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分担研究報告

エストロゲン関連受容体 γ の変異受容体による化学物質結合部位解析

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研究要旨

エストロゲン関連受容体 γ 型 (estrogen-related receptor γ : ERR γ) は、リガンドなしで活性化コンホメーションを取る「自発活性化型」オーファン核内受容体として知られているが、その内在性リガンドや生理機能は不明である。内分泌攪乱化学物質・ビスフェノールA (BPA) は、低用量で脳神経系や生殖系で悪影響を及ぼす化学物質として、そのリスクが強く懸念されている。我々は最近、こうした BPA は ERR γ と非常に強く結合するに関わらず、「初めからある自発活性に何の影響も与えない」ことを発見した。さらに、BPA/ERR γ -LBD (リガンド結合ドメイン) 複合体の X 線結晶構造解析では、この結合には ERR γ ヘリックス 12 (H12) の Phe450 および Leu454 の結合ポケット充填が必須であると示唆された。一方、ERR γ を不活性化にする 4-ヒドロキシタモキシフェン (4-OHT) は、H12 との相互作用はなく、むしろ、他の受容体構造が必要ではないと推定された。そこで、本研究では、H12 の受容体自発活性化およびリガンド結合の選択性における構造要因の解明を目指して、H12 を段階的に削除してリガンド結合性、自発活性への影響を解析した。

A. 研究目的

内分泌攪乱化学物質、いわゆる「環境ホルモン」の問題は、主として女性ホルモン受容体 (estrogen receptor; ER) における内分泌攪乱作用とされてきた。しかし、ヒト・ゲノム解析に伴い、ヒト核内受容体には ER を含めて合計 48 種類あることが判明すると、これらすべてに環境ホルモン問題が存在し、しかも、きわめて高リスクである可能性が強く懸念されるようになった。こうしたなか、我々は 2006 年に、環境ホルモンであるビスフェノールA (BPA) はヒト・エストロゲン関連受容体 γ 型 (estrogen-related receptor γ ; ERR γ) と非常に強く結合し、ERR γ の高い自発活性を保持することを明らかにした。

BPA は、女性ホルモン様作用を示すとされてきた環境ホルモンであるが、女性ホルモン

受容体である ER への直接の作用は人間の体内で作られる女性ホルモン 17 β -estradiol の 1/1,000~1/10,000 と非常に弱いものである。しかしながら、近年、BPA についてきわめて低濃度において脳の性分化、成熟、さらに生体の組織や行動に重大な影響を及ぼすという「低用量効果」の報告が相次ぎ、この作用機序、分子メカニズムの解明が急務とされている。

エストロゲン関連受容体 (estrogen-related receptor; ERR) は、ER と非常によく似たアミノ酸配列をもつことから発現され、ステロイドホルモン受容体グループに分類される。ヒトでは α 、 β 、 γ 型の3つのサブタイプが存在し、これらのいずれについても内在性リガンドや生理機能に関してはほとんど何も分かっていない。さらに、ERRはリガンドなしで活性化コンホメーションとなる、いわゆる

「自発活性化型核内受容体」(self-activated nuclear receptors, SA-NR) である。ERR γ は、ヒト成人では脳や脾臓、肺、骨髄、副腎、前立腺、胃など高濃度に発現しており、また、胎盤には最も高濃度の発現している。特に注目されるのは、ヒト胎児で脳に非常に高濃度に発現していることである。さらに、ERR γ は、脳の性分化や成熟、脳神経系の発達に関与していると推測されている。こうしたなか、BPAがERR γ に非常に強く結合するということから、BPAが「低用量で攪乱作用を示す機構」と「脳神経系の発達を攪乱する危険性」の分子機構解明のきわめて有力な端緒になる。

そこで、なぜ ERR γ が BPA と結合できるのか、構造化学的な解析が急務となった。我々はごく最近、BPA/ERR γ -LBD (LBD; リガンド結合ドメイン) 複合体の X 線結晶構造解析に成功した。この結合には、ERR γ ヘリックス 12 (H12) の Phe450 および Leu454 の結合ポケット充填が必須であると示唆された。一方、ERR γ を不活性化にする 4-ヒドロキシタモキシフェン (4-OHT) は、H12 との相互作用はなく、むしろ、他の受容体構造が必要ではないと推定された。

そこで、本研究では、H12 の受容体自発活性化およびリガンド結合の選択性における構造要因の解明を目指して、H12 を段階的に削除してリガンド結合性への影響を解析した。

B. 研究方法

(1) ERR γ 変異体の設計

ERR γ の自発活性およびリガンド結合選択性に関して H12 どのぐらい残基が必要なのか? を調べるため、ERR γ の C 端側から 3 アミノ酸残基ずつ削除して、合計 6 種類の H12 削除した変異体を設計した。

430 440 450 458

WT : ----KAVQHFFYNIKLEGGKVPMHKLFLEMLEAKV*

δ 456-458: ----KAVQHFFYNIKLEGGKVPMHKLFLEMLE*

δ 453-458: ----KAVQHFFYNIKLEGGKVPMHKLFLE*

δ 450-458: ----KAVQHFFYNIKLEGGKVPMHKL*

δ 447-458: ----KAVQHFFYNIKLEGGKVP*

δ 444-458: ----KAVQHFFYNIKLEGG*

δ 441-458: ----KAVQHFFYNIK*

("_": H11 相当する部位; "___": H12 相当する部位)

(C 端側 456~458 の 3 アミノ酸残基削除を δ 456-458 で表記、以下表記方法同じ)

(2) 変異受容体の作製

ヒト腎臓の cDNA を鋳型として、PCR の増幅より、ERR γ の LBD (222-458 aa) および全長領域のクローニングを行った。さらに、得られた WT-ERR γ -LBD および WT-ERR γ -full length を鋳型として 6 種類の LBD および full-length 変異受容体を作製した。変異された LBD および全長はそれぞれ発現ベクター-pGEX-6p-1 および pcDNA3.1(+) に組み換えた後、塩基配列を確認した。

(3) 変異受容体タンパク質の調製

得られた ERR γ -LBD/ pGEX-6p-1 変異受容体は、大腸菌 (BL21) を用いて GST との融合タンパク質として大量発現を行った。1 mM イソプロピル 1-チオ- β -D-ガラクトシド (IPTG) で 37°C、3 時間を誘導させ、グルタチオンセファロース 4B ビーズを用いてアフィニティー精製した。20 mM のグルタチオンでビーズより溶出後、Sephadex G-10 で脱塩して、Bradford 法でタンパク質濃度を測定した。

(4) 飽和結合試験

飽和結合試験は、受容体の失活を防ぐため、4°Cで行った。野生型および各種変異受容体 GST-ERR γ -LBD と各濃度の放射標識された BPA または 4-OHT を binding buffer 中で混合し、2 時間インキュベートした。非特異的な結合は過剰量の BPA または 4-OHT を放射標識された BPA または 4-OHT と併に加えることにより調べた。遊離のリガンドは 1% デキストラン被膜活性炭溶液を反応溶液に加えることにより取り除いた。受容体の結合解離定数 (K_d) および最大結合濃度 (B_{max}) はスキヤッチャードプロット解析により算出した。

(5) レポーター遺伝子アッセイ試験

ヒト子宮ガン細胞である HeLa 細胞に、野生型および各種変異受容体 ERR γ 発現プラスミドと 3xERRE ルシフェラーゼレポータープラスミドを遺伝子導入し、一過性の強制発現を行った。24 時間インキュベート後、化学物質を暴露した。さらに 24 時間後、ルシフェラーゼ活性をは発光基質を用いて測定した。WT-ERR γ が自発的に持っているルシフェラーゼ活性を 100%として、各誘導受容体の転写活性を評価した。

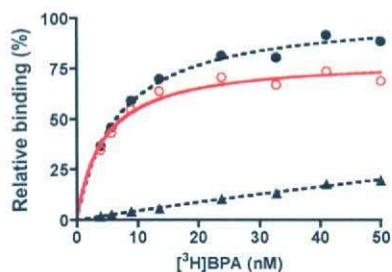
C. 研究結果

(1) BPA に対する結合試験

ERR γ -LBD-WT 受容体の飽和結合試験

ERR γ -LBD-WT はグルタチオン S-トランスフェラーゼ (GST) との融合タンパク質として大腸菌で発現した。放射標識された BPA ($[^3\text{H}]$ BPA) を用いて、飽和結合試験を行った。スキッチャードプロット解析を行った。

(A)



(B)

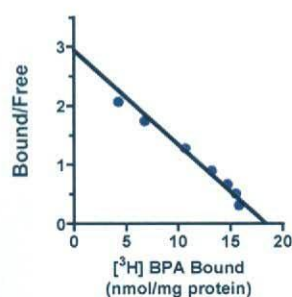


図 1. ERR γ -WT 飽和結合試験

(A) ERR γ に対する $[^3\text{H}]$ BPA の全結合(●)、特異的結合(○)、非特異的結合(▲)を示す。

(B)スキッチャードプロット解析

結果、ERR γ -LBD-WT の K_d 値は 5.7 nM であり、 B_{max} は 18.4 nmol/mg であった。これは理論値 B_{max} (18.9 nmol/mg) とほぼ一致した (図 1)。この結果より、BPA の ERR γ に対する結合は、生体内で機能するホルモンとその受容体の結合親和性に匹敵するほど強いものであることが明らかとなった。

ERR γ -LBD 変異受容体の飽和結合試験

ERR γ -LBD の C 端側から 3 残基ずつアミノ酸の削除による、BPA の結合性への影響を調べるため、6 種の変異受容体を作製した。これら 6 種変異受容体は、WT と同様にグルタチオン S-トランスフェラーゼ (GST) との融合タンパク質として大腸菌で発現・精製した。放射標識された BPA ($[^3\text{H}]$ BPA) を用いて、ERR γ -LBD-WT と同じ反応温度、時間、緩衝溶液の組成、B/F 分離の条件で飽和結合試験を

