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COMPARATIVE AFFINITY OF STEROIDAL AND NON-STEROIDAL ANTIOESTROGENS, CHOLESTEROL DERIVATIVES AND COMPOUNDS WITH A DIALKYLAMINO SIDE CHAIN FOR THE RAT LIVER ANTIOESTROGEN BINDING SITE

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Abstract—Steroidal and non-steroidal antioestrogens, steroidal compounds with (disubstituted) dialkyl amino side chain, cholesterol derivatives, histaminic and (anti)-progestational compounds were tested for their ability to compete with [^{3}H]tamoxifen for the specific antioestrogen binding site (AEBS) in the post-mitochondrial fraction of rat liver homogenates. Relative binding affinity was highest for compounds with diethylamino or pyrrolidino ethoxy side chains. Affinity decreased with shortening of this side chain. No connection could be established between the carbon backbone of the compound and affinity, except for the presence of (sometimes aromatic) ring structures. Steroidal ring structures do not seem to be necessary for binding. The cholesterol derivatives, tested in our laboratory, showed very little affinity for the rat liver AEBS. Histamine, melatonin and the (anti)-progestational compounds showed no affinity for the AEBS; and we therefore conclude that the AEBS is not identical to receptors for these compounds. Results of these experiments could be useful in other investigations on the development of resistance of breast cancer to antioestrogens.

Antioestrogens such as tamoxifen are widely used in the treatment of breast cancer. The mechanism of action of the antioestrogens is generally thought to be through binding to the oestrogen receptor competing with endogenous oestradiol [1]. There is evidence, however, to suggest that they may act through other, oestrogen-independent, processes. tamoxifen can block breast cancer cells in the G_1-phase of the cell cycle [2, 3]. It has been reported that tamoxifen is able to antagonize the action of calmodulin [4–7] and to inhibit protein kinase C [8]. In view of the relatively high concentrations of tamoxifen required to achieve these effects on calmodulin and protein kinase C it is questionable as to whether these effects play a role at therapeutic levels of this drug.

Apart from binding to the oestrogen receptor, triphenylethylene antioestrogens have been shown to bind with high affinity and specificity to another kind of binding site, which does not bind oestrogens: the antioestrogen-binding site (AEBS§) [9]. The AEBS is a membrane-bound protein located mainly in the microsomal fraction of many cells, irrespective of the presence of oestrogen receptors. The concentration is particularly high in liver [10, 11]. Reports from several investigators have demonstrated the existence of an endogenous ligand for the AEBS in rat liver [12–14]. The nature of this endogenous ligand and the biological function of the AEBS are still unknown, but muscarinic or histaminic compounds and cholesterol derivatives have been suggested as possible candidates [15–20]. In our laboratory evidence supporting the suggestion that the AEBS might be a muscarinic receptor could not be obtained [21].

Although the nature of the AEBS is presently unknown, it is conceivable that the AEBS could interfere with breast cancer treatment by scavenging the administered tamoxifen or its biologically active metabolite(s). Scavenging could explain why in some cases of recurrent breast cancer a response to increased tamoxifen administration is observed [22].

New steroidal and non-steroidal antioestrogens are being developed. It is of interest to find out whether these compounds have any affinity for the AEBS as the AEBS may play a role in the mechanism of acquiring resistance to antioestrogen therapy. It is conceivable that new antioestrogens with affinity for the oestrogen receptor but without affinity for the AEBS will be found to be more effective in the treatment of breast cancer than tamoxifen. Furthermore, the results could be used in examining the structural requirements necessary for binding to the AEBS; such information might give a clue as to the properties of the AEBS and of the endogenous ligand. The present paper describes our experiments on the binding to rat

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§ Abbreviations: AEBS, antioestrogen binding site; PMF, post-mitochondrial fraction; RBA, relative binding affinity.

liver AEBSs of these new antioestrogens and other compounds suggested as possible ligands.

