

Study on the assessment for the adverse effects of the bisphenol A analogs in the human body

袈裟丸, 仁志

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**Study on the assessment for the adverse effects of
the bisphenol A analogs in the human body**

Hitoshi Kesamaru

Laboratory of Biomolecular Chemistry

Department of Chemistry

Graduate School of Science

Kyushu University

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CONTENTS

	Page
Study on the assessment for the adverse effects of the bisphenol A analogs in the human body	
CHAPTER 1	
Influence of receptor conformations in docking calculation based endocrine disruptor risk assessment: evaluation of binding affinity and transcriptional activity of estrogen receptor binding chemicals.	
1. Introduction	2
2. Materials and Methods	6
3. Results and Discussion	8
4. Conclusion	14
5. References	15
CHAPTER 2	
Evaluation of the influence of halogenation on the binding affinity and transcriptional activity of bisphenol A to the estrogen receptors.	
1. Introduction	54
2. Materials and Methods	56
3. Results and Discussion	67
4. Conclusion	72
5. References	73
CHAPTER 3	
Effects of halogenation of bisphenol A analogs on cytotoxicity for HeLa cells: di-halogenated BPA analogs inducing different types of cell death depending on halogen atom species.	
1. Introduction	91
2. Materials and Methods	94
3. Results and Discussion	96
4. Conclusion	99
5. References	102
Acknowledgements	120

ABBREVIATIONS

The abbreviations according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9-37 (1984), are used throughout. Unless otherwise specified, the amino acids are L-stereoisomers. Additional abbreviations are follows, but generally accepted abbreviations and symbols were used without definition.

(Z)-4-OHT	(Z)-4-hydroxytamoxifen
ABC	agonist-bound conformations
AF-1/2	activation function 1/2
BPX	bisphenol X (where X is any of the following A, AF, C, and E)
cAMP	cyclic adenosine monophosphate
DCC	dextran coated charcoal
DES	diethylstilbestrol
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
E2	17 β -estradiol
EDCs	endocrine disrupting chemicals
EMEM	Eagle's minimim essential medium
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
ERR γ	estrogen related receptor γ
FBS	fatal bovine serum

GPR30	G protein-coupled receptor 30
GST	glutathione S-transferase
H11	α -helix numbered 11
H12	α -helix numbered 12
HPTE	2,2-bis(<i>p</i> -hydroxy phenyl)-1,1,1-trichloroethane
IC ₅₀	50% inhibition concentration
LBD	ligand binding domain
LBP	ligand binding pocket
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MOM	methoxymethyl
MTT	methylthiazole tetrazolium
NABC	non-agonist-bound conformations
NMR	nuclear magnetic resonance spectroscopy
NR	nuclear receptor
PBS	phosphate buffered saline
PDB	protein data bank
PFCs	perfluorinated compounds
PKA	protein kinase A
PPAR	peroxisome proliferator-activated receptor
RAL	raloxifene
RMSD	root mean square deviation
SDS-PAGE	sodium dodecyl sulfate-poly-acrylamide gel electrophoresis
S.E.	standard error

T ₃	3,3',5-Triiodo-L-thyronine
TBBPA	tetrabromobisphenol A
TCBPA	tetrachlorobisphenol A
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TMS	tetramethylsilane

CHAPTER 1

Importance of receptor conformations in docking calculation based risk assessment for endocrine disruptor: evaluation of binding potency and transcriptional activity of estrogen receptor α binding chemicals

Abstract

Risk assessment of chemical substances in environment is an important issue for the development and safe use of chemicals. To evaluate adverse effects of chemicals for living bodies in a high accuracy, it is necessary to investigate physiological activities from broad perspectives by using various kinds of assay methods. Among them, *in silico* docking calculation is one of the first-line screening methods to assess the risk of environmental chemicals, which can evaluate the binding affinity between a chemical and a receptor protein. For using *in silico* docking calculations, employment of suitable receptor conformations as templates is essential for appropriate identification of the latent receptor-binding ability of chemicals. In this study, we performed docking calculations using a number of agonist- and antagonist-bound conformations of estrogen receptor α -ligand binding domains as templates to clarify the type of receptor conformations required for reasonable identification of endocrine disruptors. In the calculation results, ER α agonists bound selectively to the agonist conformations, whereas antagonists bound selectively to the non-antagonist conformations. Furthermore, structural analyses of the ligand-binding domains and docking calculation utilizing C-terminal truncated receptors indicated that the conformation of C-terminal region of the ligand-binding domain, which altered its conformation by ligand binding, distinguished agonists from non-agonists. These results suggest that employment of appropriate receptor conformations as docking templates are necessary to predict

appropriately the receptor binding affinity and the transcriptional activity of chemicals using docking calculation-based risk assessment.

1. Introduction

At present, a number of chemical compounds exceed 100 million have been developed and reported in Chemical Abstracts Service (CAS) and many of them have been utilized for the chemical industries. However, it has been reported that chemical substances leaked from diverse consumer products and spread in the air, water, soil, and food. These chemicals, called environmental chemicals, have been concerned to exhibit various adverse effects on the living organisms. For example, early exposure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) caused decrease of sperm counts and motility in human or increase of cancers in children and young adults by endocrine disruption via receptor proteins^{1,2}. As described in these reports, receptor-mediated endocrine disruption of environmental chemicals has become a concern for adverse effects on developmental processes, especially in fetuses and infants. Therefore, it is necessary to assess risk of these chemical substances promptly including newly developed chemicals. However, a large number of chemical substances are still unevaluated their activity by conventional toxicity tests, even though many of them are expected to exhibit various biological activities. Therefore, it is necessary to develop evaluation methods to assess the risk of chemicals efficiently and reasonably.

Since receptors are thought to be a first access point of environmental chemicals, the interaction between chemicals and receptors should be evaluated rapidly. Among the receptor proteins, nuclear receptors (NRs) are receptor proteins involved in regulating a wide range of physiological functions. NRs represent a protein superfamily comprising 48 different proteins in humans³. The ligand-binding domains (LBDs) of NRs share a highly homologous structure, including 12 α -helices (H1 to H12) and one or more underlying β -sheets. The conformation of NR protein is altered by the positioning of the H12 helix, which depends on the biological characteristics of the bound-ligand (agonist or antagonist). Following the agonist-ligand binding, NRs change their conformation from the apo-form to the activated form, while the antagonist-ligand binding results in achievement of an inactive conformation

of H12 (Figure 1). Compared with the change in conformation of H12, H11 shows relatively little but apparent position changes according to agonist or antagonist binding. Such conformational changes allow NRs to differentiate chemical agonists and antagonists³⁻⁶.

