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ORIGINAL PAPER

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Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors

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Abstract This work reports the binding capacity of various chemicals (so-called endocrine disruptors) to recombinant human steroid receptors (hERα, hPR and hAR). The tested chemicals are organochlorine insecticides (DDT and its metabolites, methoxychlor, aldrin, dieldrin, chlordecone, lindane, trichlorobenzene), estrogenic insecticides (endosulfan, toxaphene, nonachlor), herbicides (alachlor and atrazine), fungicides (benomyl and vinclozolin), industrial chemicals (nonylphenol, bisphenol A, diphenylphtalate), antioxidants (butylated hydroxyanisol) and some phytoestrogens. Except for phytoestrogens, most of the tested chemicals (DDT and its metabolites, aldrin, α- and β-endosulfan, toxaphen, trans-nonachlor) show higher affinities for hPR than for hERα, indicating that the interaction with the progesterone receptor could contribute to the endocrine-disrupting effects imputed to these chemicals. We propose to use binding assays using recombinant human steroid receptors as screening tools for the detection of endocrine disruptors in various samples.

Keywords Endocrine disruptors · Steroid receptors · Binding assay · Pesticides · Radioreceptor assay

Introduction

Various chemicals such as pesticides, plasticizers and persistent pollutants are highly suspected to display endocrine-disrupting effects in animals and humans. A lot of studies have been reported. To give some examples, in animals, environmental endocrine-disrupting chemicals (EDC) could be the cause of reproductive and teratogenic effects in bald eagles of the Great Lakes [1]. Reproductive disorders have been described in animals and humans [2, 3, 4]. In humans, endometriosis [5] and testicular cancer [6] have been linked to exposure to organochlorine and plasticizers, respectively. A general decline of the male reproductive health is observed and imputed to EDC [7, 8, 9, 10, 11, 12].

Endocrine disruption is caused by the interference of chemicals at the level of steroid receptors: it is now well known that these chemicals display estrogenic effects [13, 14, 15] and/or anti-androgenic effects [16]. Much less is known about the interaction of these compounds with the progesterone receptor.

Here, we describe the utilization in hormone binding assays of human steroid receptors that have been produced in genetically modified bacteria [17]. In order to better define the mechanism of action of various endocrine disrupters and to clarify which receptor is involved, we have studied and compared their binding to human estrogen (hERα), androgen (hAR) and progesterone (hPR) receptors.

The same approach could also obviously be applied to the detection of endocrine disrupters in environmental samples, in water and food, to monitor for occupational exposure and toxicological evaluation of new industrial chemicals.

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Experimental

Materials

All the hormones (17β-estradiol [E-8875], progesterone [P-0130] and dihydrotestosterone [A-2570]) used standards were from Sigma (Bornem, Belgium) and tritiated steroids (^{3}H-17β oestradiol [TRK-322], ^{3}H-progesterone [TRK-413], ^{3}H-dihydrotestosterone [DHT, TRK-443]) were from Amersham (Rosendaal, Nederland). Other standards were from Promochem (Molsheim, France) (Alachlor [IPO 003], 99.5%; Aldrin [IPO 004], 99.1%; α-endosulfan [IPO 181], 99.8%; atrazine [IPO 005], 98.7%; β-endosulfan [IPO 182], 99.%; cis-nonachlor [PST-1200], 99%, *o*,*p*′-DDE [IPO 120], 99.8%; *o*,*p*′-DDT [IPO 125], 99.8; methoxychlor [PST-691], 98%; toxaphen [ERT 002], technical; trans-nonachlor [PST-1201], 99%;

