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と共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}）はスキャ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細胞を用いて、全長の野生型（WT）および各種変異受容体ERRγ発現プラスミドと3 × ERRE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は発光基質を用いて測定した。WT-ERRγ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

ERRγ-LBD-WT受容体の飽和結合試験

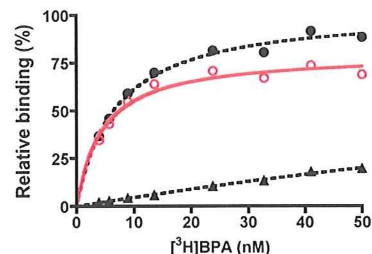
ERRγ-LBD-WTはグルタチオンS-トランスフェラーゼ（GST）との融合タンパク質として大腸菌で発現した。放射標識されたBPA（[^{3}H] BPA）を用いて、飽和結合試験を行った。スキャッチャードプロット解析を行った。

測定の結果、ERRγ-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ERRg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改めて確認された。

ERRγ-LBD変異受容体の飽和結合試験

ERRγ-LBDのC端側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、13種の変異受容体を作製した。これら13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。[^{3}H] BPAを用いて、ERRγ-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

(A)



(B)

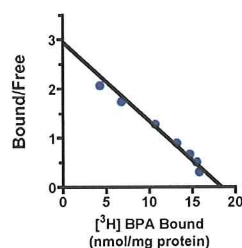


図1. ERRγ-WT飽和結合試験
(A). ERRγに対する[^{3}H]BPAの全結合(●)、特異的結合(○)、非特異的結合(▲)を示す。
(B)スキャッチャードプロット解析

で飽和結合試験を行った。その結果、C端側の3残基を1残基ずつ削除した場合—Δ458、Δ457-458、Δ456-458受容体はBPAとの結合性は遂次に弱くなった。さらにH12から1または3残基ずつ削除すると、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

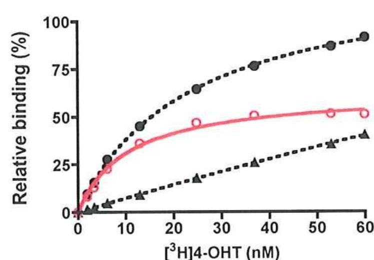
ERRγ-LBD-WT受容体の飽和結合試験

前述で得られたERRγ-LBD-WTタンパク質について、[^{3}H]4-OHTを用いて、飽和結合試験を行った。スキャ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離れることから、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。

ERRγ-LBD変異受容体の飽和結合試験

[^{3}H]4-OHTを用いてERRγ-LBD変異受容体タンパク質との飽和結合試験を行った結果、変異受容体はいずれとも4-OHTと結合することが分かった。スキャ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

(A)



(B)

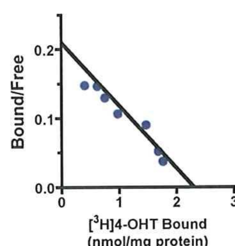


図 2. ERRγ-WT 飽和結合試験

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

(B)スキャッチャードプロット解析

体Δ456-458、Δ455-458、Δ454-458、Δ453-458、Δ450-458 は WT に対するのと同様の[^{3}H]4-OHT 結合性を示した。また、H12 と H11-H12 loop とも欠損の変異受容体Δ441-458 も同様であった。H12 全欠損に加え、H11-H12 loop 部分的削除した変異受容体Δ447-458 およびΔ444-458 は、[^{3}H]4-OHT との結合性が WT の 3～4 倍弱くなった。H11 部分的削除した変異受容体Δ438-458、Δ435-458、Δ432-458 は、[^{3}H]4-OHT と結合性が弱く、解析できなかった（図３）。

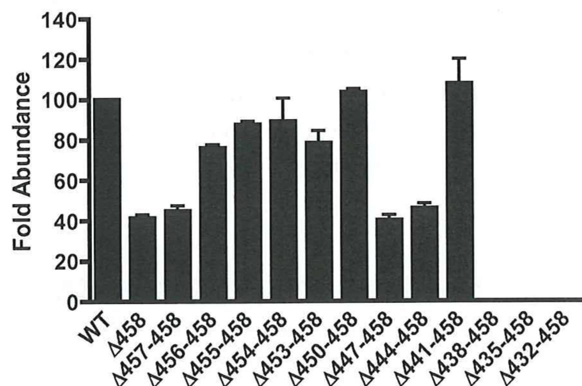


図 3. スキャッチャードプロット解析計算より、各受容体は[^{3}H]4-OHT に対する Kd 値の比較。

(WT の K_d 値は 1 とする)

以上のことから、4-OHT と ERRγ の結合に H12 は不必要なことが示された。さらに、H11 と H12 の存在はこの間の loop 構造を安定させるが、H12 が欠失すると、loop 構造は不安定になると考えられる。すなわち、H11-loop-H12 の loop 構造に何が特別な役割があることが考えられる。しかも、H11 構造は受容体と 4-OHT 強い結合に重要な構造であると考えられる。

(3) レポーター遺伝子アッセイ試験

ERRγはリガンドなしでも高い活性化能を持つ「自発活性化型」核内受容体である。そこで、H12 を部分的および全て削除して、受容体の自発活性化への影響を解析するため、HeLa 細胞を用いたレポーター遺伝子アッセイにより転写活性を評価した。pcDNA3 からベクターの活性は 1 とすると、WT-ERRγの自発活性は約 4 倍の高い活性を示した。C 端側の 3 残基は 1 残基ずつ削除しても自発活性に何の影響もないものの、H12 を一部でも削除した変異受容体は自発活性を完全に失うことが判明した（図４）。これらの結果により、ERRγのビスフェノール A 結合および自発活性には、H12 が必須な構造要因であることが証明された。

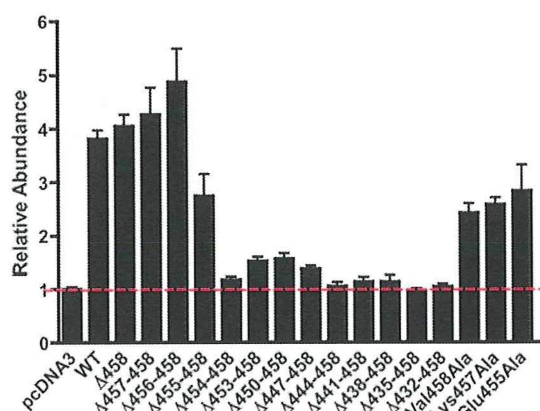


図 4. レポーター遺伝子アッセイ試験

(pcDNA3 空ベクターの活性は 1 とする)

D. 考察

BPA/ERRγ-LBD 複合体の X 線結晶構造解析より、BPA と ERRγ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、ERRγ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 と全く結合しないものの、一方で 4-OHT とは強く結合することが以前の研究から分かった（投稿中）。こうして、ERRγのインバースアゴニストである 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、ERRγ-LBD の C 端から 1 または 3 残基ずつを逐次に削除した部分構造欠損型の 13 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}H]BPA および[^{3}H]4-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、H12 部分および全欠損型受容体は BPA と全く結合しない、一方、4-OHT はいずれとも非常に強く結合することから、その結合に H12 は不要な構造であることが判明した。このことは、同じ受容体に結合する化学物質であっても、その結合様式が全く異なることを示している。また、受容体がこうした化学物質にそれぞれに対した結合構造要因を持っていることを示している。

さらに、変異受容体の自発活性化を調べたところ、ERRγは高い活性化を示し、C 端側の 3 残基は 1 残基ずつ削除しても受容体の自発活性に何の影響もないものの、H12 を一部でも削除した変異受容体は自発活性を完全に失うことが判明した。以上の結果により、ERRγのリガンド結合性の選択性および自発活性に対して、H12 が必須な構造要因であることが証明された。自発活性化型核内受容体はすべて、このような活性構造を持っているのかも知れない。

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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G. 知的財産権の出願・登録状況

特になし。

別紙４

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研究成果の刊行物・別刷

ミニレビュー

放射標識化合物を用いた受容体結合試験による特異的受容体の同定・解析：ビスフェノールAが結合する特異的核内受容体ERRγ†

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Key Words：estrogen-related receptor γ, endocrine disruptor, [^{3}H]bisphenol A, [^{3}H]4-hydroxytamoxifen, receptor-binding assay, inverse antagonist

