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Inverse antagonist activities of parabens on human oestrogen-related receptor γ (ERRγ): *In vitro* and *in silico* studies

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ABSTRACT

Parabens are *p*-hydroxybenzoic acid esters that have been used extensively as preservatives in foods, cosmetics, drugs and toiletries. These intact esters are commonly detected in human breast cancer tissues and other human samples, thus arousing concern about the involvement of parabens in human breast cancer. In this study, an *in vitro* nuclear receptor coactivator recruiting assay was developed and used to evaluate the binding activities of parabens, salicylates and benzoates *via* antagonist competitive binding on the human oestrogen-related receptor γ (ERRγ), which is known as both a diagnostic biomarker and a treatment target of breast cancer. The results showed that all of the test parabens (methyl-, ethyl-, propyl-, butyl- and benzylparaben) possessed clear inverse antagonist activities on ERRγ, with a lowest observed effect level (LOEL) of 10^{-7} M and the 50% relative effective concentrations (REC50) varying from 3.09×10^{-7} to 5.88×10^{-7} M, whereas the salicylates possessed much lower activities and the benzoates showed no obvious activity. *In silico* molecular docking analyses showed that parabens fitted well into the active site of ERRγ, with hydrogen bonds forming between the *p*-hydroxyl group of parabens and the Glu275/Arg316 of ERRγ. As the paraben levels reported in breast cancer tissues are commonly higher than the LOELs observed in this study, parabens may play some role *via* ERRγ in the carcinogenesis of human breast cancer. In addition, parabens may have significant effects on breast cancer patients who are taking tamoxifen, as ERRγ is regarded as a treatment target for tamoxifen.

Introduction

Parabens are a homologous series of *p*-hydroxybenzoic acid esters that differ in the ester group. They have been proven to be very effective antimicrobial agents, and are used extensively, whether singly or in combination, as preservatives in foods, cosmetics, drugs and toiletries. In 1974, the FAO/WHO Joint Expert Committee on Food Additives (JECFA, 1974) allocated an acceptable daily intake of 0–10 mg/kg bw (milligrams/kilogram body weight) sum total of methylparaben (MP), ethylparaben (EP) and propylparaben (PP). Over the years, paraben use has steadily increased to include more food categories such as processed vegetables, baked goods, fats and oils, seasonings, sugar substitutes, coffee extracts, fruit juices, pickles, sauces, soft drinks and frozen dairy products at concentrations between 450 and 2000 ppm (Daniel, 1986; Soni et al., 2005). Parabens have routinely functioned as preservatives in cosmetics for several decades and are reportedly used in over 13,200 formulations (Elder, 1984). Rastogi et al. (1995) examined the contents of parabens in various cosmetic products and reported a preferential use of MP, EP, PP, butylparaben (BuP) and benzylparaben (BzP). The European Union has allowed the use of parabens and their salts in cosmetic products at a maximum concentration of 0.4% each and a total maximum concentration of 0.8% (EU Cosmetics directive 76/768/EEC), and it has recently recommended a reduction in the sum of PP and BuP to a maximum concentration of 0.19% in the review of SCCS/1348/10.

Studies have recently raised concerns about the potential role of parabens in breast cancer, as these chemicals are commonly detectable in human breast cancer tissues. An analysis of 160 breast tissue samples collected from 40 mastectomies for primary breast cancer in England showed that at least one intact paraben ester was detectable in virtually all of the samples (99%), and that all five paraben esters investigated were detectable in 60% of the samples, with a total median value of 85.5 (range 0–5134.5) ng/g of tissue for all five parabens (Barr et al., 2012). Another study also showed detectable intact parabens in 20 human breast cancer tissue samples collected in Scotland at an average of 20.6 ± 4.2 ng/g of tissue (Darbre et al., 2004). Parabens are also commonly detectable in general populations. A recent analysis of urine samples from the US general population showed that MP, PP and BuP were measurable in 99.9%, 98.3% and 73.6% of women and 99.3%, 90.2% and 35.9% of men, respectively (Smith et al., 2012). Studies have similarly shown the presence of parabens in 98% of urine samples from Danish men (Frederiksen et al., 2011) and in 100% of urine samples from pregnant women and children in Spain (Casas et al., 2011).