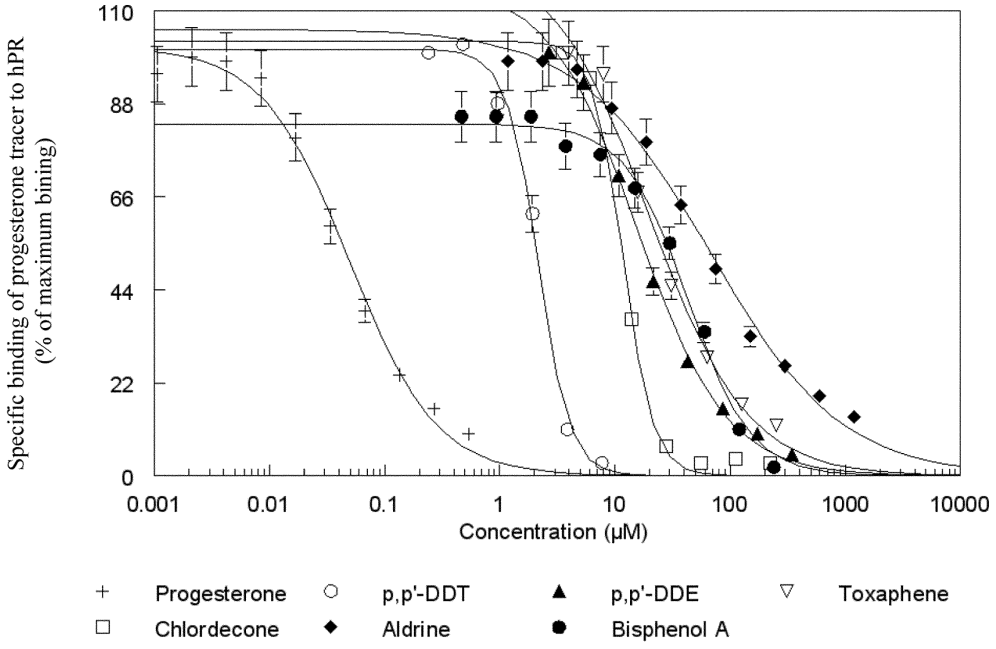

Fig. 1 Competition curves for binding of ^{3}H-progesterone to the recombinant hPR obtained with progesterone and various chemicals

vinclozolin [IPO 182], 99.5%), and Sigma (apigenin [A-3145], 95%; benomyl [381586], 95%; biochanin A [D-2016]; bisphenol A [23,965–8], 99%; butylated hydroxyanisole or BHA [B-6655], 90% 3-BHA and 8% 2-BHA; chlordecone [45379], 99.2%; *p,p′* DDT [386340], 98%; *p,p′*-DDE [12,389–7], 99%; *o,p′*-DDD or mitotane [C-3010]; *p,p′*-DDD [3,959–3], 97%; dieldrin [D-7519], 90%; diphenyl phthalate [RH 36617], 99.9%; glycitein [G-2785], 97%; kaempferol [K-0133], 90%; lindane [H-4500], 97%; luteolin [L-9283]; nonylphenol [203–199–4]; technical mixture; 1,2,3-trichlorobenzene [4–7188], 99%).

Production of steroid receptors

Recombinant human estrogen receptor (hERα) was produced in *E. coli* as a fusion of GST (glutathione-*S*-transferase) protein with the D,E and F domains of the natural receptor, encompassing the hormone binding domain. Briefly, D, E and F domains of hERα were fused in frame to the C-terminus of the *S. japonicum* glutathione-*S*-transferase gene (GST), present in the pGEX-4T-3 bacterial expression plasmid (Amersham, Rosendaal, Nederland). The resulting plasmid pGEX-ER was transformed into the *E. coli* strain BL21 (ompT-, lon-), for expression of the GST-ER fusion protein following induction with isopropyl-*β*-D-thiogalactoside (IPTG). To facilitate the folding of the GST-ER protein, expression vectors encoding the GroEL and GroES chaperone proteins were co-transformed.

Induction and lysis protocol

E. coli BL21 harbouring pGEX-ER and pGRoEL/S were grown overnight in 50 mL of Luria–Bertani medium (LB) containing 100 µg ampicillin mL^{-1} and 30 µg chloramphenicol mL^{-1}. The culture was then transferred to 1 L of fresh LB with both antibiotics. When the culture reached an OD_{600} of 0.9, synthesis was induced with 1 mM IPTG and culture was grown for an additional 15 h at 18°C. After centrifugation, bacterial pellets were resuspended and the bacteria were broken in a French cell press at 10^8 Pa. After centrifugation for 2 h at 30,000 g, the pellet was discarded and the supernatant was used for binding experiments.