1. はじめに

我々の生体内では膨大な数のレセプター（受容体）が機能しており，シグナル伝達や転写制御などを介して生命活動を司っている。受容体は，通常は不活性な状態にあり，特異的な化合物が結合すると特有な活性を発現する。このような活性発現のシグナルとして働く化合物をアゴニストと呼ぶ。生体のほとんどの受容体は内在性のアゴニストを持っている。一方，受容体に結合するものの活性化しない化合物は，アゴニストの活性を抑制・阻害することができるため，アンタゴニストと呼ばれる。受容体に結合するアゴニスト，アンタゴニストはリガンドと称され，受容体の活性化の分子機構の解明に欠かせない分子ツールであり，また，「くすり」として役立つので人工物の開発が精力的になされている。

ところが近年になって，リガンドなしに最初から100％フルに活性化されている受容体が発見され，注目されている。この「自発活性化型」受容体に内在性のリガンドが存在するのか？明らかになっていないが，自発活性を抑制し，不活性化する化合物・インバースアゴニストが知られている。一方，このインバースアゴニストを阻害し，元の活性化状態に戻す化合物が存在し，インバースアンタゴニストと呼ばれる。筆者らは，核内受容体の一つについて，このインバースアンタゴニストが存在することを初めて発見した。そして，この発見にトリチウム標識リガンドを用いた受容体結合試験が必須であった。本稿では，環境ホルモンとして本命視されている化合物ビスフェノールAをインバースアンタゴニストとして発見した，筆者らの『アイソトープを用いた受容体結合試験』の有用性について紹介したい。

2. 今なお，「環境ホルモン」問題

アメリカ厚生省所轄のNTP（国家毒性評価プログラム）は，ビスフェノールAがヒトに何らかの悪影響を及ぼす可能性があることを認め，この2008年6月に公開パネルを開催した[1)]。実はここ数年，欧米を中心にしてBisphenol A and the Baby Bottle Debate（ビスフェノールA，そして哺乳瓶をめぐる論争）[2)]がセンセーションとなっており，哺乳瓶から溶出するビスフェノールAが乳幼児に悪影響をもたらすとして，テレビ，新聞，インターネットはもちろん，地方政府，国家レベルでの大きな議論になっている。こうしたなかでのNTPの公開パネルであり，非常に注目されている。日本では，いわゆる「環境ホルモン問題」は一時に比べてすっかり影をひそめ，沈静化しているが，2008年7月上旬，厚生労働省が国立医薬品食品衛生研究所のラットでの実験結果を受けて，発生・発達段階にある胎児や子供には微量でも中枢神経や免疫系などに影響する恐れありとして内閣府の

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† Receptor Binding Characteristics of the Endocrine Disruptor Bisphenol A as Inverse Antagonist for the Human Estrogen-Related Receptor γ.
Hiroyuki OKADA and Yasuyuki SHIMOHIGASHI：Laboratory of Structure-Function Biochemistry, Department of Chemistry, The Research-Education Centre of Risk Science, Faculty and Graduate School of Sciences, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka-shi, Fukuoka Pref. 812-8581, Japan.

食品安全委員会に評価を諮問した。環境ホルモン問題は，今なお終わっていない。

3. ビスフェノール A の受容体を発見

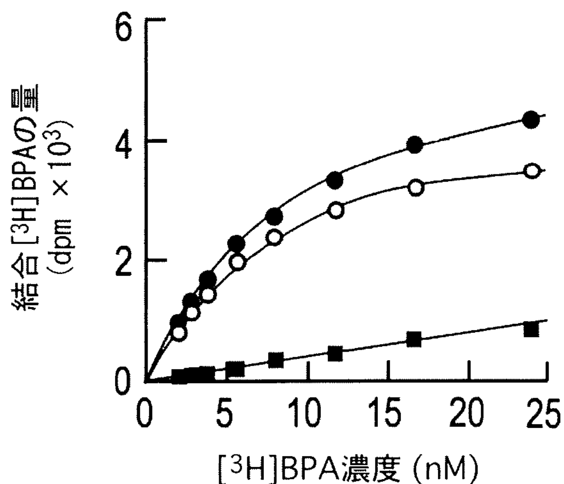
我々の生活環境中には，膨大な数の化学物質が存在し，それらの一部は生体に悪影響を及ぼす「内分泌かく乱物質（環境ホルモン）」である。その代表的なものが，ポリカーボネート製プラスチックやエポキシ樹脂の原料・ビスフェノール A である。ビスフェノール A は，女性ホルモン（エストロゲン）様の作用を示すと指摘され，プラスチック製品からの溶出基準が各国で定められている。しかしながら，2000 年前後より，溶出基準を大きく下回る低濃度において，ラット乳幼仔の行動などに悪影響を与えるという報告が相次ぎ[3]，こうした「低用量問題」が大きくクローズアップされるようになった。ビスフェノール A の内分泌かく乱作用については，これまで学界と産業界の論争という形で，問題の有無から議論されてきたが，ここに来て「作用あり」の観点から再評価する気配になってきた。そこで問題となるのがビスフェノール A が結合する受容体である。ビスフェノール A は「エストロゲン様」と形容されてきたにもかかわらず，実際にはエストロゲン受容体 (ER) への結合性がきわめて低い。筆者らは「ビスフェノール A の標的受容体が ER 以外に存在する」可能性を考え，ヒト核内受容体の 48 種類すべてについて，トリチウム標識したリガンドをトレーサに用いた競争結合試験によるスクリーニングの取り組みを開始した。その結果，ER とよく似た構造を持つものの，全く別の核内受容体・エストロゲン関連受容体 γ 型 (ERRγ) がビスフェノール A の標的受容体であることを発見した[4),5]。

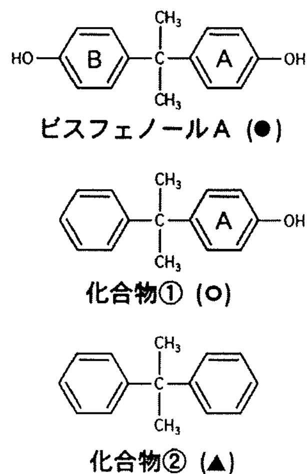
4. ERRγ は自発活性化型受容体

ERRγ はヒト核内受容体スーパーファミリーに属し，ステロイド受容体グループに分類される。このグループは一般に，ステロイドホルモンが結合すると活性化されるが，ERRγ はリガンドなしで最初から 100% フルに活性化の状態になっている。筆者らは，こうした受容体を「自発活性化型核内受容体」と呼んでいる。ERRγ の機能を制御する内因性リガンドは見つかっていないが，4-hydroxytamoxifen (4-OHT) は強く結合し，活性を低下させる。つまり，4-OHT は ERRγ のインバースアゴニスト（自発活性を抑制するリガンド）である[6]。この報告をもとに，当初，筆者らは，トリチウム標識した 4-OHT をトレーサとして ERRγ に結合する化学物質をスクリーニングしていたが，驚くほどに強く結合する化合物があることに気づき，これがビスフェノール A であった[4]。このため，すぐさま，トリチウム標識したビスフェノール A ([^{3}H]BPA) をトレーサとして結合試験系（飽和結合試験及び競争結合試験）を構築した。そして，ERRγ へのビスフェノール A の非常に強い結合特性を詳密に解析することに成功した[7]。ビスフェノール A の結合定数 K_D 値は，5.5 nM である（図 1）。この値は，生体内の一般的な天然ホルモンと受容体の結合親和性に匹敵するほど非常に高い結合親和性であることを意味している。

5. ビスフェノールA類似体の結合性と活性

ビスフェノール A を原材料にしたポリカーボネートで製造される製品は数限りない。携帯電話，コンピュータ，眼鏡レンズ，DVD，医療用チューブ。これらは，我々の生活に欠かせない。高性能ポリカーボネートとして，ビスフェノール A の類似化合物の開発も急ピッチで進められている。果たしてビスフェノール A だけが問題だろうか？きっと他にも ERRγ に結合する化合物があるはずである。そこで，ERRγ に結合するために必須な分子構造を明らかにするために，[^{3}H]BPA とビスフェノール A の構造類似体を用いて競争結合試験を行った。例えば，ビスフェノール A の OH 基の重要性を解析する場合，化合物①と化合物②の結合能をビ





