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Species specificity of triphenylethylene derivatives and of compounds with a steroidal backbone for human and rat liver antioestrogen binding site (AEBS)

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Abstract—The binding affinity of derivatives of the triphenylethylene (TPE) antioestrogen tamoxifen and of steroidal compounds for human liver antioestrogen binding sites (AEBS) was compared with their binding affinity for rat liver AEBS. Despite the observation of some quantitative differences overall a highly significant correlation between the relative binding affinity (RBA) for human and rat liver AEBS was found for all compounds tested ($r = 0.93$, $N = 19$, $P < 0.001$). This was more pronounced for TPE derivatives ($r = 0.83$, $N = 12$, $P < 0.01$) than for cholesterol derived compounds ($r = 0.64$, $N = 7$, not significant). We conclude that AEBS from rat liver can be used instead of human livers as a model to study the interactions of antioestrogens with AEBS.

Tamoxifen, a triphenylethylene (TPE*) antioestrogen, is currently the most successfully used antioestrogenic agent in the treatment of breast cancer. The mechanism of action of antioestrogens is generally thought to be through binding to the oestrogen receptor competing with endogenous oestradiol [1]. Apart from binding to the oestrogen receptor, TPE antioestrogens have been shown to bind with high affinity and specificity to another kind of binding site, which does not bind oestrogens, the microsomal antioestrogen binding site (AEBS) [2–4].

In previous experiments we have examined the structural requirements for binding of different compounds to the rat liver AEBS [5]. Relative binding affinity (RBA) appeared to be highest for compounds with diethylamino or pyrrolidino ethoxy side chains; affinity decreased with shortening of this side chain. Steroidal ring structures did not appear necessary for binding, although the presence of (sometimes) aromatic ring structures was. The purpose of the present study was to compare the ligand specificity of rat and human liver AEBS.

Materials and Methods

Materials. [*N-methyl*-^{3}H]Tamoxifen (sp. act. 87 Ci/mmol; Du Pont de Nemours, 's-Hertogenbosch, The Netherlands) was purified by Sephadex LH20 gel filtration. Purity was verified as described previously [5] and was >98%. Sources of tamoxifen and derivatives, toremifene and derivatives, estramustine, prednimustine, LS3360, LS3348 and LS3347, nafoxidine, clomiphene, CB7432, 7β-hydroxycholesterol, 7-ketocholesterol, ICI164.384, cholesterol, Org 2058 and Org 31710 and their structural formulas are given before [5]. Lathosterol was donated by Dr B. Wolthers, Academic Hospital Groningen, The Netherlands. 6-Ketocholestanol and 7-ketocholestanol were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Tissue preparation. Liver tissue from adult Wistar rats was frozen immediately at −70° until use. Human liver was obtained from the operating theatre of the Academic Hospital Utrecht. Tissue was placed on ice and frozen at −70° as soon as possible. Preparation of the postmitochondrial fraction (PMF) at 4° was carried out as described before and its AEBS content was determined as described previously [5, 6] using Scatchard plot analysis and the correction described by Blankenstein and Mulder [7 and Refs therein]. Protein concentrations were determined by the method of Bradford [8].

Competitive binding analysis. The competition experiments were performed as described previously [5, 9]. The competitive binding analysis was performed on buffer diluted PMF containing about 400 fmol human liver AEBS/incubation or 1000 fmol rat liver AEBS/incubation. The concentrations of the competitor and of radio-inert tamoxifen inhibiting [^{3}H]tamoxifen binding by 50% (IC_{50}) were calculated. The RBA was determined by using:

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

Results

Rat and human liver were tested for the presence of high affinity antioestrogen binding sites in their PMF. Scatchard plot analysis of the binding of [^{3}H]tamoxifen to this fraction revealed that rat liver PMF contained 5300 fmol AEBS/mg protein with a K_d of 1.3 nM and human liver PMF contained 9400 fmol AEBS/mg protein with a K_d of 3.0 nM. The competitive binding of tamoxifen, toremifene, CB7432, LS3360, LS3348 and 7-ketocholestanol for rat and human liver PMF AEBS is shown in Fig. 1.

