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Differential estrogen receptor binding of estrogenic substances: a species comparison

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Abstract

The study investigated the ability of 34 natural and synthetic chemicals to compete with [^{3}H]17β-estradiol (E2) for binding to bacterially expressed glutathione-*S*-transferase (GST)-estrogen receptors (ER) fusion proteins from five different species. Fusion proteins consisted of the ER D, E and F domains of human alpha (GST-hERαdef), mouse alpha (GST-mERαdef), chicken (GST-cERdef), green anole (GST-aERdef) and rainbow trout ERs (GST-rtERdef). All five fusion proteins displayed high affinity for E2 with dissociation constants (K_d) ranging from 0.3 to 0.9 nM. Although, the fusion proteins exhibited similar binding preferences and binding affinities for many of the chemicals, several differences were observed. For example, α-zearalenol bound with greater affinity to GST-rtERdef than E2, which was in contrast to other GST-ERdef fusion proteins examined. Coumestrol, genistein and naringenin bound with higher affinity to the GST-aERdef, than to the other GST-ERdef fusion proteins. Many of the industrial chemicals examined preferentially bound to GST-rtERdef. Bisphenol A, 4-*t*-octylphenol and *o*,*p*′ DDT bound with approximately a ten-fold greater affinity to GST-rtERdef than to other GST-ERdefs. Methoxychlor, *p*,*p*′-DDT, *o*,*p*′-DDE, *p*,*p*′-DDE, α-endosulfan and dieldrin weakly bound to the ERs from the human, mouse, chicken and green anole. In contrast, these compounds completely displaced [^{3}H]E2 from GST-rtERdef. These results demonstrate that ERs from different species exhibit differential ligand preferences and relative binding affinities for estrogenic compounds and that these differences may be due to the variability in the amino acid sequence within their respective ER ligand binding domains.

Keywords: Estrogen receptor; Phytoestrogens; Competitive binding; Estrogenic

1. Introduction

Estrogen influences the growth, development, behavior and regulation of reproductive tissues in all vertebrates. Many of the effects of estrogens are mediated through binding to the estrogen receptor (ER). Following estrogen binding, the ER undergoes a conformational change, which facilitates chromatin binding and the modulation of estrogen responsive gene expression. The ER exists as two subtypes, ERα and ERβ, which are distinct genes that differ in their tissue distribution, and ligand preference [1]. Both receptors are modular in structure and consist of six distinct domains (A–F) [2]. The DNA-binding domain (C domain) separates the NH_2-terminal ligand-independent activation domain (A/B domains) and the COOH-terminal region, which includes a hinge region (D domain), the ligand binding domain (E domain) and a variable F domain.

It has been suggested that exposure to natural and synthetic estrogenic chemicals may adversely affect wildlife and human health [3]. There have been controversial reports of decreases in sperm production and seminal volume in humans during the past half-century [4] and increases in reproductive abnormalities in mammals [5], reptiles [6], birds [7] and several fish species [8] following exposure to environmental contaminants. However, it has also been argued that weak estrogenic chemicals do not possess sufficient potency to elicit

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these adverse health effects when compared to the intake and potency of natural estrogenic chemicals, such as phytoestrogens [9].

The diets of humans and other species consist of several natural, non-steroidal estrogenic compounds either produced by plants (phytoestrogens) or fungal molds (mycotoxins) [10]. There are three chemically distinct classes of phytoestrogens: flavonoids (e.g. genistein and naringenin), coumestans (e.g. coumesterol) and lignans (e.g. enterdiol and enterolactone), which have been associated with reproductive abnormalities in grazing animals. However, the presence of high levels of phytoestrogens in Asian diets, combined with comparatively lower rates of hormone-induced cancers prevalent in western populations, suggests that they may have a chemoprotective effect [11]. Of the mycotoxins, zearalenone and its metabolites α- and β-zearalenol are the most commonly studied and have been shown to cause reproductive problems in swine and cattle fed contaminated grain [12].

Results from several studies suggest that estrogenic compounds may exhibit differential binding preferences and relative binding affinities for both ER subtypes [13] and for ERs from different species [14]. 17β-estradiol (E2) exhibits a ten-fold lower affinity for the rainbow trout (*Onchorhynkus mykiss*) ER (rtER) than for the human ERα (hERα) [15]. Moreover, the pig ER exhibits a significantly greater affinity for α-zearalenol than does the ER from the Leghorn chicken [16]. Although, these differences may be due to the variability in the amino acid sequence within the ER ligand binding domain among species [14,17], many of these studies used different assay conditions and examined a limited set of test chemicals, making overall comparisons difficult.

Under controlled conditions potential differences in ER binding among species were further investigated using bacterially expressed glutathione-*S*-transferase (GST)-ERdef fusion proteins consisting of the D, E and F domains of human alpha, mouse alpha, chicken, green anole and rainbow trout ERs. The ability of several endogenous, synthetic and natural compounds to compete with E2 for binding to GST-ERdef fusion proteins was examined using a semi-high throughput competitive binding assay. The hERα, considered to be the prototypical ER, was selected as the basis for all comparisons due to the information available on its ligand binding characteristics and structure of its ligand binding pocket [18,19]. The mouse (*Mus musculus*) ERα and chicken (*Gallus gallus*) ER were included as representative rodent and avian ERs, respectively. The ER from the green anole (*Anolis carolinensis*; aER), a lizard commonly found throughout the southeastern United States, was also included to investigate interactions with a representative reptilian ER. In addition the aER represents the only reported complete ligand binding domain sequence for a reptile [14], although partial sequences have been previously reported [20,21]. The rtER was also examined due to its environmental relevance and because it has a highly divergent amino acid sequence within its ligand binding domain, with percent identity and similarity of 60% and 67%, respectively, when compared to the hERα.

2. Materials and methods

The steroids 17β-estradiol (1,3,5[10]-estratriene-3,17 β-diol), 17α-ethynyl estradiol (17α-ethynyl-1,3,5[10]-estratiene-3,17β-diol), estrone (1,3,5[10]-estratrien-3-ol-17-one), estriol (1,3,5[10]-estratriene-3,16α,17β-triol), β-estradiol benzoate (1,3,5[10]-estratriene-3,17β-diol 3-benzoate), DHT (dihydrotestosterone, 5α-androstan-17β-ol-3-one) and DHEA (dehydroisoandrosterone, 5-androsten-3β-ol-17-one) were purchased from Sigma (St. Louis, MO)

Synthetic estrogens tamoxifen ([Z]-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene), 4-hydroxytamoxifen ([Z]-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-buten-4-ol), diethylstilbestrol (4,4′-(1,2-diethyl-1,2-ethene-diyl)-bisphenol) were from Sigma. The antiestrogen ICI 164,384 (*n*,*n*-butyl-11-(3,17β-dihydroxestra - 1,3,5(10)trien - 7α - yl) - *N* - methyl - undecanamide) was a gift from Alan Wakeling of AstraZeneca (Alderley Park, UK).