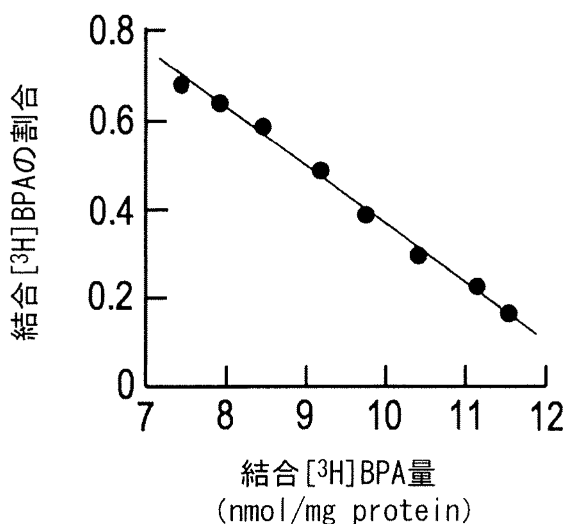


図1　[³H]BPA による ERRγ 飽和結合試験の結果
(上) ●：全結合，○：特異的結合，■：非特異的結合を示す。縦軸は，結合した [³H]BPA の壊変数，横軸は，使用した [³H]BPA の濃度を示す。(下) スキャッチャードプロット解析の結果を示す。縦軸は遊離の [³H]BPA に対する結合した [³H]BPA の比率，横軸は単位蛋白質量あたりに結合した [³H]BPA を示す。線形グラフは直線で，結合部位が一つであることを示し，その傾きは $-1/K_D$ である。また，X 切片が B_{max} 値を示し，これは蛋白質 1 mg あたりの最大結合量を意味する。(文献 7 より改変して引用) なお，結合部位の数については X 線結晶解析より証明された[8),9)]

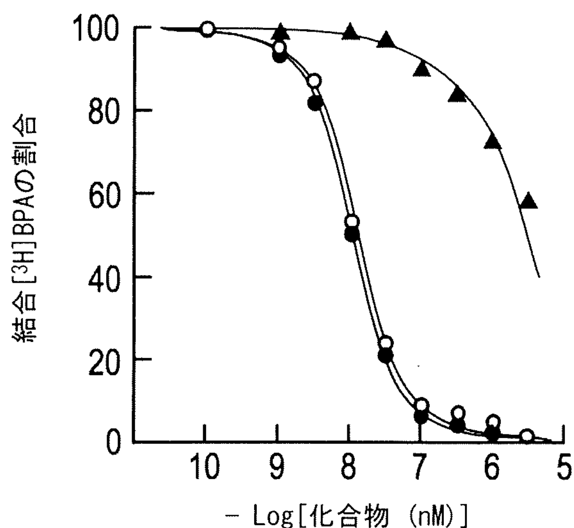


図2　[³H]BPA による ERRγ 競争結合試験の結果
ビスフェノール A，化合物①及び化合物②について，[³H]BPA の結合に対する阻害能を解析した。これにより，調べたい化合物の結合性を相対的に評価することができる。縦軸は化合物を競合させていないときの [³H]BPA 結合数と競合後の [³H]BPA 結合数の割合を百分率で示す。横軸は競合させた化合物濃度を示す。BPA と化合物①はフェノール環 A を共有し，これらは ERRγ の同じ部位に結合する[9)]

スフェノール A と比較する。これにより，一つの OH 基がきわめて重要であることがわかる（図 2)。こうした解析を網羅的に行い，ビスフェノール類とアルキルフェノール類が ERRγ に強く結合することを明らかにした[7)]。更に，筆者らは，ビスフェノール A が結合した ERRγ の結晶構造解析にも成功し[8),9)]，もはや，ビスフェノール A やその類似体の ERRγ 結合性は疑いようがない。

一方，ビスフェノール A による ERRγ の活性変化を調べたところ，驚きの結果が得られた。

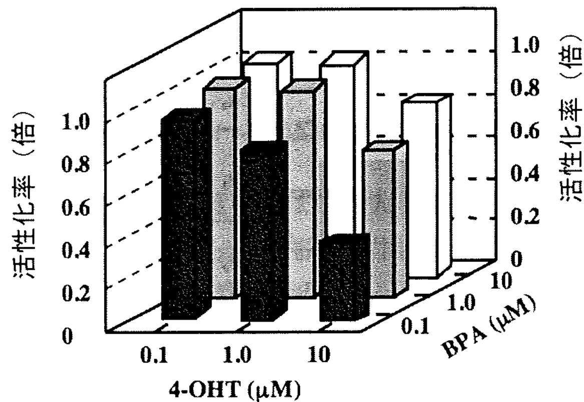


図3 インバースアンタゴニスト活性の測定結果
レポーター遺伝子アッセイでERRγの活性を測定した結果を示す。縦軸は活性の大きさ，横軸はリガンド濃度を示す。4-OHTによりERRγの自発活性が減少するが，ビスフェノールAを加えることで活性が回復した（インバースアンタゴニスト活性）。インバースアンタゴニストは，濃度依存的に作用した。（文献4より改変して引用）

ビスフェノールAは，ERRγに強力に結合するが，ERRγの自発活性に対して何の影響も示さなかった。更に，4-OHTと競合させた場合，4-OHTのインバースアゴニスト活性を顕著に阻害した（図3）。つまり，ビスフェノールAはERRγの「インバースアンタゴニスト」であることが判明した[4),5),7)]。インバースアンタゴニスト活性はこれまでにない概念であり，生理学的な意義については不明である。したがって，現在のところ，ビスフェノールAによる生体への影響，分子機構は想像もつかない。ビスフェノールAの内分泌かく乱作用にERRγが関与しているのか？を含めて，新しい研究視点で世界中の研究機関で解析が始まっている。近い将来，ビスフェノールA問題の全容が明らかにされるものと期待される。

6. おわりに

「受容体科学」の分野では，近年，分子間相互作用を様々な原理に基づく測定から解析しようとする流れがある。BIAcore，SPR，QCM，等々。しかし，筆者らの経験からすると，「受容体に強く結合する化合物を放射標識したものをリガンドとして解析し，これをトレーサとした競争結合試験で結合の構造要因を探り，更に強く結合する化合物を探索し，あるいは分子デザインする」こうした一連の解析に勝るものはないように思われる。受容体結合試験。高価であるが，結果も高価値である。ビスフェノールAのトリチウム標識体は，このように受容体結合試験に活躍できるとは夢にも思っていなかったに違いない。しかし，今，この放射標識化合物こそが，最もホットな化合物なのである。

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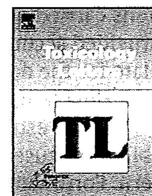

Exploration of endocrine-disrupting chemicals on estrogen receptor α by the agonist/antagonist differential-docking screening (AADS) method: 4-(1-Adamantyl)phenol as a potent endocrine disruptor candidate

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ABSTRACT

We established a novel screening method to survey endocrine-disrupting chemicals by means of *in silico* docking calculations. Endocrine disruptors target the human nuclear receptor, which bind a chemical in a pocket presenting in the ligand-binding domain (LBD). The LBD alters its conformation, depending upon the binding of either agonist or antagonist. We discovered that the chemicals can be differentiated into either agonist or antagonist by the docking calculations of the chemical for the LBD. We used the crystal structures of both agonist-bound LBDs and antagonist-bond LBDs as templates in the docking calculations, and estimated binding energies to discriminate between agonist and antagonist bindings. This agonist/antagonist differential-docking screening (AADS) method predicted, for example, 4-(1-adamantyl)phenol as an agonist of the human estrogen receptor α (hERα). Indeed, this compound, one of the essential raw materials for nanoporous organosilicate thin films, was confirmed to exhibit strong agonist activity in the reporter-gene assay for hERα with a high binding affinity. The AADS method is an approach that appears to foresee both the binding ability and the agonist/antagonist function of chemicals for the target nuclear receptors.

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1. Introduction

Nuclear receptors (NRs) play a central role as transcription factors in biological processes such as cell growth and differentiation, embryonic development, and metabolic homeostasis in metazoan organisms. Most of these NRs directly activate target genes by binding to the DNA response elements in conjunction with the hormone binding specific to the NR's ligand-binding domain (LBD). Mostly, NR-LBDs possess a pocket that is able to capture a hormone or a hormone-like chemical to build up the activation conformation (Germain et al., 2006; Heldring et al., 2007; Olefsky, 2001), while the antagonist disrupts this conformation so that the NR cannot bind the coactivator proteins. This conformation preference or discrimination by a specific agonist/antagonist is a common molecular recognition mechanism of most NRs.