Receptors hAR and hPR have similarly been produced in *E. coli* by following the same strategy, except that domains D and E of the human androgen and progesterone receptors respectively were produced in *E. coli* fused to the maltose binding protein using the pMALC2 (Biolabs, Leusden, Belgium) prokaryotic expression vector.

Receptor binding assays

Tritium-labelled ligands were used as tracers (^{3}H-17β estradiol, ^{3}H-progesterone, ^{3}H-DHT) for inhibition binding experiments to hERα, hPR and hAR, respectively, as already described elsewhere [17].

Binding competition curves were established by using non-linear regression.

Results and discussion

Characteristics of specificity and stability of the steroid receptors assays used here have been reported previously [17]. Here, we present the data concerning the binding of various non-steroidal chemicals to human recombinant estrogen, androgen and progesterone receptors.

Table 1 Binding of DHT and various chemicals to human androgen receptor. IC_{50} concentrations (µM) (concentration needed to observe a 50% inhibition of the maximum binding of [^{3}H] DHT) and relative binding affinity (RBA, potency of binding to hAR expressed as a percentage relative to that of DHT)

	IC_{50}(µM)	RBA
DHT	0.05	100.000
p,p′-DDD	15	0.333
o,p′-DDD	17	0.294
p,p′-DDT	30	0.167
o,p′-DDT	32	0.156
o,p′-DDE	38	0.132
BHA	140	0.036
p,p′-DDE	184	0.027
Vinclozolin	245	0.020

Figure 1 shows, as a typical example, the competition curves obtained for binding of progesterone tracer to the recombinant human progesterone receptor with progesterone and several chemicals suspected to present hPR binding activity. Most of the tested components were able to completely inhibit the binding of the tritiated progesterone to the progesterone receptor, indicating a relatively high binding affinity of these substances for hPR. From these inhibition curves, we calculated the IC_{50} concentrations (the concentration needed to observe a 50% inhibition of the maximal binding of the tritiated progesterone to the human recombinant progesterone receptor) for all the tested chemicals and these are reported in Table 2.

Table 2 Binding of various compounds to recombinant estrogen and progesterone receptors. In each case, the relative binding affinity (RBA) is the ratio (expressed as a percentage) between the IC_{50} (concentration needed to observe a 50% inhibition of the maximum binding of [^{3}H] ligand) of the tested chemical and that of the natural ligand of the receptor

			Binding to hPR		Binding to hERα	
			IC_{50} (μM)	RBA (%)	IC_{50} (μM)	RBA (%)
Progesterone			0.05	100		
17β-estradiol					0.002	100
Organochlorine insecticides						
		p,p′-DDT	2	2.5	610	0.0003
		o,p′-DDT	15	0.3	76	0.003
		o,p′-DDE	13	0.4	100	0.002
		p,p′-DDE	21	0.2	188	0.001
		o,p′-DDD	9	0.6	318	0.0006
		p,p′-DDD	46	0.1	82	0.002
		methoxychlor	260	0.02	125	0.002
		aldrin	70	0.07	113	0.002
		dieldrin	–[a]	–	106	0.002
		chlordecon	11	0.5	9	0.02
		lindane	214	0.02	257	0.0008
		trichlorobenzene	–	–	1,300	0.0002
Other pesticides						
	Insecticides					
		α-endosulfan	20	0.3	170	0.001
		β-endosulfan	88	0.06	156	0.001
		toxaphen	28	0.2	110	0.002
		trans-nonachlor	11	0.5	143	0.001
		cis-nonachlor	–	–	200	0.001
	Herbicides					
		alachlor	298	0.02	240	0.0008
		atrazine	–	–	358	0.0006
	Fungicides					
		benomyl	–	–	205	0.001
		vinclozolin	233	0.02	422	0.0005
Alkylphenols						
		nonylphenol	5	1	9	0.02
Industrial chemicals						
		bisphenol A	45	0.1	8	0.03
		diphenylphtalate	173	0.03	174	0.001
Food additives (antioxidants)						
		BHA	228	0.02	263	0.0008
Phyto-estrogens						
	Isoflavones					
		genistein	ND[b]	ND	0.6	0.3
		daidzein	ND	ND	131	0.002
		glycitein	–	–	17	0.01
		biochanin A	158	0.03	20	0.01
	Flavonoides					
		apigenin	3	1.7	0.4	0.5
		luteolin	–	–	25	0.008
		kaempherol	72	0.07	36	0.006

