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Studies on the Ligand Specificity and Potential Identity of Microsomal Antiestrogen-Binding Sites

COLIN K. W. WATTS and ROBERT L. SUTHERLAND

Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, New South Wales, 2010, Australia

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SUMMARY

Synthetic nonsteroidal antiestrogens are bound intracellularly by two high affinity saturable binding sites, the estrogen receptor and the microsomal antiestrogen-binding site (AEBS). In order to further define the structural requirements for ligand binding to AEBS from rat liver and the MCF 7 human breast cancer cell line, the relative binding affinities of an extensive series of structurally related ligands were investigated using competitive binding assay techniques. The groups of compounds studied were: analogues of the triphenylethylene antiestrogens, CI 628 and tamoxifen; analogues of cyclofenil; bibenzyl and stilbene derivatives; analogues of the cytochrome P-450 inhibitor SKF-525A; phenothiazine derivatives; and a series of structurally related compounds with a variety of pharmacological activities. High affinity binding to AEBS required the presence of both a hydrophilic basic aminoether side chain and a hydrophobic aromatic ring stucture (di- or tricyclic for maximal affinity). Structural modifications to either influenced binding affinity. Aromatic substitution either raised (CF_3) or lowered (OH, OCH_3) affinity, apparently by electronic effects transmitted through the benzene nucleus. Side chain structure was the major determinant of binding affinity, but its influence was complex and dependent upon terminal amino group structure, side chain branching and substitution, and tissue source of AEBS. Optimal binding affinity was shown by side chains bearing basic heterocyclic amino terminal groups. Other cellular sites that are known to bind antiestrogens with relatively high affinity include calmodulin, cytochrome P-450, and histamine, dopamine, and muscarinic receptors. Binding studies using a variety of pharmacologically active and radiolabeled ligands selective for these sites, including those for dopamine D_1 and D_2 receptors ([^{3}H]fluphenazine, [^{3}H]flupenthixol, [^{3}H]spiperone, and [^{3}H]SCH 23390) and histamine H_1 receptors ([^{3}H]pyrilamine), demonstrated that several of these compounds interact with AEBS with high affinity. However, the ligand specificity and other binding properties of the AEBS as determined by competitive binding studies and Scatchard analysis show this site to be a molecular entity truely distinct from these other cellular binding sites.

The nonsteroidal antiestrogens, synthetic compounds which comprise the most studied group of estrogen antagonists, are clinically important agents, particularly in the treatment of breast cancer. Such compounds are generally accepted to exert many of their biological activities through interaction with the ER system of estrogen target cells (as reviewed in Refs. 1–4). In addition, such compounds bind, often with high affinity, to a microsomal protein (AEBS) present in a wide variety of tissues (5–13). However, despite definition of the biochemical properties of this site (5–13), the physiological and pharmacological significance of the AEBS, particularly in antiestrogen action, remains unknown.

Structure-affinity studies, which could potentially provide insight into the functional role of the AEBS, are confined to a limited range of triphenylethylene antiestrogens, mainly analogues of TAM (10, 11, 13) and clomiphene (11, 14), and a small number of other structurally related antiestrogenic and miscellaneous compounds (15, 16). Because of the availability of a large number of antiestrogenic compounds in this laboratory, we have been able to undertake a more extensive study of the structural requirements of ligand binding to the AEBS. Analogues of TAM, CI 628 and cyclofenil, bibenzyl and stilbene derivatives, and structurally related compounds (including analogues of the cytochrome P-450 inhibitor SKF-525A and phenothiazine derivatives) were investigated. The binding properties of AEBS in rat liver and in the MCF 7 human breast cancer cell line were compared to assess any possible species/tissue differences.

There have been several recent reports of interactions of TAM and other antiestrogens with a variety of cellular receptors and binding sites distinct from ER and AEBS. These include muscarinic receptors (17), dopamine D_2 receptors (18), histamine H_1 receptors (19), cytochrome P-450 (20), calmodulin (21), and protein kinase C (22), and there has been speculation that the AEBS could represent one of these sites. Therefore, a

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ABBREVIATIONS: ER, estrogen receptor; AEBS, antiestrogen-binding site; TAM, tamoxifen (Nolvadex); ICI 46474, *trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene; CI 628, nitromifene, α-(4-pyrrolidinylethoxy)phenyl-4-methoxy-α-nitrostilbene; RBA, relative binding affinity; DPPE, *N,N*-diethyl-2-[(4-phenylmethyl)-phenoxy]-ethanamine·HCl.

further aspect of the present study was to determine whether binding to AEBS by antiestrogens and compounds with a variety of other pharmacological activities might indicate that the AEBS had identity with other known receptors of binding sites.

Materials and Methods

The structures of the compounds used in this study are illustrated in Fig. 1 and Tables 1–6 (see Results). *trans*-[*N-methyl*-^{3}H]TAM, (71–89 Ci/mmol), [^{3}H]fluphenazine (30.8 Ci/mmol), [^{3}H]pyrilamine (26 Ci/mmol), and [^{3}H]spiperone (77 Ci/mmol) were purchased from Amersham Australia (Sydney, Australia); [^{3}H]flupenthixol (10.2 Ci/mmol) and [^{3}H]SCH 23390 (87 Ci/mmol) were purchased from New England Nuclear (Boston, MA).

TAM and its analogues were supplied by I.C.I. Pharmaceuticals Division (Macclesfield, Cheshire, England) through the courtesy of Drs. B. Tait and A. Wakeling. TAM *N*-oxide was synthesized as reported by Foster *et al.* (23). CI 628 and its analogues (as 50:50 mixtures of the *cis* and *trans* isomers) were from Dr. E. Elslager of the Warner-Lambert Parke Davis Company (Ann Arbor, MI). Bibenzyl and stilbene derivatives (the H series of compounds) were synthesized by Professor C. W. Emmens, University of Sydney (Sydney, Australia). These compounds are weak antiestrogens (24). Cyclofenil and the related diphenylethylene derivatives were supplied by Dr. Guy Leclercq, Institut Jules Bordet (Brussels, Belgium). SKF-525A and its analogues were supplied by Smith, Kline and French Laboratories (Philadelphia, PA). Fluphenazine hydrochloride was supplied through courtesy of Mr. P. F. Levvey, E. R. Squibb and Sons Pty. Ltd. (Noble Park, Victoria, Australia). Other compounds were from Sigma Chemical Co. (St. Louis, MO).

Stock solutions (10^{-6}–5×10^{-2} M) were prepared in ethanol or in *N*, *N*-dimethylformamide when compounds were insoluble in ethanol and stored at −20° in glass vials. Procedures for the preparation of aqueous stock solutions of these compounds were as previously described (11–13).