The mycotoxin zearalenone (2,4-dihydroxy-6-[10-dihydroxy-6-oxo-undecyl]benzoic acid μ-lactone), its metabolites α-zearalenol (2,4-dihydroxy-6-[6α,10-dihydroxy-undecyl]benzoic acid μ-lactone), and β-zearalenol (2,4-dihydroxy-6-[6β,10-dihydroxy-undecyl]benzoic acid μ-lactone), and the flavonoids genistein (4′,5,7-trihydroxyisoflavone), naringenin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside) and quercitin (3,3′,4′,5,7-pentahydroxyflavone), and β-sitosterol (22,23-dihydrostimasterol) were from Sigma. Coumestrol (2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid lactone) was obtained from Arcos Organics (Pittsburgh, PA).

The pesticide methoxychlor (1,1,1-trichloro-2,2-bis(p-methoxphenyl)ethane) and its bis-hydroxylated metabolite HPTE (2,2-bis(p-hydroxyphenyl)-1,1,1,-trichloroethane) were provided by William Kelce (Monsanto, St. Louis, MO). *o*,*p*′-DDT (1,1,1-trichloro-2-[2-chlorophenyl]-2-[4-chlorophenyl]ethane), *p*,*p*′-DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane), *o*,*p*′-DDE 1,1-dichloro-2-[2-chlorophenyl]-2-[4-chlorophenyl]ethylene) and *p*,*p*′-DDE (1,1- dichloro-bis[4-chlorophenyl] ethylene) were purchased from AccuStandard (New Haven, CT). The alkyl phenolic compound 4-*t*-octylphenol and bisphenol A (4,4′-isopropylidenediphenol) were obtained from Aldrich (Milwaukee, WI). Atrazine, simazine and chlordecone (kepone) (decachloro - octahydro - 1,3,4 - metheno - 2H - cyclobuta

(*cd*)pentalene) were from Chem-Service (West Chester, PA). Dieldrin (1,4:5,8-dimethanonaphthalene), α-endosulfan (hexachlorohexahydromethano-2,4,3-benzo-dioxathiepin-3-oxide), β-endosulfan (hexachlorohexahydromethano-2,4,3-benzo-dioxathiepin-3-oxide) were provided by S. Safe (Texas A&M University, College Station, TX). Monsanto (St. Louis, MO) and Eastman Chemical Company (Kingsport, TN) supplied butylbenzylphthalate and dibutylbenzylphthlate, respectively.

Radiolabeled [2,4,6,7,16,17-^{3}H] 17β-estradiol ([^{3}H]E2; 123 Ci/mmol) was purchased from New England Nuclear (Boston, MA). SuperScript II reverse transcriptase and Trizol Reagent were purchased from Life Technologies (Gaithesburg, MD). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA), and restriction enzymes and Taq DNA polymerase were obtained from Roche/Boehringer Mannheim (Indianapolis, IN). All other chemicals and biochemicals were of the highest quality available from commercial sources

2.1. Recloning of chicken ER DEF domains

Total RNA from a 1-cm^3 liver section from a *G. gallus* (chicken) was isolated using Trizol Reagent according to manufacturer's instructions. One microgram of RNA was then reverse transcribed using primer PRcr-5′-aaaactcgagttatattgtattctgcatactctcctc-3′ as previously described [14]. The entire cDNA products from the reverse transcription (RT) reaction were used in a PCR mixture containing 200 μM dNTPs, 2 μM primers (PRcf - 5′ - aaaagaattccgaaatgatgaaacagaaacgtcaaag - 3′ and PRcr) and 1.25 units of Vent DNA polymerase was amplified for 30 cycles using the following conditions: 94°C for 1 min, 62°C for 1 min and 72°C for 2 min. Sequence analysis was performed using MacVector 6.5 and the GCG Wisconsin Package (Oxford Molecular Ltd., Beaverton OR).

2.2. Construction of GST-ER DEF fusion proteins

The construction of pGEX-hERαdef, pGEX-aERdef and pGEX-rtERdef vectors has already been described [14]. The pGEX-mERαdef plasmid (mERα a.a. 268–599) was generated by PCR amplification of the plasmid pJ3MOR containing the complete mouse ER cDNA (provided by M.G. Parker; Molecular Endocrinology Research Laboratory, London, UK) using primers PRmf-5′-aaaaggatccatgttgaagcacaagcgtcagagag-3′ and PRmr-5′-aaaagaattcgcgccgctcagatcgtgttggggaagccctc-3′. The pGEX-cERdef plasmid (cER a.a. 258–589) was prepared using the products of the RT-PCR reaction described above. The mERαdef and cERdef PCR fragments were digested with the *Bam*HI/*Not*I and *Eco*RI/*Xho*I restriction enzymes and ligated into the appropriately digested GST fusion protein expression vector, pGEX6p3 (Amersham/Pharmacia; Piscataway, NJ). The PCR amplification was performed using Vent DNA polymerase (New England Biolabs) as described above. The sequence of each construct was confirmed with restriction enzyme digest and ABI/Prism automated sequencing (Perkin Elmer Applied Biosystems; Foster City, CA).

2.3. Expression and purification of GST-ERdef fusion proteins

Expression and purification of GST-ERdef fusion proteins was done as previously described [14]. Partially purified fusion proteins were separated by SDS-PAGE using a 4% stacking and 10% separating gel. Proteins were visualized by coomassie brilliant blue R250 staining.

2.4. Receptor binding assays

Receptor binding assays were performed as previously described [14]. Briefly, GST-ERdef fusion proteins were diluted in TEGD buffer (10 mM Tris pH 7.6, 1.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol) containing 1 mg/ml bovine serum albumin (BSA), and incubated at 4°C for 2 h with 0.1–3.5 nM [^{3}H]E2 in 1 ml glass tubes arranged in a 96-well format (Marsh Scientific, Rochester, NY). Fusion protein preparations were diluted to ensure 10 000 dpms of total binding (varied from 750–2000-fold). Binding assays were initiated by adding 240 μl of protein preparation to glass tubes containing 5 μl of DMSO and 5 μl [^{3}H]E2, thus the concentration of solvent did not exceed 4%. Bound [^{3}H]E2 was separated from free using a 96-well filter plate and vacuum pump harvester (Packard Instruments). After drying, the filter plates were sealed and 50 μl of MicroScint 20 scintillation cocktail (Packard Instruments) was added to each well. Bound [^{3}H]E2 was measured using a TopCount luminescense and scintillation counter (Packard Instruments).