The conformational difference between the agonist-bound LBD and antagonist-bound LBD is primarily described by different positioning of the α-helix No. 12 in the NR-activation function 2 (AF2) in the C-terminal region of LBD (Brzozowski et al., 1997; Shiau et al., 1998). In the activation conformation, the AF-2-α-helix is positioned so that it covers up the ligand-binding pocket (LBP), where the ligand, and especially the agonist, usually docks. By contrast, the antagonist rejects the AF-2-α-helix binding and it pushes away the helix in other locations. It should be noted that this discrimination is definitely due to the molecular size of the antagonist being much larger than that of the agonist. This size difference substantiates the discrepancy between the volumes of agonist-bound LBP and the antagonist-bound LBP, providing different molecular surface reactants necessary for ligand–receptor interactions.

If we could forecast the compounds that would fit into the LBP, it would definitely be advantageous for the design of agonist or antagonist ligands of particular NR. It would also be very helpful to predict which chemicals would bind to NRs and as a result cause serious disruptions in the endocrine system. Knowledge of the structures of agonist-bound and antagonist-bound LBP is certainly beneficial in making these structural predictions, and computational analysis is a powerful methodology for obtaining such knowledge. In fact, there have been many reports of screening procedures for identification of endocrine-disrupting chemicals (Celik et al., 2008; Cui et al., 2009; Fukuzawa et al., 2003, 2005; Hong et al., 2002; István Virága et al., 2005; Nose and Shimohigashi, 2008; Schmieder et al., 2003; Selassie et al., 2003). However, the calcula-

Abbreviations: AADS, the agonist/antagonist differential-docking screening; AF2, activation function 2; *Cpf*, conformation preference factor; EDC, endocrine-disrupting chemical; ERα, estrogen receptor α; LBD, ligand-binding domain; LBP, ligand-binding pocket; NR, nuclear receptor.

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tions carried out as part of the screening process have not always provided accurate results, primarily due to the lack of a good template for the docking calculation. Usually there is not much choice regarding template selection.

Human estrogen receptor α (hERα) is one of 48 NRs, and its ability to cause endocrine disruption has long been a source of concern. Fortunately, the X-ray crystal structures of hERα have been described for complexes with both agonists and antagonists. These prompted us to utilize computational analysis to forecast a compound that would fit the LBP of hERα by using the agonist-bound LBD and the antagonist-bound LBD as templates. In the present study, we carried out the docking and free energy calculations, and established a method called agonist/antagonist differential-docking screening (AADS). We used hERα-LBD crystal structures, two of which were originally coupled with agonists 17β-estradiol (E2) and diethylstilbestrol (DES), respectively, and another two with antagonists raloxifene (RAL) and 4-hydroxytamoxifen (4-OHT), respectively. We here report that the AADS method allowed us to identify 4-(1-adamantyl)phenol as a strong agonist of hERα.

2. Materials and methods

2.1. Materials

E2 and 4-(1-adamantyl)phenol (ADA) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and 4-*n*-octylphenol (nOCT) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-*tert*-Octylphenol (tOCT) and 4-OHT were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals were of the best grade available.

2.2. Docking calculation by AutoDock

The automated docking method was applied to estimation of the appropriate complex structure between the test chemical and the ERα-LBD. Four different hERα-LBD three-dimensional (3D) structures were downloaded from the Protein Data Bank (PDB). These structures included 1ERE (A chain), 1ERR (A chain), 3ERD (A chain), and 3ERT (Brzozowski et al., 1997; Shiau et al., 1998). In order to prepare an appropriate receptor molecule for the docking calculation, missing hydrogen atoms were added in conjunction with the CHARMm force field by Discovery Studio 1.7 (Accelrys, San Diego, CA, USA). For this calculation, the bound ligands were removed from the 3D structures together with water molecules. All hydrogen atoms were energy-minimized using the minimization protocol of Discovery Studio (Adopted Basis NR, Max step = 2000, and Minimization RMS Gradient = 0.01). The vacant volumes of those LBDs were determined by means of the tool called the receptor–ligand interaction included in the Discovery Studio. As for antagonist-bound LBDs, the structural elements of the chemical, which are present outside of the LBP, were not involved in the vacant volume. The 3D structures of the test chemicals were prepared by Discovery Studio. The molecular volumes were also calculated by the Discovery Studio.

The docking calculation was performed using the AutoDock 3.0 software package running on a PowerMac G5 (Apple, Cupertino, CA, USA) to score ligand poses docked with a protein target (Morris et al., 1998). Polar hydrogen atoms were added, and Kollman united atom charges and atomic solvation parameters were assigned to the receptor molecule using AutoDock tools. Complete torsion of ligands was allowed to occur during docking. The grid maps representing the receptor molecule in the docking calculation were generated by the program AutoGrid. Each grid was centered at the LBP of hERα-LBD. The grid dimensions were large enough to cover the LBP and were $60\,Å \times 60\,Å \times 60\,Å$ with a spacing of 0.375 Å between the grid points. The Lennard-Jones parameters 12-10 and 12-6, supplied with the program, were used for modeling H-bonds and van der Waals interactions, respectively. A Lamarckian genetic search algorithm was used for all docking calculations by AutoDock (Morris et al., 1998). The run parameters used in this study were as follows: the number of GA runs was 100, the maximum number of energy evaluations was 2.5×10^6, and the maximum number of generations was 1.0×10^5. Other parameters were represented by default values implemented by the program.

2.3. Estrogen receptor binding assay

The estrogen receptor binding assay using purified hERα-LBD was carried out by the previously reported method (Nakai et al., 1999). Varied concentrations (1×10^{-11} to 1×10^{-4} M) of sample solutions were mixed with the recombinant hERα-LBD, and the mixture solution was incubated for 1 h at 25 °C. After removal of free radioligands, radioactivity was determined on a liquid scintillation counter (TopCount NXT; PerkinElmer Life Sciences, Boston, MA, USA). The IC_{50} values (the concentrations for the half-maximal inhibition) were calculated from the dose–response curves obtained using the nonlinear analysis program ALLFIT (DeLean et al., 1978).

2.4. Luciferase reporter-gene assay

The reporter-gene assay was carried out essentially as described previously (Liu et al., 2007; Takayanagi et al., 2006). The ERα response element (ERE)-luciferase construct was generated and ligated into pGL3-Luc (Promega, Madison, WI, USA). Both the hERα expression and luciferase reporter plasmids were transiently transfected in HeLa cells, which were exposed to various concentrations of test chemicals to detect agonist activity. The antagonist activity was determined against E2.

3. Results and discussion

3.1. The docking screening of agonist E2 for the activation and inactivation conformations

The postulated procedure, the agonist/antagonist differential-docking screening (AADS) method, was first verified for known ligands of hERα. This procedure predicts the agonist or antagonist activity of small molecules from their binding energy, which is calculated by the docking program named AutoDock 3.0 in binding to a receptor with known 3D structure. We tested the ERα receptor structures determined as complexes with E2, DES, RAL, and 4-OHT (Fig. 1). These X-ray crystal structures are registered in PDB as 1ERE, 3ERD, 1ERR, and 3ERT, respectively. E2 and DES are agonists of hERα, while RAL and 4-OHT are antagonists.

In the present study, we first prepared the 3D structures of these compounds independently using the Discovery Studio computer program. The 3D structures of hERα with no ligand, namely 1ERE-desE2, 3ERD-desDES, 1ERR-desRAL, and 3ERT-des4-OHT, were utilized individually as templates for the docking calculations. Using the computer program AutoDock 3.0, the natural ligand E2 was evaluated for its ability to bind to the hERα-LBD templates. When E2 was docked with the template 1ERE-desE2, the complex afforded almost the same conformation as 1ERE itself. As shown in Fig. 2A, docked E2 (blue) is calculated to be located close to the native E2 (red). In these complexes, E2-phenol-hydroxyl group is hydrogen-bonded to Arg394 and Glu353. The program predicted the binding energy as −10.68 kcal/mol (Table 1).