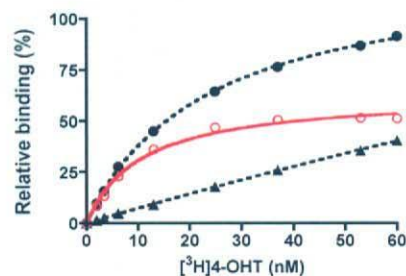
行った。その結果、全ての変異受容体は BPA と全く結合しないことが分かった。

(2) 4-OHT に対する結合試験

ERR γ -LBD-WT 受容体の飽和結合試験

前述で得られた ERR γ -LBD-WT タンパク質は、 $[^3\text{H}]$ 4-OHT を用いて、飽和結合試験を行った。スキッチャードプロット解析を行った結果、 K_d 値は 10.1 nM であった。しかし、 B_{max} は 2.20 nmol/mg であり、理論値 (18.9 nmol/mg) の約 1/10 しかなかった (図 2)。4-OHT 分子は BPA より約 2.5 倍大きく、結合によって、H12 は活性化コンホメーションよりは慣れることから、4-OHT と受容体の結合は H12 必要ではなく、逆に邪魔になると考えられた。

(A)



(B)

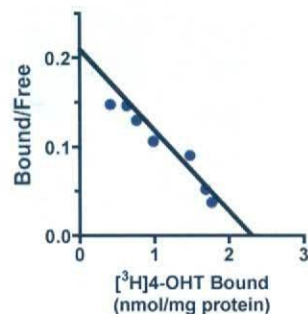


図 2. ERR γ -WT 飽和結合試験

(A) ERR γ に対する $[^3\text{H}]$ 4-OHT の全結合(●)、特異的結合(○)、非特異的結合(▲)を示す。

(B)スキッチャードプロット解析

ERR γ -LBD 変異受容体の飽和結合試験

ERR γ -LBD 変異受容体タンパク質と $[^3\text{H}]$ 4-OHT の飽和結合試験を行った結果、全ての変異受容体はいずれとも 4-OHT と強く結合することが分かった。スキッチャードプロット解析により K_d 値および B_{max} 値を算出した。

その結果、H12 部分的欠損の変異受容体 $\delta 456-458$ 、 $\delta 453-458$ 、 $\delta 450-458$ および H12 と H11-H12 loop とともに欠損の変異受容体

δ441-458 は、 $[^3\text{H}]4\text{-OHT}$ との結合性は WT とほぼ同様な強さが見られた。H12 全欠損を加え H11-H12 loop 部分的削除した変異受容体 δ447-458、δ444-458 は、 $[^3\text{H}]4\text{-OHT}$ との結合性は WT の 3~4 倍弱くなった (図 3)。以上のことから、4-OHT と $\text{ERR}\gamma$ の結合に H12 は不要なことが示された。さらに、H11 と H12 は同時に loop 構造を安定され、H12 を欠失させて、loop 構造は不安定になり、受容体と 4-OHT の結合に不利な原因となることが考えられる。

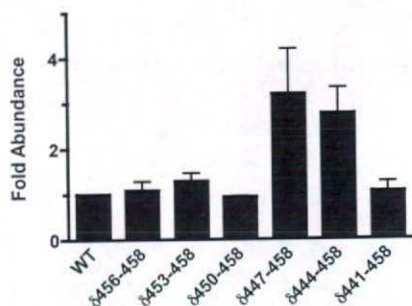


図 3. スキャッチャードプロット解析計算より、各受容体は $[^3\text{H}]4\text{-OHT}$ に対する K_d 値の比較。(WT の K_d 値は 1 とする)

D. 考察

BPA/ $\text{ERR}\gamma$ -LBD 複合体の X 線結晶構造解析より、BPA と $\text{ERR}\gamma$ の結合には、ヘリックス 12 (H12) の Phe450 および Leu454 の結合ポケット充填が必須であると示唆された。さらに、 $\text{ERR}\gamma$ の点変異解析より、Phe450 および Leu454 残基を Ala に変異させると BPA と全く結合せず、4-OHT と強く結合することが以前の研究から分かった (data not shown)。一方、 $\text{ERR}\gamma$ のインバサゴニストである 4-OHT との結合は、H12 との相互作用はなく、むしろ、他の受容体構造が必要ではないと推定された。今回は、 $\text{ERR}\gamma$ -LBD の C 端から 3 残基ずつを逐次に削除した部分構造欠損型の 6 種の変異受容体を作製し、GST 融合タンパク質として発現して、 $[^3\text{H}]$ BPA および $[^3\text{H}]4\text{-OHT}$ を用いた結合試験によりリガンド結合の選択性を詳細に解析した。その結果、変異受容体は BPA と全く結合しないことが分かった。一方、4-OHT はいずれとも非常に強く結合し、その結合に H12 は不要なことが示された。

以上の結果より、H12 はリガンド結合の選

択性に対して、重要な構造要因であることが証明された。

E. 健康危険情報

現在のところ特に該当する情報はない。

F. 研究発表

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特になし。

研究成果の刊行物・別刷

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
H. Okada., T. Tokunaga., X. Lui, S. Takayanagi, A. Matsushima, Y. Shimohigashi	Direct Evidence Revealing Structural Elements Essential for the High Binding Ability of Bisphenol A to Human Estrogen-related Receptor γ (ERR γ).	<i>Environmental Health Perspectives</i>	116	32-38	2008
A. Matsushima, Y. Shimohigashi	A strategy to explore the target receptor of endocrine disruptors: Estrogen-related receptor γ (ERR γ) as a genuine acceptor of Bisphenol A	<i>Alternatives to Animal Testing and Experimentation</i>	14 Special Issue	495-497	2008
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D. Asai, T. Tokunaga, K. Kondo, T. Kawaguchi, S. Takayanagi, T. Shinmyozu, M. Nakai, Y. Yakabe, Y. Shimohigashi	Direct Measurement of Fluorescence Intensity for Efficient Receptor-binding Assay: Conjugates of Ethinylcarboxyestradiol and 5 (and 6) -Carboxyfluorescein via α , ω -Diaminoalkanes as a Tracer for Estrogen Receptor.	<i>Journal of Biochemistry</i>	143(6)	781-792	2008

A. Matsushima, T. Teramoto, H. Okada, X. Lui, T. Tokunaga, Y. Kakuta, Y. Shimohigashi	ERR γ Tethers Strongly Bisphenol A and 4- α -Cumylphenol in an Induced-fit Manner.	<i>Biochemical and Biophysical Research Communications</i>	373	408-413	2008
Y. Takeda, X. Liu, M. Sumiyoshi, A. Matsushima, M. Shimohigashi, Y. Shimohigashi	Bisphenol A-Specific Nuclear Receptor ERR γ : Structure-Function Analysis of the Two Novel Isoforms Lacking Vital Peptide Fragment in the Ligand Binding Domain.	<i>Peptide Science 2008</i>	2008	517-518	2009
A. Matsushima, X. Liu, T. Tokunaga, T. Teramoto, Y. Kakuta, Y. Shimohigashi	Induced-fit Type Ligand Binding Guided by Free- rotatory Leu Residue Present in the 7th α -Helix Peptide in the Estrogen-related Receptor γ (ERR γ).	<i>Peptide Science 2008</i>	2008	521-522	2009
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Y. Takeda, X. Liu, M. Sumiyoshi, A. Matsushima, M. Shimohigashi, Y. Shimohigashi	Placenta expressing the greatest quantity of bisphenol A receptor ERR γ among the human reproductive tissues: Predominant expression of type-1 ERR γ isoform	<i>Journal of Biochemistry</i>		In Press	

Direct Evidence Revealing Structural Elements Essential for the High Binding Ability of Bisphenol A to Human Estrogen-Related Receptor- γ

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BACKGROUND: Various lines of evidence have shown that bisphenol A [BPA; HO-C₆H₄-C(CH₃)₂-C₆H₄-OH] acts as an endocrine disruptor when present in very low doses. We have recently demonstrated that BPA binds strongly to human estrogen-related receptor- γ (ERR- γ) in a binding assay using [³H]4-hydroxytamoxifen ([³H]4-OHT). We also demonstrated that BPA inhibits the deactivation activity of 4-OHT.

OBJECTIVES: In the present study, we intended to obtain direct evidence that BPA interacts with ERR- γ as a strong binder, and also to clarify the structural requirements of BPA for its binding to ERR- γ .

METHODS: We examined [³H]BPA in the saturation binding assay using the ligand binding domain of ERR- γ and analyzed the result using Scatchard plot analysis. A number of BPA derivatives were tested in the competitive binding assay using [³H]BPA as a tracer and in the luciferase reporter gene assay.

RESULTS: [³H]BPA showed a K_D of 5.50 nM at a B_{max} of 14.4 nmol/mg. When we examined BPA derivatives to evaluate the structural essentials required for the binding of BPA to ERR- γ , we found that only one of the two phenol-hydroxyl groups was essential for the full binding. The maximal activity was attained when one of the methyl groups was removed. All of the potent BPA derivatives retained a high constitutive basal activity of ERR- γ in the luciferase reporter gene assay and exhibited a distinct inhibitory activity against 4-OHT.

CONCLUSION: These results indicate that the phenol derivatives are potent candidates for the endocrine disruptor that binds to ERR- γ .

KEY WORDS: bisphenol A, constitutive activity, endocrine disruptor, estrogen receptor, estrogen-related receptor- γ , inverse agonist, nuclear receptor. *Environ Health Perspect* 116:32–38 (2008). doi:10.1289/ehp.10587 available via <http://dx.doi.org/> [Online 5 October 2007]

Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane] has a symmetrical chemical structure of HO-C₆H₄-C(CH₃)₂-C₆H₄-OH. BPA is used mainly in the production of polycarbonate plastics and epoxy resins. Its worldwide manufacture is approximately 3.2 million metric tons/year. BPA has been acknowledged to be an estrogenic chemical able to interact with human estrogen receptors (ER) (Dodds and Lawson 1938; Krishnan et al. 1993; Olea et al. 1996), and many lines of evidence have revealed that BPA, at even low doses, acts as an endocrine disruptor (Gupta 2000; Nagel et al. 1997; vom Saal et al. 1998; Welshons et al. 2003). However, its binding to and hormonal interaction with ER are extremely weak, 2–3 orders of magnitude lower than those of natural hormones, and thus the intrinsic significance of these low-dose effects is rather intangible and obscure (Safe et al. 2002). These facts led us to hypothesize that BPA may interact with nuclear receptors (NRs) other than ER.

We have recently demonstrated that BPA binds strongly to estrogen-related receptor- γ (ERR- γ) with high constitutive activity (Takayanagi et al. 2006). ERR- γ is a member of the human NR family and the estrogen-related receptor (ERR) subfamily of orphan NRs, which are closely related to the ERs ER- α and ER- β (Giguère 2002; Horard and

Vanacker 2003). The ERR family includes three members—ERR- α , ERR- β , and ERR- γ —with ERR- γ being the most recently identified (Eudy et al. 1998; Hong et al. 1999). The amino acid sequences are quite highly conserved among ERRs and ERs, but 17 β -estradiol (E₂), a natural ligand of ERs, does not bind to any of the ERR family members. Our discovery that BPA binds strongly to ERR- γ , but not to ERs, indicates that the effects of the so-called endocrine disruptors should be examined for all NRs without delay.