Competitive ligand binding assays were performed as described above except diluted GST-ERdef fusion protein preparations were incubated with a final concentration of 2.5 nM [^{3}H]E2 (5 μl aliquot) and increasing final concentrations of unlabeled competitor (0.1 nM–100 μM, 5 μl aliquots) at 4°C for 24 h. Each treatment was performed in quadruplicate and results are expressed as percent specific binding of [^{3}H]E2 versus log of competitor concentration. IC_{50} values were determined from non-linear regression for single site competitive binding analysis. The reported IC_{50} values represent the concentration of test compound required to displace 50% [^{3}H]E2 from the GST-ERdef fusion proteins as compared to the 50% displacement of [^{3}H]E2 achieved by unlabeled E2. Analyses were per-

formed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Recloning of chicken ERdef sequence

The recloned chicken (*G. gallus*) ERdef sequence was identical to the previously published sequence [22]. A comparison of the amino acid sequences of the cERdef with those of human, mouse, green anole and rainbow trout is shown in Fig. 1. Amino acids found to interact with E2 and/or line the ligand binding pocket are boxed. Though these residues are highly conserved among the species, there are differences. For example, Met421 in hERαdef is substituted for Phe175 in aERdef. In addition, hERαdef differs in two locations from rtERdef, in hERαdef Leu349 and Met528 replace Met93 and Ile272, in rtERdef.

3.2. Expression and saturation analysis of GST-ERdef fusion proteins

The amino acids used in the construction of the GST-ERdef fusion proteins for each species and their sequence identity compared to that of the human ERα are shown in Fig. 2. All five fusion proteins migrated according to their predicted molecular weights (MWs): GST-hERαdef (64.2 kDa), GST-mERαdef (64.4 kDa), GST-cERdef (65.2 kDa), GST-aERdef protein (64.3 kDa) and GST-rtERdef (65.5 kDa), although each appears to migrate as a doublet (Fig. 2(A)). The higher MW bands most likely represent the full-length product, whereas the lower bands may result from proteolytic cleavage [23]. In addition, higher and lower MW proteins co-purified with the proteins. The purity of the GST-ERdef fusion proteins varied among protein preparations, with yields ranging from 1 to 4 mg/l. This was evident with the GST-mERαdef preparation (Fig. 2(B) lane m) which contained lower amounts of the fusion protein, when compared to the other fusion protein preparations. However the GST-mERαdef preparations resulted in sufficient recombinant receptor to investigate the competitive binding of approximately 500 compounds per liter culture. This value varied among protein preparations, ranging from 100 to 600 compounds per liter culture.

Binding affinities of the partially purified GST-ERdef fusion proteins for E2 were determined by saturation analysis and linear transformation of the data [24] (Table 1). Differences in the amount of receptor re-

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hERαdef  264   --MLKHKRQRDDGEGRGEVGSAGDMRAANLWPSPLMIKRSKKNS-----LALSLTADQMVSALLDAEPPILYSEY  331
mERαdef  268   --.........L...N.M.AS.............V..HT....-----P...................MI....  335
cERdef   257   -E.M.Q....EEQDS.NGEA.STEL..PT..T...VV.HN....-----P......E.......E.....V....  325
aERdef    19a  --.........EENDS.N-A.ALTEA.STA.........H.....-----P..............E....VV....   85
rtERdef    1b  RVLR.D..YCGPAGD.EKPYGDLEH.T.PPQDGGRNSSS.LNGGGGWRGPRITMPPE.VLFL.QG....A.C.RQ   75

         332   DPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAP  406
         336   ..S.....................................G..N.....................................  410
         326   ..N...N.....T..........................................................T.....  400
          86   ..S...N...V.T..........................A.....................V.....I..IHC....I..Q  160
          76   KVA..YT.VT..T...SM..K.....A...K....QE.S.....Q...SS...V....I..IHC....I..Q  150

         407   NLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLDK  481
         411   .....................................................................................  485
         401   ..........................AA.....................................R.Y.......  475
         161   ......SH..V...F.....................VR.............P.I..Y.......V..R.........  235
         151   D.I...SE.D.....A.....V.....LK.KP.......A.......AFS.C.NSVE..HNSSAVESM..N  225

         482   ITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLHAPTSRG  556
         486   ....................R.....................N.....................A..M  560
         476   ............S..S.....R.....................N.....................A.RS  550
         236   .....M.....S..S.....R.....................N.....................AAK.  310
         226   ...A...HISHS.ASV...PR.Q.....................I....K.....G...QS.GKVA  300

         557   GASVEETDQSHLATAGSTSSHSLQK----YYITGEAEG-FPATV---------  595
         561   .VPP..PS.TQ...TS...A....T------...PP....-..N.I---------  599
         551   A.PM..ENRNQ.T..-PA......S----F..NSKE.ESMQN.I---------  589
         311   SPPS.DDPLNQ..VP-.PSM...LP----C.VNKQE..NEQEAI---------  349
         301   Q.GEQTEGP.TTT.TSTG..IGPMRGSQDTH.RSPGS.VLQYGSPSSDQMPIP  353
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Fig. 1. Alignment of the estrogen receptor D, E and F domains from human (hERαdef), mouse (mERαdef), chicken (cERdef), green anole (aERdef) and rainbow trout (rtERdef). Numbers refer to amino acid position in the full-length sequence. Identical amino acid residues are represented as dots while missing residues are shown as dashes. The E domains are shown in bold. Residues that line the hormone binding pocket and/or interact with bound E2 are boxed. [a]Only a portion of the green anole ER sequence was cloned and the start of the D domain corresponds to amino acid residue 19 in the cloned sequence [14]. [b]Refers to the first amino acid of a recloned rainbow trout ER partial sequence [14]. This figure was modified from Pike et al. (1999) [42].

