H]tamoxifen found AEBS with a K_d of 17.8 ± 2.4 nM and a B_{max} of 1830 ± 56 fmol/mg protein. In SK-N-SH cells, [^{3}H]tamoxifen bound AEBS with a K_d of 4.8 ± 2.3 nM and a B_{max} of 1142 ± 335 fmol/mg protein. In contrast, the amount of AEBS was low (10% of the amount found in the parent cell line MCF-7) and near the limit of detection of specific tamoxifen binding in the tamoxifen-resistant Rtx-6 cells (Table 6).

TABLE 5. Effect of AEBS and sigma ligands on Rtx-6 cells

Compound	Effect (30 μM) 6 hr	48 hr	72 hr	Notes
Tamoxifen	N	C	C	C (100 μM) 72 hr
PBPE	N	N	N	
PCPE	N	N	N	
MBPE	N	N	N	
MCPE	N	N	N	
BD1008	N	N	N	
Haloperidol	N	N	A	
(+)-3-PPP	N	N	N	
(+)-Pentazocine	N	N	N	
DTG	N	N	A	

Experiments were carried out as described in Table 2 caption.

DISCUSSION

Three-dimensional analysis of PBPE and BD1008 showed that these compounds contain common structural features. PBPE and BD1008 are 60% superimpossible, and corresponded to the diphenylmethane and aminoethoxy moiety of tamoxifen. This would suggest their possible interaction with the same binding site. To test this hypothesis, we did a competition assay on AEBS. It has been shown [13] that the presence of the cyclic amine influences affinity for AEBS [3, 14] and that the presence of a hydrophobic phenylethylenic moiety on the benzyl position contributes to the high affinity of tamoxifen for AEBS. This phenylethylenic moiety is, however, a factor in loss of specificity, since this moiety is also important to estrogen receptor, calmodulin, and protein kinase C binding [35]. In this study, we showed that the diphenylmethane compounds PBPE and MBPE had a high affinity for AEBS, the affinity of the pyrrolidinyl derivative (PBPE) being greater than that of the morpholinyl derivative (MBPE). The same observation was made for cumyl derivatives (PCPE and MCPE), thereby confirming that the structure of the amine was a high-affinity determinant. Modification of the hydrophobic part of the molecule by addition of two methyl groups induced a slight decrease in affinity, which is interesting in that this position is thought to accept the bulky phenylethylene substituent [13]. Prototypical sigma compounds displayed a low affinity for AEBS, but BD1008, which exhibits structural homology with AEBS ligands, showed, as expected, a high affinity for AEBS. This point is of particular interest, because past attention has mainly been focused on the development of triphenylethylenic-based compounds and the present data may open up a new direction for the design of new AEBS ligands. BD1008 has been described as a selective sigma receptor ligand [16]. Competition experiments showed that BD1008, pentazocine, and DTG are competitive inhibitors of tamoxifen. Interestingly, Scatchard analysis did not show any heterogeneity in the slopes obtained with or without competitors, thereby showing both qualitatively and quantitatively that sigma ligands compete on one class of tamoxifen-binding site.

As described below, prototypical sigma ligands displayed a low affinity for AEBS, and a low or negligible affinity of AEBS ligands for sigma receptors was therefore expected. However, AEBS ligands showed high-affinity binding with sigma receptors. The diphenylmethane derivatives (PBPE and MBPE) and cumyl derivatives (PCPE and MCPE) displayed a higher affinity for sigma-1 receptors than for sigma-2 receptors. The pyrrolidinyl derivatives of both series (PBPE and PCPE) showed a higher affinity than the morpholinyl derivatives (MBPE and MCPE) for sigma receptors. The affinity of PBPE for sigma-1 was 7.4 times

TABLE 6. Binding parameters of AEBS, sigma-1, and sigma-2 receptors in membranes of various tumoral cell lines