When E2 was next docked with the template 3ERD-desDES, the complex also afforded a conformation similar to that of 1ERE (Orange, Fig. 2B). The E2-phenol-hydroxy group was hydrogen-bonded to Arg394 and Glu353 of hER-LBD derived from 3ERD-desDES. The binding energy for 3ERD-desDES was −10.90 kcal/mol (Table 1), which was somewhat smaller (ca. 0.22 kcal/mol) than that calculated for 1ERE-desE2. This difference is due to the water molecules, which are usually found in the LBP, being ignored in the docking calculation.

The structures of 1ERE and 3ERD represent the activation conformation induced by agonist binding, while those of 1ERR and 3ERT characterize the inactivation conformation with antagonist binding (Brzozowski et al., 1997; Shiau et al., 1998). When 1ERR-desRAL and 3ERT-des4-OHT were docked with E2, the binding energy was calculated to be −10.06 and −9.77 kcal/mol (Table 1). The results clearly show that the agonist E2 is much more stable in the activation conformation than in the inactivation conformation.

The average binding energy of E2 in the activation conformation was −10.79 kcal/mol (Table 1), while that in the inactivation conformation was −9.92 kcal/mol. The difference between these average energies was −0.87 kcal/mol, indicating that E2 is 0.87 kcal/mol more stable in the activation conformations 1ERE-desE2 and 3ERD-desDES than in the inactivation conformations 1ERR-desRAL and 3ERT-des4-OHT.

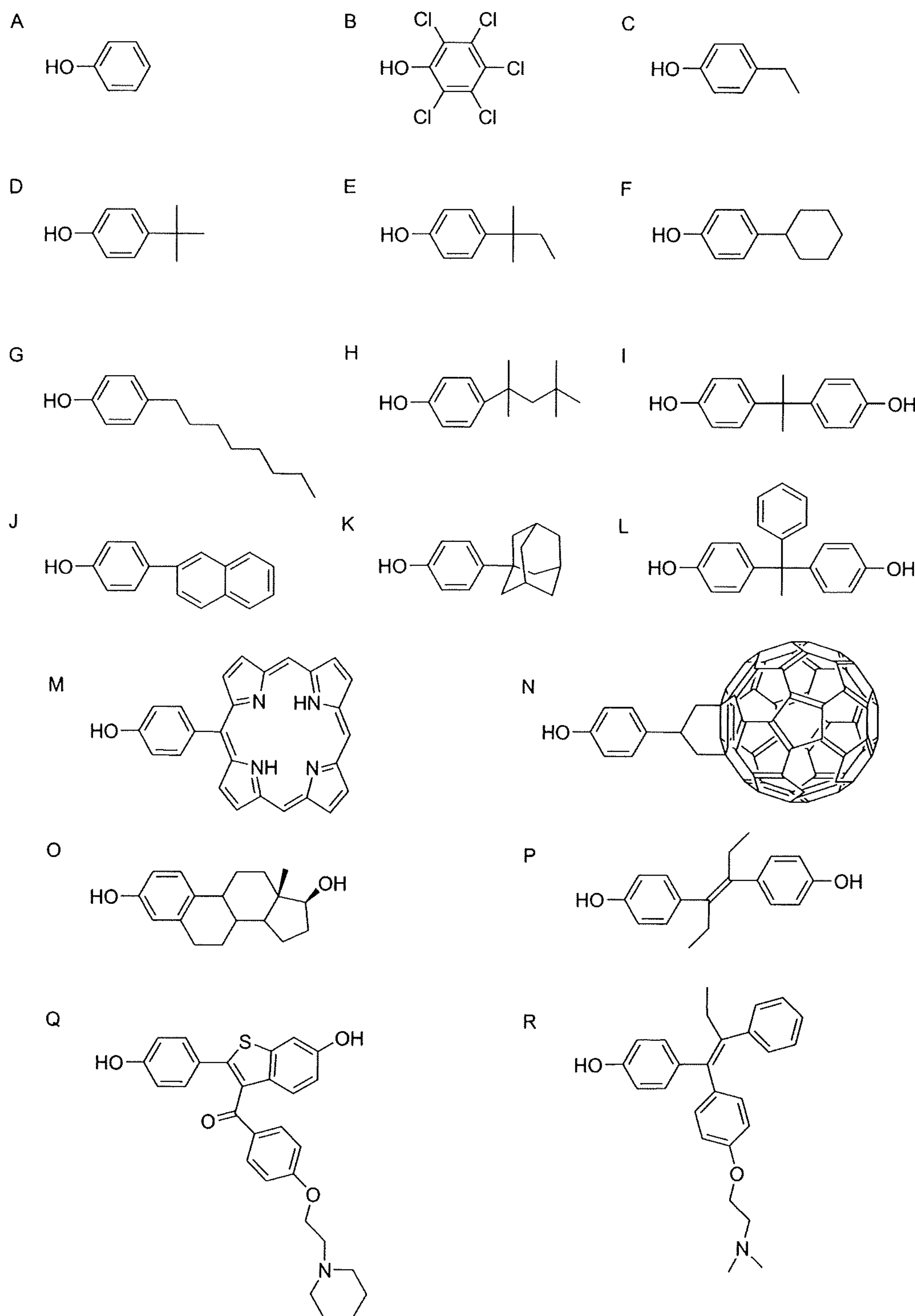

Fig. 1. Chemical structures of the phenol-related derivatives and ER-ligands used in this study. (A) Phenol, (B) pentachlorophenol (PCP), (C) 4-ethylphenol (ETP), (D) 4-*tert*-butylphenol (tBUT), (E) 4-*tert*-amylphenol (tAMY), (F) 4-cyclohexylphenol (CYC), (G) 4-*n*-octylphenol (nOCT), (H) 4-*tert*-octylphenol (tOCT), (I) bisphenol A (BPA), (J) 4-(2-naphthyl)phenol (NAP), (K) 4-(1-adamantyl)phenol (ADA), (L) bisphenol AP (BPAP), (M) 10-(4-hydroxyphenyl)porphyrin (POR), (N) C_{60}-conjugated phenol (C60), (O) 17β-estradiol (E2), (P) dietylstilbestrol (DES), (Q) raloxifene (RAL), and (R) 4-hydroxytamoxifen (4-OHT). Abbreviations of chemicals are denoted in parentheses.

3.2. Screening of DES, RAL, and 4-OHT for the activation and inactivation conformations

When DES was calculated for 1ERE-desE2 and 3ERD-desDES in the activation conformation, the binding energies were −8.95 and −9.39 kcal/mol, respectively (Table 1). The average binding energy was −9.17 kcal/mol. In contrast, those for 1ERR-desRAL and 3ERT-des4-OHT in the inactivation conformation were −8.66 and −8.91 kcal/mol, respectively, with the average binding energy being −8.79 kcal/mol (Table 1). It should be noted that −8.95 kcal/mol for 1ERE-desE2 and −8.91 kcal/mol for 3ERT-des4-OHT are almost equal to each other. The difference in the