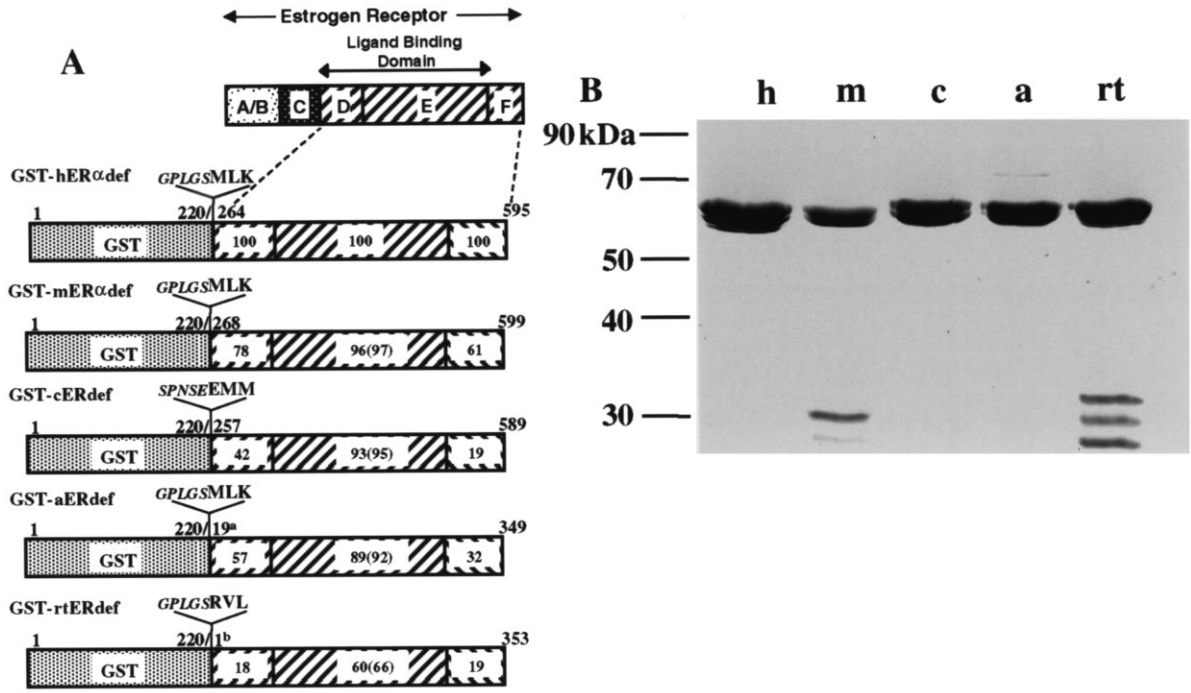

Fig. 2. (A) A schematic representation the GST-ERdef fusion proteins expressed in bacteria. Amino acid residues that link the two proteins are provided above the transition point. The residues shown in reduced font and italics represent amino acids from the linker region downstream of GST. The first three residues of the ER D domains are shown in regular text. Numbers provided above identify the amino acids used in the construction. The numbers within the domains represent the percent identity while those in parentheses represent percent similarity to hERα. [a]Only a portion of the green anole and rainbow trout ER sequences were cloned as previously described [14]. (B) SDS-PAGE analysis of the GST-ERdef fusion proteins purified using GSH affinity chromatography. Lane h: GST-hERαdef (predicted molecular weight (MW) = 64.2 kDa). Lane m: GST-mERαdef (predicted MW = 64.4 kDa). Lane c: GST-cERdef (predicted MW = 65.2 kDa). Lane a: GST-aERdef (predicted MW = 64.3 kDa). Lane rt: GST-rtERdef (predicted MW = 65.5 kDa). Each lane was loaded with 5 μg of partially purified GST-ERdef fusion protein. Proteins were analyzed using a 4% stacking and a 10% separating gel stained with coomassie brilliant blue R250.

quired to attain the desired 10 000 dpm at saturation were species-dependent and may be due, in part, to differences in protein purity, functionality and level of expression between preparations. All GST-ERdef fusion proteins exhibited high binding affinity for E2, with dissociation constants (K_d) similar to other reports (see Table 1 and references therein).

3.3. Relative binding affinities of endogenous steroids and antiestrogens

The classification of the competitive binding ability of the test compounds followed the same criteria as previously discussed [14]. Briefly, compounds were classified as non-binders (nb) if less than 10% displacement was observed or as weak binders (wb) if only 10–50% of [^{3}H]E2 was displaced at the highest concentration (100 μM) of competitor examined. For compounds that were capable of displacing greater than 50% of the [^{3}H]E2 from the GST-ERdef fusion proteins an IC_{50} value was calculated using Graphpad Prism 3.0. Concentrations greater than 100 μM were not examined due to potential solubility limitations of the test compounds.

Table 2 and Fig. 3 summarize the ability of several natural and synthetic compounds to compete with [^{3}H]E2 for binding to GST-ERdef fusion proteins. Non-binders and weak-binders were tested in a single experiment in which each treatment was performed in quadruplicate. IC_{50} values were determined for compounds that displaced at greater than 50% of the [^{3}H]E2 from the GST-ERdef fusion proteins and are shown as averages from at least two experiments. The relative binding affinities were determined for each

Table 1
Comparison of the dissociation constants (K_d) of the GST-ERdef fusion proteins with reported values

Protein	K_d (nM)	K_d reported (nM)[a]	References
GST-hERαdef	0.4 ± 0.1	0.1–1.5	[27,45]
GST-mERαdef	0.6 ± 0.2	0.1–1.4	[46,47]
GST-cERdef	0.9 ± 0.1	0.2–3.7	[16,48]
GST-aERdef	0.7 ± 0.2	0.5–17	[33,35]
GST-rtERdef	0.6 ± 0.1	0.9–6	[29,30]

[a] K_d values were derived from a variety of different ER sources including in vitro translated proteins, recombinant proteins expressed in bacteria, yeast and SF9 cells, and cytosol prepared from uteri, testis and liver tissue.

Table 2
IC_{50} values and relative binding affinities (RBAs) of natural and synthetic estrogenic chemicals for GST-ERdef fusion proteins following a 24-h incubation at 4°C