In agreement with the [^{3}H]tamoxifen concentration of 5 nM, as used in these experiments, the $IC_{[tam]50}$ was found to be 8.5 ± 1.1 nmol/L (mean ± SD; N = 7) for human liver AEBS and 6.3 ± 1.8 nmol/L (N = 11) for rat liver AEBS. The resulting RBA values of all compounds tested for both rat and human liver AEBS are summarized in Table 1.

Compounds (N = 9, see Table 1) that showed no affinity for human liver AEBS (i.e. RBA < 0.01) are not included in the correlation analysis. With some exceptions the binding affinities for rat liver AEBS of the compounds tested showed a high correlation with the binding affinities for human liver AEBS. Figure 2 gives the logarithmic correlation plot (linear regression analysis $r = 0.93$, $N = 19$). Using Spearman's rank correlation test a significant correlation between the RBA values for human and rat liver AEBS was found for all compounds tested. A significant correlation was also found considering TPE derivatives alone (closed dots in Fig. 2; $r = 0.83$, $N = 12$, $P < 0.01$), but this was not true for compounds with a steroidal backbone (open dots in Fig. 2; $r = 0.64$, $N = 7$, not significant).

Discussion

The present experiments clearly demonstrate that rat and human liver AEBS have largely similar properties in terms of ligand specificity. Nevertheless, some differences were found as well. As was previously shown for rat liver AEBS, shortening of the dialkylamino ethoxy side chain of tamoxifen derived compounds also results in a gradual

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

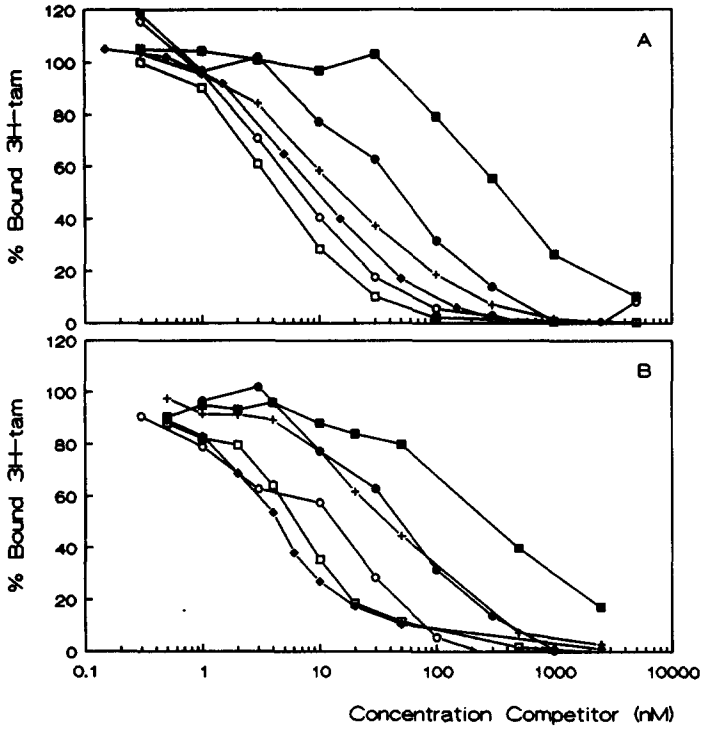

Fig. 1. Competitive binding experiment with [^{3}H]tamoxifen for the AEBS in the PMF of human (A) and rat liver (B). (□) Tamoxifen; (◆) CB7432; (○) toremifene; (+) LS3360; (■) LS3348; (●) 7-ketocholestanol.