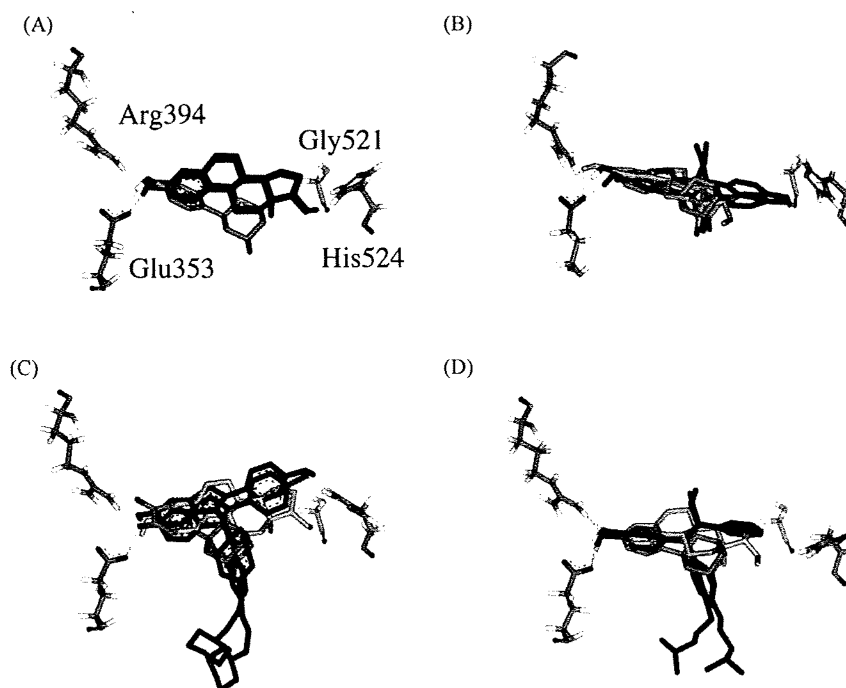

Fig. 2. Results of the docking calculations on four different ERα-LBDs. The docking templates used in this study were 1ERE-desE2 (A), 3ERD-desDES (B), 1ERR-desRAL (C), and 3ERT-des4-OHT (D), respectively. Amino acid residues (Arg394, Glu353, Gly521, and His524) that showed hydrogen-bonding (green lines) with docked ligands are shown. Ligands originally found in complexes, (A) E2, (B) DES, (C) RAL, and (D) 4-OHT, are depicted in red and the calculated ones in blue. Calculated E2 in the models (B), (C), and (D) is colored in orange. The root mean square deviation (RMSD) values of heavy atoms between the molecules calculated by AutoDock and the molecule determined by X-ray crystallography were as follows: (A) 0.74 Å, (B) 1.41 Å, (C) 1.47 Å, and (D) 1.65 Å. 4-(1-adamantyl)phenol is shown as a stick model with atomic color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

average binding energies was −0.38 kcal/mol, indicating that DES is 0.38 kcal/mol more stable in the activation conformation than in the inactivation conformation. The partial similarity of molecular structures between DES and the antagonists RAL and 4-OHT might explain these results (Fig. 1).

Antagonists RAL and 4-OHT were also evaluated for their stability in the activation and inactivation conformations. RAL was demonstrated to be 0.79 kcal/mol more stable in the inactivation conformation (the average binding energy = −11.86 kcal/mol) than in the activation conformation (−11.07 kcal/mol) (Table 1). 4-OHT

Table 1
Docking of phenol derivatives and ER-ligands into the ERα-LBP.

Chemicals	Estimated free energy of binding (kcal/mol)						ΔG[a]	$\Delta\Delta G$[b] (*Cpf*)[c]
	Agonist-bound receptor			Antagonist-bound receptor				
	1ERE	3ERD	Av.	1ERR	3ERT	Av.		
Agonist								
E2	−10.68	**−10.90**	−10.79	−10.06	−9.77	−9.92	−10.35	−0.87
DES	−8.95	**−9.39**	−9.17	−8.66	−8.91	−8.79	−8.98	−0.38
Antagonist								
RAL	−11.28	−10.86	−11.07	**−13.25**	−10.47	−11.86	−11.47	+0.79
4-OHT	−7.23	−8.50	−7.87	−10.54	**−10.77**	−10.66	−9.26	+2.79
Test chemicals								
Phenol	−4.16	**−4.54**	−4.35	−4.27	−4.18	−4.23	−4.29	−0.12
PCP	−6.13	**−6.48**	−6.31	−6.10	−5.33	−5.72	−6.01	−0.59
ETP	−4.99	**−5.29**	−5.14	N.D.	−4.75	−4.75	−4.95	−0.39
tBUT	−5.69	**−6.38**	−6.04	−5.55	N.D.	−5.55	−5.80	−0.49
tAMY	−5.86	**−6.53**	−6.20	−5.69	−5.57	−5.63	−5.92	−0.57
CYC	−6.93	**−7.27**	−7.10	−6.75	−6.48	−6.62	−6.86	−0.48
nOCT	−6.79	**−7.05**	−6.92	−6.77	−6.69	−6.73	−6.83	−0.19
tOCT	−7.45	**−7.91**	−7.68	−6.86	−6.77	−6.82	−7.25	−0.86
BPA	−7.37	**−7.45**	−7.41	−7.00	−7.10	−7.05	−7.23	−0.36
NAP	−7.63	**−8.25**	−7.94	−7.59	−7.30	−7.45	−7.69	−0.49
ADA	−8.96	**−9.50**	−9.23	−8.46	−8.43	−8.45	−8.84	−0.78
BPAP	**−8.10**	−7.02	−7.56	−7.85	−7.57	−7.71	−7.64	+0.15
POR	+5.27	+13.7	+9.49	**+4.05**	N.D.	+4.05	+7.68	−5.44
C60	>	>	–	>	>	–	>	–

N.D.: not determined. The docked chemical did not find in the LBP. Bold means the best score for each chemical in the four different calculations. –: not calculated; >: larger than +100 kcal/mol.

[a] ΔG: average of binding energies from four docking calculations.

[b] $\Delta\Delta G$: difference between average energies from the calculations used by agonist-bound receptor and antagonist-bound receptor.

[c] *Cpf*: conformation preference factor.

Table 2
Calculated molecular volume of ER-ligands and phenol derivatives.

Chemicals	Molecular volume (Å^3)	Relative volume (%)
Agonist		
E2 (17β-estradiol)	229.1	(100)
DES (diethylstilbestrol)	220.4	96.2
Antagonist		
4-OHT (4-hydroxytamoxifen)	319.9	140
RAL (raloxifene)	355.0	155
Test chemicals		
Phenol	75.7	33
PCP (pentachlorophenol)	155.3	67.8
ETP (4-ethylphenol)	104.4	45.6
tBUT (4-*tert*-butylphenol)	133.5	58.3
tAMY (4-*tert*-amylphenol)	148.0	64.6
CYC (4-cyclohexylphenol)	155.1	67.7
nOCT (4-*n*-octylphenol)	191.1	83.4
tOCT (4-*tert*-octylphenol)	191.5	83.6
BPA (bisphenol A)	187.6	81.9
NAP (4-(2-naphthyl)phenol)	176.2	76.9
ADA (4-(1-adamantyl)phenol)	198.7	86.7
BPAP (bisphenol AP)	235.2	103
POR (10-(4-hydroxyphenyl)porphyrin)	304.9	133
C60 (C_{60}-conjugated phenol)	581.6	254

Relative volume: molecular volume of E2 was used as the standard (100%).

was also judged to be 2.79 kcal/mol more stable in the inactivation conformation (−10.66 kcal/mol) than in the activation conformation (−7.87 kcal/mol) (Table 1). In conclusion, antagonists RAL and 4-OHT are more stable in the inactivation conformation.

The results for agonists E2 and DES show that they prefer to bind into the activation conformation of ERα-LBD. In contrast, the antagonists RAL and 4-OHT prefer the inactivation conformation. Such a feature for agonists or antagonists was also identified based on the best estimated free energy of binding score for each test chemical (Table 1). In addition to the four LBDs used here, if we could use the LBD structure, which best fits each test chemical, in the docking calculation, the calculation will provide the real best score to indicate both the binding potency and the biological activity of the chemical.

As to agonists and antagonists, what is the distinction between the two in hERα-LBDs? First, it was expected that the size of the LBPs must differ greatly, and the vacant volumes of the LBPs were found to vary considerably. The estimated volumes were 455 Å^3 for 1ERE-desE2, 339 Å^3 for 3ERD-desDES, 417 Å^3 for 1ERR-desRAL, and 468 Å^3 for 3ERT-des4-OHT. By contrast, the calculated molecular volumes of the ER-ligands were considerably different: 220–230 Å^3 for the agonists E2 and DES, while 320–355 Å^3 for the antagonists RAL and 4-OHT (Table 2). Antagonists are approximately 50% bigger than agonists.

3.3. The agonist/antagonist differential-docking screening (AADS) method

Agonists E2 and DES were found to be more stable in the activation conformation, while antagonists RAL and 4-OHT were more stable in the inactivation conformation. Antagonists (320–355 Å^3) were found to be approximately 50% bigger than agonists (220–230 Å^3). If these two issues, namely the suitable receptor conformation and the molecular size of chemicals, could be illustrated appropriately, differentiation between agonist and antagonist would become feasible. To this end, we defined the parameter as agonist/antagonist factor *Cpf* for the conformation preference.