The toxicology of parabens and their role as endocrine-disrupting chemicals have been documented in recent years. The antiandrogenic properties and spermatotoxic effects of PP and BuP were reported

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by Satoh et al. (2005) and Oishi (2001, 2002a,b), respectively. The oestrogenic properties of parabens have aroused high concern since 1998, generating debate over the role of parabens in the increased incidence of breast cancer (Darbre and Harvey, 2008; Routledge et al., 1998; Soni et al., 2005). *In vitro* and *in vivo* assays have since shown that all of the widely used parabens possess oestrogenic properties (Darbre and Harvey, 2008). Because oestrogen is known to play a central role in the development, growth and progression of breast cancer (Miller, 1996), some researchers argue that parabens are involved in its increased incidence. Others, however, maintain that the oestrogenic activities of parabens are too weak to have a significant effect, with the most potent (BuP) being 10,000 times less potent than 17β-estradiol (E_2) (Golden and Gandy, 2004; Golden et al., 2005; Routledge et al., 1998). Using expression microarrays, studies have shown some similarities in the global gene expression patterns of parabens and E_2 in MCF-7 human breast cancer cells (Pugazhendhi et al., 2007; Terasaka et al., 2006). However, it has also been noted that the majority of genes, whether up- or down-regulated, do not follow the same pattern of regulation under parabens and E_2 treatments (Pugazhendhi et al., 2007). This raises the question of whether parabens use other pathways in the regulation of gene expression. Darbre and Harvey (2008) reviewed more than 150 studies on paraben toxicology and observed that 'whether the parabens can also bind to the oestrogen-related receptors (ERRs) will be important in the light of the recent description of the binding of bisphenol A (BPA) to human oestrogen-related receptor-γ (ERRγ)'. ERRs are a subfamily of orphan nuclear receptors most closely related to oestrogen receptors (ERs), with which they share target genes, coregulatory proteins, ligands and sites of action (Giguere, 2002). Oestrogen-related receptor-γ (ERRγ) is a member of the ERR subfamily. A recent study reported that ERRγ could be detected in 79% of human invasive breast cancer cases, and that it may play an important role as a modulator of oestrogen signalling in breast cancer cells (Ijichi et al., 2011). Some earlier studies indicated that ERRγ may be a biomarker of the development of hormone-sensitive breast cancer and a breast cancer treatment target for tamoxifen (Ariazi et al., 2002; Coward et al., 2001). In a recent study on uterine endometrial cancer, Yamamoto et al. (2012) showed that a selective ERRγ agonist, DY131, inhibited the growth of ERα-positive cancer cells but promoted that of ERα-negative cancer cells. Therefore, clarification of the binding activities of parabens on ERRγ is necessary to better understand their role in breast cancer.

Materials and methods

Chemicals

The compounds 17β-estradiol (>98%, E_2), bisphenol A (>98%, BPA), 4-hydroxy-tamoxifen (>98%, 4-OH-TAM), daidzein (>98%), genistein (>98%) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MP (>99%), EP (>99%) and PP (>99%) were obtained from Hengye Zhongyuan Chemical Co (Beijing, China). The BuP (>99%), methyl benzoate (>99%, MB), ethyl benzoate (>99%, EB), methyl salicylate (>99.5%, MS) and ethyl salicylate (>99%, ES) were purchased from Aladdin Chemistry Co Ltd. (Shanghai, China). The BzP (>98%) was obtained from Tokyo Kasei Kogyo Co Ltd. (Tokyo, Japan). All of the chemicals and reagents used in this study were of molecular biology grade unless otherwise described.

Methods

Plasmid construction and glutathione S-transferase (GST)–human ERRγ LBD protein expression. A cDNA fragment encoding human ERRγ LBD (residues 184–435) was generated by reverse transcription polymerase chain reaction (RT-PCR) using mRNA from an OVCAR-3 ovarian cancer cell. The primer sets used for amplification were as follows: forward primer, 5′-CCGGAATTCCGGCAGAAGTACAAGCG-3′ and reverse primer, 5′-CCGCTCGAGTTAGTCAGACCTTGGCCTCCAA-3′. The entire sequence of the amplified fragment was confirmed by sequencing after subcloning into a pMD18-T vector (Takara, Dalian, China). The confirmed sequence of human ERRγ LBD was digested with *Eco*RI and *Xho*I and ligated into the *Eco*RI–*Xho*I sites of a pGEX-4T-1 glutathione S-transferase (GST) expression vector (Amersham Bioscience, Uppsala, Sweden). The GST/ERRγ LBD fusion gene was confirmed by sequencing and the resulting plasmid was designated as pGEX-4T1-GST–ERRγ LBD (Fig. 1A). The GST–ERRγ LBD fusion protein was expressed in *Escherichia coli* BL21 (Chaperone Competent Cells BL21). Purification of the GST–ERRγ LBD fusion protein was performed according to the standard procedure (Amersham Bioscience, Uppsala, Sweden). Briefly, the bacteria were grown at 25 °C to an optical density of 0.4–0.6 and then grown for a further 3 h in the presence of 1 mM IPTG at 25 °C, and finally, the cells were disrupted by sonication and then centrifuged to obtain the crude *E. coli* extracts. The GST–ERRγ LBD fusion protein was purified by Glutathione Sepharose 4B (Amersham Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. To determine the presence of the fusion protein, a 10% SDS-polyacrylamide gel electrophoresis analysis was performed.