ERR- γ is expressed in a tissue-restricted manner—for example, very strongly in the mammalian brain during development, and then in the brain, lung, and many other tissues during adulthood (Eudy et al. 1998; Heard et al. 2000; Lorke et al. 2000). Our preliminary results have shown that the highest expression is brought about in the placenta (Takeda Y, Sumiyoshi M, Liu X, Matsushima A, Shimohigashi M, Shimohigashi Y, unpublished data). Strong binding of BPA to ERR- γ would affect not only the physiologic functions but also the metabolism of this NR as a transcription-activating factor. Although the intrinsic physiologic functions of ERR- γ have not yet been clarified, it is crucial that a structure–function study be performed to

clarify the structural requirements for the binding of BPA to ERR- γ .

In a previous study (Takayanagi et al. 2006), we used tritium (³H)-labeled 4-hydroxytamoxifen (4-OHT) as a tracer in a receptor binding assay for ERR- γ . 4-OHT binds strongly to ERR- γ and deactivates it as an inverse agonist, decreasing the very high level of spontaneous constitutive activity (Coward et al. 2001). As a substitute for [³H]4-OHT, BPA was found to be as potent as 4-OHT in this binding assay. Furthermore, BPA was found to retain or rescue ERR- γ 's high basal constitutive activity in the reporter gene assay for ERR- γ using HeLa cells. These results indicated that BPA and 4-OHT bind to ERR- γ with equal strength, but have structural differences that affect their occupation of ERR- γ 's ligand binding pocket. In the complex formed between 4-OHT and the ERR- γ –ligand binding domain (LBD), 4-OHT remained at the ligand binding pocket of ERR- γ –LBD, but the α -helix 12 of the receptor was repositioned from the activation conformation (Greschik et al. 2004; Wang et al. 2006). In contrast, BPA was suggested to bind to the pocket without changing the positioning of helix 12, and thus preserved the high receptor constitutive activity of ERR- γ .

It is evident that the binding ability of BPA to ERR- γ should be examined by means of tritium-labeled BPA. Fortunately, [³H]BPA is now commercially available; thus, in the present study we performed the first saturation binding assay for direct exploration of the binding characteristics of BPA. We then established a competitive receptor binding assay in which chemicals were assessed for their ability to displace [³H]BPA from the receptor binding pocket. In particular, industrial chemical products of BPA analogs were inspected structurally in order to better understand the

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structural elements of BPA that are required for binding to the ERR. Here we describe the structural elements of BPA that are required for the binding to ERR- γ -LBD and for maintaining the receptor in an active conformation.

Materials and Methods

Chemicals. We purchased 2,2-bis(4-hydroxyphenyl)propane and 4,4-isopropylidenediphenol, both denoted as BPA, from Tokyo Kasei Kogyo Co. (Tokyo, Japan), Nakarai Tesque (Kyoto, Japan), Aldrich (Madison, WI, USA), Junsei Chemical (Tokyo, Japan), Acros (Geel, Belgium), Lancaster Synthesis (Windham, NH, USA), Merck (Darmstadt, Germany), and Fluka (Buchs, Switzerland). The purity designated on the labels varied from 95 to 99%. We also obtained the following analogs of BPA: bisphenol AF [2,2-bis(4-hydroxyphenyl)hexafluoropropane; Tokyo Kasei], bisphenol AP [4,4'-(1-phenylethylidene)bisphenol; Tokyo Kasei], bisphenol B [2,2-bis(4-hydroxyphenyl)butane; Tokyo Kasei], bisphenol E [2,2-bis(4-hydroxyphenyl)ethane; Aldrich], and bisphenol F [bis(4-hydroxyphenyl)methane; Tokyo Kasei].

4- α -Cumylphenol [2-(4-hydroxyphenyl)-2-phenylpropane], 4-*tert*-amylphenol, 4-*tert*-butylphenol, 4-isopropylphenol, and 4-ethylphenol were obtained from Tokyo Kasei. 2,2-Diphenyl propane, and 4-*tert*-octylphenol were obtained from Aldrich, and *p*-cresol and phenol from Kishida Chemical (Osaka, Japan).

Preparation of receptor protein GST-fused ERR- γ -LBD. ERR- γ -LBD was amplified from a human kidney cDNA library (Clontech Laboratories, Mountain View, CA, USA) by polymerase chain reaction (PCR) using gene-specific primers and cloned into pGEX6P-1 (Amersham Biosciences, Piscataway, NJ, USA). Glutathione *S*-transferase (GST)-fused receptor protein expressed in *Escherichia coli* BL21 α was purified on an affinity column of glutathione-sepharose 4B (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA) to obtain GST-ERR- γ -LBD. The glutathione used for elution of GST-ERR- γ -LBD from the column was removed by gel filtration on a column of Sephadex G-10 (15 \times 100 mm; GE Healthcare Bio-Sciences Co.) equilibrated with 50 mM Tris-HCl (pH 8.0), and the protein content (506.24 μ g/mL) was estimated by the Bradford method using a Protein Assay CBB Solution (Nakarai Tesque). Preparation of GST-fused ER- α -LBD was carried out as described previously (Takayanagi et al. 2006).

Radioligand binding assays for saturation binding. The saturation binding assay for GST-ERR- γ -LBD was conducted at 4°C using [³H]BPA (5 Ci/mmol; Moravak Biochemicals, Brea, CA, USA) with or without BPA (10 μ M in the final solution). Purified protein (0.32 μ g/mL) was incubated

with increasing concentrations of [³H]BPA (2.1–24.3 nM) in a final volume of 100 μ L of binding buffer [10 mM HEPES (pH 7.5), 50 mM sodium chloride, 2 mM magnesium chloride, 1 mM EDTA, 2 mM CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}, and 2 mg/mL γ -globulin]. Nonspecific binding was determined in a parallel set of incubations that included 10 μ M nonradiolabeled BPA. After incubation for 2 hr at 4°C, all the fractions were filtered by the direct vacuum filtration method (MultiScreen_{HTS} HV, 0.45 μ m pore size; Millipore, Billerica, MA, USA) for the B/F separation (the separation of receptor-bound ligand from free ligand) (Nakai et al. 1999). Filtration was carried out on a multiscreen separation system (Millipore). Before filtration, 100 μ L of 1% dextran-coated charcoal (DCC) (Sigma) in phosphate buffer (pH 7.4) was added to the assay vessels, and the mixture was incubated for 10 min on ice. The radioactivity of the filtered solution was counted on a liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA, USA). The saturation assay was performed in triplicate. The specific binding of [³H]BPA was calculated by subtracting the nonspecific binding from the total binding.

Radioligand binding assays for competitive binding. BPA and the BPA-related chemicals were dissolved in a binding buffer containing 0.3–1.0% *N,N*-dimethylsulfoxide (DMSO). These compounds were examined for their ability to inhibit the binding of [³H]BPA (3 nM in the final solution) to GST-ERR- γ -LBD (0.32 μ g/mL in the final solution). The reaction mixtures were incubated for 2 hr at 4°C and free radioligand was removed with 1% DCC by filtration as described above. Radioactivity was determined on a liquid scintillation counter (TopCount NXT; PerkinElmer Life Sciences Tokyo, Japan). The IC₅₀ values (the concentrations for the half-maximal inhibition) were calculated from the dose–response curves obtained using the nonlinear analysis program ALLFIT (De Lean et al. 1978). Each assay was performed in duplicate and repeated at least three times. The competitive binding assay for GST-ER- α -LBD was carried out as described above using [³H]E₂ (5.74 TBq/mmol; Amersham Biosciences, Buckinghamshire, UK).

Cell culture and transient transfection assays. HeLa cells were maintained in Eagle's MEM (EMEM; Nissui, Tokyo, Japan) in the presence of 10% (vol/vol) fetal bovine serum at 37°C. For luciferase assays, HeLa cells were seeded at 5 \times 10⁵ cells/6-cm dish for 24 hr and then transfected with 4 μ g of reporter gene (pGL3/3 \times ERRE) and 3 μ g of ERR- γ expression plasmids (pcDNA3/ERR- γ) by Lipofectamine Plus reagent (Invitrogen Japan,

Tokyo, Japan) according to the manufacturer's protocol. Approximately 24 hr after transfection, cells were harvested and plated into 96-well plates at 5 \times 10⁴ cells/well. The cells were then treated with varying doses of chemicals diluted with 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS, vol/vol). To measure the antagonistic activity, a fixed concentration of compounds (10⁻⁵ M to 10⁻¹⁰ M in the final solution) was added along with 4-OHT. After 24 hr, luciferase activity was measured with the appropriate reagent using a Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Light emission was measured using a Wallac 1420 ARVO α s multi-label counter (PerkinElmer). Cells treated with 1% BSA/PBS were used as a vehicle control. Each assay was performed in triplicate and repeated at least three times.

Results and Discussion

Highly specific binding of BPA to ERR- γ . To demonstrate the direct binding of BPA to ERR- γ , we first attempted to establish a saturation receptor binding assay using radiolabeled BPA. We analyzed the saturation binding of [³H]BPA against the recombinant ERR- γ -LBD protein, to which GST was fused at the N-terminus. In the actual receptor binding assay, we used [³H]BPA (2.0–24 nM) against purified protein at a concentration of 0.32 μ g/mL, which corresponds to a concentration of 6.3 nM. The removal of receptor-free [³H]BPA was carried out with 1% DCC. In this procedure, DCC mixtures were transferred to a 96-well HV-plate with a filter (0.45- μ m pore size) for direct vacuum.

As shown in Figure 1A, the binding of BPA to ERR- γ was specific and saturated. Specific binding of [³H]BPA to ERR- γ was estimated to be approximately 80%, which we judged to be a very high value. In other words, the level of nonspecific binding of [³H]BPA was very low (Figure 1A). The high level of specific binding of [³H]BPA clearly demonstrated that BPA has no structural elements for nonspecific binding to the receptor protein and exclusively occupies the binding pocket of ERR- γ -LBD. GST did not bind [³H]BPA at all. It should be noted that the specific binding of [³H]4-OHT was only about 50% (Takayanagi et al. 2006).