Agonists prefer to bind the templates of agonist-bound LBD, and antagonists prefer to bind the templates of antagonist-bound LBD. Such preferences are thought to be a parameter to help identify the biological activity of ligands. To set a parameter to determine the agonist/antagonist specificity, the factor ($Cpf = \Delta\Delta G$) was defined as follows:

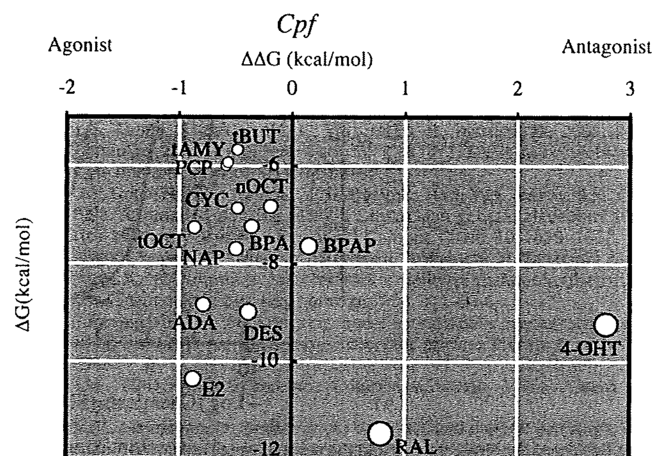

Fig. 3. Plotting analysis of the AADS method. ΔG versus ΔΔG plotting analysis. The agonist/antagonist factor (ΔΔG) was calculated as the difference between average energies from each docking calculation used by agonist-bound receptor and antagonist-bound receptor. The size of each plot corresponds to the relative molecular size of the test chemical, as listed in Table 2.

$$Cpf = \Delta G(\text{activation conformation}) - \Delta G(\text{inactivation conformation}) \quad (1)$$

Here ΔG(activation conformation) is the average ΔG value from the docking calculations using agonist-bound hERα-LBD (1ERE and 3ERD) as templates. ΔG(inactivation conformation) is the average ΔG value from the docking calculations using antagonist-bound hERα-LBD (1ERR and 3ERT) as templates.

When the total average binding energy (ΔG) and agonist/antagonist factor ($Cpf = \Delta\Delta G$) of E2, DES, 4-OHT, and RAL were plotted (vertical, ΔG; horizontal, *Cpf*), it was clearly shown that the former two compounds, E2 and DES, are in the agonist area and the latter two, 4-OHT and RAL, are in the antagonist area (Fig. 3). It is clear that E2 is the best agonist with regard to both the binding energy and the agonist/antagonist preference. In contrast, among the antagonists, RAL exhibits the largest binding energy, while 4-OHT exhibits the biggest antagonist preference (Fig. 3). When the absolute values of agonist/antagonist factor were compared, 4-OHT exhibited the largest value and DES the smallest.

In the figure, the size of the molecules is indicated by their circle size (Fig. 3). It should be noted that the plots in the antagonists area are larger than those in the agonist area, indicating that ER-antagonists are much larger than the ER-agonists. We have called this line of analysis agonist/antagonist differential-docking screening (AADS). All the results obtained here imply that the AADS plotting analysis represents specific characteristics of chemicals, especially with regard to their reactivity to the nuclear receptor ER.

3.4. The AADS method for test compounds

In order to evaluate the ease of use of the AADS method, we tested a series of chemicals, 14 in total, as listed in Table 1. The average binding energies of the chemicals were obtained from the results of the docking calculations using four LBDs. Thus, the total average binding energy (ΔG) and the agonist/antagonist factor ($Cpf = \Delta\Delta G$) were estimated as in Table 1. The calculated free energies of binding (ΔG) of ER-ligands ranged from −4.29 to −8.84 kcal/mol. Apparently, the value for 10-(4-hydroxyphenyl)porphyrin (POR) cannot be corroborated because

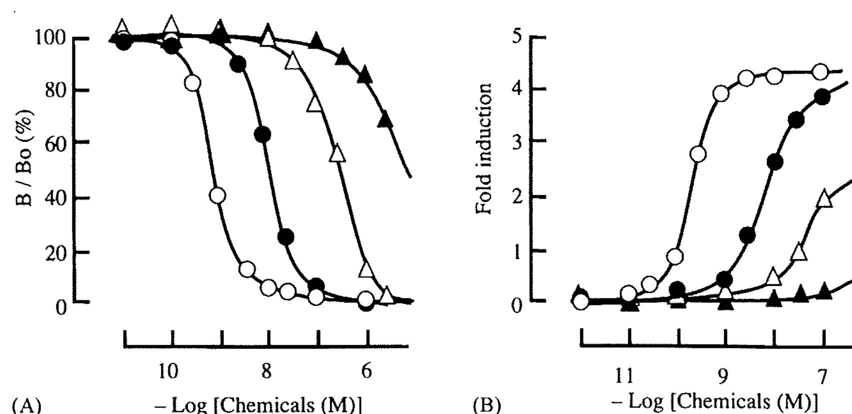

Fig. 4. Results of the binding assay and the reporter-gene assay. (A) Binding assay using purified hERα-LBD. (B) Reporter-gene assay. E2 (open circle), 4-(1-adamantyl)phenol (closed circle), 4-*tert*-octylphenol (open triangle), and 4-*n*-octylphenol (closed triangle). Data are presented as the mean from one representative experiment.

of its extreme instability in docking to any of the hERα-LBDs. This instability was further enhanced by C_{60}-conjugated phenol (C60), affording no convergent values (Table 1).

Among the 12 remaining compounds for which ΔG values were estimated, there was only one putative antagonist (Table 1 and Fig. 3). Because of its minus $\Delta\Delta G$ value, it is possible that BPAP has antagonist-like activity. However, as BPAP shows the best score against the agonist-form LBD, 1ERE, it may not work as an antagonist. On the other hand, we identified 4-(1-adamantyl)phenol (ADA) as a relatively strong agonist for hERα. ADA attained a binding energy (−8.84 kcal/mol) greater than that of the other test chemicals. The AADS method predicted that ADA is almost as active as DES (Table 1). In the calculated hERα-LBD–ADA complexes, the ADA-hydroxy group was found to form hydrogen bonds with Glu353 and Arg394 of hERα-LBD (Fig. 2). As shown in Fig. 3, ADA definitely excels with regard to both its binding energy and its agonist conformation preference.

3.5. Binding assay and reporter-gene assays for 4-(1-adamantyl)phenol (ADA)

By using the AADS calculation, 4-(1-adamantyl)phenol, ADA, was estimated as a putative ERα agonist. To confirm its binding and biological activities by other assays, we conducted the receptor binding assay and the luciferase reporter-gene assay. In the binding assay, ADA exhibited the most potent binding activity among the test chemicals used here, except for the known ERα-ligands, E2, DES, RAL, and 4-OHT (data not shown). Therefore, ADA was carefully tested in the binding and reporter-gene assays, and the results were compared with those for the agonist (E2), the antagonist (4-OHT), the weak ligand (*tert*-octylphenol), and the very weak ligand (*n*-octylphenol).

As shown in Fig. 4A, ADA showed a clear dose-dependent binding curve in the assay for hERα-LBD. Although ADA was approximately 60-fold weaker than the native agonist E2, its receptor binding activity (IC_{50} = 82.3 nM) was not as bad as a ligand of the receptor (Table 3). As a result, the prediction value of the AADS method was actually substantiated by the *in vitro* assay carried out to examine the receptor binding ability.

We next carried out the reporter-gene assay to determine the reporter activation potency of the chemicals. In this assay, ADA exhibited full agonist activity (Fig. 4B) as well as E2. When the concentration necessary to estimate full maximal activation, namely the EC_{50} value, was calculated, ADA exhibited significantly high activity at a concentration of 9.0 nM (Table 3). Indeed, ADA functioned as an agonist, as reported independently by Endo et al. (1999) for rat ERα. ADA is expected to be an excellent raw material for items such as nanoporous organosilicate thin films, and one should therefore be cautious with regard to its potential leakage (Cha et al., 2006). It is now clear that the AADS method can predict or foresee the activity of chemicals for human ERα by calculating the binding energy in the *in silico* docking modeling.

Table 3
Results of the competitive binding assay on ERα and biological activity through ERα activation by means of the luciferase reporter-gene assay of alkylphenols.