MATERIALS AND METHODS

Materials. Dextran-T70 was obtained from Pharmacia (Uppsala, Sweden) and charcoal (Norit A) from Merck (Darmstadt, Germany). Oestradiol was purchased from Makor Chemicals Ltd (Jerusalem, Israel). [*N*-methyl-^{3}H]Tamoxifen (sp. act. 87 Ci/mmol) was a product of Du Pont de Nemours ('s Hertogenbosch, The Netherlands); its purity was verified by TLC on silica gel plates (Riedel-de Haën, Seelze, Germany) using diethylether:triethylamine (98:2 v/v) as solvent system. Purity of the [^{3}H]tamoxifen was >90%.

Cholesterol was from BDH Chemicals Ltd (Poole, U.K.). 3α,7α,12α-Trihydroxycholic acid was from Serva (Heidelberg, Germany) and histamine, pyrilamine and melatonin from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

All other competitors were generously donated as follows: 4,4,10β-trimethyl-*trans*-decal-3β-ol was a gift of Prof. T. A. Spencer (Dartmouth College, Hanover, NH, U.S.A.); CB7432 of Drs M. Dowsett and M. Jarman (London U.K.). Dr A. Wakeling, ICI Pharmaceuticals Ltd (Macclesfield, U.K.), supplied tamoxifen (ICI46.474),*N*-desmethyltamoxifen(ICI55.548),4-OH- tamoxifen(ICI79.280), cyanotamoxifen (ICI99.311), monophenoltamoxifen (ICI141.389) and ICI 164.384. 3-OH-Tamoxifen (Droloxifene) was provided by Dr M. Hasmann (Klinge Pharmaa, Germany). Prednimustine, estramustine, LS3360, LS3348 and LS3347 were products of Kabi-Pharmacia LEO-Therapeutics AB (Helsingborg, Sweden). Toremifene and derivatives were obtained from Dr L. Kangas, Farmos (Turku, Finland). Nafoxidine was from Upjohn (Kalamazoo, MI, U.S.A.); clomiphene from Merrell Toraude (The Netherlands). Dr J. Stekar from Asta Pharmaa (Frankfurt, Germany) provided zindoxifene, deacetylated zindoxifene and D 18954. 7-Ketocholesterol and 7β-hydroxycholesterol were gifts from Prof. H. Degenhart, Sophia Children Hospital (Rotterdam, The Netherlands). RU486 was a product of Roussel (Romainville, France). Org 2058, and the antiprogestins Org 31376 and Org 31710 were obtained from Dr E. W. Bergink (Organon International, Oss, The Netherlands). ZK.119.010 was obtained from Schering AG (Berlin, Germany). Structural formulas of the different compounds are given in Results.

Tissue preparation. All procedures were carried out at 4° except where indicated. Liver was removed from adult Wistar rats and frozen immediately at −70° until use. Liver was minced and homogenized (12.5% w/v) in buffer [10 mM Tris–HCl, 1.5 mM EDTA, 10% glycerol (v/v; Merck), 0.1% α-monothioglycerol (v/v; Sigma), 3 mM sodiumazide, pH 7.4] using a Teflon/glass Potter tube (5 strokes 800 rpm). The homogenate was centrifuged at 12,000 *g* for 30 min and the supernatant, the post-mitochondrial fraction (PMF), was used for binding experiments. The AEBS content of the PMF was determined as described by Blankenstein *et al.* [21], using Scatchard plot analysis and the correction as described in Ref. 23 and references therein.