(Figure 1)

Under physiological conditions, NRs are susceptible to endocrine disruption by environmental chemicals⁷, including so-called endocrine-disrupting chemicals (EDCs).⁸ EDCs can bind to human NRs, disrupt gene transcription, and cause adverse effects on development, reproduction, cell differentiation, nervous system function, and immune responses in mammals and especially in fetuses and infants⁹⁻¹¹. A representative example of an EDC is bisphenol A (BPA), which is used to manufacture polycarbonate plastics, epoxy resins, and other products. Exposure to BPA in mice and rats leads to early onset of sexual maturation in females¹²⁻¹⁴, increased prostate size in male offspring¹⁵⁻¹⁷, and altered immune function¹⁸⁻²⁰. Therefore, to avoid health damage by BPA, several bisphenol analogs known as “next-generation bisphenols” have been developed and used as alternatives to BPA²¹. However, some BPA analogs, such as bisphenol AF (BPAF²⁰) have been receiving much attention, since it was revealed that these analogs also bind to NRs and could induce endocrine disruption similar to BPA. For example, it was reported that bisphenol AF (BPAF), which is a fluorinated analog of BPA, strongly binds to estrogen receptors. Surprisingly, it was also revealed that BPAF acted as a full agonist for estrogen receptor α , while exhibiting strong antagonist activity for estrogen receptor β ²². These results clearly indicate that some BPA analogs act as ligands with variety of activities via different NRs. Furthermore, it is highly likely that latent chemicals will emerge as EDCs similar to BPA; therefore, all NRs are regarded as candidate targets of these yet-undetermined EDCs²³. Based on this likelihood, it is important to develop reliable assays capable of predicting the risk of NR-related endocrine disruption associated with various chemicals.

The recent emphasis on implementing strict ethical standards in research has increased the demand to switch from *in vivo* to *in vitro* or *in silico* methods of risk assessment of chemical substances. For example, according to the OECD guidelines, *in vitro* assays such as receptor binding assay and transcriptional activation assay to NRs are conducted as level

2 assessment of EDCs, which is early level for quick evaluation of a large number of substances, whereas *in vivo* evaluation is used for subsequent assessment for refined target substance²⁴. Since *in vitro* and *in silico* assays can be carried out quickly and readily compared to *in vivo* methods, they are applied early stage of the assessment for a number of compounds as shown in the OECD guidelines. In addition, environmental chemicals exhibit adverse effects for living organisms not only by endocrine disruption via binding to nuclear receptors but also inducing several other types of biological toxicity including induction of mitochondrial injury and apoptotic death in several types of cells. Therefore, it is necessary to evaluate the risk of chemical substances from broad perspectives by using various *in vitro* and *in silico* methods. Regarding the *in silico* techniques, diverse computational method including quantitative structure-activity relationship models²⁵, pharmacophore models²⁶, docking calculations^{27,28}, and molecular dynamics simulations²⁹, have been frequently used as appropriate methods for assessment of the physiological impacts induced by a number of chemicals via various kinds of receptors. Among these techniques, docking calculations represent a possible first-line screening method for EDCs. To implement docking calculations, it is essential to utilize a receptor's 3D-structure as a template for the calculations. To date, 3D-structures of the LBDs determined by X-ray crystallography are necessary to perform docking calculations. However, the quality and choice of the structures greatly affecting the results. In most cases, the receptor structure has been treated as rigid (i.e. all atoms of the protein structure are fixed) during docking calculation using common docking programs such as Autodock, DOCK, and Autodock Vina³⁰. Under the rigid conditions, if the receptor structure is not appropriately selected, binding affinity of the chemical will not be estimated reasonably, since the conformations of each residue on the ligand-binding pocket (LBP) are incapable of adapting to all structure of ligands possessing binding activity. For example, accurate antagonist-binding affinity cannot be calculated from a structure of an activated (agonist-bound) form of an NR, as H12 and H11 positioning in the activated form of the NR hinders antagonist binding (Figure 2). This renders an antagonist incapable of altering the active conformation of NR structures that obtained in the presence of an agonist by crystallography, without extensive structure optimization of the whole

antagonist-bound complex along with docking calculations. Therefore, it is necessary to select suitable NR-template structures, including agonist-bound conformations (ABCs) and non-agonist-bound conformations (NABCs), to enable accurate evaluation of binding affinities between NRs and ligands via docking calculations^{28,31}. Nevertheless, it is not always possible to select an appropriate receptor structure for *in silico* studies. Thus, docking calculation studies using only one receptor structure may not be appropriate to evaluate the binding affinity of various chemicals. In this context, statistical analyses of the binding energy values obtained by docking calculations using multiple receptor structures might be an effective method to reduce prediction errors.

(Figure 2)

In this study, we performed docking calculations using estrogen receptor alpha (ER α) to examine whether the physiological activity of ER ligands could be predicted with high accuracy by statistical analyses of the obtained binding energy values. ER α is a member of the steroid-receptor family and a representative target of EDCs⁵. Since numerous agonist-bound and non-agonist-bound structures of ER α /ligand complexes have been reported in the Protein Data Bank (PDB; www.rcsb.org), ER α is suitable to use for statistical analysis of EDC binding. Therefore, some *in silico* docking studies using different conformations of ER α have been reported previously. For example, Nose *et al.* reported on an agonist/antagonist differential-docking screening method using a total of 4 ER α -LBDs, including 2 agonist-bound conformations and 2 antagonist-bound conformations²⁵. Celik *et al.* also reported *in silico* study using 3 newly found quasi-stable structures of the ER α and LBD complexes and 3 crystallographic conformations of the protein³². In order to further advance these studies using multiple receptor structures and to clarify the optimal method for selecting the receptor structures in docking calculation, we tested eight known ER α agonists and antagonists against crystal structures representing 83 human ER α -LBDs in the present study. By statistically comparing the calculations results, we investigated whether docking calculations were capable of accurately predicting ER α chemical-binding activity. Moreover, using modified receptor structures in which H11 and H12 were truncated, we also investigated how H11 and H12 positioning affect the docking calculation.

2. Material and Methods

2.1. Preparation of receptor structures for docking calculations

As templates for docking calculations, 83 ER α -LBD structures reported by 2015 were prepared using crystal structures deposited in the PDB (Table 1). Preparation of the three-dimensional structures as docking templates was performed using Discovery Studio software (v4.0; Accelrys, San Diego, CA, USA). All calculations were performed using a Dell Precision T3610 workstation (Dell, Round Rock, TX, USA). In all cases, co-crystallized ligands and other small molecules except for water molecules were removed. The size of the LBP in ER α -LBDs and root mean square deviation (RMSD) between receptor structures were evaluated using Discovery Studio software. Receptor structures were classified as ABC or NABC (including both the antagonist-bound and apo conformations). Receptor structures that belong to ABC were categorized on the condition of the distance between the amide nitrogen of Leu540 and the oxygen atom on Asp351 side chain in wild type ER α , or alternatively Ser357 and Asp351 in Y537S mutant, were within hydrogen bond distance (~ 3.5 Å). On the other hand, NABC was composed of receptor structures other than that classified as ABC.