[a] – no binding
[b] *ND* not determined

Similar binding experiments were also performed using hERα and hAR recombinant receptors. The results concerning the binding of a limited number of compounds to hAR are shown in Table 1 in terms of IC_{50} concentrations in our set-up and as relative binding affinity (RBA) compared to DHT. Table 2 compares the results obtained in the experiments performed with hPR and hERα.

A prolific amount of literature exists about the binding of chemicals to estrogen and androgen receptors from various animal species (including humans). Table 3 gives some references reporting the in vitro activity (steroid agonist or antagonist) determined for the chemicals tested here. By using the hAR and hERα binding assay, the results and relative affinities were roughly similar to those obtained previously by others [14, 16, 18]. In contrast, very little is known about the binding of potentially endocrine-disrupting chemicals to progesterone receptors. We will thus mostly focus on the binding to the progesterone receptor.

When analysing the comparative results in Tables 2 and 3, it clearly appears that the affinities for hPR (evaluated from IC_{50} values) are generally higher than those for hERα, except for some substances such as methoxychlor, alachlor and phytoestrogens.

Among all the tested chemicals, *p*,*p*′-DDT displays the highest "affinity" (IC_{50}=2 μM) for hPR. Its potency of binding to hPR is only 40 times lower than that of progesterone. *p*,*p*′-DDE, the persistent metabolite of DDT known

Table 3 Binding potency of chemicals versus known effect described in the literature[a]

Chemical		Reported in vitro activity	Binding to hPR	Binding to hERα	Binding to hAR
Organochlorine insecticides					
	p,*p*′-DDT	estrogenic [14]	++++	+/-	+++
	o,*p*′-DDT	estrogenic [34], anti-androgenic [34]	+++	+++	+++
	o,*p*′-DDE	estrogenic [14]	+++	++	+++
	p,*p*′-DDE	estrogenic [34], androgenic [34], anti-androgenic [16, 33, 34, 35]	+++	++	+
	o,*p*′-DDD	estrogenic [37]	++++	+	++++
	p,*p*′-DDD	estrogenic [37]	+++	+++	++++
	methoxychlor	anti-androgenic [35, 36]	+	++	ND[c]
	aldrin		++	++	ND
	dieldrin	estrogenic [14]	–	++	ND
	chlordecon	estrogenic [14]	++++	++++	ND
	lindane	hPR antagonist [24]	+	++	ND
	trichlorobenzene		–b	+/-	ND
Other pesticides					
Insecticides					
	α-endosulfan	hPR antagonist [24]	+++	++	ND
	β-endosulfan	hPR antagonist [24]	++	++	ND
	toxaphen	estrogenic [13, 39]	+++	++	ND
	trans-nonachlor	estrogenic [37]	++++	++	ND
	cis-nonachlor	estrogenic [37]	–	++	ND
Herbicides					
	alachlor	estrogenic [37]	+	++	ND
	atrazine		–	+	ND
Fungicides					
	benomyl		–	++	ND
	vinclozolin	estrogenic [34], anti-androgenic [35, 40]	+	+	+
Alkylphenols					
	nonylphenol	estrogenic [22], hPR antagonist [25]	++++	++++	ND
Industrial chemicals					
	bisphenol A	estrogenic [36, 38], anti-androgenic [34]	+++	++++	ND
	diphenylphtalate		++	++	
Food additives (antioxidants)					
	BHA	estrogenic [14]	+	++	++

[a]The relative binging affinity (RBA) from Table 2 is expressed with a different number of + signs, from ++++ (high RBA) to + (low RBA)

[b]– no binding

[c]*ND* not determined

for its anti-androgenic properties [16], also binds rather strongly to hPR (IC_{50}=21 µM and RBA=0.2%). In contrast, our results confirm that the affinity of *p*,*p*′-DDE for hER α and for hAR is rather low (IC_{50}=188 and 184 µM, respectively). As expected, metoxychlor bound poorly to hERα due to the fact that only the demethylated metabolite binds to ER [19]. In the same manner, it bound poorly to hPR. Klotz and collaborators [20] have already shown, using a combination of in vitro assays, that DDT metabolites interact with hPR and function as hPR antagonists. However, in contrast to our data only *o*,*p*′-DDT and *o*,*p*′-DDE were able to bind to hPR in a whole cell competition binding assay using [^{3}H]R5020 [20]. In one published bioassay based on reporter gene assay, neither *o*,*p*′-DDT nor metoxychlor displayed any hPR agonistic or antagonistic activity [21].