RBAs for the AEBS were determined by competitive binding assays conducted in the presence of 1 μM prostaglandin E_2 to eliminate interaction with ER, using either KCl-washed rat liver microsomes (12, 13) or MCF 7 postmitochondrial fraction (11) as a source of AEBS. In outline, [^{3}H]TAM at a final concentration of 4–5 nM was incubated for 16 hr at 0° or for 1–2 hr at 22° with increasing concentrations of unlabeled ligand over the range 2.5 nM–10 μM. The incubation mixture consisted of 50 μl of 16–20 nM [^{3}H]TAM in 10 mM Tris-HCl, 25 mM KCl, 0.25 M sucrose, pH 7.4 at 22° buffer (TSK) containing 1 mg/ml bovine serum albumin (TSKB), 50 μl of the unlabeled competing ligand at concentrations of up to 40 μM in TSKB, and 100 μl of MCF 7 postmitochondrial fraction or rat liver microsomal suspension in TSK. Bound and free [^{3}H]TAM were separated by charcoal/dextran adsorption. In some experiments the unlabeled competing ligands were added as ethanol stock solutions in which case, instead of 50 μl of unlabeled ligand in TSKB, 40 μl of TSKB and 10 μl of ethanol stock solution were added. This concentration of ethanol had no significant effect on the determination of RBA. The use of ethanol stocks allowed high concentrations of ligands with limited solubility in aqueous solutions to be used. Stock solutions prepared in dimethylformamide could also be used in the same way. Data were plotted as percentage total [^{3}H]TAM bound versus log ligand concentration. RBA was calculated as (IC_{50} of TAM/IC_{50} of the test compound) × 100%, where IC_{50} = the concentration of ligand required to displace 50% of the specifically bound [^{3}H]TAM. The RBA of TAM for AEBS is defined as 100%.

In competition studies with [^{3}H]fluphenazine and [^{3}H]pyrilamine the final concentration of radiolabel was 7–14 nM and 15 nM, respectively. Otherwise the assays were performed as described above for [^{3}H]TAM.

For Scatchard analysis (25), 100-μl aliquots of microsomal suspension in TSK containing 1 μM prostaglandin E_2 were incubated with 50 μl of increasing concentrations of radiolabeled ligand in TSKB, and either 50 μl of TSKB or 50 μl of unlabeled ligand (400 nM) in TSKB giving a total reaction volume of 200 μl. After incubation and separation of bound and free ligand as described above, correction for nonspecific binding was applied using the method of Chamness and McGuire (26), and binding site capacities and dissociation constants (K_D) were calculated.

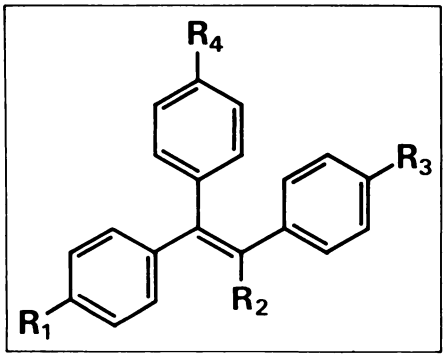


Fig. 1. Generalized structure of substituted triphenylethylenes.

Results

Triphenylethylene antiestrogens. The RBAs of CI 628 analogues for rat liver and MCF 7 AEBS are shown in Table 1. The order of binding affinity as determined by the terminal amino group structure was: —*c*—$N(CH_2CH_2)_2CH_2$ (piperidino—) > —*c*—NC_4H_8 (pyrrolidinyl) > —$N(C_2H_5)_2$ > —$N(CH_3)_2$ > —$NHCH_3$ > —*c*—NC_3NH_3 (imidazolyl) > —*c*—NC_2H_4 (aziridine) (compound **4** versus **3** versus **2** versus **1** versus **5**; **9** versus **14**; **13** versus **6** versus **12**; **20** versus **7**; **31** versus **27** versus **22**). Compounds with nonbasic side chains had no affinity (not shown).

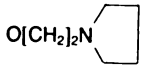
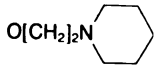
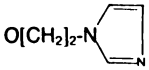
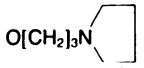
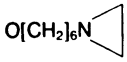
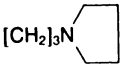
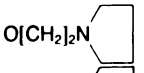
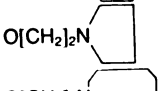
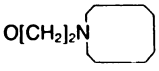
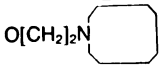
The effect of hydrocarbon side chain length on binding affinity was dependent upon the terminal amino group structure. For compounds with ether-linked side chains terminating in dimethylamino groups, the order of binding affinity as determined by the indicated number of side chain methylene units was 3 > 4 > 5 = 6 > 2 in the case of rat liver AEBS (compound **6** versus **7** versus **8** versus **9** versus **1**). This order was the same for MCF 7 AEBS except that compound 1 with 2 methylene units did not have decreased affinity. In fact, this was the only CI 628 analogue to show major differences in RBA between MCF 7 and rat liver AEBS. A slightly different order was observed when no ether side chain linkage was present (compound **17** versus **19** versus **18**), in which case the binding order was **3** = **5** > 4 methylene units (rat liver) or 3 > 5 > 4 methylene units (MCF 7). For pyrrolidinyl or diethylamino terminal groups the same binding affinity was found for compounds with either 2 or 3 methylene units (compound **3** versus **13**, and **15** versus **16**, respectively). Side chain branching increased binding affinity (compound **1** versus **10** and **11**).

The nature of the bond linking the side chain to the remainder of the molecule influenced binding affinity. Thus, a C—C bond, instead of an ether linkage, produced compounds of higher affinity (compounds **17**–**19** versus **1** and **6**–**8**; **20** versus **3** or **13**). However, a thioether bond caused no change in affinity (compound **2** versus **15**).

A comparison of CI 628 analogues also enabled the effects of aromatic substitution to be studied. The apparent order of influence of various substituents (from compounds of highest to lowest affinity) was: CF_3 > H > CH_3 > 2 × CH_3 = Cl > 2 × Cl > OCH_3 > OC_2H_5.

TABLE 1

Relative binding affinities of CI 628 analogues for MCF 7 and rat liver microsomal AEBS[a]