Competitive binding analysis. The competition experiments were performed essentially as described by Kon [24]. All procedures were carried out at 4° except where indicated. The competitive binding analysis was performed on a dilution of the PMF containing 1–2 pmol AEBS/incubation, based on binding capacity for tamoxifen. The PMF was incubated for 1 hr with 1 μM oestradiol to eliminate binding of tamoxifen to the oestrogen receptor. The oestradiol-treated PMF was incubated 16–20 hr with 5 nM [^{3}H]tamoxifen in the presence of different concentrations of competitor or radio-inert tamoxifen in a final volume of 500 μL of buffer containing 5% ethanol. Competitors were dissolved at 0.005 M in ethanol (except for prednimustine and droloxifene which were dissolved in dimethylsulfoxide) and stored at 4 or −20° as indicated by the manufacturers. Further dilutions were made with ethanol. Aspecific binding was determined using 2500 nM radio-inert tamoxifen. Bound and free ligand were separated by adding an equal volume of dextran-coated charcoal suspension (1% charcoal, 0.1% dextran T70 in buffer). After 15–30 min the charcoal was pelleted by centrifugation at 1900 *g* for 15 min. The AEBS-bound radioactivity was counted in the supernatant. The concentrations of the competitor and of radio-inert tamoxifen inhibiting [^{3}H]tamoxifen binding by 50% (IC_{50}) were calculated. The relative binding affinity (RBA) was determined by using:

$$RBA = 100 \times (IC_{[TAM]_{50}}/IC_{[COMP]_{50}}).$$

Each compound studied was tested in at least two different binding experiments.

RESULTS

The presence of a high concentration of AEBSs in the PMF of the rat liver homogenate was verified. An example of the resulting Scatchard plot, shown in Fig. 1, indicates that a single class of high affinity binding sites (K_d = 1.0 nM) is present abundantly ([AEBSs] = 3100 fmol/mL incubation volume). Based on these findings the preparation was judged suitable for the competition studies.

Figure 2 shows the competitive binding of several triphenylethylene derivatives for rat liver PMF AEBSs. In all experiments tamoxifen served as an internal standard to calculate the RBA values for the compounds tested. In agreement with the [^{3}H]-tamoxifen concentration of 5 nM, as used in these experiments, the $IC_{[TAM]_{50}}$ was found to be 5.8 ± 0.7 nmol/L (mean ± SD; N = 8) indicating a coefficient of variation of 12%.

Table 1 shows the structural formulas and the relative binding affinities of the triphenylethylene antioestrogens and their derivatives tested. When substituents at R1 are considered, it is clear that deletion of one of the terminal methyl groups (*N*-desmethyltamoxifen and *N*-desmethyltoremifene) results in a considerable loss of binding affinity. Two compounds were found to have a higher RBA than tamoxifen, i.e. clomiphene and CB7432; these compounds both have a longer dialkyl amino group, which is cyclic in the case of CB7432.

Modifications at R2 as in cyanotamoxifen and

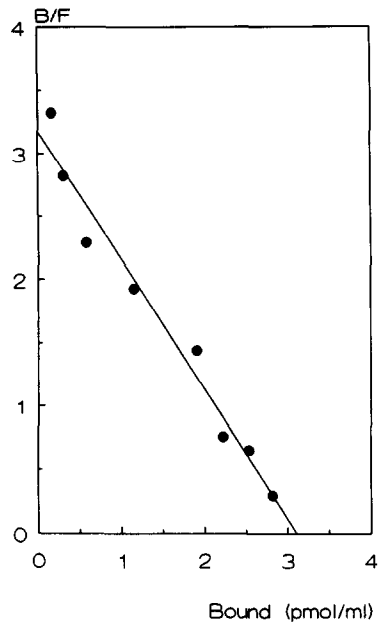


Fig. 1. Scatchard plot of the binding of [^{3}H]tamoxifen to the PMF of rat liver. The concentration of AEBSs is 9070 fmol/mg PMF protein; K_d = 1.0 nM.

toremifene also result in a loss of binding affinity, which is most pronounced for cyanotamoxifen. It can not be evaluated from these data whether substitution of R2 by a chlorine atom (clomiphene) has an effect on RBA. Substitution of a hydroxyl group at R3 or R4 also results in a decrease in RBA. The introduction of an iodine atom at R3 as in CB7432 could potentiate or decrease the affinity for AEBSs. Similarly to clomiphene, studies with other compounds are necessary.