The Scatchard plot analysis showed a distinct single binding mode (Figure 1B). From the slope, the binding affinity constant (K_D) was calculated to be 5.50 nM. The receptor density (B_{max}) was estimated to be 14.4 nmol/mg protein, which is roughly compatible with the calculated value of 18.9 nmol/mg protein. The B_{max} value of [³H]4-OHT is much smaller than that of [³H]BPA. These results further demonstrate that ERR- γ binds [³H]BPA very specifically and exclusively.

Binding ability of BPA to ERR- γ . We performed the competitive receptor binding assay using [3 H]BPA (3 nM in the final solution) for GST-ERR- γ -LBD (0.32 μ g/mL in the final solution). To confirm that BPA is a truly specific ligand for ERR- γ , we tested all nonradiolabeled BPA compounds available in Japan, which we obtained from seven different reagent companies. Because the compounds all had different levels of purity (95–99%), we adjusted their initial concentration, 1.0×10^{-2} M, based on the purity indicated on the label.

We found that BPA displaces [3 H]BPA in a dose-dependent manner. Its binding curve

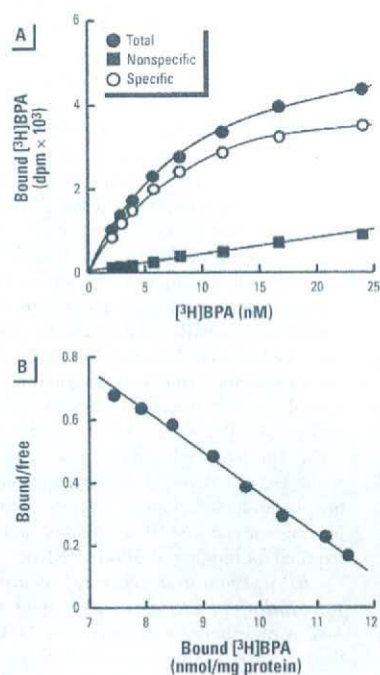


Figure 1. The saturation binding analysis of BPA for ERR- γ . (A) Saturation binding curve of [3 H]BPA for the recombinant human ERR- γ -LBD showing total, nonspecific, and specific binding. Determination of nonspecific binding was carried out by excess unlabeled BPA (10 μ M). (B) Binding data analyzed by Scatchard plot analysis to estimate the dissociation constant (K_D) and the receptor density (B_{max}). The plot was linear, the K_D value was estimated to be 5.50 ± 0.87 nM, and B_{max} was 14.4 nmol/mg protein. The saturation binding analysis was performed in duplicate and repeated four times.

Table 1. Receptor binding affinity (mean \pm SE) of BPA and its analogs, and 4-OHT for ERR- γ .

Chemical	Binding affinity (IC_{50} , nM)
BPA	9.78 ± 0.87
Bisphenol AF	358 ± 30.5
Bisphenol AP	123 ± 15.1
Bisphenol B	26.3 ± 2.65
Bisphenol E	8.14 ± 0.83
Bisphenol F	131 ± 17.9
4-OHT	10.9 ± 0.91

was sigmoidal in a single binding mode (slope = -1), which afforded an average IC_{50} value of 9.78 nM. We found all BPA compounds purchased to be equally potent. These results clearly demonstrate that BPA binds very strongly to the NR ERR- γ .

4-OHT as a potent displacer of BPA in ERR- γ . 4-OHT has been reported to potently displace [3 H]4-OHT in the binding to ERR- γ (Greschik et al. 2004; Takayanagi et al. 2006). In the present study, 4-OHT very potently displaced [3 H]BPA ($IC_{50} = 10.9$ nM) (Table 1). BPA and 4-OHT yielded sigmoidal binding curves indistinguishable from each other (data not shown), indicating that the two are almost equipotent. These results obtained using the [3 H]BPA tracer were almost identical to those obtained by [3 H]4-OHT (Takayanagi et al. 2006).

BPA and 4-OHT share only a phenol group, and thus the phenol groups of these

compounds are highly likely to occupy the same binding site in the ERR- γ receptor. Because the phenol group of 4-OHT is anchored by hydrogen bonds to Glu275 and Arg316 of ERR- γ (Greschik et al. 2004), the phenol group of BPA may also bind to these ERR- γ residues. Indeed, this has been proven by our recent X-ray crystal structure analysis of the complex between BPA and human ERR- γ -LBD (Matsushima et al. 2007). Hereafter, we designate the benzene ring of this phenol group of BPA as the A-ring and the additional benzene ring as the B-ring.

BPA-methyl as a structural requirement for binding to ERR- γ . We evaluated the role of the two methyl (CH_3) groups on the sp^3 -C atom of BPA in binding to ERR- γ by a series of analogs of BPA, HO-C $_6$ H $_4$ -C(CH_3) $_2$ -C $_6$ H $_4$ -OH. First, we examined the effect of incorporation of the methyl group on the binding affinity of BPA. When CH_3 was

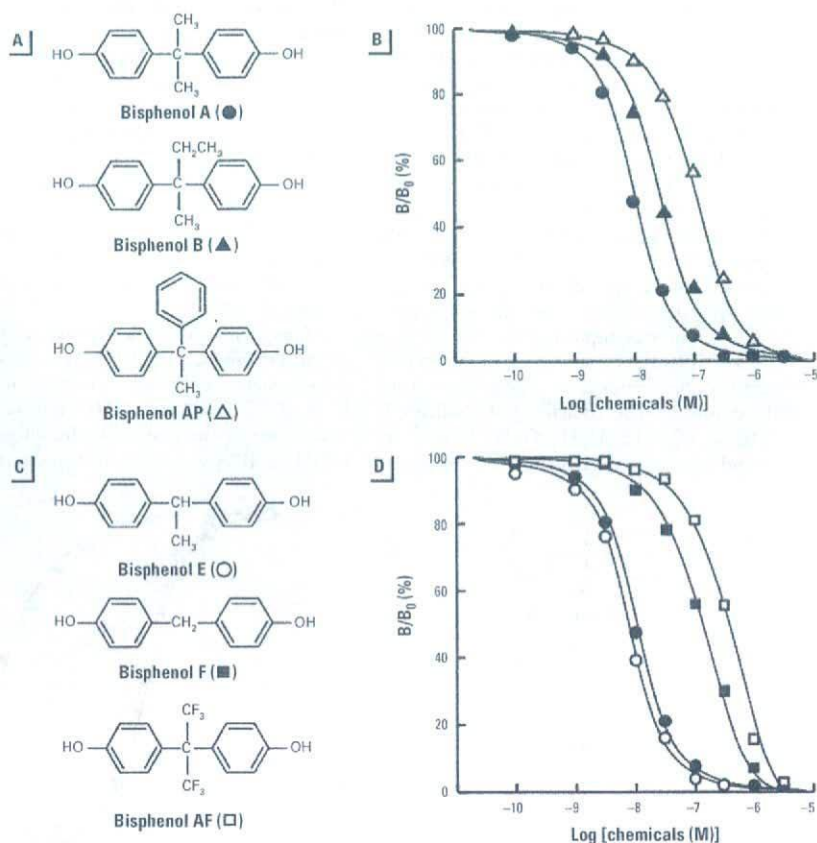


Figure 2. Chemical structure of BPA and its derivatives and their dose-response curves in the radioligand receptor binding assay for ERR- γ . (A) Chemical structures of BPA (two methyl groups) and its derivatives: bisphenol B (a methyl group and an ethyl group) and bisphenol AP (a methyl group and a phenyl group). (B) Binding activities of BPA, bisphenol B, and bisphenol AP examined by the competitive binding assay using [3 H]BPA and GST-ERR- γ -LBD. (C) Chemical structures of bisphenol E (one methyl group) and its derivatives, bisphenol F and bisphenol AF (two trifluoromethyl groups (CF_3)). (D) Binding activities of BPA, bisphenol E, bisphenol F, and bisphenol AF examined by the competitive binding assay. (B) and (D) each show representative curves with the IC_{50} values closest to the mean IC_{50} from at least five independent assays for each compound. B/B_0 is the relative inhibitory activity estimated from the calculation of the percentage of displacement by the chemical tested (B) against the specific binding ($B_0 = 100\%$) of [3 H]BPA.

incorporated into the parent methyl group to produce HO-C₆H₄-C(CH₃)(CH₂CH₃)-C₆H₄-OH (Figure 2A), we found the resulting bisphenol B to be approximately half as potent (IC₅₀ = 26.3 nM) as BPA (Table 1). This result clearly indicates that a bulky group on the central sp³-C atom is obviously disadvantageous in terms of the binding of BPA to ERR- γ 's binding pocket.

On the other hand, an enhancement of activity was observed when one of the methyl groups was eliminated from BPA. The resulting bisphenol E [HO-C₆H₄-CH(CH₃)-C₆H₄-OH] (Figure 2C) exhibited slightly better binding activity (IC₅₀ = 8.14 nM) than BPA (Table 1). Bisphenol E is indeed the most potent chemical to date for the NR ERR- γ (Figure 2D). The maximal activity was attained when one of the methyl groups was removed from BPA. Apparently, the concomitance of two methyl groups on the central sp³-C atom of BPA is disadvantageous and unfavorable.

The fact that a single methyl group had the best fit for ERR- γ was further demonstrated by the diminished activity of bisphenol AP, which has a phenyl group in place of the hydrogen atom that is found in bisphenol E (Figure 2A). Bisphenol AP exhibited approximately 15-fold weaker binding affinity for ERR- γ than bisphenol E, with IC₅₀ = 123 nM (Figure 2B, Table 1). Steric hindrance by the benzene ring, as well as its electron-rich characteristics, might be responsible for this drop in the receptor binding affinity of bisphenol AP.

The importance of the remaining methyl group in bisphenol E became evident from the drastically reduced activity of bisphenol F [HO-C₆H₄-CH₂-C₆H₄-OH]. This compound was approximately 16-fold less potent

than bisphenol E, exhibiting an IC₅₀ value of 131 nM (Table 1). All of these results clearly indicate that one of the two methyl groups is involved in the intermolecular interaction with the receptor residue(s). The interaction involving the CH₃ group is a kind of hydrophobic interaction, such as CH₃-alkyl and CH/ π interactions.