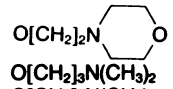
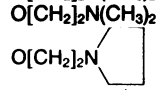
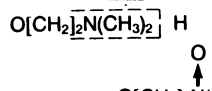
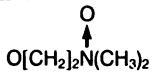
Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA	
						AEBS (Rat liver)	AEBS (MCF 7)
1	65,071	OCH_3	NO_2	H	$O[CH_2]_2N(CH_3)_2$	5 ± 0.2	18 ± 0.5
2	55,325	OCH_3	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	35 ± 4	38 ± 3
3	CI 628	OCH_3	NO_2	H		51 ± 4	82 ± 9
4	56,295	OCH_3	NO_2	H		95 ± 13	140 ± 7
5	63,356	OCH_3	NO_2	H		5 ± 0.3	
6	62,501	OCH_3	NO_2	H	$O[CH_2]_3N(CH_3)_2$	32 ± 3	21 ± 3
7	64,983	OCH_3	NO_2	H	$O[CH_2]_4N(CH_3)_2$	12 ± 2	15 ± 1
8	64,984	OCH_3	NO_2	H	$O[CH_2]_5N(CH_3)_2$	12 ± 0.1	10 ± 0.5
9	65,070	OCH_3	NO_2	H	$O[CH_2]_6N(CH_3)_2$	19 ± 3	11 ± 3
10	66,640	OCH_3	NO_2	H	$OCH(CH_3)CH_2N(CH_3)_2$	33 ± 4	23 ± 3
11	61,665	OCH_3	NO_2	H	$OCH_2C(CH_3)_2N(CH_3)_2$	107 ± 2	134 ± 12
12	63,557	OCH_3	NO_2	H	$O[CH_2]_3NHCH_3$	6 ± 0.5	
13	57,788	OCH_3	NO_2	H		57 ± 12	
14	66,419	OCH_3	NO_2	H		0.8 ± 0.3	
15	65,470	OCH_3	NO_2	H	$S[CH_2]_2N(C_2H_5)_2$	27 ± 2	
16	66,843	OCH_3	NO_2	H	$S[CH_2]_3N(C_2H_5)_2$	27 ± 5	
17	70,117	OCH_3	NO_2	H	$[CH_2]_3N(CH_3)_2$	64 ± 4	64 ± 7
18	65,969	OCH_3	NO_2	H	$[CH_2]_4N(CH_3)_2$	31 ± 0.9	21 ± 2
19	66,113	OCH_3	NO_2	H	$[CH_2]_5N(CH_3)_2$	61 ± 5	31 ± 3
20	70,347	OCH_3	NO_2	H		99 ± 8	133 ± 14
21	65,912	H	NO_2	H	$OCH(CH_3)CH_2N(CH_3)_2$	40 ± 4	
22	67,013	Cl	NO_2	H	$O[CH_2]_3N(CH_3)_2$	49 ± 4	
23	63,591	H	NO_2	Cl_2[b]	$O[CH_2]_2N(CH_3)_2$	14 ± 1	
24	55,067	H	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	114 ± 2	
25	55,169	CH_3	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	91 ± 1	
26	55,220	CH_3	NO_2	CH_3	$O[CH_2]_2N(C_2H_5)_2$	80 ± 11	
27	55,368	Cl	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	76 ± 12	81 ± 0.8
28	55,752	Cl	NO_2	Cl[c]	$O[CH_2]_2N(C_2H_5)_2$	55 ± 3	
29	56,422	CF_3[d]	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	175 ± 20	205 ± 17
30	56,252	OC_2H_5	NO_2	H		33 ± 0.6	
31	63,052	Cl	NO_2	H		119 ± 1	127 ± 2
32	56,152		NO_2	Cl[c]		8 ± 2	

[a] R_1–R_4 refer to Fig. 1.
[b] 2,4-Chloro.
[c] *ortho*-.
[d] *meta*-.

The binding properties of a number of TAM analogues to MCF 7 and rat liver AEBS have been previously reported by this laboratory (11–13). For comparative purposes the data have been combined with those for several additional analogues in Table 2. As was the case for CI 628 analogues, RBA is influenced by structure of both the hydrophobic portion of the molecule and, in particular, the hydrophilic basic aminoether side chain. When ligand specificity is compared between rat liver and MCF 7 AEBS, both quantitative and, in some cases, qualitative differences are apparent. In general, the rat liver AEBS has more rigorous requirements with regard to ligand structure, and alterations to the ligand, especially to the terminal amino group and to the hydrophobic triphenylethylene portion of the molecule, cause larger changes in binding affinity than for MCF 7 AEBS (e.g., TAM versus compounds **35**, **42**, and **43**). In contrast, binding affinity for the MCF 7 AEBS is more sensitive to changes involving the alkyl portion of the alkyl amino ether side chain (TAM versus compounds **37** and **40**), but not when the terminal group is $—N(C_2H_5)_2$ (compound **36** versus **38**).

Bibenzyl and stilbene derivatives. The affinities of bibenzyl and stilbene derivatives for rat liver and MCF 7 AEBS were generally low (Table 3), perhaps not unexpectedly because of the presence of aromatic hydroxy and methoxy substituents which, as has been shown for triphenylethylenes, decrease RBA. Compounds with aromatic methoxy substituents had

TABLE 2
Relative binding affinities of TAM analogues for MCF 7 and rat liver microsomal AEBS[a]

Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA AEBS (Rat liver)	RBA AEBS (MCF 7)
33	Tamoxifen (*trans*)	H	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	100	100
34	55,548[b]	H	C_2H_5	H	$O[CH_2]_2NHCH_3$	12 ± 1	19 ± 2
35	79,280[c]	OH	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	30 ± 5	80 ± 7
36	45,960	H	C_2H_5	H	$O[CH_2]_2N(C_2H_5)_2$	21 ± 5	46 ± 4
37	47,108	H	C_2H_5	H	$O[CH_2]_3N(CH_3)_2$	127 ± 15	36 ± 1
38	47,590	H	C_2H_5	H	$O[CH_2]_4N(C_2H_5)_2$	119 ± 26	54 ± 7
39	47,699 (*cis*)	H	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	73 ± 7	98 ± 9
40	94,230	H	C_2H_5	H	$OCH_2CHOHCH_2N(CH_3)_2$	14 ± 3	2 ± 0.3
41	132,802	H	C_2H_5	OCH_3	$O[CH_2]_2N(CH_3)_2$	22 ± 0.2	35 ± 5
42	148,067	H	C_2H_5	F	$O[CH_2]_2N(CH_3)_2$	31 ± 3	97 ± 1
43	47,399	H	C_2H_5	H		1993 ± 924	219 ± 14
44	46,414	H	CH_3	H	$O[CH_2]_3N(CH_3)_2$	163 ± 29	32 ± 5
45	49,500	H	C_3H_7	H	$O[CH_2]_2N(CH_3)_2$	42 ± 10	73 ± 7
46	133,312	H	C_2H_5	CH_3	$O[CH_2]_2N(CH_3)_2$	58 ± 9	96 ± 6
47	129,817	OH	C_2H_5	CH_3	$O[CH_2]_2N(CH_3)_2$	7 ± 1	49 ± 14
48	129,351	OH	C_2H_5	H		28 ± 5	106 ± 9
49	133,055	H	C_2H_5			17 ± 2	69 ± 16
50	Tamoxifen *N*-oxide	H	C_2H_5	H		19 ± 3	5 ± 3

[a] R_1–R_4 refer to Fig. 1.
[b] *N*-desmethylTAM.
[c] 4-hydroxyTAM.

higher affinities than compounds with hydroxy substituents (compound **51** versus **52**; **53** versus **54**; **56** versus **57**). Side chain terminal amino group structure was the most important determinant of binding affinity and for rat liver the order was: —*c*—$N(CH_2CH_2)_2CH_2$ = —*c*—NC_4H_8 > —$N(C_2H_5)_2$ > —*c*—$N(CH_2CH_2)_2O$ = —$N(CH_3)_2$ (compounds **58–62** and **64–66**). A similar order was observed for MCF 7 AEBS. Dibasic aminoether derivatives had decreased affinity (compound **62** versus **63**; **66** versus **67**).

Structural variations in that part of the molecule connecting the two benzene rings (i.e., presence or absence of unsaturation, number and positions of methyl substituents) had little or no significant effect on affinity for rat liver AEBS. Methyl substitution had a variable effect on affinity for MCF 7 AEBS (compound **54** versus **64**; **52** versus **64**; **52** versus **54**; **51** versus **53**) and unsaturation caused a general decrease in RBA for MCF 7 AEBS (compound **51** versus **68**; **59** versus **64**; **52** versus **57**).

Cyclofenil and diphenylethylene derivatives. Cyclofenil derivatives (Table 4) had markedly lower affinities for rat liver AEBS than for MCF 7 AEBS, with the exception of compound **69** which carries a terminal diethylamino group in the side chain. However, the effects on RBA of differing terminal groups were qualitatively the same for both tissues. The order of binding was —*c*—$N(CH_2CH_2)_2O$ > —*c*—$N(CH_2CH_2)_2CH_2$ = —$N(CH_3)_2$ > —$N(C_2H_5)_2$.