Preparation of his-tagged human SRC2–BAP fusion protein. The *E. coli* BL21 strain containing the expression plasmid pET28a, which carried the 6×histidine (6×his)-tagged nuclear receptor interaction domain of steroid receptor coactivator 2–bacterial alkaline phosphatase (6×his-SRC2 NID–BAP), was a gift from Dr. J. Nishikawa (Osaka University, Japan). The preparation of the 6×his-SRC2 NID–BAP fusion protein was conducted according to a study by Kanayama et al. (2003).

Human ERRγ coactivator recruiting assay. A crude *E. coli* extract containing a GST–ERRγ LBD fusion protein was used for fixation on a Glutathione Immobilizer™ microplate (EXIQON A/S, Vedbaek, Denmark). The micro-well plate was incubated at 4 °C overnight in a total volume of 100 μl per well. The prepared micro-well plate was rinsed twice with 120 μl of suspension buffer per well (Kanayama et al., 2003). Next, 100 μl of purified 6×his-SRC2 NID–BAP fusion protein (30 μg/ml) was added to the well with the test chemical or DMSO solvent only. After 1 h of incubation at 4 °C, the plate was washed three times with 120 μl of wash buffer per well (Kanayama et al., 2003). After removal of the wash buffer, the enzyme reaction was started by adding 100 μl of a 10 mM p-nitrophenyl phosphate (p-NPP) solution (Kanayama et al., 2003). After incubation at 37 °C for 1 h, the absorbance at 405 nm was measured to evaluate the BAP activity in the 6×his-SRC2 NID–BAP binding on the GST–ERRγ LBD fusion protein. The wells without the crude *E. coli* extract and 6×his-SRC2 NID–BAP fusion protein were used as background. The wells without the 6×his-SRC2 NID–BAP fusion protein were used as control A ($Ctrl^a$). The wells without the crude *E. coli* extract were used as control B ($Ctrl^b$). The chemicals were diluted in DMSO to prepare the appropriate concentrations for the tests. The wells with DMSO were used as solvent control C ($Ctrl^c$). The known ERRγ agonist BPA and antagonist 4-OH-TAM were used along with the oestrogen receptor agonist E_2 and oestrogen-related receptor α (ERRα) agonists daidzein and genistein as standards to develop the ERRγ coactivator recruiting assay (Coward et al., 2001; Suetsugi et al., 2003; Takayanagi et al., 2006). For a comparison with the constitutive transcriptional activity of the GST–ERRγ-LBD fusion protein, a human oestrogen receptor α (ERα) coactivator recruiting assay was performed to show the ligand-dependent transcriptional activity of the nuclear receptor according to our previous method (Zhang et al., 2012). To illustrate the ERRγ binding activities of the chemicals, the ERRγ coactivator recruiting assay was developed *via* antagonist-competitive binding, which was carried out by mixing 10^{-6} M of 4-OH-TAM with the diluted test chemicals; therefore, the activity is referred to as inverse antagonist activity. Finally, the inverse antagonist activities of the test chemicals, including MP, EP, PP, BuP, BzP, MS, ES, MB and EB, were analysed by the developed ERRγ coactivator recruiting assay *via* antagonist (4-OH-TAM)-competitive binding. The 50% relative effective concentration (REC50) of the chemical that offset

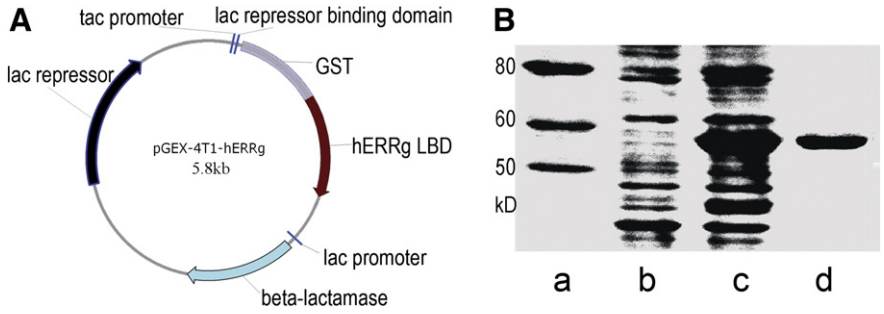

Fig. 1. Expression of the GST–human ERRγ LBD fusion protein. (A) The GST–ERRγ LBD fusion protein expression vector. (B) 10% SDS-PAGE analysis of the induced and purified GST–ERRγ LBD fusion protein. Lane a: size markers; lane b: uninduced *E. coli* BL21 whole cells; lane c: 1 mM IPTG induced whole cells; and lane d: purified GST–ERRγ LBD fusion protein by Glutathione Sepharose 4B (Amersham Bioscience).

50% of the antagonistic activity of 4-OH-TAM (1×10^{-6} M) was calculated with the ERRγ-LBD activity in the solvent control ($Ctrl^c$) as 100%. Sigmoidal concentration–effect curves for the test chemicals were calculated using the GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA).