The fundamental nature of this interaction involving the CH₃ group became rather apparent from the binding result of bisphenol AF [HO-C₆H₄-C(CF₃)₂-C₆H₄-OH]. The CH₃→CF₃ substitution in BPA creates this compound (Figure 2C), which has two electron-rich trifluoromethyl CF₃ groups instead of the rather electron-poor methyl CH₃ group. The molecular size of CF₃ is almost equal to that of CH₃. A drastically reduced activity of bisphenol AF, about 35-fold less potent (358 nM) than BPA (Table 1), thus demonstrates that the BPA's CH₃ group is in an electrostatic interaction with the electron-rich residue(s) of the receptor. Replacement of CH₃ with CF₃ is definitely disadvantageous, because CF₃ is very electron-rich and thus brings about a strong repulsion with such electron-rich residues of the receptor. One of the electron-rich candidates of the receptor is the aromatic ring of Phe, Tyr, His, and Trp. Based on the reported X-ray crystal structure of ERR- γ , feasible candidates are Phe-435 and Phe-450 (Greschik et al. 2002, 2004; Matsushima et al. 2007; Wang et al. 2006).

A single phenol-hydroxyl group is enough for BPA to bind to ERR- γ . BPA has a very simple symmetrical chemical structure of HO-C₆H₄-C(CH₃)₂-C₆H₄-OH (Figure 2A). When one of the phenol-hydroxyl groups (-OH) of BPA was eliminated, the resulting

4- α -cumylphenol (HO-C₆H₄-C(CH₃)₂-C₆H₅; Figure 3A) still bound very strongly to ERR- γ . 4- α -Cumylphenol was as potent as BPA (Figure 3B), having an IC₅₀ value of 10.6 nM (Table 2). Contrary to the expectation that both of the phenol-hydroxyl groups of BPA would participate in the hydrogen bonds, this result indicates that the second hydroxyl group does not necessarily participate in the hydrogen bonding. Given that this hydroxyl group forms a hydrogen bond with the ERR- γ receptor residue, the bond would be considered extremely weak, as suggested by the X-ray crystal analysis of 4- α -cumylphenol-ERR- γ complex (Matsushima A, Teramoto T, Okada H, Liu X, Tokunaga T, Kakura Y, Shimohigashi Y, unpublished data).

When both of the phenol-hydroxyl groups were eliminated from BPA, the resulting 2,2-diphenylpropane [C₆H₅-C(CH₃)₂-C₆H₅] was almost completely inactive (Figure 3B, Table 2). This compound elicits only about 30% inhibition of the binding of [³H]BPA at the 1- μ M concentration, whereas BPA almost completely inhibits the binding of [³H]BPA at this concentration (Figure 3B). It is clear that one of the phenol-hydroxyl groups of BPA is indispensable for the interaction with a binding pocket of ERR- γ . These results, together with the fact that 4- α -cumylphenol and BPA are equipotent, emphasizes the significance of one of the two phenol groups in the interaction of BPA with ERR- γ . As described above, this hydroxyl group should be attached to the benzene A-ring. It became apparent that the phenol-hydroxyl group attached to another phenol-benzene ring (B-ring) is not necessarily required for binding of BPA to ERR- γ .

BPA-phenol as a structural requirement for binding to ERR- γ . As described above, 4- α -cumylphenol is as active as BPA. The importance of the benzene B-ring can be examined by replacing the B-ring with the alkyl groups. When the benzene B-ring of 4- α -cumylphenol was substituted with either methyl or ethyl, the resulting 4-*tert*-butylphenol [HO-C₆H₄-C(CH₃)₂-CH₃] and

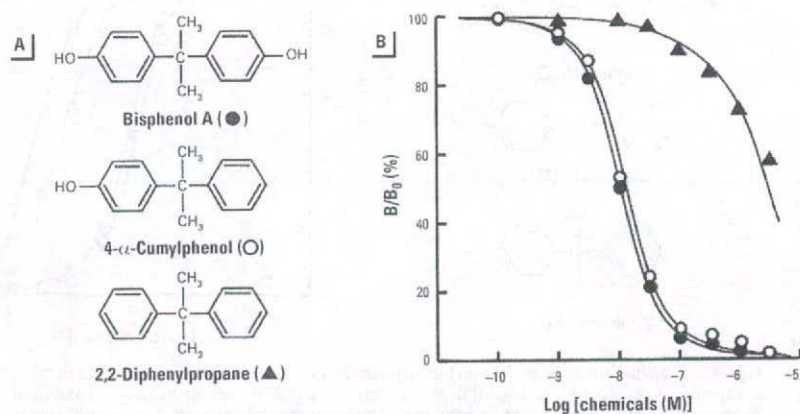


Figure 3. Chemical structure of BPA and its derivatives lacking the hydroxyl group(s) and their dose-response curves in the radioligand receptor binding assay for ERR- γ . (A) Chemical structure of BPA and its derivatives lacking the hydroxyl group(s): 4- α -cumylphenol (without one hydroxyl group from BPA), and 2,2-diphenylpropane (without either hydroxyl groups from BPA). (B) Binding activities of BPA, 4- α -cumylphenol, and 2,2-diphenylpropane examined by the competitive binding assay using [³H]BPA and GST-ERR- γ -LBD; representative curves indicate the IC₅₀ value closest to the mean IC₅₀ from at least five independent assays for each compound.

Table 2. The receptor binding affinity (mean \pm SE) of BPA and its derivatives lacking of the phenol group for human ERR- γ .

Chemical	Binding affinity (IC ₅₀ , nM)
BPA	9.78 \pm 0.87
4- α -Cumylphenol	10.6 \pm 0.87
2,2-Diphenylpropane	ND
4- <i>tert</i> -Butylphenol	26.1 \pm 2.45
4- <i>tert</i> -Amylphenol	33.2 \pm 2.85
4-Isopropylphenol	71.1 \pm 7.73
4- <i>tert</i> -Octylphenol	238 \pm 28.1
4-Ethylphenol	289 \pm 45.9
<i>p</i> -Cresol	1,290 \pm 72.5
Phenol	ND

ND, not determined (IC₅₀ value could not be calculated because of extremely weak binding activity, even at a 10 μ M concentration).

4-*tert*-amylphenol [$\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}_2\text{CH}_3$] (Figure 4A) were considerably potent (Figure 4B), with values of 26.1 nM and 33.2 nM, respectively (Table 2). This reveals that alkyl groups can be substituted for the aromatic benzene ring without affecting the basal binding capability.

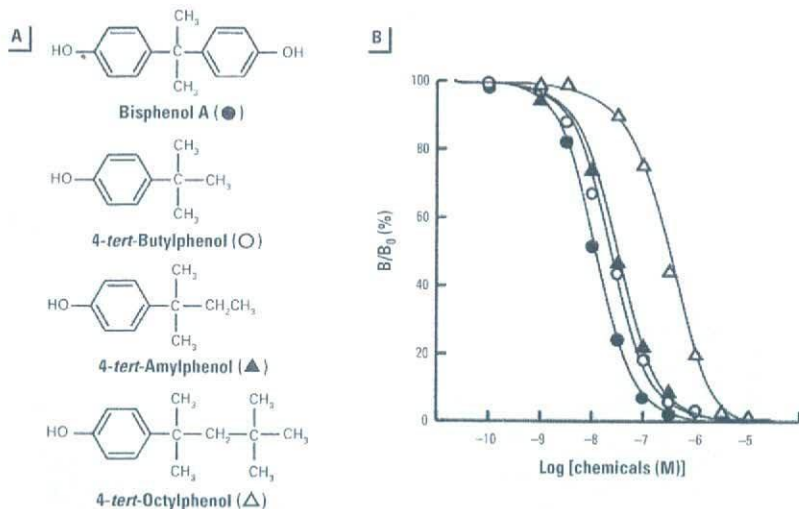


Figure 4. Chemical structure of BPA and its derivatives lacking the phenol group and their dose–response curves in the radioligand receptor binding assay for ERR- γ . (A) Chemical structure of BPA and its derivatives with the alkyl group at the position of phenol group: 4-*tert*-butylphenol (a methyl group); 4-*tert*-amylphenol (an ethyl group); and 4-*tert*-octylphenol (a *tert*-butyl methyl group). (B) Binding activities of BPA, 4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-*tert*-octylphenol examined by the competitive binding assay using [^3H]BPA and GST-ERR- γ -LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

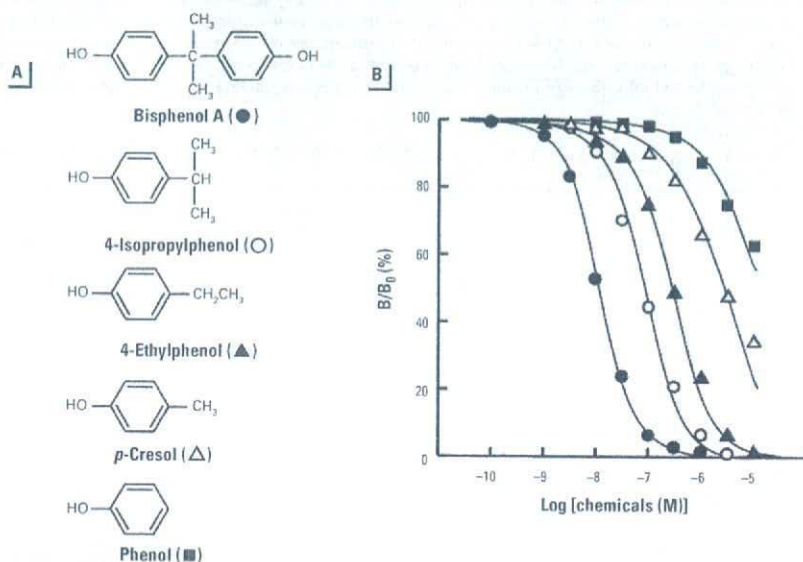


Figure 5. Chemical structure of BPA and a series of alkyl phenols and their dose–response curves in the radioligand receptor binding assay for ERR- γ . (A) Chemical structure of BPA and its derivatives with the alkyl group at the *para* position: 4-isopropylphenol (a 4-isopropyl group); 4-ethylphenol (an ethyl group); *p*-cresol (a methyl group); and phenol (a hydrogen atom). (B) Binding activities of BPA, 4-isopropylphenol, 4-ethylphenol, *p*-cresol, and phenol examined by the competitive binding assay using [^3H]BPA and GST-ERR- γ -LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

However, because both 4-*tert*-butylphenol and 4-*tert*-amylphenol are still a few times less active than 4- α -cumylphenol, a specific binding site of ERR- γ appears to prefer the aromatic benzene ring to the alkyl groups. This suggests that BPA's second phenol-phenyl group (benzene B-ring) is in the π interaction with

the receptor residue(s), that is, either a XH/ π interaction (X = N, O, and C) or a π/π interaction. The most plausible candidate for the receptor residue in this interaction is the Tyr residue at position 326 of ERR- γ . Indeed, the phenol-hydroxyl group of this Tyr-326 was found in the OH/ π interaction with the B-ring of BPA (Matsushima et al. 2007).