In the related diphenylethylene series (Table 4, compounds **73–75**) the effect of diethylamino and dimethylamino groups was reversed. Removal of the basic side chain abolished binding affinity (compounds **68** and **73**).

Analogues of SKF-525A. Affinities of the SKF-525A series were generally higher for rat liver AEBS (Table 5). For both sites aromatic substitution at R_1 or R_3 with Cl (compound **77**) or NO_2 (compound **76**) groups resulted in low affinity. In the remainder of the compounds the effects of aromatic substitution depended upon the nature of the substituent at R_2 and upon the tissue source of AEBS.

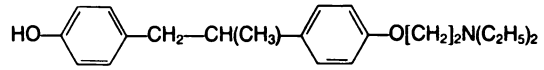
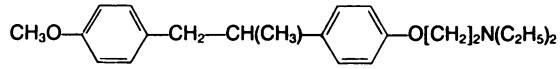
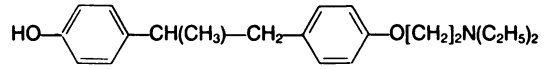
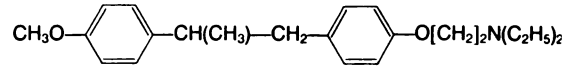
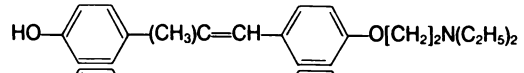
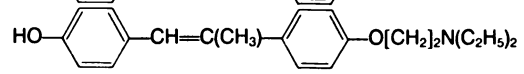
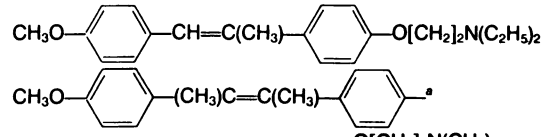
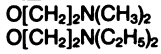
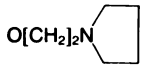
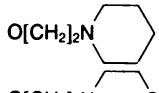
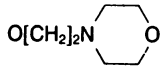
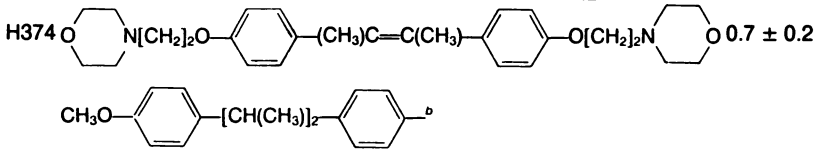
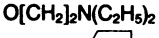
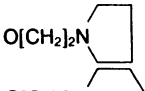
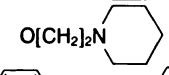
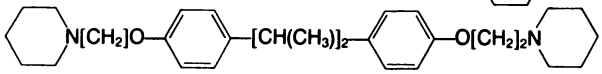
Phenothiazine derivatives. A series of phenothiazine derivatives (Table 6) has relatively high affinity for the AEBS. A basic side chain was necessary for binding, and affinity was determined both by the side chain structure (perphenazine versus prochlorperazine versus chlorpromazine) and by aromatic substitution (trifluoperazine versus chlorpromazine, rat liver; perphenazine versus fluphenazine, MCF 7). Tissue differences were also evident (fluphenazine). The structurally related thioxanthene derivative flupenthixol had a high affinity for AEBS (Table 6).

Possible identity of AEBS with other binding sites. The observation that phenothiazine derivatives bind with high affinity to AEBS is potentially significant given that these compounds are also known to interact with a variety of cellular binding sites including: calmodulin (27), dopamine D_1 and D_2, serotonin (5-HT_2), α_1- and α_2-adrenergic, muscarinic cholinergic, and histamine receptors (28, 29). To determine whether the AEBS was in fact one of these receptors, the binding of a variety of additional ligands, of known specificity for some of these receptor sites, to the AEBS was investigated.

First, the binding properties of [^{3}H]fluphenazine and the structurally related thioxanthene derivative [^{3}H]flupenthixol were directly studied in tissue microsomal fractions (Table 7, Fig. 2). Both [^{3}H]fluphenazine (Fig. 2) and [^{3}H]flupenthixol bound with high affinity to rat liver microsomes. [^{3}H]TAM and [^{3}H]flupenthixol bound to an equal number of sites, whereas [^{3}H]fluphenazine bound to additional sites from which it was displaced to a greater extent by 100 nM fluphenazine than by 100 nM TAM.

TABLE 3

Relative binding affinities of stilbene and bibenzyl derivatives for MCF 7 and rat liver microsomal AEBS

Compound No.	Code No.		RBA AEBS (Rat liver)	RBA AEBS (MCF 7)
51	H233		0.7 ± 0.2	1.4 ± 0.2
52	H237		9 ± 3	33 ± 4
53	H223		1.4 ± 0.5	0.6 ± 0.2
54	H228		8 ± 1	12 ± 2
55	H279			1.1 ± 0.2
56	H288			0.3 ± 0.1
57	H166			3.3 ± 0.1
58	H369		2.2 ± 0.2	3.3 ± 0.1
59	H246		8 ± 1	7 ± 1
60	H315		12 ± 1	19 ± 2
61	H336		13 ± 1	12 ± 1
62	H385		4 ± 0.1	7 ± 0
63	H374		0.7 ± 0.2	
64	H310		10 ± 1	15 ± 0
65	H730		20 ± 4	43 ± 1
66	H731		23 ± 7	30 ± 3
67	H725		1.9 ± 0.1	5 ± 0

[a] *trans*, compounds 58–62.
[b] Compounds 64–67.

The lack of affinity of TAM for the population of apparently fluphenazine-specific sites was confirmed in competition studies (Figs. 3 and 4) which showed that unlabeled fluphenazine fully competed for [^{3}H]TAM binding at high concentrations but displaced [^{3}H]fluphenazine to a greater extent than TAM at all unlabeled ligand concentrations > 40 nM. The identity of these fluphenazine-specific binding sites, which are not competed for by (+)-butaclamol (see below), is unknown.

Both fluphenazine and flupenthixol are unselective antagonists for dopamine D_1 and D_2 receptors (28, 29). To investigate whether the AEBS could be either of these receptors, a series of dopamine receptor ligands was tested for competitive binding to the AEBS (Table 8). (+)-Butaclamol, which has high affinity for D_1 and D_2 receptors (28, 30), was unable to displace significant amounts of either [^{3}H]TAM or [^{3}H]fluphenazine binding in rat liver microsomes (Figs. 3 and 4). In addition, the selective D_1 antagonist [^{3}H]SCH 23390 showed no specific binding to such preparations. The butyrophenones, haloperidol and spiperone, which are selective D_2 antagonists (29), also had no affinity for AEBS, and no specific binding of [^{3}H]spiperone to rat liver microsomes was detected in the present study.

Similarly, although fluphenazine and flupenthixol bind to the serotonin 5-HT_2 receptor (29), several other serotonin antagonists and uptake (amine pump) inhibitors including (+)-butaclamol, spiperone, haloperidol, amitryptyline, imipramine, and trimipramine (Table 8) had little or no affinity for AEBS.