The structures of other steroidal and non-steroidal antioestrogens tested and the RBA values obtained are shown in Fig. 3. With the exception of nafoxidine, most compounds displayed little or no affinity for the rat liver AEBS. Like CB7432, nafoxidine displayed a higher RBA than tamoxifen. Both compounds have a pyrrolidino group and it is tempting to attribute a major part of the affinity to this group. If this were true, however, ZK119.010 which also has such a group might be expected to have a higher RBA as well.

Considering reports in the literature [25] on a microsomal progestin binding protein, which at least with respect to its localization could resemble the AEBS, the RBA of a number of steroidal (anti)-progestational compounds and other steroidal compounds with (substituted) dialkylamino side chains was also determined. The results depicted in Fig. 4 show that antiprogestins with this particular side chain have no measurable affinity for the AEBS; other progestagens (including progesterone and several Org compounds) also showed no affinity for the AEBS (12 compounds tested: Org 2058 in Fig. 5, other data not shown). Three of the LEO compounds appeared to have affinity for the AEBS. LS3360, like clomiphene, with a terminal diethyl amino group showed the highest affinity; LS3347 with the shorter (dimethyl) amino group is less potent in binding to the AEBS. Introduction of the double bond at C5 of the steroid increased the affinity of LS3347 in comparison with LS3348. In these three compounds the dialkyl amino group is linked through an ethoxy group to C3 of the steroid skeleton; examining other compounds for this feature it appears that all those showing affinity for the AEBS have dialkyl amino ethoxy side chains. The inactive (anti)-progestational compounds with substituted dialkyl amino groups tested all lack this "ethoxy" bridge.

Because the suggestion was made that the natural ligand for the AEBS could be an oxygenated cholesterol metabolite, several cholesterol-derived compounds have been tested. In our experiments 7-ketocholesterol, 7β-hydroxycholesterol and cholesterol itself all showed some affinity for the AEBS

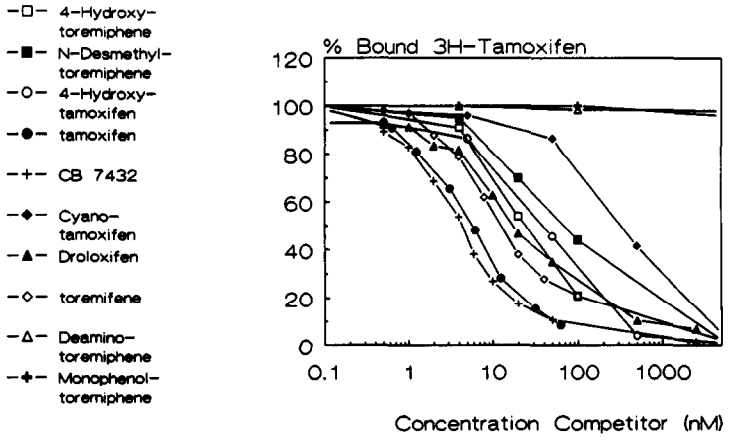
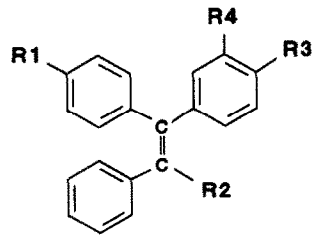


Fig. 2. Competition of triphenylethylene antioestrogens with [^{3}H]tamoxifen for the AEBS in the PMF of rat liver.

Table 1. Structural formulas and RBAs of triphenylethylene antioestrogens and derivatives