(Table 1)

2.2. *In silico* preparation of C-terminal-truncated ER α -LBDs

To assess the effects of deletions of the C-terminal regions of LBDs on ligand selectivity, C-terminal-truncated ER α -LBDs were prepared using Discovery Studio software. A series of Δ K529C mutations, resulting in deletion of H12 (from K529 to the C-terminus), were computationally prepared for 83 ER α -LBD structures. Each prepared structure was energy minimized by Discovery Studio software. A series of Δ N519C mutants that deleted both H11 and H12 (from N519 to the C-terminus), were prepared in the same manner.

2.3. *In silico* ligand preparation for docking calculation

The ligands tested were as follows: 17 β -estradiol (E2) and diethylstilbestrol (DES) as agonists for ER α , (Z)-4-hydroxytamoxifen [(Z)-4-OHT] and raloxifene (RAL) as antagonists for ER α , BPA and bisphenol C (BPC) as partial agonists for ER α , and bisphenol AF (BPAF) and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) as EDCs act as agonists for ER α . All chemical structures of the ligands were constructed using Accelrys Draw (v4.0; Accelrys) and energy minimized using the minimization protocol of Discovery Studio software. Volume of ligands was evaluated using the analytical tools included in Discovery Studio software. The list of chemical structures used in this study is provided in Table 2^{33–36}.

(Table 2)

2.4. Docking calculations

The binding affinities of ligands were evaluated by docking calculations using Autodock version 4.2 (Scripps Research Institute, San Diego, CA, USA) on a Dell Precision T3500 workstation (Dell). The grid maps representing the receptor molecule were generated by AutoGrid (Scripps Research Institute), with each grid centered at the LBP. The docking-calculation area was set within a 126 x 126 x 126 grids at 0.197 Å per grid cube to include the entire LBP. Docking calculations were performed 100 times using the algorithm Lamarkian GA³⁷. The run parameters used in this study were as follows: the number of GA runs was 100, the maximum number of energy evaluations was 2.5×10^7 , and the maximum number of generations was 1.0×10^6 . Other parameters were represented by default values implemented by the program. In the calculation, the free energy change (ΔG) associated with each conformation was defined as the sum of the free energy changes, van der Waals forces, electrostatic interactions, hydrogen-bonding events, desolvation activity, and torsion energetics as designed by the developer. Dissociation constant (K_d) was calculated from ΔG using a theoretical formula (i).

$$\Delta G = RT \ln K_d \quad (i)$$

The structure resulting from 100 rounds of free energy minimization was adopted as the calculation result. The lowest binding energy value among the calculation results from

the 100 runs was used for the analysis. A total of 83 ER α structures (Table 1), and 8 ligands (Table 1) were used in this study. The calculation results were analyzed and compared according to their ΔG values between ABCs and NABCs. Data normalization was determined using a normal quantile-quantile plot (Figure 3)³⁸. Subsequently, comparison of the means and standard errors of the mean was performed using Welch's *t*-test³⁹.

(Figure 3)

3. Results & Discussion

3.1. Analysis and classification of receptor structures

First of all, crystal structures of ER α were classified by the positioning of the H12 helix. The LBD of NRs share a highly homologous structure, including 12 α -helices (H1 to H12) and one or more underlying β -sheets. Within the LBD, an LBP mainly composed of multiple hydrophobic residues and fewer hydrophilic residues is responsible for ligand binding. Upon ligand binding, the LBD attains an altered conformation of H12 depending upon the structure of the ligand (Figure 4a). The conformation of H12 was primarily categorized as representing either an activated conformation (ABC) or a non-activated conformation (NABC). When an agonist binds to wild type ER α -LBD, the amide hydrogen atom of Leu540 on H12 forms a hydrogen bond with the oxygen atom of Asp351 side chain on H3⁴⁰. Additionally, in case of the Y537S mutant of ER α , it was reported that Asp351 formed a hydrogen bond with Ser537, forcing it to form an activation conformation⁴¹. Therefore, ABC and NABC were classified by whether the distance between Leu540 and Asp351 for the wild type ER α and Ser537 and Asp351 for the Y537S mutant ER α were within a typical hydrogen bond distance of ~ 3.5 Å or not as indexes (Figures 4b and 4c). By applying these criteria to 83 ER α -LBD (Table 1), we assigned 56 ER α -LBDs to the ABC group and 27 ER α -LBDs to the NABC group.

(Figure 4)

The ratio of ER α -LBD structures in the ABC group to those in the NABC group was almost 2:1. Therefore, we assumed that docking calculations using all LBDs would result in

a higher identification rate of chemicals that shows high-affinity to ABC as compared with chemicals lacking affinity to ABC. Thus, it was concerned that calculation results by using ABC and NABC should be evaluated separately.

We also calculated the volume of each LBP on LBD structure, as it was reported that differences in LBP volume could affect ligand selectivity *in vitro*^{2, 42, 43}. It was estimated that the average LBP volume in structures from the ABC (55 structures) and NABC (10 structures) groups were $632 \pm 67 \text{ \AA}^3$ and $707 \pm 89 \text{ \AA}^3$, respectively. Thus, structures from the NABC group had larger LBPs than those from the ABC group. This difference in LBP volume might provide a reason for differences in ligand selectivity observed *in silico* docking calculations.

3.2. Effects of water molecules on LBP/ligand-docking calculations

To investigate the effects of water molecules observed in the crystal structures, docking calculations were performed using the same LBDs in the presence or absence of water molecules, as those water molecules might serve as spacers or mediators of hydrogen bonds between a ligand and residues of the LBP. Ratios of the two calculated K_d values [K_{dw} (water preserved) and K_{dnw} (water removed)] are shown in Table 3, and in the calculations, E2 and (Z)-4-OHT were used as one of the representative agonists and antagonists, respectively. For structures in the ABC group, the K_{dw} : K_{dnw} ratio was 0.96 in the presence of E2, and 1.5 in the presence of (Z)-4-OHT. For structures in the NABC group, the K_{dw} : K_{dnw} ratio was 0.97 for E2, and 1.5 for (Z)-4-OHT, indicating highly similar ratios between the two groups. E2 showed relatively high affinity for structures in the ABC group, whereas (Z)-4-OHT showed high affinity for structures in the NABC group. These results suggested that the presence of water molecules in and around the LBD did not significantly influence the results of docking calculations performed on structures in either the ABC or NABC group. Thus, water molecules in the LBP crystal structures were preserved in the subsequent analysis.

(Table 3)

3.3. Comparison of ligand-binding energy between ABC and NABC of ER α -LBD

structures

The eight chemicals included agonists, antagonists, and partial agonists (Table 4) were investigated their binding affinity to ER α -LBD structures by docking calculation using the classified structures as mentioned above. Using the average of calculated ΔG values against each test chemical as listed in Table 4, we obtained the dissociation constant (K_d) values associated with their receptor-binding activity against the target structures. To confirm the reproducibility of the docking calculation, the calculated average ΔG values were plotted against $\log K_d$ values previously determined using wet assays, and Pearson's product-moment correlation (r) was calculated⁴⁴. As a result, strong correlation ($r = 0.737$) was observed between the calculated average ΔG values and K_d values determined by wet assays (Figure 5).