As noted above, fluphenazine and flupenthixol have α_1-adrenergic antagonist activity (29). However, haloperidol and

TABLE 4

Relative binding affinities of cyclofenil analogues for MCF 7 and rat liver microsomal AEBS

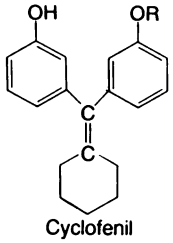
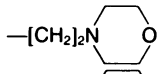
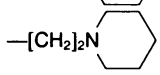
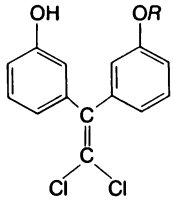
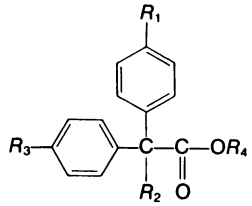
Compound No.	R	RBA AEBS (Rat liver)	RBA AEBS (MCF 7)
	 Cyclofenil		
68	—H	0	
69	—$[CH_2]_2N(C_2H_5)_2$	6 ± 1	0.4 ± 0
70	—$[CH_2]_2N(CH_3)_2$	17 ± 3	99 ± 9
71	—$[CH_2]_2N$ 	25 ± 1	122 ± 12
72	—$[CH_2]_2N$ 	10 ± 2	73 ± 11
			
73	—H	0	
74	—$[CH_2]_2N(C_2H_5)_2$	8 ± 1	
75	—$[CH_2]_2N(CH_3)_2$	2 ± 0.4	

TABLE 5

Relative binding affinities of SKF-525A analogues for MCF 7 and rat liver microsomal AEBS

Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA AEBS (Rat liver)	RBA AEBS (MCF 7)
							
76	80533	NO_2	CH_3	NO_2	$CH_2CH(CH_3)N(C_2H_5)_2$	0.6 ± 0.1	0.5
77	19086A	Cl	$[CH_2]_2CH_3$	Cl	$[CH_2]_2N(C_2H_5)_2$	2.5 ± 0.2	0.5
78	490	H	$CH_2CH(CH_3)_2$	H	$[CH_2]_2N(C_2H_5)_2$	5 ± 0.5	4
79	485A	H	$CHCH_2$	H	$[CH_2]_2N(C_2H_5)_2$	10 ± 2	1.2
80	19500A	CH_3	$[CH_2]_2CH_3$	CH_3	$[CH_2]_2N(C_2H_5)_2$	11 ± 1	2.6
81	525A	H	$[CH_2]_2CH_3$	H	$[CH_2]_2N(C_2H_5)_2$	12 ± 1	2
82	10108A	CH_3	CH_3	CH_3	$[CH_2]_2N(C_2H_5)_2$	33 ± 3	3

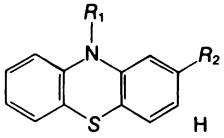
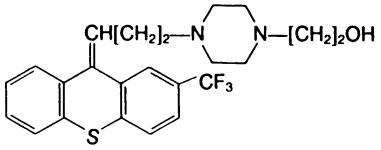
spiperone share this property and, like a series of other α- (and β-)adrenergic ligands (Table 8; Refs. 10 and 19), were found to have little or no affinity for the AEBS.

Binding of clomiphene to muscarinic cholinergic receptors has been reported (17). Fluphenazine and flupenthixol also have limited affinity for the muscarinic receptor (29), but other muscarinic and nicotinic cholinergic receptor ligands tested in the present study, including atropine, quinuclidonyl benzoate, benactyzine, acetylcholine, and tetracaine, had no affinity for the AEBS.

Fluphenazine and flupenthixol act as histamine receptor antagonists (29), and this was also investigated as a possible clue to the identity of the AEBS. Of several histamine H_1 antagonists (Table 8) only hydroxyzine had high affinity for AEBS. Histamine agonists and H_2 antagonists including cimetidine and ranitidine had no affinity.

TABLE 6

Relative binding affinities of phenothiazine and thioxanthine derivatives for MCF 7 and rat liver microsomal AEBS

Compound	R_1	R_2	RBA AEBS (Rat liver)	RBA AEBS (MCF 7)
Phenothiazine		H	0	0
Trifluoperazine	$[CH_2]_3$—$N(CH_3)_2$	CF_3	31 ± 6	18 ± 3
Perphenazine	$CH[_2]_3$—N N—$[CH_2]_2OH$	Cl	39 ± 4	33 ± 5
Prochlorperazine	$[CH_2]_3$—N N—CH_3	Cl	21 ± 2	12 ± 1
Chlorpromazine	$[CH_2]_3$—$N(CH_3)_2$	Cl	1.5 ± 0.2	
Fluphenazine	$[CH_2]_3$—N N—$[CH_2]_2OH$	CF_3	41 ± 1	11
Flupenthixol			100[a]	

[a] Calculated from K_D, Table 7.

TABLE 7

Specific binding of [^{3}H]TAM, [^{3}H]fluphenazine, and [^{3}H]flupenthixol to rat liver microsomes

Ligand	K_D	C
	nM	pmol/mg protein
[^{3}H]TAM[a]	1.1 ± 0.2	18.54 ± 1.71
[^{3}H]Fluphenazine[a]	2.5 ± 0.6	25.85 ± 3.66
[^{3}H]Fluphenazine[b]	3.1 ± 0.5	30.49 ± 2.68
[^{3}H]Flupenthixol[a]	1.1 ± 0.3[c]	17.32 ± 0.24
[^{3}H]Flupenthixol[b]	1.4 ± 0.1	17.08 ± 1.71

[a] Nonspecific binding determined in the presence of 100 nM TAM.
[b] Nonspecific binding determined in the presence of 100 nM fluphenazine.
[c] Apparent RBA = 100%.

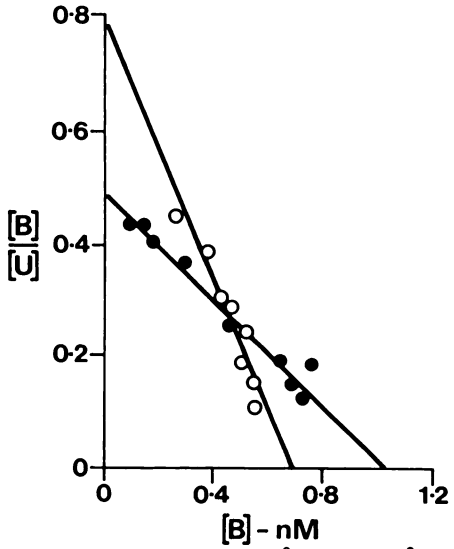


Fig. 2. Scatchard analysis of the binding of [^{3}H]TAM and [^{3}H]fluphenazine to rat liver microsomes. Rat liver microsomes were labeled with increasing concentrations of [^{3}H]TAM (○) and [^{3}H]fluphenazine (●) as described in Materials and Methods. Nonspecific binding was determined in the presence of 100 nM unlabeled TAM and fluphenazine, respectively. Data were plotted after correction for nonspecific binding.