Automated molecular docking. Scigress (Ultra Version 3.0.0, Fujitsu) was used to perform *in silico* molecular docking analyses. The chemical structures of the test chemicals and original ligands were drawn, cleaned and energy optimised using Scigress-integrated procedures. Crystallographic three-dimensional (3D) structures of ERRγ-LBD were downloaded from the Protein Data Bank website (http://www.rcsb.org/pdb). The hydrogen atoms of the target proteins and the original ligands were subsequently added through the Scigress Workspace module. Automated blind docking of the flexible ligand into the rigid protein was then carried out to discover the active binding sites of ERRγ. This method allowed the ligands to bind anywhere on the surface without bias or in the interior of the target protein. Unliganded structures (PDB ID codes 1KV6, 1TFC, 2GPO and 2ZBS) and liganded structures (2E2R, 2ZAS, 2ZKC and 2P7A) were used as the ERRγ templates. The 3D structures were refined by removing the ligand molecules, water molecules and other molecules adsorbed on the surface of the protein. Valences were added to the protein targets to achieve the correct ionisation and tautomeric states of amino acid residues such as histidine. The calculations for docking the flexible ligands into the rigid protein were evaluated using a genetic algorithm. The procedure was set to run 30,000 generations with an initial population size of 50, an elitism of 7, a crossover of 0.8, a mutation of 0.2 and a convergence of 1.0. Other parameters were left at their default values. The best fits were identified by analysing the Potential of Mean Force (PMF) scores of all of the chromosomes to evaluate the possible binding sites of the test chemicals in ERRγ. Automatic docking of the flexible ligand into the known active site in the liganded structure was

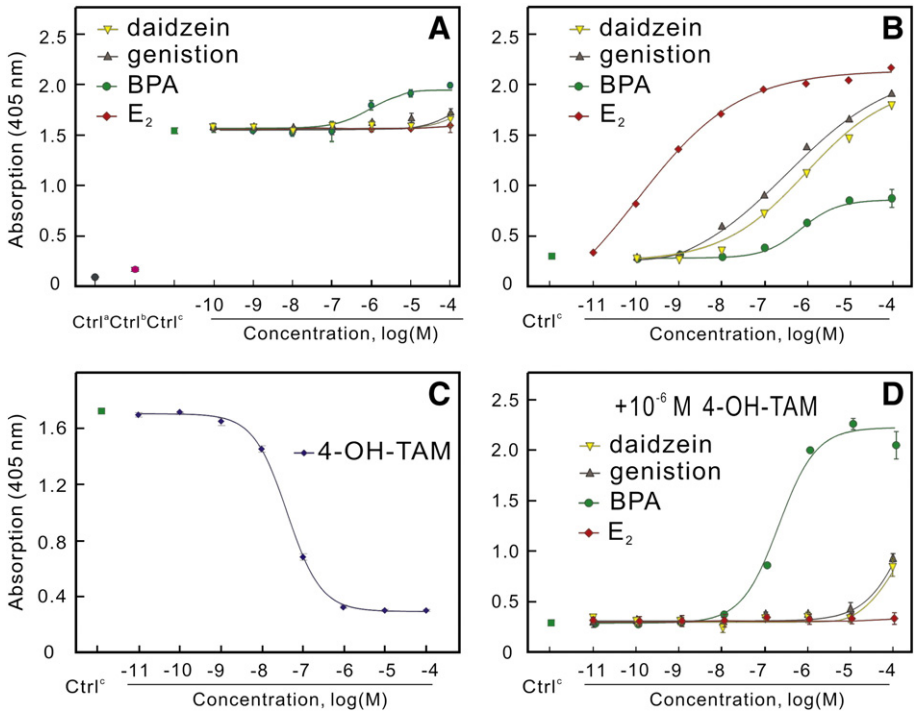

Fig. 2. Development of the ERRγ coactivator recruiting assay for inverse antagonist activity evaluation, based on the activity of the BAP fused to the coactivator. (A) Agonist activities of test chemicals on ERRγ. (B) Agonist activities of test chemicals on hERα according to the previous method (Zhang et al., 2012). (C) Antagonist activity of 4-OH-TAM on ERRγ. (D) Inverse antagonist activity of test chemicals *via* antagonist competitive binding. The test chemicals include the known ERRγ agonist (BPA), ERRγ antagonist (4-OH-TAM), ER agonist (E_2) and ERRα agonists (daidzein and genistein). $Ctrl^a$: wells used as control without 6×his-SRC2 NID–BAP fusion protein; $Ctrl^b$: wells used as control without crude *E. coli* extract; and $Ctrl^c$: wells used as solvent control without test chemical added. Results are expressed as the mean ± standard deviation (SD) of the triplicate assays. The sigmoidal concentration–effect curves for the test chemicals were calculated using the GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA).