Test compound	GST-hERαdef		GST-mERαdef		GST-cERdef		GST-aERdef		GST-rtERdef	
	IC_{50}[a] (M)	RBA[b]	IC_{50} (M)	RBA	IC_{50} (M)	RBA	IC_{50} (M)	RBA	IC_{50}	RBA
4-Hydroxytamoxifen	$1.9 \pm 0.1 \times 10^{-9}$	155	$1.2 \pm 0.4 \times 10^{-9}$	212	$1.9 \pm 0.3 \times 10^{-9}$	168	$1.3 \pm 0.1 \times 10^{-9}$	243	$1.2 \pm 0.9 \times 10^{-9}$	272
Ethynyl estradiol	$2.3 \pm 0.1 \times 10^{-9}$	127	$2.2 \pm 0.7 \times 10^{-9}$	118	$1.9 \pm 0.1 \times 10^{-9}$	171	$2.2 \pm 0.1 \times 10^{-9}$	139	$3.1 \pm 0.9 \times 10^{-9}$	108
17β-Estradiol	$2.9 \pm 0.5 \times 10^{-10}$	100	$2.7 \pm 0.4 \times 10^{-9}$	100	$3.2 \pm 0.5 \times 10^{-9}$	100	$3.1 \pm 0.5 \times 10^{-9}$	100	$3.3 \pm 0.5 \times 10^{-9}$	100
Diethylstilbestrol	$3.2 \pm 0.1 \times 10^{-9}$	91	$3.2 \pm 0.5 \times 10^{-9}$	84	$2.5 \pm 0.6 \times 10^{-9}$	130	$2.9 \pm 1.0 \times 10^{-9}$	107	$2.0 \pm 0.1 \times 10^{-9}$	165
α-Zearalenol	$6.1 \pm 0.2 \times 10^{-9}$	48	$5.1 \pm 0.5 \times 10^{-9}$	53	$4.6 \pm 0.9 \times 10^{-9}$	70	$8.6 \pm 2.1 \times 10^{-9}$	36	$1.3 \pm 0.1 \times 10^{-9}$	267
Estrone	$6.5 \pm 0.3 \times 10^{-9}$	45	$9.5 \pm 0.8 \times 10^{-9}$	28	$6.4 \pm 0.1 \times 10^{-9}$	50	$5.1 \pm 0.1 \times 10^{-9}$	60	$2.4 \pm 0.2 \times 10^{-8}$	14
ICI 164,384	$7.0 \pm 0.3 \times 10^{-9}$	42	$5.9 \pm 0.3 \times 10^{-9}$	45	$5.2 \pm 1.0 \times 10^{-9}$	62	$1.1 \pm 0.2 \times 10^{-8}$	28	$1.0 \pm 0.7 \times 10^{-9}$	327
Estriol	$1.0 \pm 0.3 \times 10^{-8}$	28	$2.1 \pm 0.5 \times 10^{-8}$	13	$2.9 \pm 0.1 \times 10^{-8}$	11	$1.0 \pm 0.1 \times 10^{-8}$	30	$9.0 \pm 0.6 \times 10^{-8}$	3.7
β-Zearalenol	$2.3 \pm 0.3 \times 10^{-8}$	13	$2.4 \pm 1.6 \times 10^{-8}$	11	$1.4 \pm 0.1 \times 10^{-8}$	23	$7.3 \pm 1.8 \times 10^{-8}$	4.2	$3.7 \pm 0.3 \times 10^{-9}$	91
Tamoxifen	$2.8 \pm 0.4 \times 10^{-8}$	11	$2.6 \pm 0.1 \times 10^{-8}$	10	$2.1 \pm 0.1 \times 10^{-8}$	16	$3.0 \pm 0.3 \times 10^{-8}$	10	$1.3 \pm 0.1 \times 10^{-8}$	25
Estradiol benzoate	$2.8 \pm 0.5 \times 10^{-8}$	10	$2.3 \pm 0.2 \times 10^{-8}$	12	$2.2 \pm 0.1 \times 10^{-8}$	15	$2.4 \pm 0.3 \times 10^{-8}$	13	$3.7 \pm 0.5 \times 10^{-9}$	9.0
Zearalenone	$3.1 \pm 0.3 \times 10^{-8}$	9.3	$2.3 \pm 0.5 \times 10^{-8}$	12	$9.9 \pm 1.1 \times 10^{-9}$	33	$2.7 \pm 0.3 \times 10^{-8}$	12	$4.1 \pm 0.8 \times 10^{-9}$	82
HPTE	$2.5 \pm 0.8 \times 10^{-7}$	1.2	$2.2 \pm 0.2 \times 10^{-7}$	1.2	$6.8 \pm 2.2 \times 10^{-8}$	4.8	$6.4 \pm 1.7 \times 10^{-8}$	4.8	$2.4 \pm 0.1 \times 10^{-8}$	14
Coumestrol	$3.6 \pm 0.3 \times 10^{-7}$	0.81	$8.0 \pm 3.2 \times 10^{-7}$	0.33	$4.6 \pm 1.0 \times 10^{-7}$	0.70	$1.0 \pm 0.4 \times 10^{-7}$	3.1	$1.4 \pm 0.1 \times 10^{-6}$	0.24
Genistein	$6.3 \pm 0.7 \times 10^{-7}$	0.46	$8.1 \pm 0.4 \times 10^{-7}$	0.33	$4.1 \pm 0.5 \times 10^{-7}$	0.78	$2.4 \pm 0.1 \times 10^{-7}$	1.3	$7.5 \pm 0.8 \times 10^{-7}$	0.44
4-*t*-Octylphenol	$2.4 \pm 0.7 \times 10^{-6}$	0.12	$1.6 \pm 0.1 \times 10^{-6}$	0.17	$5.6 \pm 0.1 \times 10^{-7}$	0.57	$3.9 \pm 1.6 \times 10^{-6}$	0.079	$1.1 \pm 0.2 \times 10^{-7}$	3.2
Dihydrotestosterone	$5.9 \pm 0.9 \times 10^{-6}$	0.049	$6.6 \pm 1.4 \times 10^{-6}$	0.040	$3.8 \pm 0.6 \times 10^{-5}$	0.0085	$8.2 \pm 1.2 \times 10^{-7}$	0.38	$1.0 \pm 0.3 \times 10^{-5}$	0.034
Bisphenol A	$3.6 \pm 1.6 \times 10^{-5}$	0.0080	$3.1 \pm 0.7 \times 10^{-5}$	0.0086	$7.3 \pm 1.9 \times 10^{-6}$	0.044	$2.4 \pm 1.6 \times 10^{-6}$	0.13	$1.6 \pm 0.3 \times 10^{-6}$	0.21
Kepone	$4.2 \pm 1.8 \times 10^{-5}$	0.0069	$6.4 \pm 0.3 \times 10^{-5}$	0.0035	$3.0 \pm 0.1 \times 10^{-5}$	0.011	$2.7 \pm 0.7 \times 10^{-5}$	0.011	$6.2 \pm 0.4 \times 10^{-6}$	0.054
Naringenin	wb[c]	–	wb	–	$3.9 \pm 0.4 \times 10^{-5}$	0.0082	$4.7 \pm 0.8 \times 10^{-6}$	0.065	$8.7 \pm 1.3 \times 10^{-6}$	0.039
DHEA	wb	–	wb	–	wb	–	wb	–	$1.2 \pm 0.2 \times 10^{-5}$	0.028
Quercitin	wb	–	wb	–	$8.2 \pm 2.2 \times 10^{-5}$	0.0039	$1.9 \pm 0.2 \times 10^{-5}$	0.016	$8.0 \pm 2.0 \times 10^{-6}$	0.042
o,p′-DDT	wb	–	$3.6 \pm 3.5 \times 10^{-5}$	0.0073	$3.7 \pm 1.2 \times 10^{-6}$	0.086	wb	–	$7.8 \pm 0.1 \times 10^{-7}$	0.43
o,p′-DDE	wb	–	wb	–	wb	–	wb	–	$3.2 \pm 1.0 \times 10^{-6}$	0.11
p,p′-DDE	wb	–	wb	–	nb	–	wb	–	$8.0 \pm 0.6 \times 10^{-6}$	0.042
p,p′-DDT	wb	–	wb	–	wb	–	wb	–	$2.0 \pm 0.4 \times 10^{-6}$	
Butylbenzylphthalate	wb	–	wb	–	wb	–	wb	–	wb	–
Dibutylbenzylphthalate	wb	–	wb	–	wb	–	wb	–	$1.7 \pm 2.3 \times 10^{-6}$	0.20
α-Endosulfan	wb	–	wb	–	nb	–	wb	–	$2.8 \pm 1.4 \times 10^{-5}$	0.012
Methoxychlor	nb[d]	–	nb	–	wb	–	wb	–	$3.5 \pm 0.4 \times 10^{-6}$	0.95
β-Endosulfan	nb	–	nb	–	nb	–	nb	–	wb	–
Atrazine	nb	–	nb	–	nb	–	nb	–	nb	–
Simazine	nb	–	nb	–	nb	–	nb	–	nb	–
β-Sitosterol	nb	–	nb	–	nb	–	nb	–	nb	–