Compound	R1	R2	R3/R4*	RBA
Tamoxifen	-O-$(CH_2)_2$-N-$(CH_3)_2$	-CH_2CH_3		100
4-OH-Tamoxifen	-O-$(CH_2)_2$-N-$(CH_3)_2$	-CH_2CH_3	R3 = -OH	24
3-OH-Tamoxifen	-O-$(CH_2)_2$-N-$(CH_3)_2$	-CH_2CH_3	R4 = -OH	30
N-Desmethyltamoxifen	-O-$(CH_2)_2$-NH-CH_3	-CH_2CH_3		9
Monophenoltamoxifen	-OH	-CH_2CH_3		0.2
Cyanotamoxifen	-O-$(CH_2)_2$-N-$(CH_3)_2$	-C≡N		3
CB 7432		-CH_2CH_3	R3 = -I	140
Toremifene	-O-$(CH_2)_2$-N-$(CH_3)_2$	-CH_2CH_2-Cl		48
4-OH-Toremifene	-O-$(CH_2)_2$-N-$(CH_3)_2$	-CH_2CH_2-Cl	R3 = -OH	27
N-Desmethyltoremifene	-O-$(CH_2)_2$-NH-CH_3	-CH_2CH_2-Cl		9
Monophenoltoremifene	-OH	-CH_2CH_2-Cl		<0.1
Deaminotoremifene	-O-$(CH_2)_2$-OH	-CH_2CH_2-Cl		<0.1
Clomiphene	-O-$(CH_2)_2$-N-$(C_2H_5)_2$-Cl			222

* Where not indicated otherwise R = -H.

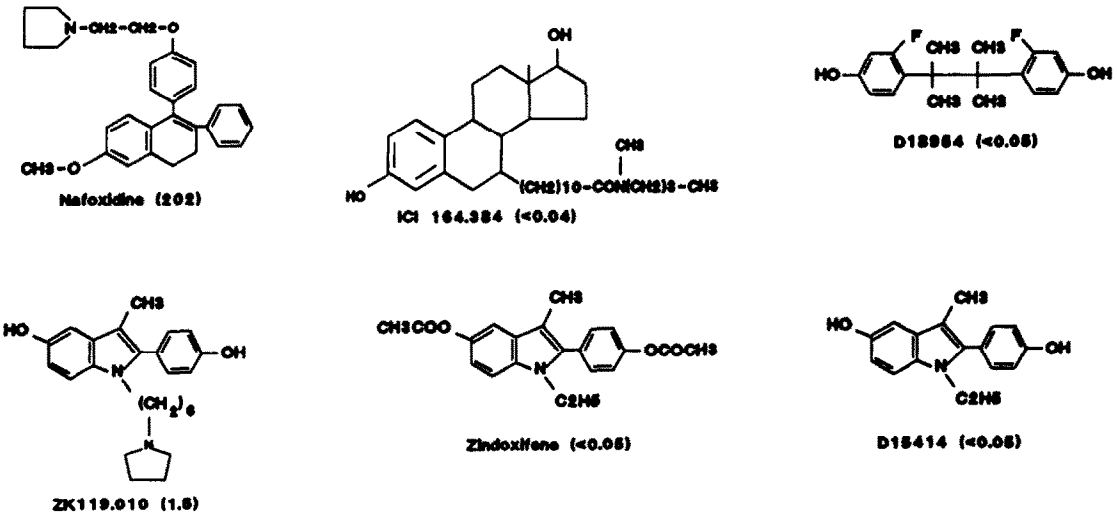


Fig. 3. Structural formulas and RBAs for the rat liver AEBS of non-triphenylethylene antioestrogens.

(Fig. 5), but the RBA values for these compounds were all <1%. From our experiments there is no clue that the steroidal backbone by itself is necessary for binding to the AEBS. Another compound tested in our experiments was 4,4,10β-trimethyl-*trans*-decal-3β-ol, an inhibitor of cholesterol biosynthesis. This compound showed no affinity for the AEBS.

The RBA of several histaminic compounds (Fig.