In a BPA molecule, two $\text{C}_6\text{H}_4\text{-OH}$ (phenol) groups are connected to the sp^3 carbon atom ($\text{sp}^3\text{-C}$) together with two CH_3 (methyl) groups. The most simple structure–activity study is to compare the activity of compounds lacking one of these groups. The compound that lacks the phenol group is 4-isopropylphenol [$\text{HO-C}_6\text{H}_4\text{-CH}(\text{CH}_3)_2$] (Figure 5A), and this *para*-isopropyl phenol was fairly potent at displacing [^3H]BPA (Figure 5B), with an IC_{50} value of 71.1 nM (Table 2). However, 4-isopropylphenol was still approximately 7-fold less active than BPA, indicating that the phenol backbone structure is an essential structural element for the binding to ERR- γ .

When one of the two methyl groups was eliminated from 4-isopropylphenol, the resulting 4-ethylphenol [$\text{HO-C}_6\text{H}_4\text{-CH}_2\text{-CH}_3$] (Figure 5A) was found to be very weakly active (289 nM) (Table 2). Elimination of another methyl group still afforded a compound of inactive *p*-cresol [$\text{HO-C}_6\text{H}_4\text{-CH}_3$], but with the IC_{50} value being approximately 1.3 μM . Phenol [$\text{HO-C}_6\text{H}_5$] tended to bind to ERR- γ (Figure 5B). These results clearly indicate that the phenol group is a core structure for the attachment of BPA to ERR- γ .

4-Alkyl phenols as putative potent binders to ERR- γ . Attachment of the methyl group to 4-isopropylphenol [$\text{HO-C}_6\text{H}_4\text{-CH}(\text{CH}_3)_2$] to create 4-*tert*-butylphenol [$\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_3$] considerably facilitates the binding of the phenol derivative to ERR- γ (Table 2). 4-*tert*-Amylphenol [$\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}_2\text{CH}_3$] is almost as active as 4-*tert*-butylphenol. However, 4-*tert*-octylphenol [$\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}_2\text{-C}(\text{CH}_3)_3$] (Figure 4B) was significantly weaker (approximately 10 times less potent) than 4-*tert*-butylphenol (Table 2). Thus, the activities of $\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}(\text{CH}_3)_2$, $\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}(\text{CH}_3)_3$, $\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}_2\text{-CH}_2\text{-CH}_3$, and $\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}_2\text{-CH}(\text{CH}_3)_2$ are expected to be intermediate between those of 4-*tert*-amylphenol and 4-*tert*-octylphenol, although these molecules are not commercially available. It appears that, among the 4-alkylphenols of $\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-C}_n\text{H}_{2n+1}$ ($n = \text{R}$), 4-*tert*-butylphenol ($\text{R} = \text{CH}_3$) and 4-*tert*-amylphenol ($\text{R} = \text{CH}_2\text{-CH}_3$) show the maximum competitive activity with the binding of ERR- γ .

The structural comparison of $\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}_3$ (4-*tert*-butylphenol), $\text{HO-C}_6\text{H}_4\text{-C}(\text{CH}_3)_2\text{-CH}_2\text{CH}_3$ (4-*tert*-amylphenol),

and BPA HO-C₆H₄-C(CH₃)₂-C₆H₄-OH clearly indicated that the R group should not be bulky for high receptor binding activity. A plain π electron-rich benzene aromatic ring is thus optimal for interaction with the receptor residue of ERR- γ -Tyr326.

Inhibitory activity of BPA derivatives for ERR- γ . We found that BPA retained a high constitutive basal activity of ERR- γ in the luciferase reporter gene assay (Figure 6A). ERR- γ is in a full activation with no ligand; it is one of the self-activated NRs and is deactivated by the so-called "inverse agonists" such as 4-OHT (Greschik et al. 2004; Takayanagi et al. 2006). Although BPA shows no apparent effect on the high basal activity of ERR- γ , BPA evidently antagonizes or inhibits the deactivation activity of 4-OHT in a dose-dependent manner (Figure 6B), as reported by Takayanagi et al. (2006). This neutral antagonist is a distinct inhibitor or suppressor of the inverse agonist, reversing the deactivation conformation to the activation conformation.

All of the potent BPA derivatives (i.e., bisphenol E, bisphenol AF, 4- α -cumylphenol, and 4-*tert*-butylphenol) were found, just like BPA, to retain a high constitutive basal activity of ERR- γ in the same luciferase reporter gene assay (Figure 6C). In addition, these compounds inhibited the inverse agonist activity of 4-OHT and thus were specific inhibitors against the inverse agonist 4-OHT. Their abilities to antagonize 4-OHT are approximately one order lower than their binding potencies to ERR- γ (Figure 6B,D). This discrepancy is probably caused by the inclusion of a number of co-effector proteins for eliciting a gene expression in the luciferase reporter gene assay.

Receptor selectivity of BPA derivatives for ERR- γ over ER- α . We classified BPA and its derivatives into the four groups, depending on their receptor binding affinity for ERR- γ : that is, group A, BPA and chemicals as potent as BPA; group B, chemicals considerably potent; group C, chemicals moderately potent; and group D, inactive chemicals. All chemicals were then examined for their ability to bind to ER- α , and the affinity measured was compared respectively with that for ERR- γ (Table 3). As reported previously (Takayanagi et al. 2006), BPA is highly selective for ERR- γ . It binds to ER- α only weakly; we calculated BPA's receptor selectivity to be 105, which suggests that BPA prefers ERR- γ 105 times more strongly than ER- α . Other group A compounds, namely, bisphenol E and 4- α -cumylphenol, were also greatly selective for ERR- γ (Table 3). In particular, bisphenol E was found to be exclusively selective and specific for ERR- γ because it was almost completely inactive for ER- α .

para-Alkyl phenols in group B (IC₅₀^{ERR- γ} = of 26–71 nM) were also almost completely inactive for ER- α (Table 3). Those include

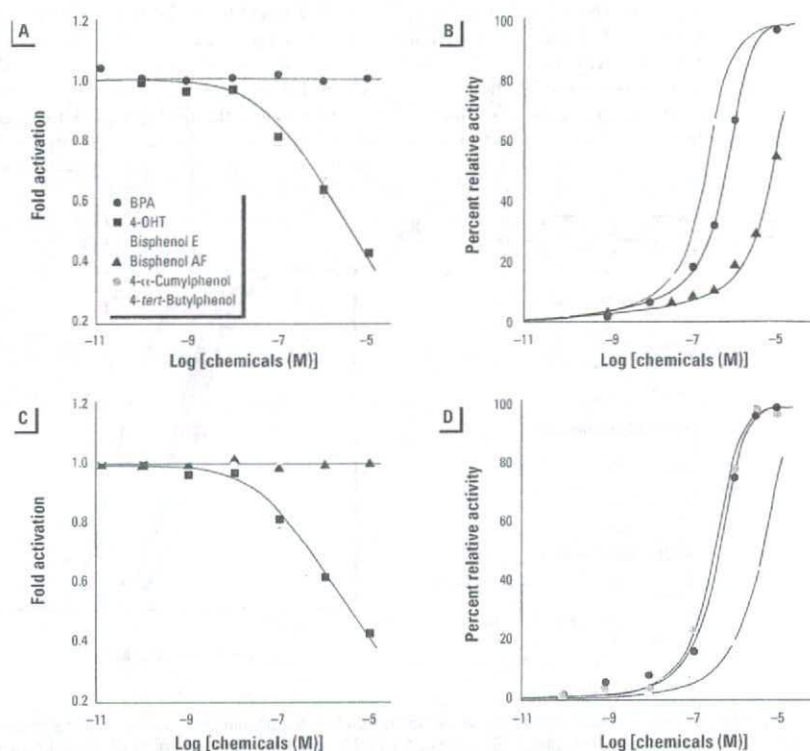


Figure 6. Luciferase-reporter gene assay of BPA and its derivatives for human ERR- γ . (A) Deactivation of the fully activated human ERR- γ by the inverse agonist 4-OHT and sustenance by BPA. (B) Reversing activity of BPA, bisphenol E, and bisphenol AF against the inverse agonist activity of 1.0 μ M 4-OHT; 1.0 μ M 4-OHT exhibited approximately 0.4-fold deactivation, and the inhibitory activities are shown by the percentage of relative activity. (C) Sustenance of the fully activated human ERR- γ by bisphenol E and bisphenol AF together with inverse agonist activity by 4-OHT. (D) Reversing activity of BPA, 4- α -cumylphenol, and 4-*tert*-butylphenol; the inverse agonist activity of 4-OHT was clearly reversed by all bisphenols tested in a dose-dependent manner. Data are from a single experiment performed in triplicate; two additional experiments gave similar results. High basal constitutive activity of ERR- γ was evaluated with the luciferase-reporter plasmid (pGL3/3 \times ERRE), and the highest activity was estimated in a cell preparation of 1.0×10^6 HeLa cells/well.

Table 3. Receptor binding affinity (mean \pm SE; $n = 3$) of BPA and its analogs for ER- α and their receptor selectivity for ERR- γ over ER- α .

Chemical	Binding affinity for ER- α (IC ₅₀ , nM)	ERR- γ receptor selectivity ER- α (IC ₅₀ , nM)/ERR- γ (IC ₅₀ , nM)
E ₂	0.88 \pm 0.13	Exclusively ER- α
Group A (chemicals as active as BPA for ERR- γ)		
Bisphenol E	ND	Exclusively ERR- γ
BPA	1,030 \pm 146	105
4- α -Cumylphenol	4,770 \pm 510	450
Group B (chemicals considerably potent for ERR- γ)		
Bisphenol B	246 \pm 29.7	9.46
4- <i>tert</i> -Butylphenol	ND	Exclusively ERR- γ
4- <i>tert</i> -Amylphenol	ND	Exclusively ERR- γ
4-Isopropylphenol	ND	Exclusively ERR- γ
Group C (chemicals moderately potent for ERR- γ)		
Bisphenol AP	361 \pm 22.6	2.93
Bisphenol F	ND	Exclusively ERR- γ
4- <i>tert</i> -Octylphenol	925 \pm 83.9	3.89
4-Ethylphenol	ND	Exclusively ERR- γ
Bisphenol AF	53.4 \pm 7.28	0.15
Group D (chemicals extremely weak or inactive for ERR- γ)		
2,2-Diphenylpropane	ND	Inactive for both receptors
<i>p</i> -Cresol	ND	Almost inactive for both receptors
Phenol	ND	Inactive for both receptors

ND, not determined (IC₅₀ value could not be calculated because of extremely weak binding activity even at a 10- μ M concentration).