[a] IC_{50} values were determined from competitive binding experiments following a 24-h incubation at 4°C as described in Section 2 and represents the average and standard deviation from at least two independent experiments.

[b] Relative binding affinity (RBA) = (IC_{50} 17β-estradiol/IC_{50} compound X) × 100.

[c] Denotes weak binder (wb) since 10–50% displacement of radiolabeled 17β-estradiol was observed at the highest examined dose (100 μM).

[d] Denotes non-binder (nb) since no significant displacement of radiolabeled 17β-estradiol was observed at the highest examined dose (100 μM).

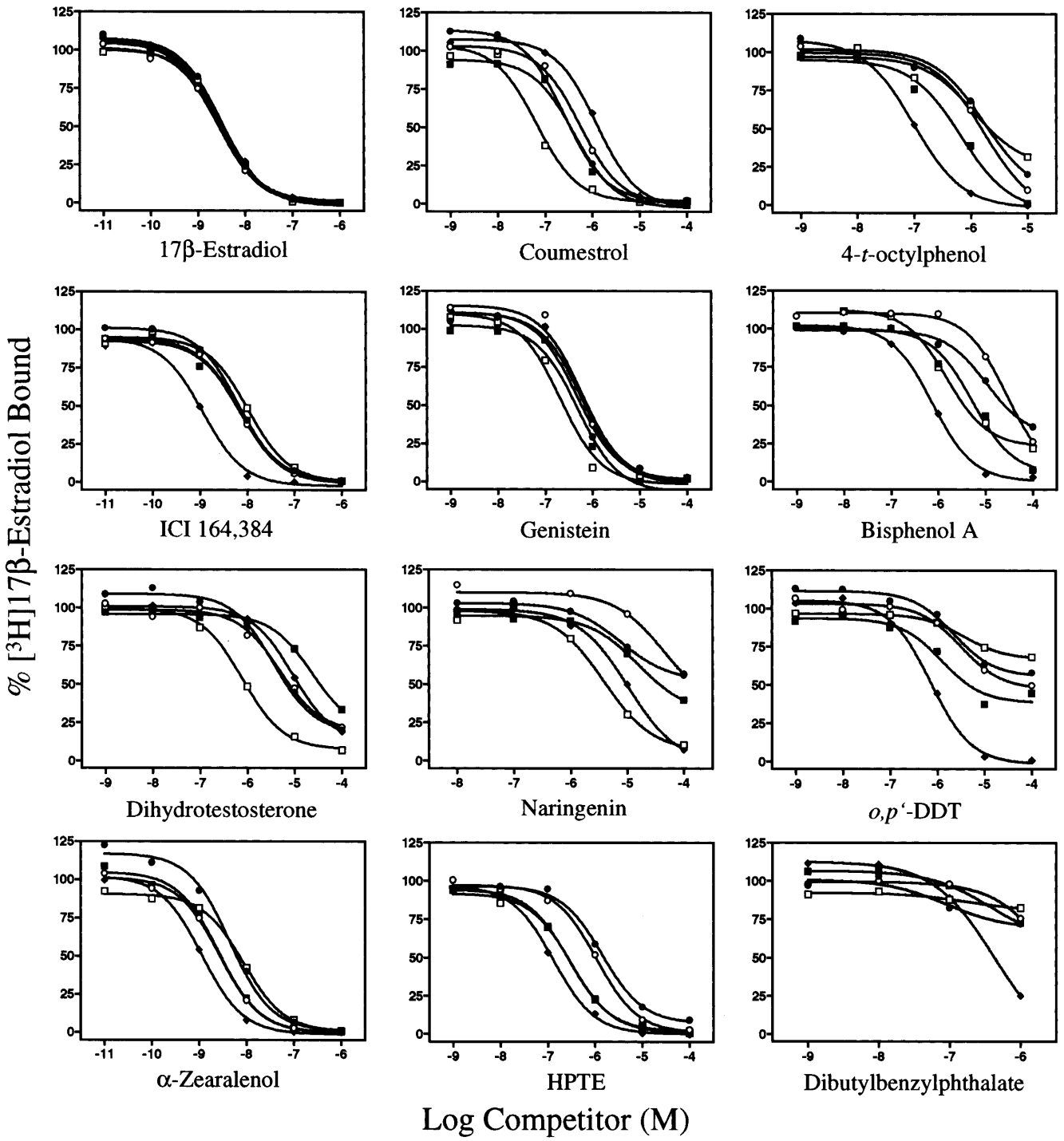

Fig. 3. Representative competitive binding curves of selected test chemicals to ● GST-hERαdef, ○ GST-mERαdef, ■ GST-cERdef, □ GST-aERdef, and ◆ GST-rtERdef fusion proteins. An aliquot of partially purified GST-ERdef fusion proteins was incubated with 2.5 nM [^{3}H]17β-estradiol and increasing concentrations of unlabeled test chemical and incubated for 24 h at 4°C as described in Section 2. The results are from a representative experiment that was repeated at least two times. Standard deviations for points on graph ranged between 5 and 15% of the mean.

compound as compared to the IC_{50} value of E2 for each GST-ERdef fusion protein. The compounds have been arranged in order of potency in comparison to GST-hERαdef. Chemicals were tested at concentrations ranging from 0.1 nM to 100 μM. This range varied depending on the competitive binding ability of the test compound.