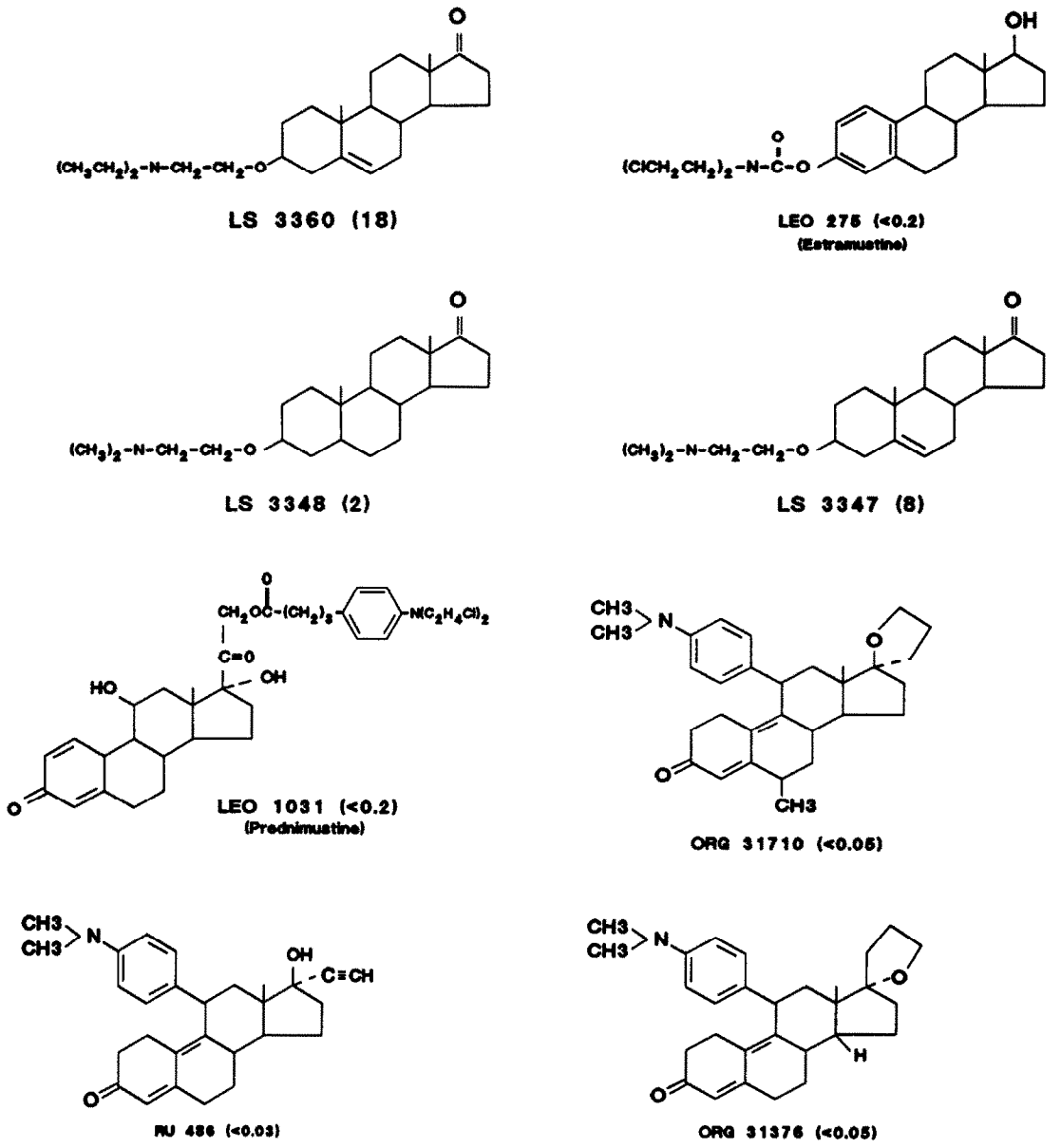


Fig. 4. Structural formulas and RBAs for the rat liver AEBS of compounds with a dialkyl amino side chain.

5) has been determined, because of the suggestion that the natural ligand for the AEBS might have a histaminic nature. Histamine and melatonine displayed no measurable affinity for the AEBS; pyrilamine showed some affinity. Pyrilamine however has one of the characteristics which has been shown here to be present in other compounds with affinity for the AEBS: a dimethyl amino ethyl side chain; therefore we believe it is this side chain instead of the histaminic character of this compound that is responsible for the affinity of pyrilamine for the AEBS. It could be interesting to find out whether substitution by an oxygen of the nitrogen connecting this chain to the rest of the compound increases the RBA of this compound.