Table 1. RBA of triphenylethylene antioestrogens and cholesterol derivatives for the AEBS

Compound	RBA rat liver	RBA human liver
Clomiphene	222	164
Nafoxidine	144	724
CB7432	140	97
Tamoxifen	100	100
Toremifene	42	79
4OH-Tamoxifen	31	101
4OH-Toremifene	24	75
3OH-Tamoxifen	22	14
LS3360	18	21
N-Desmethyltoremifene	9.0	16
N-Desmethyltanoxifen	9.0	12
7-Ketocholestanol	5.9	2.0
LS3347	5.4	3.4
Cyanotamoxifen	2.6	6.6
LS3348	2.0	1.4
7β-OH-Cholesterol	0.7	0.3
6-Ketocholestanol	0.5	3.1
Lathosterol	0.2	0.3
Monophenoltamoxifen	0.2	0.2
Cholesterol	0.11	<0.01
7-Ketocholesterol	0.25	<0.01
Deamino-OH-toremifene	<0.1	<0.01
Monophenoltoremifene	<0.1	<0.01
Estramustine	<0.2	<0.01
Prednimustine	<0.2	<0.01
ICI164.384	<0.04	<0.01
Org2058	<0.04	<0.01
Org31.710	<0.05	<0.01

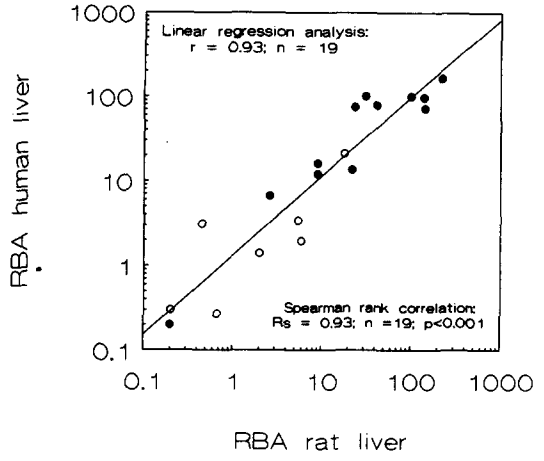

Fig. 2. Correlation plot of the RBA for rat liver AEBS and for human liver AEBS. (○) RBA of steroidal compounds; (●) RBA of TPE derivatives.

loss of binding affinity for the human liver AEBS; tamoxifen and toremifene have higher affinity than their *n*-desmethyl derivatives, which in their turn have a higher affinity than the monophenol form. The presence of a diethyl amino ethyl side chain in comparison with a dimethyl amino ethyl side chain, as seen in LS3360 and clomiphene, increases the affinity for human liver AEBS analogous to the rat liver site. LS3360 has a significantly higher affinity for the AEBS than LS3347 and differs only in this aspect. However, considering the pyrrolidino side chain of nafoxidine and CB7432, the increase in affinity as found with rat liver AEBS (where both compounds had very high affinity) was not found for human liver AEBS.

Steroidal compounds have lower affinity for both rat and human AEBS than the TPE derivatives as can be seen from Table 1 and Fig. 2. The calculated Spearman correlation coefficient ($r = 0.64$, $N = 7$) does not appear to indicate a statistically significant relationship. When the steroidal compounds having no appreciable RBA for either binding site (Table 1) are taken into consideration, it is clear that such a relationship does occur.

Steroidal compounds with a dialkyl amino ethoxy side chain (LS3360, LS3348 and LS3347) showed a relatively higher affinity for AEBS from both species than compounds without this group (except for 7-ketocholestanol which has a relatively high affinity), suggesting that the presence of this side chain is favorable for binding to the AEBS.

Derivatives of tamoxifen and toremifene (except for 3OH-tamoxifen) all showed a higher affinity relative to tamoxifen for human liver AEBS than for rat liver AEBS. Based on the differences in the observed affinities, we concluded that relatively minor differences in the ligand binding site of rat and human liver AEBS must exist. This does, in our opinion, not preclude the use of rat liver AEBS as a more general model to study the interaction of antioestrogens with AEBS.

Other compounds that have been investigated for their affinities for AEBS, but were not used in the present experiments, include benzofurans [10]. The RBA of these compounds was evaluated in EL4 lymphoid cells and not in liver tissue or cells. Hence it is difficult to compare these data with our data on TPE and steroidal compounds [5 and present study]. In terms of the putative biological effect through AEBS of these compounds, it is remarkable that the benzofurans have a much lower antiproliferative effect than might be expected on the basis of their RBA for the EL4-AEBS. In addition, antiproliferative effects were also observed in Rtx6 cells which are devoid of AEBS. Therefore, the conclusion of these authors that benzofuranic compounds may influence cell proliferation through AEBS may not be valid.

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