Overall the steroidal and antiestrogenic compounds exhibited similar binding preferences and relative binding affinities for GST-ERdef fusion proteins. The E2

binding was similar across species with IC_{50} values ranging from 2.7 to 3.3 nM (Fig. 3). 4-Hydroxytamoxifen, the hydroxylated metabolite of tamoxifen, bound with greatest affinity to all 5 of the fusion proteins, with a ten- to 25-fold greater affinity than the parent compound. Diethylstilbestrol and ethynyl estradiol bound with similar affinities, 1.9–3.1 nM and 2.0–3.2 nM, respectively, to the five fusion proteins.

Though the rank order of these chemicals was similar among species, some notable differences were observed. The pure ER antagonist ICI 164,384 bound to GST-hERαdef, -mERαdef, -cERdef and -aERdef proteins with a five- to ten-fold lower affinity than to GST-rtERdef (Fig. 3). In addition, ICI 164,384 bound with higher affinity than E2 to GST-rtERdef, which was not observed with the other four fusion proteins. DHT bound to GST-aERdef with a seven- to 12-fold greater affinity than to GST-hERαdef, -mERαdef and -rtERdef, but bound with a 42-fold greater affinity than to GST-cERdef (Fig. 3), which shares 91% amino acid sequence identity to aERdef within their respective ER E, ligand binding, domains. DHEA, a precursor in the endogenous synthesis of estrogens and androgens, weakly bound to the ERs of human, mouse, chicken and green anole; however it exhibited an IC_{50} value of 12 ± 2 μM with GST-rtERdef.

3.4. Differential binding of phytoestrogens and mycotoxins

Differences in ligand preferences and binding affinities were also seen with some mycotoxins and phytoestrogens. α-Zearalenol, a hydroxylated metabolite of the mycotoxin zearalenone, consistently bound with greater affinity to the GST-rtERdef than to any other fusion proteins (Fig. 3). Similarly, the parent compound zearalenone and another metabolite, β-zearalenol, bound with greater affinity to the GST-rtERdef than to ERs from other species (Table 2). Interestingly, α-zearalenol bound with greater affinity than E2 to GST-rtERdef (2.6-fold) but bound to the other 4 fusion proteins with approximately half the affinity of E2.

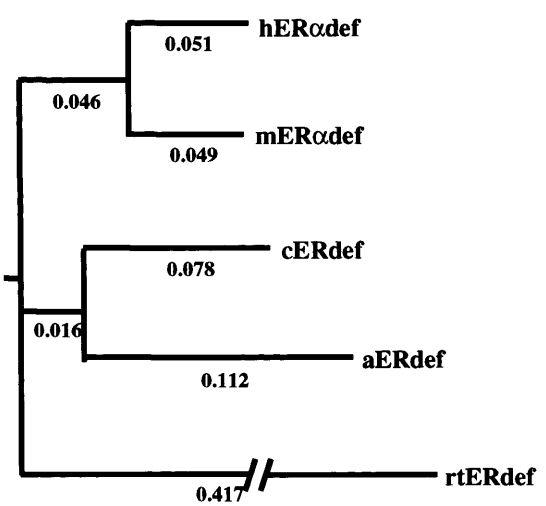

Fig. 4. Dendogram generated from the aligned ERdef sequences. The distance between nodes reflects the degree of sequence identity when doing pairwise alignments. The value of 0.1 corresponds to a difference of 10% between two sequences. This figure was generated using the ClustalW alignment function in MacVector 6.5 (Oxford Molecular Ltd.).

Overall the phytoestrogens displayed higher affinity for GST-aERdef than the other GST-ERdef fusion proteins. However, quercitin bound with slightly greater affinity to GST-rtERdef and β-sitosterol was unable to displace [^{3}H]E2 from any of the GST-ERdef fusion proteins at the highest concentration examined (100 μM; Table 2). Coumestrol bound with greatest affinity to GST-aERdef ($IC_{50} = 0.10 \pm 0.04$ μM). It exhibited similar affinity to the human, mouse, chicken and ERdef proteins (IC_{50} values ranging from 0.36 to 0.80 μM) and bound with a 14-fold lower affinity to the rainbow trout ER ($IC_{50} = 1.4 \pm 0.1$ μM; Fig. 4). Genistein exhibited similar binding affinities for all five GST-ERdef fusion proteins, but consistently bound with higher affinity to GST-aERdef (Fig. 4). Moreover, naringenin bound with highest affinity to GST-aERdef ($IC_{50} = 4.7 \pm 0.8$ μM) and slightly lower affinity to GST-rtERdef (8.7 ± 1.3 μM). However it bound with approximately a ten-fold lower affinity to GST-cERdef and bound weakly to GST-hERαdef and GST-mERαdef (Fig. 4).

3.5. Differential binding of synthetic chemicals

Overall, this class of compounds bound with greater affinity to GST-rtERdef than to any other fusion protein. Bisphenol A, 4-*t*-octylphenol and *o*,*p*′-DDT bound with approximately a 10-fold greater affinity to GST-rtERdef than to the ERs of the other species (Fig. 4). Complete displacement of [^{3}H]E2 by these compounds was only observed with GST-rtERdef at the highest concentration examined (100 μM). Methoxychlor, *p*,*p*′-DDT, *o*,*p*′-DDE, *p*,*p*′-DDE, α-endosulfan and dieldrin were found to bind weakly to the ERs from the human, mouse, chicken and green anole. In contrast, these compounds completely displaced [^{3}H]E2 from GST-rtERdef (Table 2). Although, α-endosulfan effectively displaced [^{3}H]E2 from GST-rtERdef, its isomer β-endosulfan did not displace greater than 30% [^{3}H]E2 and was therefore classified as a weak binder. Butylbenzylphthalate and dibutylbenzylphthalate bound weakly to the ERs of the different species. However, dibutylbenzylphthalate was found to displace of 75% [^{3}H]E2 from GST-rtERdef ($IC_{50} = 1.7 \pm 2.3$ μM) at the highest concentration (1 μM) examined (Fig. 3). A visible precipitate was observed at concentrations greater than 10 μM and 1 μM, for butylben-

zylphthalate and dibutylbenzylphthalate, respectively, thus higher concentrations were not examined. Finally, two chloro-*S*-triazines (Atrazine and Simazine) were unable to displace [^{3}H]E2 from any of the GST-ERdef fusion proteins at the highest concentration examined (100 μM).

The time to equilibration for steroid receptor:competitor complexes has been reported to be greater than 16 h at lower temperatures [25]. Since the competitive ligand-binding assay used a 24-h incubation time it is unlikely that the observed differences in binding affinities were due to a lack of equilibration. However when this data set was examined following a 2-h incubation at 4°C, the binding profiles of most chemicals and differences in ligand preferences, and binding affinities were similar to that of the 24 h incubation time, though there were some exceptions (unpublished data).