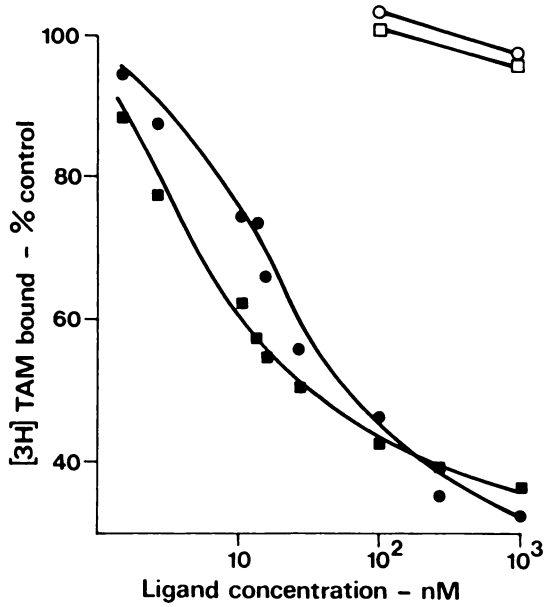


Fig. 3. Competitive binding assay for the rat liver microsomal AEBS. Rat liver microsomes were labeled with 5 nM [^{3}H]TAM together with increasing concentrations of unlabeled ligands as described in Materials and Methods. Data are expressed as the amount of [^{3}H]TAM bound (as a percentage of the total bound [^{3}H]TAM in the absence of unlabeled ligand) versus the concentration of unlabeled ligand. ■, TAM; ●, fluphenazine; □, (+)-butaclamol; ○, haloperidol.

The possible relationship between AEBS and histamine-receptor was further investigated by studying the binding of the H_1 ligand [^{3}H]pyrilamine to the rat liver microsomes. Linear Scatchard plots of [^{3}H]pyrilamine binding were obtained after correction for nonspecific binding (Fig. 5). The concentration of binding sites (i.e., pyrilamine-binding sites; 28.95 ± 0.88 pmol/mg of microsomal protein) was similar to the AEBS concentration determined by [^{3}H]TAM binding (32.17 ± 2.93 pmol/mg of protein). The AEBS content of the

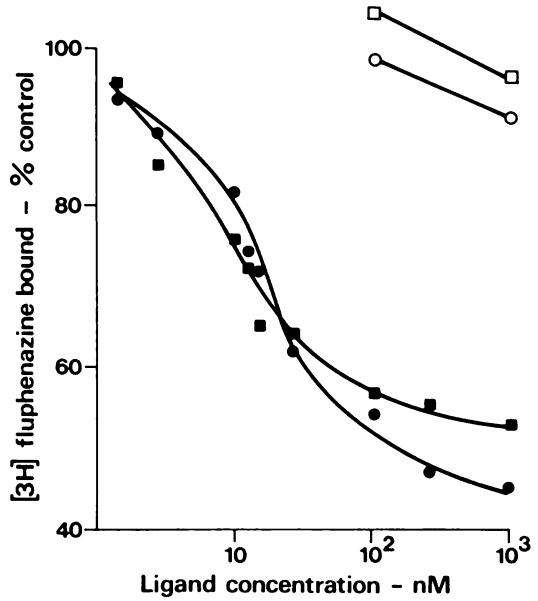


Fig. 4. Competitive binding assay for rat liver microsomal fluphenazine-binding sites. Rat liver microsomes were labeled with 7 nM [^{3}H]fluphenazine together with increasing concentrations of unlabeled ligands as described in Materials and Methods. Data are expressed as the amount of [^{3}H]fluphenazine bound (as a percentage of the total bound [^{3}H] fluphenazine in the absence of unlabeled ligand) versus the concentration of unlabeled ligand. ■, TAM; ●, fluphenazine, □, (+)-butaclamol; ○, haloperidol.

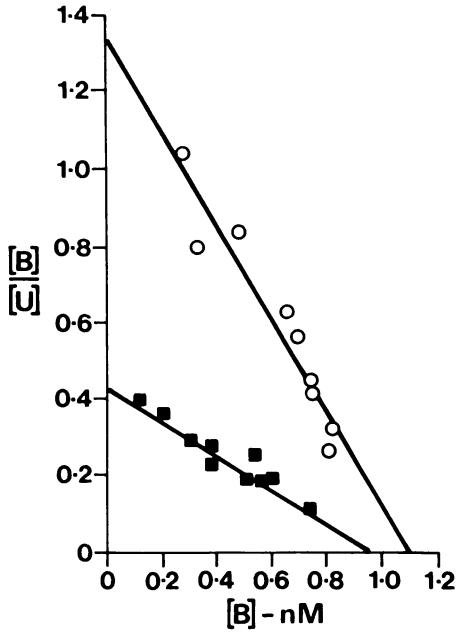


Fig. 5. Scatchard analysis of the binding of [^{3}H]TAM and [^{3}H]pyrilamine to rat liver microsomes. Rat liver microsomes were labeled with increasing concentrations of [^{3}H]TAM (○) and [^{3}H]pyrilamine (●) as described in Materials and Methods. Nonspecific binding was determined in the presence of 100 nM unlabeled TAM and pyrilamine, respectively. Data were plotted after correction for nonspecific binding.

TABLE 8

RBA of compounds with miscellaneous pharmacological activity for the AEBS

Compound	Receptor selectivity	RBA	
		Rat liver	MCF 7
		%	
(+)-Butaclamol	D_1, D_2 antagonist[a]	0	0.3
Spiperone	D_1, D_2 antagonist[a]	0	0
Haloperidol	D_1, D_2 antagonist[a]	0	0
Amitryptyline	serotonin antagonist[b]	0.2	0.4 ± 0.2
Imipramine	serotonin antagonist[b]	0.5	
Trimipramine	serotonin antagonist[b]	0.4 ± 0.2	0.4 ± 0.2
Desmethylimipramine	α-adrenergic antagonist[c]	0.05	
Propranalol	β-adrenergic antagonist[d]	1.4	
Hydroxyzine	H_1 antagonist	25 ± 3	25
Pyrilamine	H_1 antagonist	0.2 ± 0.1	
Phentoloxamine	H_1 antagonist	0.8 ± 0.3	0.7
Diphenhydramine	H_1 antagonist	≤0.05	

[a] Also has serotonin and α_1-adrenergic antagonist activity.
[b] Also has serotonin uptake inhibitory, antihistamine, anticholinergic, and antiadrenergic properties.
[c] Also a norepinephrine uptake (amine pump) inhibitor.
[d] Ref. 9.

microsomes used in this series of experiments was higher than in those used to determine [^{3}H]fluphenazine binding. [^{3}H] Pyrilamine binding (K_D 2.17 ± 0.07 nM) was of slightly lower affinity than that of [^{3}H]TAM (0.92 ± 0.05 nM; Ref. 12). Despite a similar concentration of specific binding sites for these two ligands, competitive binding studies indicated separate identities. When [^{3}H]pyrilamine was used as a radiolabel, TAM had an RBA for the pyrilamine binding site of 0.3% (compared to the RBA of pyrilamine for AEBS of 0.2 ± 0.1%).

Discussion

This study has provided the most detailed information yet available on the AEBS structure-affinity relationship. It is clear from the above results that high affinity binding to the AEBS requires a hydrophobic di- or tricyclic aromatic ring structure to which is attached a hydrocarbon side chain terminating in one of a number of basic amino groups. Structural changes at several positions in the ligand can affect binding affinity for AEBS, although it appears that the influence of a given substituent is variable and is dependent upon the structural features of the remainder of the ligand and on the tissue source of the AEBS.