performed to obtain a detailed analysis of the test chemical/ERRγ protein interaction. The PDB entry 2E2R was selected due to its high resolution (1.6 Å). The structures were refined, and all of the water molecules were removed from the protein except two (237 and 592) that are important to the ligand-binding pocket composition. The docking calculations were evaluated on a 0.25 Å grid in a 15 × 15 × 15 Å box, which contained the active site for the original ligand. The local search parameters included 300 maximum iterations at a rate of 0.06. The other parameter settings were the same as for the automated blind docking. After deleting the original ligand, a geometry optimisation calculation in Mechanics using augmented MM3 parameters was performed to refine the docked complex structures of the receptor with the test chemical to the lowest stable energy state. This energy minimisation procedure continued until the energy change was less than 0.001 kcal/mol; otherwise, the molecules were updated 300 times. During the calculation, both the receptor and ligand were allowed to interact flexibly. Finally, a PMF score was determined and used to evaluate the binding energy between the ligand and the active site. To validate this method, an automatic docking of ERRγ agonist 4-chloro-3-methyl-phenol (43M) into the known active site of the ERRγ structure (2E2R) was performed.

Results

Development of human ERRγ coactivator recruiting assay

As shown in Fig. 1B, the SDS-polyacrylamide gel electrophoresis analysis showed a 55.4 kDa protein that was consistent with the expected size of the GST–ERRγ LBD fusion protein. Purification from a crude *E. coli* extract using Glutathione Sepharose 4B indicated that this protein was efficiently expressed with the addition of 0.1 mM IPTG and was present in the soluble portion. To test the activity of the GST–ERRγ LBD fusion protein, we performed a nuclear receptor coactivator recruiting assay for the known ERRγ agonist (BPA), oestrogen receptor agonist (E_2) and ERRα agonists (daidzein and genistein) (Coward et al., 2001; Suetsugi et al., 2003; Takayanagi et al., 2006). As shown in Fig. 2A, there was a strong interaction between the protein and SRC2 NID–BAP with or without the addition of chemicals. In comparison, a significant interaction between the ERα and the SRC2 NID was observed only in the presence of sufficient oestrogenic chemical levels (Fig. 2B). We then performed an ERRγ coactivator recruiting assay for 4-OH-TAM, a known ERRγ antagonist, and found that 4-OH-TAM significantly weakened the interaction of GST–ERRγ LBD with SRC2 NID–BAP in a concentration-dependent manner (Fig. 2C); the 50% effective concentration (EC50) was 5.85×10^{-8} M. This demonstrated that the constitutive transcriptional activity of the GST–ERRγ-LBD fusion protein was consistent with expectation (Hong et al., 1999; Xie et al., 1999). To illustrate the ERRγ binding activities of the chemicals, the ERRγ coactivator recruiting assay was developed *via* antagonist-competitive binding. The BAP activity shown in Fig. 2D confirmed that the BPA had a strong inverse antagonist activity on ERRγ, effectively resuming the interaction of ERRγ with SRC2 in a concentration-dependent manner, and its EC50 value was 1.93×10^{-7} M. No obvious activities were observed for E_2, daidzein or genistein at levels up to 10^{-5} M. These results are consistent with existing knowledge (Okada et al., 2008; Suetsugi et al., 2003; Takayanagi et al., 2006).

Inverse antagonist activity of parabens, salicylates and benzoates on ERRγ

Using the developed assay, the inverse antagonist activities of MP, EP, PP, BuP and BzP were determined in addition to those of MS, MB, ES and EB. As shown in Fig. 3, all of the test parabens had obvious inverse antagonist activities, which increased the BAP activity from 10^{-7} M (the LOEL) in a concentration-dependent manner. The REC50 values of MP, EP, PP, BuP and BzP were 4.79×10^{-7}, 3.73×10^{-7}, 3.45×10^{-7}, 3.09×10^{-7} and 5.88×10^{-7} M, respectively. In comparison, the salicylates possessed lower activities than their isomeric parabens, and the REC50s