(Figure 5 and Table 4)

The statistical difference between ΔG values from the ABC group and those from the NABC group was determined by Welch's t -test. It was revealed that the ΔG values of agonist-ligand that bind to ABC group was statistically smaller than that observed in the NABC group, whereas the ΔG values of antagonist binding to NABC group was smaller than that observed in the ABC group. Based on these values, K_d ratios, i.e. the $K_{d(nabc)}/K_{d(abc)}$, were calculated for each chemical to investigate the selectivity of the conformations that indicated which receptor group preferentially bound to the chemical (Table 4). Usually, E2 (a potent mammalian estrogenic steroid hormone) binds as an agonist to induce an activated conformation in ER α . Our results showed that E2 bound to ER α structures in the ABC group with 1.7-fold greater affinity as compared with structures in the NABC group on an average. Although E2 and DES share similar molecular features, including molecular volumes (E2 = 234.51 Å³; DES = 245.02 Å³), two characteristic hydroxyl groups, and ER α agonistic activity, DES exhibited only 1.2-fold greater affinity for the ABC group as compared with those for the NABC group. In contrast with the antagonists, we observed no significant differences in agonist binding between the ABC and NABC groups (Table 4). Additionally, the calculated binding affinity of DES for structures in the ABC group ($\Delta G = -8.84$ kcal/mol) was weaker than that of E2 ($\Delta G = -10.2$ kcal/mol), despite a similar 50% effective concentrations (EC₅₀;

E2 = 32 pM; DES = 13 pM)⁴⁵. To discuss the relatively low selectivity of DES, we analyzed DES/ER α -LBD complex structures determined by X-ray crystallography and docking calculations performed in this study. The complex structure that was determined by X-ray crystallography of DES and ER α -LBD (PDB: 3ERD) was clearly different from the calculated complexes using ABC in this study. Among the amino acid residues of ER α -LBDs, Met421 and His524 of 3ERD changed their conformation to make preferable interaction with DES (Figure 6). Thus, this discrepancy in the calculated binding affinity may be caused by the lack of the specific DES-accepting conformation of ER α -LBDs except for 3ERD. It was considered that ER α -LBD can induce such a unique conformation only as DES binding occurred.

(Figure 6)

BPA is a known ER α ligand; however, its binding affinity is 500- to 15,000-fold lower than that of natural ligand E2^{16,46–48}, and it also shows 160,000-fold lower induction of *in vivo* estrogenic activity against ethynyl-estradiol following oral administration⁴⁹. In this study, we estimated that the BPA-binding affinity for all ER α -LBDs were 39-fold lower than that of E2. This estimated weak binding affinity agreed with the previous results cited above and also suggest that BPA is a very weak or partial ER α -agonist. According to K_d -ratio analysis, BPA exhibited a 1.4-fold higher affinity for structures in the ABC group relative to those in the NABC group (Table 4), indicating that BPA may act as an ER α -agonist. Other bisphenol analogs, BPC, BPAF, and HPTE, bound to structures in the ABC group with 1.4-, 1.3-, and 1.5-fold higher affinity relative to their binding to LBDs in the NABC group, respectively (Table 4). These results confirmed that the BPA analogs may act as ER α -agonists, in agreement with previous *in vitro* results^{22,34,35}.

In this study, we used RAL and (Z)-4-OHT as representative antagonists among the chemicals tested in this *in silico* calculation study. RAL bound to ER α -LBDs in the NABC group with 70-fold higher affinity relative to its binding to LBDs in the ABC group. We also observed that the antagonist (Z)-4-OHT bound to ER α -LBDs in the NABC group with 3.3-fold higher affinity relative to its binding to LBDs in the ABC group. The observed NABC

selectivity of antagonists may be due to the large LBP volumes of the LBDs in NABC, which is able to properly accept relatively large antagonist ligands.

Overall, our docking calculation studies predicted that ER α agonists preferentially bound to ABC group of LBD structure, whereas antagonist ligands showed stronger binding affinity to NABC than that of ABC. Since these results were consistent with previous reports^{28,31}, the necessity of proper selection of ABC/NABC for docking calculation was appropriately confirmed by the results of this study. In addition, we suggest that the possible transcriptional activity of chemicals could be predicted by investigating the selectivity for ABC or NABC using the docking calculation described in this study.

3.4. Importance of C-terminal moiety on ER α -LBD for docking calculation

The C-terminal region of ER α -LBDs demonstrated an apparent variety of conformations between the structures determined as ligand/receptor complexes (Figure 1). To investigate the effects of conformational variance on the *in silico* docking calculations, we compared the RMSD values of all full-length ER α -LBD structures (Table 5). The mean RMSD for all full-length ER α -LBD structures was 2.55 ± 0.03 Å, whereas the values for structures within the ABC and NABC groups were 1.22 ± 0.01 and 1.40 ± 0.02 Å, respectively. As expected, a large RMSD value (4.09 ± 0.09 Å) was derived from comparisons between the ABC and NABC groups.

(Table 5)

Furthermore, to consider the importance of the C-terminal region in the docking calculation, we prepared two *in silico* C-terminal-curtailing mutants involving truncation at K529 (Δ K529C) and at N519 (Δ N519C). As shown in Figure 7, Asn519 is located in the middle of H11, and K529 is located at the end of H11 in close proximity to the loop between H11 and H12 (Figure 7). A series of Δ K529C (lacking the H12 moiety) and Δ N519C (lacking the H11 and H12 moiety) mutants was prepared for all 83 ER α -LBDs that were categorized in the ABC and NABC groups, respectively. To evaluate the effects of the C-terminal region of the LBDs in regard to ligand binding, docking calculations of E2, BPA, (Z)-4-OHT, and RAL were performed using Δ K529C series as templates. Using the obtained ΔG values, $\Delta\Delta G$

was defined as the difference in ΔG values between the ABC and the NABC groups (Figure 8). When the $\Delta K529C$ variants were used as templates for the docking calculation of agonist ligands (E2 and BPA), $\Delta\Delta G$ values were slightly increased compared to those with full-length structures (Figure 8). On the other hand, in the presence of antagonist ligands ((*Z*)-4-OHT and RAL), $\Delta\Delta G$ with the $\Delta K529C$ variants significantly decreased compared to those with the full-length structures. Decreasing of $\Delta\Delta G$ values could indicate that the selectivity of antagonist ligands for the $\Delta K529C$ variants was lower than that for the full-length LBDs. Thus, H12 seems to be important for ligand selectivity particularly in case of antagonists in receptor binding. Here, we defined “ligand selectivity” as the ratio selectivity of agonist to that of antagonist in ligand binding to LBP. Additional docking calculations were performed using the $\Delta K519C$ series to determine the importance of H11, in addition to that of H12. We observed that $\Delta\Delta G$ with the $\Delta N519C$ variants from both the ABC and NABC groups showed low ligand selectivity for E2 and BPA ($\Delta\Delta G = 0.02$ kcal/mol and 0.10 kcal/mol, respectively). Therefore, in the case of agonists, H11 was judged to be important for ligand selectivity. By contrast, in the presence of (*Z*)-4-OHT and RAL, the ligand selectivity was lower in both $\Delta K529C$ and $\Delta N519C$ than in the full-length LBD (Figure 8). The relative decrease in the ABC/NABC selectivity of ligands using the C-terminal-truncated LBDs in the docking calculation indicate that ligand selectivity is chiefly determined by the C-terminal region of ER α -LBD (Figure 8). Therefore, the docking calculations were unable to estimate ligand selectivity in the absence of the region that included H11 and H12 of ER α -LBD (from N519 to the C-terminus).