4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-isopropylphenol, and they were fully selective and specific for ERR- γ . In contrast, bisphenol B was very weakly active (246 nM) for ER- α , although it was still selective (about 9.5 times) for ERR- γ .

Among group C chemicals ($IC_{50}^{ERR-\gamma}$ = 120–350 nM), bisphenol F was almost completely inactive for ER- α , making it fully selective for ERR- γ (Table 3). This was also true for 4-ethylphenol. Bisphenol AP showed a weak binding affinity (361 nM) for ER- α , but it was still selective (about 3 times) for ERR- γ . However, bisphenol AF emerged as a ligand selective for ER- α with a selectivity ratio of 0.15 (Table 3). The reciprocal of 0.15 [i.e., $ERR-\gamma$ (IC_{50})/ER- α (IC_{50}) = 6.67] denotes a selectivity ratio of bisphenol AF for ER- α .

The results clearly indicate that the alkyl groups on the central sp^3 -C atom of bisphenol derivatives play a key role in selection of the NR ERR- γ and ER- α . When we checked the receptor binding activities of one series of bisphenol derivatives (i.e., bisphenol E, BPA, bisphenol B, bisphenol AP, and bisphenol AF), we found this line-up to be the order of compounds with increasing affinity to ER- α . At the same time, it was the order of compounds with decreasing affinity to ERR- γ . ERR- γ prefers the less bulky and less electrophilic alkyl groups, whereas ER- α appears to prefer the bulkier and more electrophilic alkyl groups.

4-*tert*-Octylphenol is a well-known endocrine disruptor candidate, but it was only moderately potent for ERR- γ (IC_{50} = 238 nM; Table 2). However, it was considerably weak for ER- α , with an IC_{50} of 925 nM; thus, we judged 4-*tert*-octylphenol to be somewhat selective (approximately 4 times) for ERR- γ . Another representative endocrine disruptor candidate is 4-nonylphenol, which was moderately active for ERR- γ (Takayanagi et al. 2006). Thus, 4-nonylphenol was slightly more selective for ERR- γ . However, some 4-alkyl phenols are distinctly more potent for ERR- γ than 4-*tert*-octylphenol and 4-nonylphenol: 4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-isopropylphenol. These 4-alkyl phenols are definitely novel candidates of the endocrine disruptor specific for ERR- γ .

Conclusion

In the present study we have shown that all the structural elements of BPA—the phenol and

methyl groups and the phenyl group on the central sp^3 -C atom—are prerequisite for binding to the NR ERR- γ . Furthermore, we have shown that the phenol derivatives are potent candidates for the endocrine disruptor that binds to ERR- γ . The binding affinity of [3 H]BPA to ERR- γ -LBD is extremely high, with a K_D value of 5.50 nM. Thus, it appears to be important to evaluate whether the previously reported effects of BPA at low doses are mediated through ERR- γ and its specific target gene(s).

At the same time, it is necessary to clarify the physiologic roles of ERR- γ and to examine the degree and ways in which BPA may influence these. This is particularly important because ERR- γ is expressed in a tissue-restricted manner—for example, it is expressed very strongly in the mammalian fetal brain and placenta—at sites that could have important outcomes for newborns. Recently, many lines of evidence have indicated that low doses of BPA affects the central nervous system (reviewed by vom Saal and Welshons 2005; Welshons et al. 2003, 2006). The molecular mechanism for these effects could involve, at least in part, the high affinity binding of BPA to ERR- γ . A similar phenomenon may be observed for other NRs, and the exploration of such chemical–receptor interactions requires a specific assay system or concept applicable to all the NRs.

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A strategy to explore the target receptor of endocrine disruptors: Estrogen-related receptor γ (ERR γ) as a genuine acceptor of Bisphenol A

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Abstract

Bisphenol A (BPA) has been acknowledged as an estrogenic chemical able to interact with an estrogen receptor (ER), and many lines of evidence have revealed that BPA has an impact as an endocrine disruptor functioning at very low doses. However, its binding to ER and consequent hormonal activity are extremely weak, making the intrinsic significance of low-dose effects intangible and obscure. Based on the idea that BPA is accessible to all 48 human nuclear receptors, we searched BPA receptor(s) and eventually discovered the estrogen-related receptor γ (ERR γ). BPA functions as an inverse antagonist of ERR γ , and this disruptor activity is expected to shed light on the molecular mechanisms of self-activated orphan ERR γ receptor. To elucidate the physiological functions of these mechanisms it will be important to undertake the sophisticated excision of *in vivo* mechanisms and a profound analysis of their molecular subtleties *in vitro*. In this paper we review the current understandings about ligand-receptor interaction modes of both the ligand-regulated nuclear receptors and the self-activated orphan nuclear receptors, and propose a sophisticated test scheme for comprehensive elucidation of their molecular mechanisms.

Keywords: bisphenol A, endocrine disruptor, estrogen-related receptor γ , inverse antagonist, nuclear receptors

1. Nuclear receptors as the target of endocrine disruptors

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, has long been recognized as an estrogenic chemical that interacts with human estrogen receptor (ER). This xenoestrogen, BPA is used mainly in the production of polycarbonate plastics and epoxy resins and is found in many plastic products, including food can linings and dental sealants. Its annual worldwide manufacture is approximately 3.2 million metric tons. The widespread industrial and household use, economic importance, and near ubiquitous presence of BPA in the environment emphasize its prospective risk as an endocrine disruptor.

Bisphenol A has been acknowledged as one of the so-called endocrine disruptors, or endocrine disrupting chemicals (EDC), that are able to alter the normal functioning of the endocrine and reproductive systems by mimicking or inhibiting endogenous hormone action, thereby modulating the production of endogenous hormones, or altering hormone receptor populations. A major mechanism of endocrine disruption is the action of chemicals that act as receptor *agonists* or *antagonists* through

direct interaction with hormone receptors, thus altering endocrine function. In particular, chemicals mimicking endogenous estrogen via ER have been the focus of research for the last two decades.

Bisphenol A has also been assumed to be an anti-androgen, or an antagonist that blocks the action of dihydrotestosterone at a human androgen receptor (AR). In addition, various so-called "low-dose effects" of BPA have recently been reported *in vivo* for many organ tissues and systems in mice and rat (Welshons *et al.*, 2003; vom Saal and Welshons, 2005; Welshons *et al.*, 2006). Since BPA's binding to ER and AR and its hormonal activity are extremely weak--1,000 to 10,000 times weaker than for natural hormones--it is unlikely that BPA interacts directly with ER and AR to achieve its effects at low doses.

All these circumstances prompted us to assume that the target of BPA may be nuclear receptor(s) (NR) other than ER and AR. Recently, with the completion of the human genome project, it became clear that nuclear receptors form a superfamily of proteins, which consists of forty-eight different NR proteins. This superfamily consists of seven families called respectively class-0, and 1 - 6. Within these, ER and

AR belong to the class-3 NR family that consists of nine steroid hormone receptors. We searched these class-3 NRs to explore an actual receptor to which BPA binds, and eventually found recently that BPA binds very strongly to the estrogen-related receptor γ (ERR γ).

In addition to the ligand-regulated NRs such as ER and AR, the class-3 NR family includes a number of orphan receptors the physiological ligands of which remain to be identified. This group, the estrogen-related receptors (ERRs) (Giguère, 2002; Horard and Vanacker, 2003), consist of three members α , β , and γ (ERR α , ERR β , and ERR γ). ERRs and ERs show a considerable level of amino acid sequence similarity and identity in both the DNA binding domain and the ligand-binding domain.

2. Ligand-regulated nuclear receptors and self-activated nuclear receptors

ERRs are almost fully active with no ligands, and we refer to such orphan receptors as self-activated NRs. There are 13 self-activated NRs amongst 48 human NRs. 17 β -Estradiol (E2), a natural *agonist* ligand of ER α and ER β , does not bind to any of the ERRs. On the other hand, 4-hydroxytamoxifen (4-OHT), which is an *antagonist* of ERs, deactivates ERRs in, for example, the luciferase reporter gene assay. Such deactivating activity against the self-activated NRs is characterized as the activity of an *inverse agonist*. We found that BPA inhibits and reverses in a dose-dependent manner the inverse agonist activity of 4-OHT to the originally high basal activation state, and thus acts as an *inverse antagonist* of ERR γ .

We also achieved the crystallization and X-ray structural analysis of the BPA/ERR γ -LBD complex (Matsushima *et al.*, 2007). In the complex, a single molecule of BPA stays at the ligand-binding pocket of each ERR γ -LBD protein molecule, whose α -helix 12 (H12) is stabilized in an activation conformation (Fig. 1). The structural elements important for the ligand-receptor interactions were elucidated by us from studies to clarify the structure-activity relationships between BPA-related compounds and ERR γ -LBD mutated receptors (Liu *et al.*, 2007; Okada *et al.*, 2008).

3. Schematic discrimination of intrinsic types of ligand-receptor interactions

For endocrine disrupting chemicals, it is important for us to know and evaluate the type of interactions these have with NRs. All the chemicals should first be evaluated in a receptor-binding assay. Those chemicals that bind should then each have their biological activities examined to determine whether they have an *agonist* or *antagonist* action, when bound to the ligand-regulated NRs. In the case that

the compound is neither an *agonist* nor *antagonist*, it should then be examined in the assay to determine whether it is an *inverse agonist* or *inverse antagonist*. In such cases, the NR is likely to be one of the self-activated NRs. Fig. 2 shows a scheme for exploring intrinsic types of interactions.