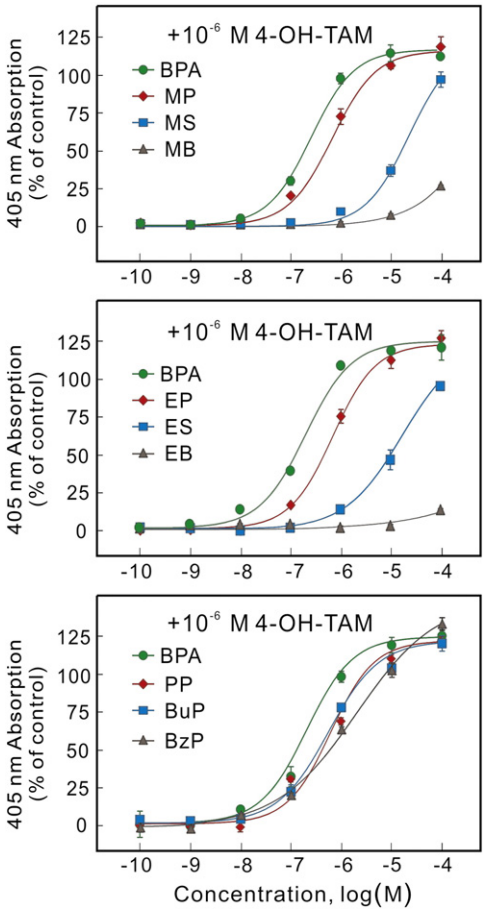

Fig. 3. Inverse antagonist activities of test chemicals on ERRγ using the developed ERRγ coactivator recruiting assay *via* antagonist competitive binding. In analysing the BAP activities of the wells, only the added 10^{-6} M 4-OH-TAM was used as a background (0%), and of these wells, only the added DMSO solvent was used as a control (100%), presenting the full constitutive activation of ERRγ. The test chemicals, including parabens (methylparaben (MP), ethylparaben (EP), propylparaben (PP), butylparaben (BuP) and benzylparaben (BzP)), salicylates (methyl salicylate (MS) and ethyl salicylate (ES)) and benzoates (methyl benzoate (MB) and ethyl benzoate (EB)), were mixed with 10^{-6} M 4-OH-TAM in DMSO. The results are expressed as the mean ± standard deviation (S.D.) of the triplicate assays. The sigmoidal concentration–effect curves for the test chemicals were calculated using the GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA).

of MS and ES were 1.55×10^{-5} and 1.07×10^{-5} M, respectively. The benzoates showed no obvious activity at concentrations of 10^{-5} M and lower.

Automated docking of parabens, salicylates and benzoates in ERRγ

To understand the nature of the interaction between the test chemicals and the ERRγ protein, automated blind docking of the flexible ligand into a rigid protein and automated docking of the flexible ligand into known active sites were performed. The possible binding sites of the test chemicals in ERRγ were analysed from the results of the automated blind docking. The parabens preferred to dock into the active site, *i.e.*, the ligand-binding pocket of ERRγ, for known agonists such as BPA, bisphenol Z (BPZ), 43M and 4-alpha-cumylphenol (1OH), whereas the salicylates and benzoates were more likely to bind into pocket 2 (Wang et al., 2006), which is known to have no physiological significance in the transcription process. The results of the automated docking of the flexible ligand into known active sites revealed the interactions between the parabens and the ligand-binding pocket (Fig. 4). The important feature of the interaction was the formation of hydrogen bonds between the *p*-hydroxyl paraben groups and the Glu275/Arg316 of the pocket. In addition, the phenyl ring and alkyl group of parabens produced van der

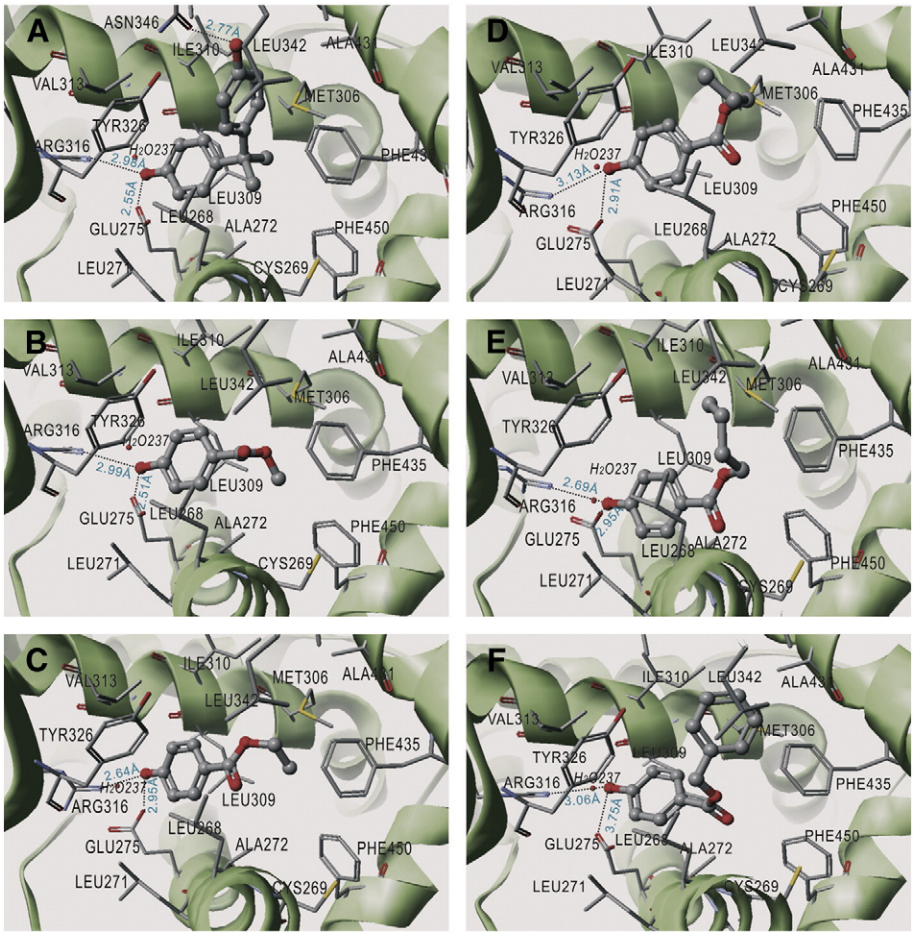

Fig. 4. Binding poses of (A) bisphenol A (BPA), (B) methylparaben (MP), (C) ethylparaben (EP), (D) propylparaben (PP), (E) butylparaben (BuP) and (F) benzylparaben (BzP) at the ERRγ active site. The dotted lines indicate hydrogen bonds between the chemicals and amino acid residues.