(Figures 7 and 8)

For further evaluation of the importance of H12 and H11 on ligand orientation in the docking calculation, docked conformation of E2, (*Z*)-4-OHT, and RAL were analyzed. For the analysis, the complex structures consisting of ligands and full-length-, $\Delta K529C$ -, or $\Delta N519C$ -LBDs that were constructed from 1GWR (ABC), and 3ERT and 1ERR (NABC) were utilized (Figure 9). By the deletion of both H11 and H12 of 1GWR, binding conformation of E2 was changed. In contrast, when the H11 was preserved, conformation of

E2 was almost identical to that observed in the full-length LBD. Therefore, the binding conformation and the ligand orientation of E2 may be mainly affected by H11. On the other hand, conformations of antagonists, (Z)-4-OHT and RAL, were quite different between full-length-1GWR and the truncated LBDs, especially between full-length-1GWR and 1GWR-ΔK529C (Figure 9). Furthermore, binding conformation of (Z)-4-OHT and RAL in the truncated LBDs belonging to ABC, 1GWRΔK529C and 1GWRΔN519C, were almost similar to those in the NABC group. In contrast, conformations of antagonists, (Z)-4-OHT and RAL, exhibited only little change in the NABC regardless of existence of H11 and H12 (Figure 10). Thus, the conformation of antagonist ligands in the LBPs is affected depending on the orientation or existence of H12.

(Figures 9 and 10)

4. Conclusion

In this study, we performed *in silico* ligand-docking studies using multiple ERα-LBDs as templates to assess whether the physiological activity of ER ligands could be predicted with high accuracy by statistical analyses of the calculated binding energy values using ABC and NABC of LBDs. In particular, we focused on and tested bisphenol analogs in the docking calculation and found weak agonistic activity of the bisphenol analogs via ERα. Our results suggested that receptor-binding affinity and potential transcriptional activity for various bisphenol analogs could be estimated properly by the calculation. Moreover, we revealed that the C-terminal regions of ERα-LBDs involving H11, H12, and an intermediate loop structure were important for estimation of ligand binding affinity by the docking calculation. Our study highlights that for accurate discrimination of EDCs that exhibit receptor-binding properties and presumed biological activity, it is necessary to analyze statistically results of the docking calculation equipped with different receptor conformations. Acquisition of both ABC and NABC structures of NRs will enable the further development of *in silico* docking-calculation-based risk assessments capable of identification of EDCs.

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Table 1. 3D-structures of ER α used in this study.

PDB ID	Ligand	Mutation	Resolution (Å)	Chain	Reference
1A52	estradiol	Q502E	2.8	A	50
1ERE	estradiol		3.1	A	4
1ERR	raloxifene		2.6	A	4
1G50	estradiol		2.9	A	51
1GWQ	raloxifene core		2.45	A	52
1GWR	estradiol		2.4	A	52
1L2I	(<i>R,R</i>)-5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol	C417X	1.95	A	53
1R5K	(2 <i>E</i>)-3-{4-[(1 <i>E</i>)-1,2-diphenylbut-1-enyl]phenyl} acrylic acid		2.7	A	54
1SJ0	(2 <i>S</i> ,3 <i>R</i>)-2-(4-(2-(piperidin-1-yl)ethoxy)phenyl)-2,3-dihydro-3-(4-hydroxyphenyl)benzo[<i>b</i>][1,4]oxathiin-6-ol		1.9	A	55
1UOM	2-phenyl-1-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-1,2,3,4-tetrahydro-isoquinolin-6-ol	C381S, C417S, C530S	2.28	A	56

Continued Table 1

1X7E	[5-hydroxy-2-(4-hydroxyphenyl)-1-benzofuran-7-yl]acetonitrile	2.8	A	57
1X7R	genistein	2	A	58
1XP1	(2 <i>S</i> ,3 <i>R</i>)-2-(4-{2-[(3 <i>R</i> ,4 <i>R</i>)-3,4-dimethylpyrrolidin-1-yl]ethoxy}phenyl)-3-(4-hydroxyphenyl)-2,3-dihydro-1,4-benzoxathiin-6-ol	1.8	A	59
1XP6	(2 <i>S</i> ,3 <i>R</i>)-2-(4-{2-[(3 <i>S</i> ,4 <i>S</i>)-3,4-dimethylpyrrolidin-1-yl]ethoxy}phenyl)-3-(4-hydroxyphenyl)-2,3-dihydro-1,4-benzoxathiin-6-ol	1.7	A	59
1XP9	(2 <i>S</i> ,3 <i>R</i>)-3-(4-hydroxyphenyl)-2-(4-{[(2 <i>S</i>)-2-pyrrolidin-1-ylpropyl]oxy}phenyl)-2,3-dihydro-1,4-benzoxathiin-6-ol	1.8	A	59
1XPC	(2 <i>S</i> ,3 <i>R</i>)-3-(4-hydroxyphenyl)-2-(4-{[(2 <i>R</i>)-2-pyrrolidin-1-ylpropyl]oxy}phenyl)-2,3-dihydro-1,4-benzoxathiin-6-ol	1.6	A	59