In general, triphenylethylene derivatives have higher RBAs than compounds with fewer aromatic rings. Other studies have shown that compounds with a single benzene ring have little or no affinity (Refs. 15 and 16; unpublished observations). Two compounds with detectable affinity are *tert*-butyl-phenoxyethyl diethylamine (31), which had an RBA of 6% for MCF 7 AEBS, and bromophenoxyethyl dimethylamine, which had an RBA for rat liver AEBS of 0.7% (16). The local anesthetics tetracaine, procaine, benzocaine, and lignocaine have no affinity (11).

As well as the H compounds (Table 3) and SKF-525A analogues (Table 5), several other compounds containing two benzene rings in a variety of configurations bind with low affinity to the AEBS including: propranalol (RBA = 1.4%; Table 8); DPPE (RBA = 8–35%; Refs. 19, 32, and 33); the histamine H_1 antagonists hydroxyzine and pyrilamine (Table 8); and a miscellany of other compounds as reported by Lyman and Jordan (15) and Ruenitz and Bagley (16).

Apart from triphenylethylenes, several classes of compounds containing three aromatic or heterocyclic rings (including condensed ring structures) bind to AEBS if the appropriate side chain is also present. These include: benzothiophene antiestro-

gens [e.g., LY 117018 (RBA = 2%; Ref. 10) and LY 156758 (RBA = 10%, mouse liver AEBS; Ref. 15)]; dibenzazepines (e.g., imipramine and derivatives, RBA ≤1%; Table 8); phenothiazines (e.g., trifluoperazine; Table 6), and thioxanthenes (e.g., flupenthixol; Table 6).

The effects of aromatic substitution on RBA are dependent not only upon the substituents themselves but also on other structural features of the molecule, in particular the vinyl substituents of triphenylethylenes (32) and the basic side chain. In general, aromatic substituents which are electron donating because of resonance effects or high electron density (e.g., OH, OCH_3, Cl) or inductive effects (CH_3) result in reduced binding affinity, whereas an electron-withdrawing group has the opposite effect (e.g., CF_3). Hydrogen-bonding effects probably do not contribute to decreased binding affinity, given the high affinity produced by the latter group. Generally, the effects on binding affinity of aromatic substitution are qualitatively similar for MCF 7 and rat liver AEBS, but are quantitatively greater in the latter tissue.

cis-/trans-Isomerism about the triphenylethylene double bond either has no effect (compound **33** versus **39**, MCF 7 AEBS) or results in moderately reduced affinity of the *cis* form (compound **33** versus **39**, rat liver AEBS, enclomiphene versus zuclomiphene, Ref. 11).

The substituent on the triphenylethylene vinyl bond (R_2, Fig. 1) influences RBA as demonstrated elsewhere by a series of hydroxylated clomiphene derivatives (32). Similar effects are observed in nonhydroxylated triphenylethylenes, and comparisons of compounds **24, 36**, and enclomiphene (11) show that both C_2H_5 and NO_2 substituents cause reduction in affinity relative to Cl. The effects of vinyl substituents on RBA do not appear to correlate with molecular size (e.g., groups with large size, e.g., CN, NO_2, and C_2H_5, have both low and high affinities), although steric factors might play a role in some circumstances as shown by the reduced affinity of a propyl-substituted TAM analogue (compound **33** versus **45**). A correlation is observed, with their respective electronic effects in the benzene nucleus, i.e., their *ortho-*, *para-*, or *meta*-directing properties. NO_2 and CN groups, which have low affinity for AEBS, are *meta*-directing and are strongly electron withdrawing, whereas the C_2H_5 group has intermediate RBA, is electron donating, and is moderately *ortho-*, *para*-directing. The halogens Br and Cl are strongly electronegative and hence electron withdrawing, but resonance effects result in these groups being strongly *ortho-*, *para*-directing, and presumably also results in chloro-substituted triphenylethylenes, in particular, having high affinity for the AEBS by allowing optimal interaction with the binding site. It is uncertain whether these effects on RBA result from local interactions (i.e., between the substituent and its binding site environment) or whether they are due to the transmission of electronic effects through the extended conjugated system of the triphenylethylene molecule, as is the case in benzene substitution. Such an explanation has been proposed to account for differences in binding affinity for ER of another series of hydroxylated triphenylethylenes through changes in phenolic hydroxyl group acidity (33). Interestingly, the influence of substituents at the R_2 position of triphenylethylenes on binding affinity is the opposite to that observed for aromatic substitution at R_1.

An alkyl-substituted basic amino terminal side chain group appears to be an absolute requirement for binding to the AEBS, with the possible exception of certain cholesterol metabolites (34, 35). Compounds with nonbasic side chains, including the natural and synthetic steroids, have no affinity. This suggests that an ionic interaction, perhaps with acidic amino acid or phospholipid residues, is important for the formation of the AEBS-ligand complex. Although it has been concluded that base strength is not correlated with affinity for AEBS among a series of TAM analogues (10), additional data suggest that such a relationship may exist. Thus, TAM analogues with terminal amino groups with lower basicity relative to the dimethylamino group (pK_a = 10.8) of TAM (e.g., morpholino-, pK_a = 8.7, compound **43**; and methyl-piperazinyl, pK_a = 9.8, Ref. 10) had higher RBAs, whereas those with more basic groups (secondary amines, e.g., desmethyl-, compound **34**; and guanidino-, pK_a = 13.7, ref. 10) had lower affinity. Furthermore, the presence of a highly basic quarternary amino group abolishes affinity (16), as does the very weakly basic pyrrolidinyl group, pK_a = −3.8 (not shown). Weak bases, e.g., aziridine- (pK_a = 8, compound **14**, Ref. 36) and imidazole- (pK_a = 7.0, compound **5**) groups produced very low affinity analogues. These observations suggest that there may be an optimal ligand basicity, with lower or higher base strengths resulting in decreased affinity.

Steric and other factors must also determine the influence of the terminal group, as groups with similar base strengths can produce widely differing RBAs. In addition, the structure of the remainder of the ligand must be taken into account. Thus, dimethylamino derivatives of TAM and cyclofenil have greater affinity than the corresponding diethylamino derivatives (compound **33** versus **36; 69** versus **70**), whereas the reverse is true of CI 628 (compound **1** versus **2**) and diphenylethylene (compound **74** versus **75**) analogues. A morpholino-containing side chain results in a high affinity TAM analogue (compound **43**) but has little effect on the RBA of the corresponding stilbene compound (compound **62**) or cyclofenil derivative (compound **71**). In addition, the presence of a pyrrolidinyl group, which generally results in high RBA (compounds **3, 31,** and **48**), has little influence on RBA of analogues with aromatic hydroxyl substituents (compound **35** versus **48**).

Two compounds which contribute to the AEBS-inhibitory activity of ethanol extracts of human serum have been tentatively identified as 5-cholestene-3β-ol-7-one (7-ketocholesterol), and 4-cholesten-3-one (34). These cholesterol metabolites are the only known compounds without basic side chains which bind, albeit with low affinity, to the AEBS as assessed by competitive binding assays. It may be that such compounds are not true competitive inhibitors of TAM binding, but act through noncompetitive mechanisms, e.g, by disruption of the binding site lipid environment. It has been shown that high concentrations of crude serum extracts inhibit TAM binding noncompetitively (35).

In summary, for all classes of compounds tertiary basic heterocyclic amino terminal groups (e.g., pyrrolidinyl, piperidino-, methylpiperazino-, and morpholino-), result in a generally higher affinity than tertiary basic alkyl amino groups (e.g., dimethylamino-, diethylamino-, and dipropylamino-) which in turn have higher affinity than secondary amino groups (methylamino-, ethylamino-).