Waal interactions with the core hydrophobic moiety provided by PHE435, PHE450, LEU268, LEU271, LEU309, LEU342, ALA272, MET306, VAL313 and ILE310. For BzP, we also observed π–π interactions between the phenyl ring in the benzyl group and those in PHE435 and PHE450. The PMF values of MP, EP, PP, BuP and BzP were calculated as −55.5, −56.1, −56.8, −61.2 and −65.7 kcal/mol, respectively. These values were less than the −73.7 kcal/mol of BPA but higher than the −38.587 kcal/mol of 43M. The method was also validated by automatic docking of 43M into the known active site of the ERRγ structure (2E2R). As shown in Fig. 5, the calculated conformations of 43M and the ligand-binding pocket were close to those in the X-ray structure (2P7A) with a root mean square (RMS) error of 0.9103 Å, indicating the reliability of the method.

Discussion

The oestrogen agonist properties and androgen antagonist activities of parabens have been well documented by a variety of *in vitro* and *in vivo* assay systems over the past decade (Chen et al., 2007; Darbre and Harvey, 2008; Golden et al., 2005). To the best of our knowledge, this is the first study to explore the binding activities of parabens to ERRγ. ERRγ is the most recently identified member of the ERR subfamily by yeast two-hybrid screening, using SRC2 as bait (Hong et al., 1999). It is known as a constitutive regulator that can interact with transcriptional coactivators, resulting in transcriptional activation in the absence of ligands (Hong et al., 1999; Xie et al., 1999). In this study, the developed *in vitro* ERRγ coactivator recruiting assay clearly showed the constitutive transcriptional activity of ERRγ, as evidenced by the BAP activity (Fig. 2A). Through antagonist-competitive binding, this *in vitro* assay also demonstrated the binding activity of BPA with ERRγ (Fig. 2D), suggesting that the method could be used to analyse the binding activity of chemicals on ERRγ. Recent studies have shown strong ERRγ binding activity of compounds containing phenolic hydroxyl groups in a *para* position, such as BPA, BPZ, 43M and 1OH, indicating the potential and intrinsic importance

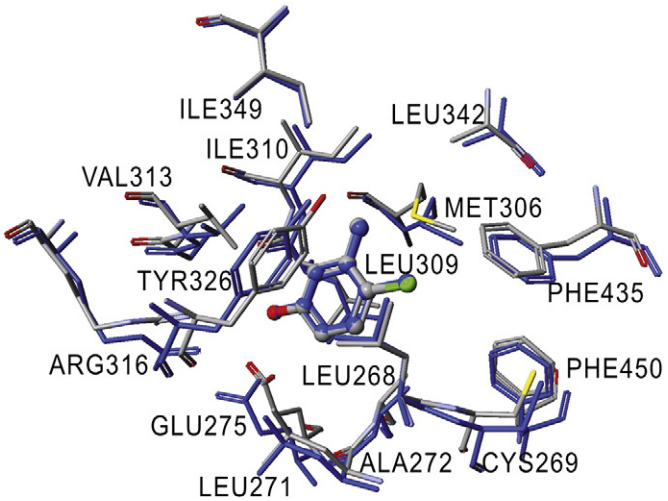

Fig. 5. Comparison between the calculated conformation (blue) of the ligand-binding pocket of ERRγ-43M by molecular docking using 2E2R (PDB ID), and the conformation (coloured according to element type) in the X-ray structure (2P7A). The root mean square deviation between the two is calculated at 0.9103 Å.

of these bindings (Abad et al., 2008; Matsushima et al., 2008; Okada et al., 2008; Takayanagi et al., 2006). Furthermore, the concentrations of these known ERRγ binding compounds were much lower than those found in the human body. In this study, we found that parabens possessed high inverse antagonist activities on ERRγ that were close to that of BPA (Fig. 3), whereas salicylates and benzoates possessed lower and no activities, respectively (Fig. 3). Automated molecular docking revealed that parabens fitted well into the active site of ERRγ, and that hydrogen bonds could be formed between the *p*-hydroxyl groups in parabens and the Glu275/Arg316 of ERRγ. Although the PMF values of MP, EP, PP, BuP and BzP were less than that of BPA, they were higher than that of 43M. This confirmed the strong ERRγ binding activities of parabens and the importance of the phenolic hydroxyl group in a *para* position for ERRγ agonists (Fig. 4).