Continued Table 1

1XQC	(1 <i>S</i>)-1-{4-[(9 <i>aR</i>)-octahydro-2 <i>h</i> -pyrido [1,2- <i>a</i>]pyrazin-2-yl]phenyl}-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol1-[4-(octahydro-pyrido[1,2- <i>a</i>]pyrazin-2-yl)- phenyl]-2-phenyl-1,2,3,4-tetrahydro-isoquinolin- 6-ol	C381S, C417S, C530S	2.05	A	60
1YIM	(2 <i>R</i> ,3 <i>R</i> ,4 <i>S</i>)-3-(4-hydroxyphenyl)-4-methyl-2- [4-(2-pyrrolidin-1-ylethoxy)phenyl]chroman- 6-ol		1.9	A	61
1YIN	(2 <i>R</i> ,3 <i>R</i> ,4 <i>S</i>)-5-fluoro-3-(4-hydroxyphenyl)-4-methyl-2-[4-(2-piperidin-1-ylethoxy)phenyl]chroman- 6-ol		2.2	A	61
1ZKY	4-[(1 <i>S</i> ,2 <i>S</i> ,5 <i>S</i>)-5-(hydroxymethyl)-6,8,9-trimethyl- 3-oxabicyclo [3.3.1]non-7-en-2-yl]phenol	C381X, C417X, C530X, Y537S	2.25	A	62
2B1V	4-[(1 <i>S</i> ,2 <i>S</i> ,5 <i>S</i>)-5-(hydroxymethyl)-8-methyl- 3-oxabicyclo [3.3.1]non-7-en-2-yl]phenol	C381X, C417X, C530X	1.8	A	62

Continued Table 1

2B1Z	17-methyl-17-alpha-dihydroequilenin	C381X, C417X, C530X, Y537S	1.78	A	63
2B23	none	C381X, C417X, C530X, Y537S	2.1	A	41
2FAI	4-[(1S,2S,5S,9R)-5-(hydroxymethyl)-8,9-dimethyl- 3-oxabicyclo[3.3.1]non-7-en-2-yl]phenol	C381X, C417X, C530X, Y537S	2.1	A	62
2G44	4-[(1S,2R,5S)-4,4,8-trimethyl-3-oxabicyclo[3.3.1]non-7-en-2-yl]phenol	C381X, C417X, C530X, Y537S	2.65	A	Unpublishe d data (Thesis)
2G5O	(9alpha,13beta,17beta)-2-[(1Z)-but-1-en-1-yl]estra-1,3,5(10)-triene-3,17-diol	C381X, C417X, C530X, Y537S	1.65	A	To be published
2IOG	n-[(1R)-3-(4-hydroxyphenyl)-1-methylpropyl]- 2-[2-phenyl-6-(2-piperidin-1-ylethoxy)-1h-indol-3-yl]acetamide		1.6	A	64
2IOK	n-[(1R)-3-(4-hydroxyphenyl)-1-methylpropyl]- 2-(2-phenyl-1h-indol-3-yl)acetamide		2.4	A	64

Continued Table 1					
2JF9	4-hydroxytamoxifen		2.1	A	65
2JFA	raloxifene		2.55	A	65
2OCF	estradiol	C381X, C417X, Y537S	2.95	A	66
2OUZ	(5 <i>R</i> ,6 <i>S</i>)-6-phenyl-5-[4-(2-pyrrolidin-1-ylethoxy)phenyl]-5,6,7,8-tetrahydronaphthalen-2-ol		2	A	67
2P15	(17β)-17-[(<i>E</i>)-2-[2-(trifluoromethyl)phenyl]vinyl]estra-1(10),2,4-triene-3,17-diol	Y537S	1.94	A	68
2Q70	(3 <i>aS</i> ,4 <i>R</i> ,9 <i>bR</i>)-2,2-difluoro-4-(4-hydroxyphenyl)-1,2,3,3 <i>a</i> ,4,9 <i>b</i> -hexahydrocyclopenta[<i>c</i>]chromen-8-ol	C381S, C417S, C530S	1.95	A	69
2QE4	(3 <i>aS</i> ,4 <i>R</i> ,9 <i>bR</i>)-4-(4-hydroxyphenyl)-6-(methoxymethyl)-1,2,3,3 <i>a</i> ,4,9 <i>b</i> -hexahydrocyclopenta[<i>c</i>]chromen-8-ol	C381S, C417S, C530S	2.4	A	70
2QZO	4-[1-allyl-7-(trifluoromethyl)-1 <i>H</i> -indazol-3-yl]benzene-1,3-diol	Y537S	1.72	A	71

Continued Table 1

2R6W	[6-hydroxy-2-(4-hydroxyphenyl)-1-benzothien-3-yl]{4-[2-(4-methylpiperidin-1-yl)ethoxy]phenyl}methanone	C381S, C417S, C530S	2	A	72
2R6Y	[6-hydroxy-2-(4-hydroxyphenyl)-1-benzothien-3-yl][4-(2-pyrrolidin-1-ylethoxy)phenyl]methanone	C381S, C417S, C530S	2	A	72
2YAT	estradiol-pyridinium tetraacetic acid	C381S, C417S, C530S	2.6	A	73
2YJA	estradiol		1.82	B	74
3DT3	5-(4-hydroxyphenoxy)-6-(3-hydroxyphenyl)-7-methylnaphthalen-2-ol		2.4	A	75
3ERT	4-hydroxytamoxifen		1.9	A	43
3HLV	(9beta,13alpha,16beta)-3,16-dihydroxyestra-1,3,5(10)-trien-17-one	Y537S	3	A	To be published
3HM1	(9beta,13alpha)-3-hydroxyestra-1,3,5(10)-trien-17-one	Y537S	2.33	A	To be published
3L03	(14beta,15alpha,16alpha,17alpha)-estra-1,3,5(10)-trien-3,15,16,17-tetrol	Y537S	1.9	A	To be published

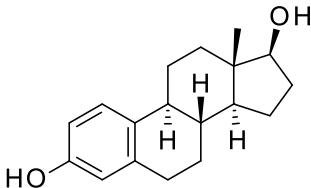
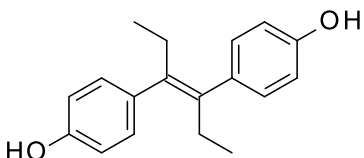
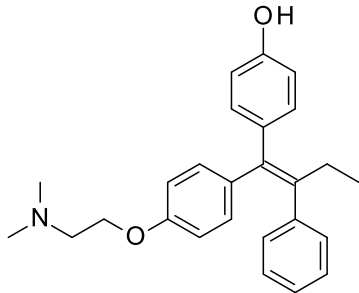
Continued Table 1					
3OS8	4-[1-benzyl-7-(trifluoromethyl)-1h-indazol-3-yl]benzene-1,3-diol	L372R, L536S	2.03	A	35
3OS9	4-[1-allyl-7-(trifluoromethyl)-1h-indazol-3-yl]benzene-1,3-diol	L372R, L536S,	2.3	A	35
3OSA	4-[1-(3-methylbut-2-en-1-yl)-7-(trifluoromethyl)-1h-indazol-3-yl]benzene-1,3-diol	L372R, L536S	2.3	A	35
3Q95	estriol	Y537S	2.05	A	To be published
3Q97	4,4'-[2-(4-ethoxyphenyl)but-1-ene-1,1-diyl]diphenol	Y537S	2.1	A	To be published
3UU7	bisphenol A	Y537S	2.2	A	76
3UUA	bisphenol AF	Y537S	2.05	A	76
3UUC	4,4'-(2,2-dichloroethene-1,1-diyl)diphenol		2.1	A	76
3UUD	estradiol	Y537S	1.6	A	76
4DMA	2'-bromo-6'-(furan-3-yl)-4'-(hydroxymethyl)biphenyl-4-ol	C530A	2.3	A	77
4IU7	4-[2-ethyl-7-(trifluoromethyl)-2h-indazol-3-yl]benzene-1,3-diol	Y537S	2.29	A	78