DISCUSSION

The present experiments show that the binding of tamoxifen-derived compounds to the AEBS decreases with the removal of one CH_3-group of the dialkyl amino ethyl side chain (R1, Table 1). Deletion of the side chain results in an almost complete loss of activity. Derivatives with diethyl or pyrrolidino side chain have higher RBAs than tamoxifen. Replacement of the ethyl side chain at R2 of tamoxifen by a cyano or ethylchloride group results in a decrease in the RBA. Addition of a hydroxyl group at R3 or R4 also lowers the affinity for the AEBS. Nafoxidine, a non-steroidal non-triphenylethylene antioestrogen which also possesses a pyrrolidino side chain, has a two-fold higher RBA than tamoxifen.

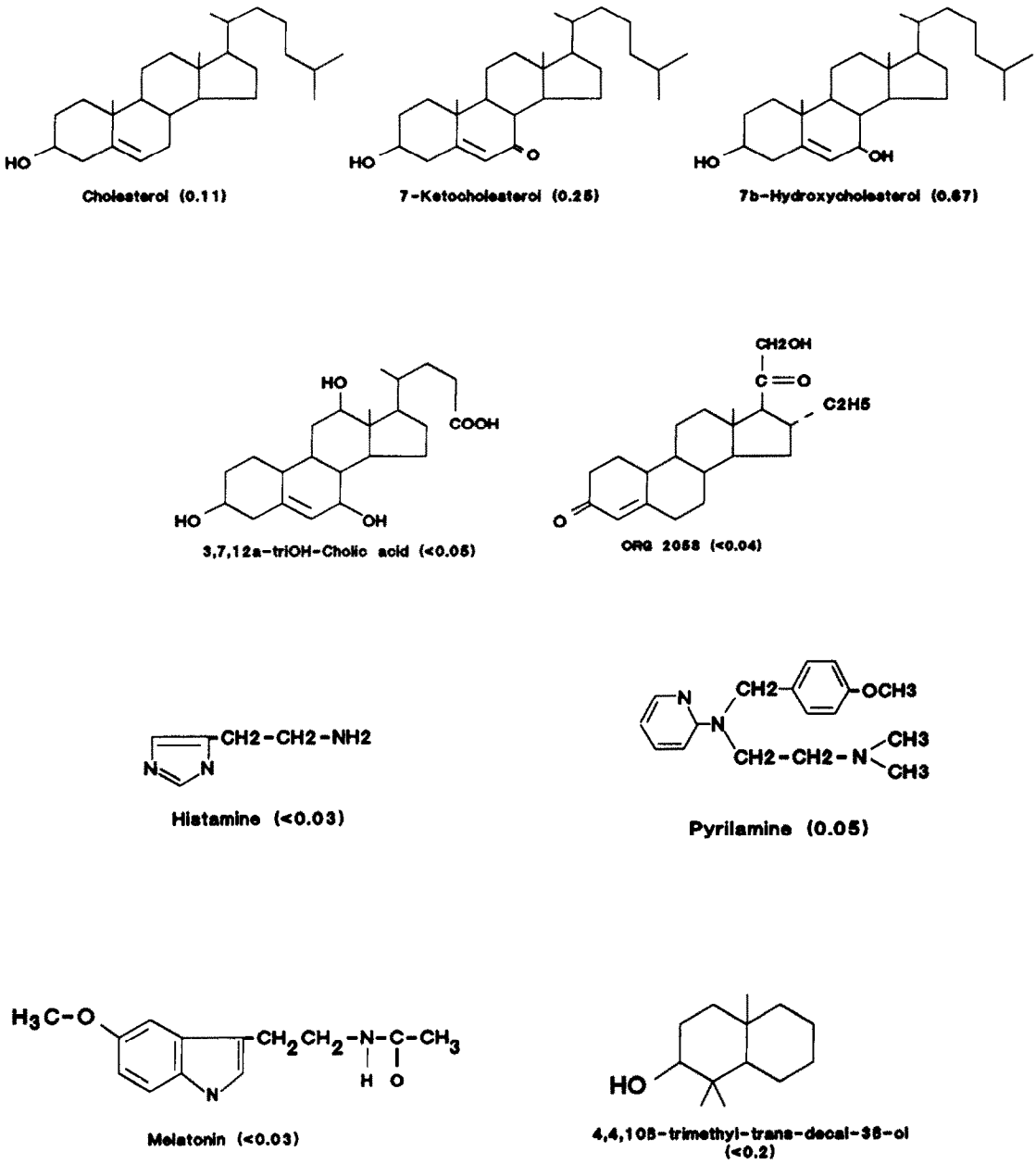


Fig. 5. Structural formulas and RBAs for the rat liver AEBS of steroidal compounds, histaminic compounds and an inhibitor of cholesterol biosynthesis.

Three steroidal LEO compounds with a dialkylamino side chain appear to have affinity for the AEBS. LS3360, the most potent of these three in binding the AEBS, has a diethyl amino group; the other two compounds have dimethyl amino groups. All compounds showing affinity for the AEBS appear to have the ethyl ether linkage between the dialkyl amino group and the "backbone".

ZK119.010, a new non-steroidal antioestrogen which also has a pyrrolidino group, shows little affinity for the AEBS; in this compound, however, this group is linked to the backbone through a hexyl group instead of an ethyl group, which could cause the low affinity of this compound for the AEBS. It would be interesting to find out whether shortening of this chain increases the affinity for the AEBS.

None of the progestins and antiprogestins tested have affinity for the AEBS, which shows that the AEBS is not identical to the progestin binding protein.

According to Hwang and Matin [26], in order to bind to the AEBS steroidal compounds are required to have: (i) a hydrocarbon side chain at C17; (ii) an oxygen function at C7; (iii) a hydroxyl group at C3; and (iv) the absence of a double bond between C5 and C6 [26]. Our results are, at least partially, in