It should be noted that BPA is neither an *agonist* nor an *antagonist* of ER and AR. BPA binds extremely weakly to these receptors, and thus is almost completely inactive against them. BPA binds to ERR γ very strongly, on the other hand, but at this receptor is neither an *agonist* nor an *antagonist*. BPA is also not an *inverse agonist*, simply sustaining

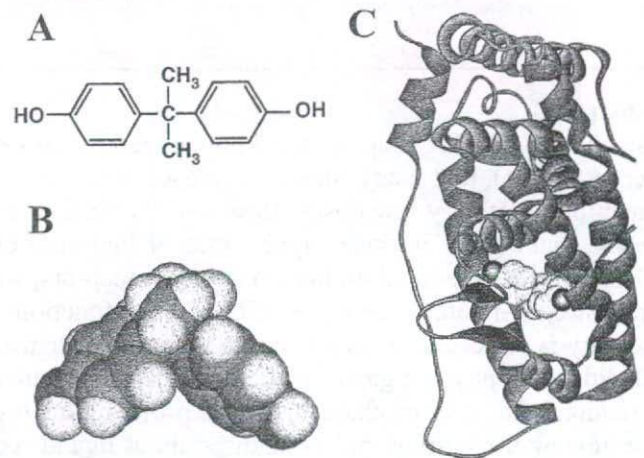


Fig. 1. Structure of bisphenol A (BPA) and its complex with estrogen-related receptor γ (ERR γ). (A) Chemical structure of bisphenol A, 2,2-bis(4-hydroxyphenyl)propane. (B) Space-filling three-dimensional structure of bisphenol A. (C) X-ray crystal structure of BPA/ERR γ -LBD complex.

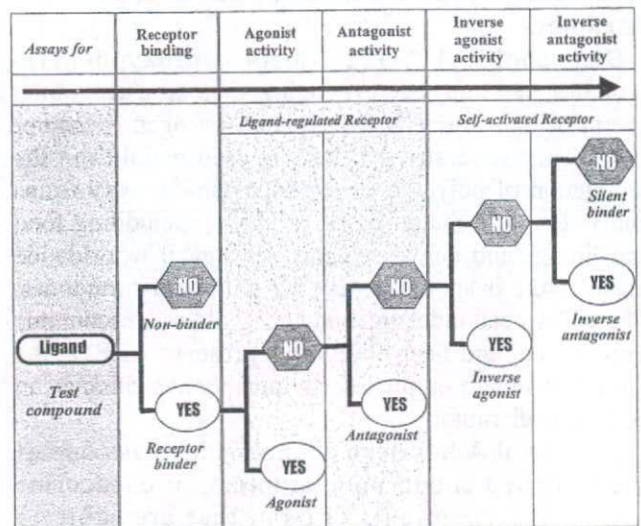


Fig. 2. Schematic flow diagram of receptor assays to discriminate the characteristic receptor responses. After initiating receptor-binding assay, the resulting binder should be assayed to test its either agonist or antagonist activity. If the compound is neither agonist nor antagonist, it should be assayed to evaluate its either inverse agonist or inverse antagonist activity.

ERR γ 's high basal constitutive activity, but reverses 4-OHT's *inverse agonist* activity, thus showing very effective *inverse antagonist* activity. Consequently, BPA is a strong binder of ERR γ , but quite silent without the inverse agonist to reveal its action. This kind chemical poses a particular problem because it would be excluded from an assay or other test as simply an inactive or insensitive ligand, and thus it is very important to include an assay that examines the receptor binding capability in any assay scheme.

After these detailed *in vitro* examinations, it is definitely necessary to clarify what the physiological roles of ERR γ are, and to examine the degree and ways in which BPA may influence these. This is particularly important because ERR γ is expressed in a tissue-restricted manner—for example, very strongly in the mammalian placenta and fetal brain—at sites that in humans could have important outcomes for newborn children.

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Relationship between the results of *in vitro* receptor binding assay to human estrogen receptor α and *in vivo* uterotrophic assay: Comparative study with 65 selected chemicals

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Abstract

For screening chemicals possessing endocrine disrupting potencies, the uterotrophic assay has been placed in a higher level in the OECD testing framework than the ER binding assay to detect ER-mediated activities. However, there are no studies that can demonstrate a clear relationship between these assays. In order to clarify the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays and to determine meaningful binding potency from the ER binding assay, we compared the results from these assays for 65 chemicals spanning a variety of chemicals classes. Under the quantitative comparison between logRBAs (relative binding affinities) and logLEDs (lowest effective doses), the log RBA was well correlated with both logLEDs of estrogenic and anti-estrogenic compounds at $r^2 = 0.67$ ($n = 28$) and 0.79 ($n = 23$), respectively. The RBA of 0.00233% was found to be the lowest ER binding potency to elicit estrogenic or anti-estrogenic activities in the uterotrophic assay, accordingly this value is considered as the detection limit of estrogenic or anti-estrogenic activities in the uterotrophic assay. The usage of this value as cutoff provided the best concordance rate (82%). These findings are useful in a tiered approach for identifying chemicals that have potential to induce ER-mediated effects *in vivo*.
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Keywords: Binding assay; Uterotrophic assay; Concordance

1. Introduction

After a variety of studies showed that certain chemicals might disrupt the sex hormonal systems of wildlife and humans, the Organization for Economic Cooperation and Development (OECD), European countries, the Uni-

ted States and Japan initiated efforts to develop and validate assays and screen chemicals for their potential to disrupt the endocrine system of human and wildlife (CEC, 2004; EPA, 1998; Gelbke et al., 2004). Under the OECD activity, “Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals” was developed. This framework has five components or levels each corresponding to a different level of biological complexity (OECD, 2002). The *in vitro* assays such as receptor binding assay are placed in level 2 in this framework to provide mechanistic information and serve for screening purposes. As a tool to detect hormonal effects mediated

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thorough specific hormone receptors, especially for estrogen receptors (ERs), the application of the ER binding assay has been investigated as a quick and cost-effective method. Furthermore, the rodent uterotrophic assay, which has been placed in level 3 to provide data about single endocrine mechanism and effects, has also been recognized as an *in vivo* assay to detect estrogen receptor mediated effects (Kanno et al., 2001; Owens and Koeter, 2003).

Since the uterotrophic assay and ER binding assay both detect ER-mediated activities, a clear relationship between the results from both two assays is expected. However, there are no studies that can demonstrate a clear relationship between two assays on a variety of chemical structures, although studies comparing at limited number of chemical structures has been available (Takemura et al., 2005; Hong et al., 2005; Legler et al., 2002; Yamasaki et al., 2004). Since the receptor binding assay is considered as one of the screening assays to determine if a chemical has potential to trigger receptor-mediated endocrine disruption such as ER and androgen receptor (AR). The results of receptor binding studies need to be related to biological relevance. The purpose of this study is to clarify the relationships between the results obtained in the *in vitro* ER binding assay and *in vivo* uterotrophic assay, and to determine the meaningful cutoff value from the ER binding assay. For this purpose, we compared the results of receptor binding assay using human ER α (hER α) with immature rat uterotrophic assay for 65 selected chemicals.

2. Materials and methods

2.1. Chemicals

The chemical names, CAS numbers and sources of 65 test chemicals subjected in this study are listed in Table 1. Parabenes, benzophenones, biphenyls, diphenylmethanes, diphenylethylenes, phthalates, phenols and other chemical structure classes were included. All test chemicals used in this study had more than 95% purity. Tris(hydroxymethyl)aminomethane (Tris), phenylmethylsulfonyl fluoride (PMSF) and 17 β -Estradiol were purchased from Sigma-Aldrich. Dimethylsulfoxide (DMSO), leupeptin hemisulfate monohydrate, sodium metavanadate, (NaVO₃) dithiothreitol (DTT), glycerol and bovine serum albumin (BSA) were obtained from Wako Pure Chemical Industries, Ltd. and disodium salt dihydrate, ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis(β -aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA) were from Dojindo Laboratories Inc.

2.2. *In vitro* ER binding assay

The receptor binding assay using recombinant human estrogen receptor α (hER α) was conducted by the method previously reported (Nakai et al., 1999; Yamasaki et al., 2004). Briefly, a recombinant hER α ligand binding domain

(hER α -LBD) fused with glutathione-S-transferase (GST) was expressed in *E. coli* and purified using affinity chromatography technique. After the addition of sample solution (10 μ L) of varied concentration (1×10^{-11} to 1×10^{-4} M as final concentrations) and [2,4,6,7,16,17-³H] 17 β -estradiol (³H-E2, 10 μ L; final conc. 0.5 nM, 155 Ci/mmol, Amersham Biosciences Corp.) in Tris-HCl (pH 7.4, 50 μ L) containing 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₃, 1 mM DTT, 10% glycerol, 10 mg/mL BSA, 0.5 mM PMSF, and 0.2 mM leupeptin, a solution of recombinant hER α -LBD (30 μ L; final conc. 0.2 nM) was gently mixed. This mixture solution was incubated for 1 h at 25 °C. Free radiolabelled ligands were removed by incubation with 0.4% dextran-coated charcoal (Sigma) (100 μ L) for 10 min at 4 °C followed by filtration. The radioactivity of residual radiolabelled ligands bound to receptors in filtrate were measured by liquid scintillation counting. The assay was repeated more than three times for each test chemical.

2.3. Immature rat uterotrophic assay

The chemicals listed in Table 1 were tested in the immature rat uterotrophic assay according to the OECD draft test guideline as previously reported (Yamasaki et al., 2004) in compliance with good laboratory practice (GLP).

Crj:CD (SD) IGS female rat pups (10-day old) purchased from Charles River Japan Inc. (Shiga, Japan) were weaned with their dams and individually housed until 19-days old. These immature female rats were weighted and weight-ranked to assign to each of the treated and control groups (6 rats/group). Three doses were used for each test chemical and the highest dose was set at the maximum tolerance dose based on the results of dose-range finding test. The limit dose was set at 1000 mg/kg/day. Each group of six immature, 20-days old female rats received subcutaneous injections of a test chemical into their back for three consecutive days (4 mL/kg/day) for evaluation of estrogenic activity. The vehicle control group treated with olive oil (s.c.) and the positive control group for estrogenic activity (s.c. 0.6 μ g/kg/day of 17 α -ethynylestradiol (EE, Sigma)) were concurrently run for each test chemical. To evaluate anti-estrogenic activity in another set of treatment groups, EE (0.6 μ g/kg/day) was co-administered with the test chemical. The positive control group for anti-estrogenic activity received subcutaneous co-administration of 1 mg/kg/day of tamoxifen (TAM, Sigma) and 0.6 μ g/kg/day of EE. The animals were sacrificed by bleeding from the abdominal vein under deep ether anesthesia 24 h after the final administration, and body weight and uterine weight of each animal were recorded.

2.4. Data analysis

The resulting data from the ER binding assay were analyzed using GraphPad Prism®, Version 4 (GraphPad Software, Inc.), and the IC₅₀ value for each test chemical was obtained by logistic equation. The relative binding affinity