Cell line	AEBS ([^{3}H]tamoxifen)	sigma-1 ([^{3}H](+)pentazocine)	sigma-2 ([^{3}H]DTG)
MCF-7	K_d = 5.3 ± 1.9	K_d = 45.56 ± 13.74	K_d = 19.32 ± 6.2
	B_{max} = 1534 ± 69	B_{max} = 369 ± 25	B_{max} = 2723 ± 52
C6	K_d = 17.8 ± 2.4	K_d1 = 5.7 ± 1.3	K_d = 101 ± 7.0
	B_{max} = 1830 ± 56	$B_{max}1$ = 42.0 ± 17.4	B_{max} = 5507 ± 537
		K_d2 = 287 ± 65	
		$B_{max}2$ = 942 ± 136	
SK-N-SH	K_d = 4.8 ± 2.3	K_d = 27.99 ± 5.78	K_d = 32.35 ± 3.36
	B_{max} = 1142 ± 335	B_{max} = 975 ± 225	B_{max} = 944 ± 104
Rtx-6	K_d = 4.8 ± 2.1	K_d = 36.04 ± 3.76	K_d = 42.19 ± 7.71
	B_{max} = 152 ± 34	B_{max} = 287 ± 32	B_{max} = 1136 ± 64

Fifteen concentrations were used over a range of 0.1 to 25 nM for [^{3}H]tamoxifen, over a range of 0.1 to 500 nM for [^{3}H](+)-pentazocine, and over a range of 0.1 to 400 nM (in the presence of 1 μM dextrallorphan) for [^{3}H]DTG. Data were analyzed using the iterative curve-fitting program BDATA (EMF Software); or GraphPad Prism (version 2.0). Values are the average of 2 to 3 experiments ± SEM, each carried out in duplicate. Values for [^{3}H](+)-pentazocine and [^{3}H]DTG in C6 glioma cells and neuroblastoma SK-N-SH cells were taken from Bowen and colleagues [23] and are the average of 3–5 experiments ± SEM, each carried out in duplicate. K_d values are in nM and B_{max} values are in fmol/mg protein.

greater than MBPE, and that of PCPE was 14.1 times greater than MCPE. For sigma-2, the increase in affinity in the diphenylmethane series compared to the cumyl series was 15.3 and 11.2, respectively. The presence of the two methyl groups at the benzyl position decreased the affinity for sigma-1 receptors by a factor of 4.3 for the pyrrolidinic compounds and a factor of 8.47 for morpholinic compounds, showing that the effect was qualitatively the same as on AEBS. This structural modification had no significant effect on the pyrrolidinic series and produced a 1.3 factor increase in affinity for sigma-2 receptors. Tamoxifen bound to sigma receptors with moderate affinity. Tamoxifen bound sigma-2 with a 2-fold greater affinity than sigma-1, showing that the hydrophobic phenylethylene side chain and perhaps the presence of a different amine side chain did not allow a distinction between sigma receptors and did not increase the affinity for sigma-2. One would have expected little or no affinity for sigma receptors because DTG and pentazocine have an extremely low affinity for AEBS. This showed that the binding sites were not mutually interchangeable. The binding sites might differ in essence but may still be related. Phenytoin, the sigma-1 allosteric regulator, is a diphenylmethane derivative that has a hydrophobic diphenylmethane moiety in common with tamoxifen. Three-dimensional structure analysis of this moiety with the tamoxifen hydrophobic core and using molecular modeling showed that both molecules were superimposable in their lowest energy conformation. The phenytoin diphenylmethane moiety matched up with the diphenylmethane moiety of tamoxifen. Intersection of their van der Waals volumes showed that they shared a volume of 123.58 Å^3, which represents 66% of the phenytoin molecule. Phenytoin was found to be a weak competitive inhibitor of tamoxifen binding to AEBS. One would have expected a higher affinity for AEBS, but phenytoin does not contain the protonable side chain, which is the high-affinity determinant for AEBS binding. Further, phenytoin interacts differently with AEBS and sigma-1 receptors, since it has been found to be a positive allosteric modulator of ligand binding to sigma-1 sites.