discordance with their results. LS3360, for instance, lacks all of the criteria formulated by Hwang and Matin [26], yet has a RBA of 18. On the other hand, 3α, 7α, 12α-trihydroxy cholic acid, which fulfils three of the four criteria, does not bind (RBA <0.05). Apparently, other criteria in addition to those formulated by Hwang and Matin [26] determine the affinity of compounds for the AEBS. Our experiments on the structural requirements necessary for binding to the rat liver AEBS have, like those by Hwang and Matin [26], to some extent been limited by the availability of the compounds.

The RBAs in our experiments have been determined with AEBSs in the PMF from rat liver. The mean concentration of AEBSs in our rat liver preparations was 6600 fmol/mg PMF protein; K_d = 1.5 nM. The mean concentration in the human liver PMF measured in our laboratory was 870 fmol/mg PMF protein; K_d = 2.8 nM. Results from our laboratory demonstrate that the occurrence of AEBSs in human breast cancer tissue is not related to the presence or content of oestrogen receptors in this tissue [21]. (AEBS concentration is 360 fmol/mg protein; K_d = 2.1 nM.) The subcellular localization of AEBS was highly comparative in human breast cancer and liver tissue. Small tissue and species variations have been reported with respect to the RBA of tamoxifen and several derivatives [10, 14, 27]. Despite these differences we could not find any indications in the literature that the AEBS might have different tissue-dependent functions. Therefore, we consider the rat liver PMF as a suitable source of AEBSs in our experiments and we plan to prove that the affinities of the new compounds tested on rat liver PMF are comparable with the affinities for AEBS from human tissues.

If the AEBS does play a role in the development of resistance to tamoxifen of breast cancer, there is a possibility that replacement of tamoxifen by an antioestrogen with affinity for the oestrogen receptor, but without affinity for the AEBS, will cause regression of the tumour. At this moment no predictions can be made on the results of such experiments; further investigations on *in vitro* systems are required. The results of the present experiments could be used for selecting compounds fit for future investigations.

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