Recent studies have shown that parabens are commonly detectable in human breast cancer tissues at relatively high levels (Barr et al., 2012; Darbre et al., 2004). These findings have raised concern about the involvement of parabens in the increased incidence of human breast cancer, as all of these chemicals possess oestrogenic properties and oestrogen is known to play a central role in the development, growth and progression of breast cancer (Miller, 1996). However, it has also been argued that the effect of parabens may not be important (Golden and Gandy, 2004; Golden et al., 2005) because the most widely used parabens have been found to be weakly oestrogenic, with the most potent (BuP) being 10,000 times less potent than E_2 (Routledge et al., 1998). As ERRγ is expressed in most human breast cancers (Ijichi et al., 2011), there is concern over the role that parabens may play in breast cancer *via* ERRγ (Darbre and Harvey, 2008). By converting the molar concentration into ppb (ng/g), we found that the LOEL values of parabens (10^{-7} M ≈ 15 ppb for MP) observed from the ERRγ coactivator recruiting assay were lower than the levels detected in breast cancer tissues. Barr et al. (2012), for instance, reported a median value of 85.5 (range 0–5134.5) ppb for five measured parabens in breast tissue samples collected from mastectomies for primary breast cancer in England between 2005 and 2008. These values suggest that the levels in most breast cancer patients are sufficient to have an effect *via* ERRγ. Worse yet, the levels detected in breast cancer tissues were often higher than the REC50s observed in this study. Although ERRγ is known as a constitutively active orphan nuclear receptor, the agonist may not be able to alter ERRγ function. However, Yamamoto et al. (2012) showed recently that a selective ERRγ agonist, DY131, promoted the growth of ERα-negative uterine endometrial cancer cells. ERRγ is known as a modulator of oestrogen signalling in breast cancer cells, and shares target genes, coregulatory proteins, ligands and sites of action with ERs (Giguere, 2002). Ijichi et al. (2011) showed that ERRγ can stimulate oestrogen response element (ERE)-driven transcription in MCF-7 cells and 293T cells, and that exogenously transfected ERRγ can increase MCF-7 cell proliferation. Charles and Darbre (2013) very recently investigated the extent to which proliferation of MCF-7 cells could be increased by exposure to MP, EP, PP, BuP and isobutyl paraben at concentrations in 160 human breast tissue samples reported by Barr et al., 2012, and demonstrated that parabens, either alone or in combination, were present in human breast tissue at concentrations sufficient to stimulate the proliferation of MCF-7 cells *in vitro*. Therefore, we believe that parabens play some role in the carcinogenesis of human breast cancers. Parabens may have significant effects in breast cancer patients who are taking tamoxifen because ERRγ, in addition to ER, is considered as a treatment target for tamoxifen (Coward et al., 2001). Additionally, although to date there has been no report on paraben levels in uterine tissue, they undoubtedly exist in most uteri due to the high levels of parabens detected in human urine, serum/plasma, seminal plasma and milk samples (Meeker et al., 2011; Sandanger et al., 2011; Ye et al., 2008, 2009). As ERRγ agonists, parabens may possess similar effects to those of DY131 on uterine endometrial cancers, and a detailed study is required.

A recent analysis of 2721 urine samples collected from 245 men and 408 women across the US general population between 2005 and 2010 showed that MP, PP and BuP were measurable in 99.9%, 98.3% and 73.6% of samples from women and 99.3%, 90.2% and 35.9% of samples from men, respectively (Smith et al., 2012). A study by Calafat et al. (2010) similarly detected MP (99.1%), EP (42.4%), PP (92.7%) and BuP (47%) in 2548 urine samples taken from the US general population *via* the 2005–2006 National Health and Nutrition Examination Survey. European studies have also demonstrated the presence of parabens in urine samples from the general population, with parabens detectable in 98% of urine samples from 60 Danish men (Frederiksen et al., 2011) and in 100% of urine samples from pregnant women and children in Spain (Casas et al., 2011). As such, the effects of parabens on the general population should also be of concern. ERRγ is expressed very strongly in a tissue-restricted manner in the mammalian placenta and foetal brain during development, and in the brain, lung and many other tissues during adulthood (Eudy et al., 1998; Heard et al., 2000; Lorke et al., 2000). BPA is known as a strong agonist of ERRγ, and many of its neural effects have been documented, including altered explorative activity, impaired social interaction/activity, compromised learning and memory, increased anxiety and aggression, decreased male sexual behaviour, modified or lost brain sex differences, increased number of oxytocin neurones in the paraventricular nucleus, loss of sex differences in the AVPV volume and tyrosine hydroxylase levels, loss of sex difference in the locus coeruleus volume, altered nitric oxide synthase signalling and advanced puberty (Frye et al., 2012). Okada et al. (2008) suggested a strong binding of BPA to ERRγ as a reason for BPA's known effects on the central nervous system. Thus, the possible neurotoxicity of parabens on general populations, especially foetuses and children, deserve more attention.

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Conflict of interest statement

The authors declare that they have no actual or potential competing financial interests.

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