Chemicals	Binding assay IC_{50} (nM)	Reporter-gene assay EC_{50} (nM)
nOCT	N.D.	N.D.
tOCT	884 ± 175	33.3 ± 8.68
ADA	82.3 ± 12.4	9.00 ± 3.01
E2	1.40 ± 0.322	0.279 ± 0.035
4-OHT	0.720 ± 0.266	Inactive[a]

N.D.: not determined.
[a] 4-OHT exhibited antagonist activity in this assay.

tert-Octylphenol (tOCT) is a weak binder of hERα, while nOCT is almost completely inactive for hERα (Tabira et al., 1999). tOCT is intrinsically active in various biological assays, while nOCT is almost completely inactive in those assays (Miyawaki et al., 2008). Similar results were obtained in the present study, as shown in Fig. 4 and Table 3.

In conclusion, it should be noted that these results were well-predicted by the AADS method, as shown in Fig. 3. This procedure will provide a general method by using the LBD of each nuclear receptor as a template. It simultaneously provides the parameters of ΔG (binding potency) and *Cpf* ($\Delta\Delta G$, conformation preference factor), which can lead to prediction of the latent capabilities of EDCs.

Conflict of interest statement

None declared.

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ERα/ERRα Nuclear Receptor Heterodimer Directly Linked by A Flag Peptide

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It has recently been suggested that, in the tissues where both ERα and ERRα are present together, these nuclear receptors form a heterodimer to function specifically for characteristic physiological role with some efficacy. In the present study, we prepared expression vectors, which produce a recombinant protein of human ERα and ERRα cDNAs cross-linked directly with the FLAG peptide (DYKDDDDK). It was found that ERα in this heterodimer retains the ordinary characteristics of free ERα.

Keywords: estrogen receptor (ER), estrogen-related receptor (ERR), heterodimer, FLAG peptide, nuclear receptor

Introduction

Estrogen receptor α (ERα) and estrogen-related receptor α (ERRα) belong to steroid hormone receptors, or group III of nuclear receptor superfamily. These nuclear receptors function as a transcription factor, binding to their response element present in their target genes and facilitating their transcriptional role in the mRNA biosynthesis. ERRα binds to the ERR-response element (ERRE) as a monomer. ERRα also has an ability to form a homodimer for binding to functional estrogen response elements (EREs) in ER target genes. On the other hand, ERα can bind to ERRE *in vitro* as a dimer. These suggest that the transcriptional activation functions of ERRα and ERα must be overlapped *in vivo* [1].

There are some tissues where both ERα and ERRα are present together. In those tissues, ERα and ERRα have been suggested to form a heterodimer to function specifically for a certain characteristic physiological efficacy. In order to clarify such a dimer-specific effectiveness, it is essential to prepare a concrete heterodimer between ERα and ERRα in the cell nucleus. When they were merely co-expressed, homodimers of each ERα and ERRα would be produced simultaneously. Thus, in the present study, we prepared a gene construct bearing these two receptor proteins successively in a single chain.

Results and Discussion

Since the nuclear receptor protein biosynthesized should be transported from cytoplasm to nucleus to function as a transcription factor, it is necessary to ensure the synthesis of heterodimer and the transcriptional activity of each unit of nuclear

receptors. To this end, we prepared two expression vectors in which human ERα and ERRα cDNAs are cross-linked together via cDNA of FLAG peptide (DYKDDDDK), producing the cDNA conjugates ERα-FLAG-ERRα and ERRα-FLAG-ERα. These vectors were transiently transfected into the HeLa cells, and the expression of dimer protein was confirmed by the Western blotting analysis using anti-FLAG antibody. Intracellular localization of the heterodimers was also ascertained by fluorescence microscopy observation using anti-FLAG antibody. The heterodimers as well as ERα and ERRα were found both in the cytoplasm and nucleus (Fig. 1).

Functional activities of receptors were evaluated by the binding assay for specific ligand interaction and the luciferase reporter gene assay for biological activity. We first examined ERα by using its endogenous agonist ligand 17β-estradiol (E2). In the radio-labeled receptor binding assay, [^{3}H]E2 was found to bind strongly to the heterodimers as in the case of ERα, with the K_D value of about 2 nM (Fig. 2). We also analyzed the transcriptional activity of these heterodimers against ERE or ERRE by reporter gene assays. Transcriptional ability of the heterodimers was found to increase by addition of E2 in a dose-dependent manner. These results indicated that ERα in this heterodimer retains its original characteristics of free ERα. ERα in the heterodimer is probably the influencer of ERRα.

Fig. 1. Intracelluar localization of the nuclear receptor monomers and their heterodimers. ERα (A), ERRα (B), ERα-FLAG-ERRα (C), and ERRα-FLAG-ERα (D). All the receptors were stained by the indirect immunofluorescence method using fluorescein isothiocyanate (FITC)-conjugated secondary antibody with anti-FLAG antibody.

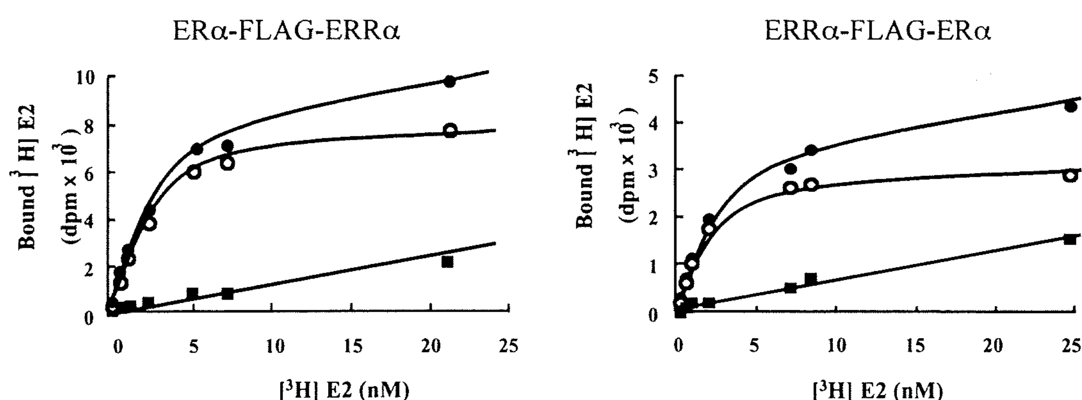

Fig. 2. The results of saturation binding assay for the nuclear receptor heterodimers.

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Induced-fit Type Ligand Binding Guided by Free-rotatory Leu Residue Present in the 7th α-Helix Peptide in the Estrogen-related Receptor γ (ERRγ)

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4-α-Cumylphenol, which lacks an OH group from endocrine disruptor bisphenol A (BPA), binds to ERRγ very strongly and specifically. X-ray crystal structural analysis of the 4-α-cumylphenol/ERRγ complex was performed and the structure was compared in detail with the BPA/ERRγ complex. The result clearly indicated that the strong binding of 4-α-cumylphenol is due to the structural flexibility of Leu-side chain at position 345, resulting in a formation of the ligand binding site in an induced-fit manner.

Keywords: bisphenol A, nuclear receptor, X-ray crystal structural analysis

Introduction

Estrogen-related receptor γ (ERRγ) is an orphan nuclear receptor that belongs to the steroid hormone receptor family. This family contains the estrogen receptors α and β, and androgen receptor, considered to be the main targets of endocrine disruptors. Bisphenol A (BPA) has long been recognized as an estrogenic endocrine disruptor, but BPA binds to estrogen receptor very weakly. We have recently discovered that BPA binds to another nuclear receptor named ERRγ very strongly and specifically [1, 2].

Almost all of the nuclear receptors have a unique ligand binding domain, in which the ligand binding pocket is constructed with 12 α-helices (H1-H12) and 2 β-strands in a sheet (S1). These helices play extremely important role to select a specific ligand for the receptor activation. BPA was found to assemble the amino acid residues from H3, H5, H7, H11, H12, and S1 of ERRγ. BPA binds to ERRγ strongly because of the formation of three hydrogen bonds between the phenol-OH groups of BPA and Glu275 from H3, Arg316 from H5, and also Asn346 from H7, respectively. More recently, we found that, in spite of the lack of one of the two BPA-phenol-OH groups, 4-α-cumylphenol exhibits a strong binding ability to ERRγ as well as BPA [2]. In this study, we performed the X-ray crystal structural analysis of 4-α-cumylphenol/ERRγ complex, and compared in detail with the BPA/ERRγ complex.