As well as the terminal amino group, the structure of the remainder of the side chain influences binding affinity. Changes in the length of the hydrocarbon chain produce effects on RBA, the magnitude and direction of which are dependent in a complicated manner on the structural features of the compound (in particular, the terminal amino group structure) and on the tissue source of the AEBS. In general, chain length influences

RBA most when the terminal group is —$N(CH_3)_2$, and with regard to tissue differences, MCF 7 AEBS has a preferred chain length of 2 methylene units whereas, for rat liver AEBS, compounds with side chains of 3 or more methylene units have RBAs greater than or equal to compounds with 2 methylene units. Compounds with hydrocarbon chains up to 5 and 6 methylene units may still have relatively high affinity for AEBS (compounds **8, 9,** and **19**), presumably because such chains are able to adopt a conformation which maintains interaction of the terminal amino group with the AEBS. Other permissible side chain configurations are illustrated in the miscellaneous series of diphenyl derivatives identified by Lyman and Jordan (15) as binding to the AEBS. Binding affinity is also influenced by substitution or branching of the side chain and by the nature of the chemical bond between the side chain and the aromatic portion of the ligand, which presumably not only has local effects but also determines side chain orientation.

The above observations indicate both quantitative and some qualitative differences between rat liver and MCF 7 AEBS. Whether these differences are due to the structures of the respective binding sites or to the influence of other microsomal components, in particular those which bind ligands with low affinity and high capacity (e.g., cytochrome P-450, calmodulin, and other unidentified protein and lipid sites), has yet to be determined.

This study shows the AEBS to be a unique intracellular binding site, distinct from a number of other receptor sites with which ligands binding to AEBS cross-react. The binding of phenothiazine derivatives (and TAM) to AEBS appears to be unrelated to the activity of these compounds as calmodulin antagonists. Thus, the RBA values of phenothiazines predict apparent K_D values for the AEBS of approximately 1–70 nM, which are quite different from the corresponding K_D values for calmodulin (e.g., trifluoperazine, 1–1.5 μM; chlorpromazine, 5 μM; Ref. 27). Next, the IC_{50} values for inhibition of cAMP phosphodiesterase (e.g., TAM, 2 μM; chlorpromazine, 6–47 μM; fluphenazine, 5 μM; trifluoperazine, 5–10 μM; Refs. 21, 27, and 37) are consistent with the affinities of these compounds for calmodulin rather than for AEBS. Several other compounds which are weak calmodulin antagonists (27) also have low affinity for AEBS, e.g., SKF-525A (Table 5), and amitryptyline, imipramine, desmethylimipramine, and propranolol (Table 8). However, many potent calmodulin antagonists (27) have no affinity for AEBS. These include the very potent antagonist R24571, the naphthalene-sulfonamide derivative W-7, haloperidol, and (+)-butaclamol. Thus, the binding specificities and affinities of these two sites are very different. Further support for the conclusion that the AEBS and calmodulin are distinct entities are observations that antagonist binding to calmodulin is inhibited in the absence of Ca^{2+} (27), unlike binding to the AEBS.

Although Hiemke and Ghraf (18) have shown that TAM, CI 628, enclomiphene, and zuclomiphene are able to bind to D_2 receptor, the present study shows that the AEBS is not the D_1 or D_2 dopamine receptor.

The failure of several serotonin antagonists to bind to AEBS rules out the possibility of equivalence between AEBS and the serotonin receptor. In support of this conclusion, TAM and other antiestrogens are unable to inhibit either [^{3}H]hydroxytryptamine or [^{3}H]spiperone binding in rat frontal cerebral cortex (18), showing that these antiestrogens do not bind to 5-HT_1 or 5-HT_2 receptors. Similar arguments hold for the adrenergic receptors, and antiestrogens do not inhibit the binding of the adrenergic receptor antagonists [^{3}H]dihydroalprenolol (α_1-, α_2-adrenergic) or [^{3}H]dihydroergocryptine (β-adrenergic) (18).

The findings regarding the binding of histamine antagonists to the AEBS are in good agreement with those presented by Brandes *et al.* (19). Despite the similar concentrations of their respective binding sites, TAM and pyrilamine show only limited cross-reactivity, demonstrating the separate nature of the two sites. In support of this conclusion no [^{3}H]pyrilamine binding was detected in MCF 7 microsomes stored at −20° for 1–4 months despite normal levels of AEBS as determined by [^{3}H]TAM binding. Furthermore, histamine receptors have been detected in high concentrations in purified rat liver plasma membrane (38), whereas previous studies have shown that the AEBS is most likely located in the endoplasmic reticulum (12, 13).

Brandes and colleagues (19, 39, 40) have proposed that the AEBS is a histamine or "histamine-like" receptor. This conclusion was reached on the basis that H_1 antagonists including DPPE (41, 42) show cross-reactivity with AEBS, and that TAM acts as a histamine (H_1) antagonist in histamine-induced smooth muscle contraction at very high concentrations (30 μM optimal concentration). Because their studies also showed that the AEBS was unlikely to be the H_1 receptor, it was concluded the AEBS may be an unknown "H_3" receptor. These observations are at variance with those of Morris (43), who found that the effects of high concentrations of TAM on smooth muscle contractility are nonspecific and noncompetitive. Moreover, it is unlikely that the AEBS mediates this activity given that the concentrations of TAM required are several orders of magnitude greater than necessary for binding site saturation.

SKF-525A is a potent inhibitor of cytochrome P-450, and several other inhibitors structurally related to TAM (including type I and type II binders) also bind to the AEBS (15). However, other cytochrome P-450 inhibitors (metyrapone and ellipticine) have no significant affinity. Other evidence supports the conclusion that the AEBS is not an inhibitory or other binding site on cytochrome P-450. Although TAM and other antiestrogens are known to interact with cytochrome P-450 and competitively inhibit oxidative metabolism of type I substrates (44, 45), the k_i values for such inhibition (6.8–22.9 μM for ethylmorphine demethylase and aminopyrine demethylase activities by TAM, desmethylTAM, and 4-hydroxyTAM; Ref. 45) suggest a much lower affinity of these ligands for cytochrome P-450 than for AEBS. The total concentration of cytochrome P-450 in the rat liver microsomes used in the present study (0.5–0.6 nmol/mg of protein) is considerably higher than that of the AEBS (30.5 pmol/mg of protein). Furthermore, in rat liver, cytochrome P-450 and AEBS have different submicrosomal distributions and these two sites are differentially extracted from rat liver microsomes by sodium cholate (unpublished observations). However, such evidence cannot rule out the possibility that the AEBS is a cytochrome P-450 isoenzyme with unique properties. Although metabolism of TAM in rabbit liver microsomes occurs through the selective involvement of such isoenzymes (20), the absence of TAM metabolism in MCF 7 cells despite high levels of AEBS also argues against identity with cytochrome P-450.

This study provides the most detailed information yet available on the ligand specificity of the AEBS and demonstrates that it is a distinct entity separate from other reported high affinity binding sites for antiestrogens, i.e., ER, calmodulin, cytochrome P-450, and D_1, H_1, and muscarinic receptors. Fur-

ther information on the functional significance of this molecule must await further investigation.

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Send reprint requests to: Dr. Robert L. Sutherland, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, N.S.W., 2010, Australia.