Continued Table 1					
4IUI	4-[1-butyl-7-(trifluoromethyl)-1h-indazol-3-yl]benzene-1,3-diol	Y537S	2.3	A	78
4IV2	4-[1-(2-methylpropyl)-7-(trifluoromethyl)-1h-indazol-3-yl]benzene-1,3-diol	Y537S	2.14	A	78
4IV4	4-[2-(2-methylpropyl)-7-(trifluoromethyl)-2h-indazol-3-yl]benzene-1,3-diol	Y537S	2.3	A	78
4IVW	4-[2-benzyl-7-(trifluoromethyl)-2h-indazol-3-yl]benzene-1,3-diol	Y537S	2.06	A	78
4IVY	4-[1-(but-3-en-1-yl)-7-(trifluoromethyl)-1h-indazol-3-yl]benzene-1,3-diol	Y537S	1.95	A	78
4IW6	4-[2-(but-3-en-1-yl)-7-(trifluoromethyl)-2h-indazol-3-yl]benzene-1,3-diol	Y537S	1.98	A	78
4IW8	4-[1-(3-methylbut-2-en-1-yl)-7-(trifluoromethyl)-1h-indazol-3-yl]benzene-1,3-diol	Y537S	2.04	A	78
4IWC	4,4'-thiene-2,5-diylbis(3-methylphenol)	Y537S	2.24	A	78
4IWF	2-chloro-3'-fluoro-3-[(<i>E</i>)-(hydroxyimino)methyl]biphenyl-4,4'-diol		1.93	A	78

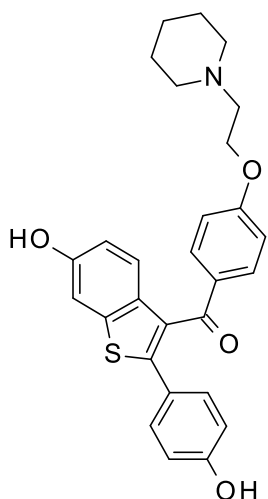
Continued Table 1					
4MG5	chlordecone	Y537S	2.05	A	79
4MG6	benzyl butyl benzene-1,2-dicarboxylate	Y537S	2.1	A	79
4MG7	ferutinine	Y537S	2.15	A	79
4MG8	alpha-zearalanol	Y537S	1.85	A	79
4MG9	butyl 4-hydroxybenzoate	Y537S	2.0	A	79
4MGA	4-(2,4,4-trimethyl pentan-2-yl)phenol	Y537S	1.8	A	79
4MGB	4,4'-propane-2,2-diylbis(2,6-dichlorophenol)	Y537S	1.85	A	79
4MGC	bis(2,4-dihydroxyphenyl) methanone	Y537S	2.15	A	79
4MGD	HPTE	Y537S	1.9	A	79
4PP6	resveratrol	Y537S	2.2	A	80
4PPP	5-[(E)-2-(3-fluoro-4-hydroxyphenyl)ethenyl] benzene- 1,3-diol(fluoro-resveratrol)	Y537S	2.69	A	80
4PPS	(1S,3aR,5R,7aS)-5-(4-hydroxyphenyl)-7a-methyloctahydro- 1h-inden-1-ol	Y537S	1.93	A	80
4PXM	estradiol	D538G	1.9	A	81
4Q13	none	D538G	2.24	B	81
4Q50	4-hydroxytamoxifen	D538G	3.07	A	81

Continued Table 1					
4TUZ	(3 <i>S</i> ,7 <i>R</i> ,11 <i>E</i>)-7,14,16-trihydroxy-3-methyl-3,4,5,6,7,8,9,10-octahydro-1 <i>H</i> -2-benzoxacyclotetradecin-1-one	Y537S	1.9	A	82
4TV1	propyl 4-hydroxybenzoate	Y537S	1.85	A	82
4ZN9	cyclohexa-2,5-dien-1-yl (1 <i>S</i> ,2 <i>R</i> ,4 <i>S</i>)-5,6-bis(4-hydroxyphenyl)-7-oxabicyclo[2.2.1]hept-5-ene-2-sulfonate	Y537S	2.21	A	43

Table 2. Chemical structure of compounds utilized in this study.

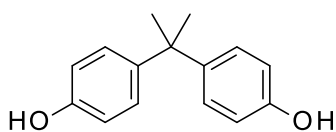
Structure	Name	K_d reported in the literatures
	IUPAC name	
	17β-estradiol (E2)	
	(8 <i>R</i> ,9 <i>S</i> ,13 <i>S</i> ,14 <i>S</i> ,17 <i>S</i>)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[<i>a</i>]phenanthrene-3,17-diol	0.13 nM ³¹
	diethylstilbestrol (DES)	
	4,4'-(3 <i>E</i>)-hex-3-ene-3,4-diylbisphenol	0.04 nM ³¹
	4-hydroxytamoxifen (4-(<i>Z</i>)-OHT)	
	4-[(<i>Z</i>)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-2-phenylbut-1-enyl]phenol	0.1 nM ³¹

Continued Table 2



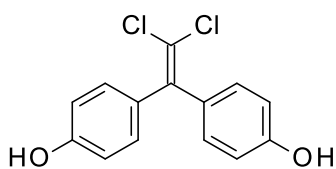
Raloxifene (RAL)

[6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophen-3-yl]-[4-(2-piperidin-1-ylethoxy)phenyl]methanon

0.38 nM³⁰

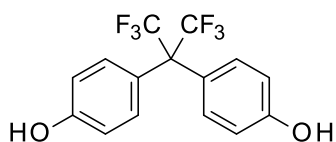
bisphenol A (BPA)

4-[2-(4-hydroxyphenyl)propan-2-yl]phenol

984 nM³⁵

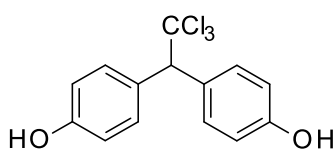
bisphenol C (BPC)

1,1-bis(4-hydroxyphenyl)-2,2-dichloroethylene

42.3 nM³⁵

bisphenol AF (BPAF)

4-[1,1,1,3,3,3-hexafluoro-2-(4-hydroxyphenyl)propan-2-yl]phenol

103 nM³⁵

HPTE

4-[2,2,2-trichloro-1-(4-hydroxyphenyl)ethyl]phenol

51 nM³⁴

Table 3. Calculated binding energy of E2 and (Z)-4-OHT to ER α in the presence or absence of water molecules.