The cytotoxicities of AEBS ligands and sigma ligands were tested on different tumoral cell lines. Tamoxifen induced cytotoxicity in a dose-dependent manner on MCF-7 cells, on neuroblastoma SK-N-SH, and on rat C6 glioma. The MCF-7 cells were more sensitive than the other cell lines. As pointed out in other studies, the presence of estrogen receptors might increase the sensitivity of MCF-7 cells to tamoxifen. Tamoxifen cytotoxicity has previously been described on C6 cells [36] and on SK-N-SH [37]. The proposed mechanism for cytotoxic activity involved a protein kinase C (PKC) inhibition which was consistent with its IC_{50} on PKC [38]. Interestingly, the cytotoxic efficiency with specific AEBS ligands and BD1008 was comparable to that of tamoxifen. Moreover, it had previously been shown that diphenylmethane AEBS ligands are not inhibitors of PKC [39, 40], which would suggest that the observed cytotoxic activity could be related to AEBS or sigma receptor binding. The amount of membranous AEBS in the three cell lines sensitive to the test compounds was greater than 1 pmol/mg protein (Table 6). In the tamoxifen-resistant Rtx-6 cells, the amount of AEBS was 8.3 to 13% of the amount found in tamoxifen-sensitive cells, which would confirm earlier observations [3, 32].

Sigma receptor-active neuroleptic drugs were initially shown to be cytotoxic to C6 glioma cells [27]. Later studies showed that a variety of structurally diverse compounds with sigma-binding affinity were cytotoxic to C6 glioma cells and other lines in culture [24]. Initially, a general correlation was made between cytotoxic potency and affinity at sigma-1 sites in C6 glioma cells labeled using [^{3}H](+)-pentazocine [24]. However, later studies have shown a much better correlation of the morphologic and cytotoxic effects of sigma ligands with affinity at sigma-2 sites [26]. Sigma-1-selective ligands were found to have little or no effect, whereas sigma-2-selective ligands and those binding to both sigma subtypes produced morphologic changes and apoptotic cell death in rat cerebellar granule cells and human SK-N-SH neuroblastoma cells [26]. The current data may also support a role for sigma-2 receptors in the action of AEBS ligands. For example,

haloperidol, which binds with very low affinity to AEBS but high affinity to sigma-2 sites, is able to produce varying degrees of toxicity in these cells. Also, it is noteworthy that, in C6 glioma cells and SK-N-SH neuroblastoma cells, there is a relatively large difference in morphologic and cytotoxic potency demonstrated by the pyrrolidinyl derivatives (PBPE and PCPE) compared with the morpholino derivatives (MBPE and MCPE) (Tables 4 and 5). The moderate difference in affinity of these compounds at AEBS sites (or sigma-1 sites) cannot adequately account for this difference in potency. However, the 15.3- and 11.2-fold lower sigma-2 affinity of MBPE and MCPE, respectively, compared to their pyrrolidinyl counterparts could well account for their decreased potency. This would indicate a cytotoxic role for sigma-2 in these cells. On the other hand, the difference in potency in the Rtx-6 cell line, which is about 10% of the AEBS sites, was comparable to the other cell lines (Table 6). Taken together, the data indicate that both AEBS and sigma-2 may contribute to the cytotoxic effects of tamoxifen and the other compounds tested. However, the contribution of these sites may be cell type-specific, where AEBS sites may play more of a role in the breast tumor cell lines. The possible contribution of AEBS and sigma-2 receptors to cytotoxicity in different cell types will need to be investigated further.

Altogether, these results show that AEBS and sigma receptors may be physically related binding sites, and that binding to AEBS might be indicative of the cytotoxicity of compounds on cells of different origins, which might be useful in the screening of new antitumoral drugs.

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