Chlordecone showed a high affinity for both hERα and hPR (9 and 11 µM, respectively) while the so-called estrogenic insecticides such as endosulfan, toxaphen and nonachlor [13] displayed higher affinities for hPR than for hERα. The high binding potency of nonylphenol to hERα is in agreement with its described estrogenic activity [22]. It is clear from Table 2 that it also binds with high affinity to hPR (IC_{50}=5 µM). A previous study [23] investigating the binding of chemicals to the progesterone receptor prepared from the oviduct of the American alligator (aPR) reported a binding activity to aPR for chlordecone and atrazine, a poor binding to aPR for DDT metabolites and no binding to aPR for dieldrin, endosulfan, and alachlor.

In contrast with our data, Jin and collaborators [24] found no competitive binding of lindane and endosulfan to hPR; however, they reported an hPR antagonistic activity for these chemicals. In another study, the same authors showed that nonylphenol, which displays a high affinity for hPR in our data, is an hPR antagonist [25].

As expected, vinclozolin, a fungicide known for the anti-androgenic effects of its metabolites M1 and M2 [16], displayed a poor affinity for the three receptors hPR, hER α and hAR.

Finally, the well-known substance bisphenol A, which is widely used in the polymer industry, also displayed a high binding potency to hPR and a high affinity for hERα, whereas diphenylphtalate and BHA showed poor affinity for both receptors (IC_{50}=173 and 174 µM, respectively).

Among the phytoestrogens tested, only one, the flavonoid apigenin, showed a high binding potency to hPR (IC_{50}= 3 µM and RBA=1.7%). IC_{50} for the binding to hERα ranged from 0.6 µM for genistein to 131 µM for daidzein. Slightly different results (generally higher IC_{50}) were reported by Collins et al. [26] for the binding of phytoestrogens to a recombinant hER produced in insect cells.

It is obvious that a discrimination between agonistic and antagonistic biological effects cannot be performed using receptor binding assays. To obtain functional information, cell-based assays based on the reporter gene technology can be used, such as those presented elsewhere in this issue [27]. The authors show that binding of *p*,*p*′-DDE and other DDT metabolites, nonylphenol, bisphenol A and apigenin to hPR results in an antagonistic effect.

Nevertheless, receptor binding assays represent very useful tools for the rapid screening of chemicals in various matrices (environmental, biological or food samples) containing endocrine-disrupting chemicals in low amounts, as well as for testing novel industrial products.

The useful range of concentrations that can be explored by using the technique described here is relevant to determine for example DDT in human fat [28] and serum lipids [29, 30].

Conclusion

These radioreceptor assays using three recombinant human steroid receptors hPR, hERα and hAR are proposed as tools for the screening of endocrine disruptors. From the affinities of the tested chemicals for the three types of receptors, a first characterization of their endocrine-disrupting potential can be obtained and the appropriate bioassay can then be selected. As an example, we show here the strong binding of some estrogenic compounds to the hPR. By using only the "binding assay", it is not possible to determine whether the binding is preliminary to an agonistic or to an antagonistic effect of an interacting chemical. Nevertheless, the interactions with hPR described here probably contribute to the hormonal disorders described in humans [4, 5, 6, 7, 8, 9, 10, 11, 12].

The originality of this screening strategy, compared to the already described binding assays, is the use of human recombinant receptors which are easily produced in bacteria. Receptors prepared from cytosol of cells collected from target tissues such as the prostate gland or uterus are not very adequate to use in standardized assays [31] and are more laborious to produce as are recombinant receptors produced in insect cells [32].

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