4. Discussion

Previous studies using a variety of protein preparations and assay conditions suggest that there may be differences in the absolute and relative binding affinities of structurally diverse estrogenic chemicals to ERs from different species. In order to investigate species-specific ligand preferences, and differences in relative and absolute binding affinities, GST-ERdef fusion proteins consisting of mammalian (human and mouse), avian (chicken), reptilian (green anole) and fish (rainbow trout) ERs were constructed.

It has been reported that truncated forms of the glucocorticoid receptor can effect protein stability and receptor function [26]. However, truncated forms of nuclear receptors overexpressed in heterologous expression systems have been previously shown to exhibit comparable affinities and ligand preferences relative to their native forms [27,28]. Heterologous expression of GST-ERdef facilitates purification of these fusion proteins and allows for precise control of the competitive binding assay conditions (e.g. protein concentration, metabolism, non-specific binding, background/accessory proteins), making direct comparisons possible.

The affinity of the bacterially expressed GST-ERdef fusion proteins for E2 was similar to the K_d values reported for full length ERs (Table 2). However, the affinity of the GST-rtERdef for E2 was approximately ten-fold higher than that reported for full length rainbow trout ER [29], but was in agreement with reports using [^{3}H]moxestrol [30]. In general, the reported K_d values for some species vary considerably, which may be due to the use of different assays such as dextran-coated charcoal and hydroxylapatite methods. For example, the K_d value determined from *Xenopus* liver cytosol ER has been reported to vary from 0.5 to 15 nM [31,32]. Similarly, the K_d value determined from turtle ER using two different receptor sources, hepatic and testis cytosol, varies from 0.7 to 17 nM, respectively [33,34]. This suggests that differences in protein preparation, assay conditions and assay methods may contribute to the variability in the reported K_d values.

The ER has been shown to bind several structurally diverse chemicals. This property appears to be unique among nuclear receptors and is also true for mammalian ERα and ERβ subtypes [13] as well as for ERs from non-mammalian species [15,35]. Crystal structures of hERα E domain in complex with E2 [18,19] support ER-E2 interaction models generated from binding studies, structure activity relationships and three-dimensional homology models using crystallographic data from other nuclear receptors [36–38]. E2 binding is achieved by a combination of specific hydrogen bonding interactions and the hydrophobic nature of the binding pocket. The promiscuity of the ER has been partially attributed to the size of the ligand binding pocket, which is almost twice the volume of E2 [19]. Despite the differences in sequence identity among species [14,17], ERs from all species harbor the same three equivalent amino acids to hERα (Glu353, Arg394 and His524) that participate in direct hydrogen bonds and stabilize E2 in the binding pocket [18,19]. However, differential binding of several natural and synthetic chemicals to hERα and hERβ, as well as to ERs from different species have been reported [14–16,35,39–41]. This suggests that additional amino acid residues may also play a role in determining ligand preference and relative binding affinity.

Genistein has been shown to preferentially bind with 30-fold greater affinity to hERβ than hERα [13]. The recent report of the crystal structure of ERβ in complex with genistein has suggested that this ligand preference may be attributed to two conservative mutations within the binding pocket that may be responsible for further stabilizing the hERβ-genistein complex [42].

Although many of the compounds examined in this study, including E2, bound with similar affinity to all five GST-ERdef fusion proteins, some notable differences were reported. The most striking differences in relative binding affinities were seen with GST-aERdef and GST-rtERdef. Comparison of the amino acid differences within their respective ligand binding domains suggests that aERdef:Phe175, rtERdef:Met93 and rtERdef:Ile272 may contribute to the observed differences in ligand preference and relative binding affinities. Preliminary mutagenesis studies indicate that these residues influence relative and absolute binding affinities of a subset of estrogenic compounds (Matthews, J.B. et al. manuscript in preparation). These residues may change the hydrophobicity and volume of the binding pocket as well as result in unique ligand-residue

interactions. Recent crystallography data has demonstrated that each of these parameters can influence ligand binding. For example, the volume of the probe-occupied ligand pocket of ERα-E2 crystal complex has been determined to be 490 Å while that of ERβ-genistein is 390 Å with the reduction being primarily due to the replacement of the Leu384 in hERα with a bulkier Met336 residue in hERβ [42]. This allows the residues that line the pocket to pack more tightly around genistein, stabilizing the ligand in the binding pocket in ERβ [42].

There were no obvious relationships between sequence identity and binding affinity. GST-rtERdef has the lowest sequence identity compared to GST-hERdef, but this was not predictive of the binding affinity of a compound for GST-rtERdef. For example, 4-*t*-octylphenol bound with greater affinity to GST-rtERdef ($IC_{50} = 0.11 \pm 0.02$ μM) compared to GST-hERαdef ($IC_{50} = 2.4 \pm 0.7$ μM) while the rank order binding affinities were reversed for coumestrol (IC_{50} values of 0.36 ± 0.03 μM vs. 1.4 ± 0.1 μM for GST-hERαdef and GST-rtERdef, respectively). However, some patterns in the relative binding affinity data were observed. Cluster analysis based on amino acid sequence identity suggested that hERαdef and mERαdef shared greater similarity than cERdef and aERdef, with rtERdef being the most divergent when compared to the other ER sequences (Fig. 4). In general, ERdef proteins with greater similarity exhibited similar relative binding affinities as illustrated in Fig. 3, with some notable exceptions. Although cERdef and aERdef shared the greatest similarity, DHT exhibited a 42-fold difference in relative binding affinity between the two species. The difference was only seven-, eight- and 12-fold for GST-hERdef, GST-mERdef and GST-rtERdef, respectively. GST-rtERdef, which has the most divergent amino acid sequence according to the cluster analysis, exhibited the greatest promiscuity in its ligand preference, further supporting the hypothesis that structural differences within the ligand binding domains among ERs of different species influences ligand preference and relative binding affinity.

The results demonstrate that ERs from human, mouse, chicken, green anole and rainbow trout exhibit differential ligand preferences and relative binding affinities for a number of natural and synthetic compounds. This data can be used to further develop ER quantitative structure activity relationships (QSARs) [43] and to evaluate the feasibility of species-specific ER QSARs [44]. Although the majority of substances examined in this study exhibited comparable relative binding affinities across ERs, a significant number of differences were observed. The relative binding affinities of the GST-rtERdef, which has the greatest amino acid variation in its E domain relative to the other species examined, exhibited the most striking differences. The rtER also had the greatest ligand promiscuity, binding a significantly greater number of structurally diverse estrogenic compounds. However, pharmacokinetic and pharmacodynamic differences between species make it unlikely that differences in binding affinities for individual estrogenic compounds would be observed in vivo. Nevertheless, in the absence of structural data for natural and synthetic ligands, this cross species comparison provides valuable insights into potentially important residues that may play critical roles in the interaction between structurally diverse ligands and the ER binding pocket.

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