Chemicals	ABC or NABC	ΔG (kcal/mol)			
		Water preserved		Water removed	
E2	ABC	-10.2	± 0.1	-10.2	± 0.1
	NABC	-9.89	± 0.07	-9.89	± 0.06
(Z)-4-OHT	ABC	-10.8	± 0.1	-11.0	± 0.1
	NABC	-11.5	± 0.1	-11.8	± 0.1

Values are Mean $\pm$ S. E.

Table 4. Calculated binding energy between the test chemicals and ER α .

Chemicals	ΔG (kcal/mol)						$K_{\text{dnabc}}/K_{\text{dabc}}$
	All		ABC		NABC		
	Mean	S.E.	Mean	S.E.	Mean	S.E.	
E2 [†]	-10.1	± 0.1	-10.2	± 0.1	-9.90	± 0.07	1.7
DES	-8.80	± 0.04	-8.84	± 0.07	-8.73	± 0.06	1.2
(Z)-4-OHT [†]	-11.1	± 0.1	-10.8	± 0.2	-11.5	± 0.1	0.30
RAL [†]	-11.5	± 0.2	-10.7	± 0.3	-13.2	± 0.2	0.014
BPA [†]	-7.95	± 0.03	-8.01	± 0.04	-7.83	± 0.06	1.4
BPC [†]	-8.31	± 0.03	-8.38	± 0.05	-8.17	± 0.06	1.4
BPAF [‡]	-7.36	± 0.03	-7.41	± 0.04	-7.24	± 0.06	1.3
HPTE [†]	-8.58	± 0.04	-8.66	± 0.06	-8.42	± 0.06	1.5

Mean: Means of calculated ΔG value. S.E.: Standard error.

[†]: $P < 0.05$ in Welch's t -test between ΔG values of ABC and NABC.

[‡]: $0.05 < P < 0.1$ in Welch's t -test between ΔG values of ABC and NABC.

Table 5. RMSD analysis for each receptor conformation group of ER α .

Compared conformation	RMSD of Full-length LBD (Å)
All conformations	2.55 $\pm$ 0.03
ABC vs. ABC	1.22 $\pm$ 0.01
NABC vs. NABC	1.40 $\pm$ 0.02
ABC vs. NABC	4.09 $\pm$ 0.03

Values are Mean $\pm$ S. E.

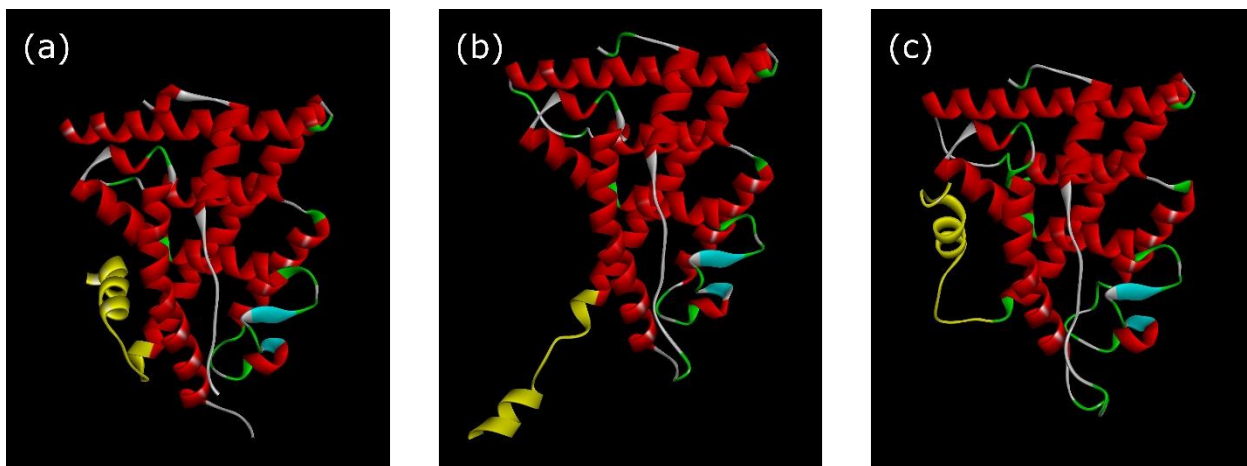


Figure 1. Different spatial positioning of H12 on ER α -LBDs determined by X-ray crystallography.

C-terminal tail of ER α -LBDs from Lys529 to its end are colored in yellow. (a) ABC (agonist-bound conformation); 1GWR, (b) NABC (apo-conformation); 1A52, and (c) NABC (antagonist-bound conformation); 3ERT.

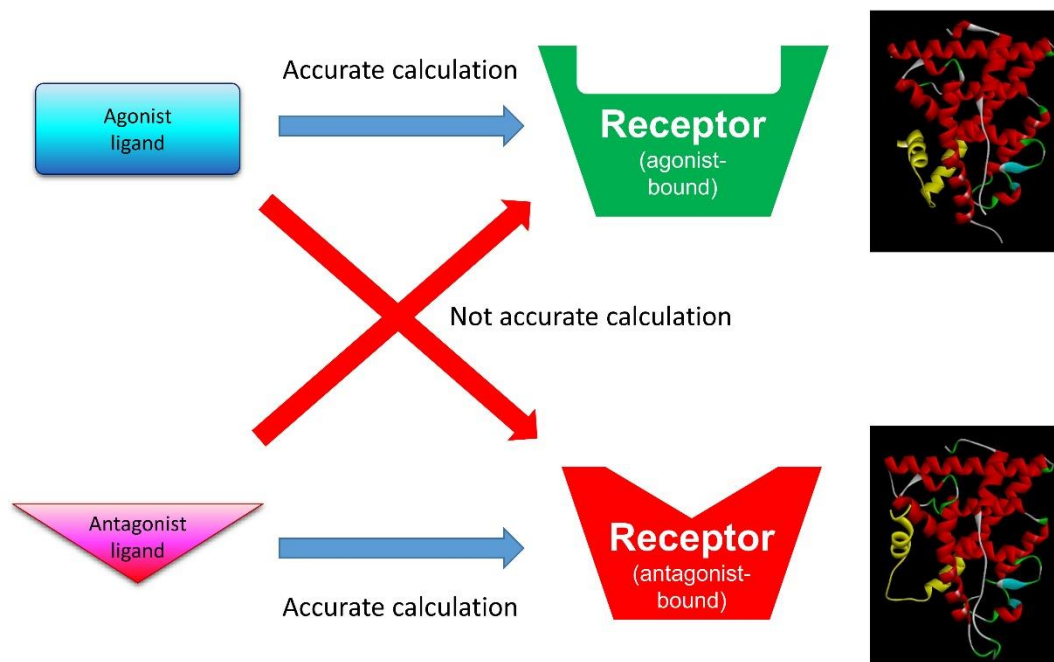


Figure 2. Importance of receptor conformation in docking calculation using rigid receptor conformation.

The C-terminal tail of ER α -LBDs from Asn519 to its end is colored in yellow.

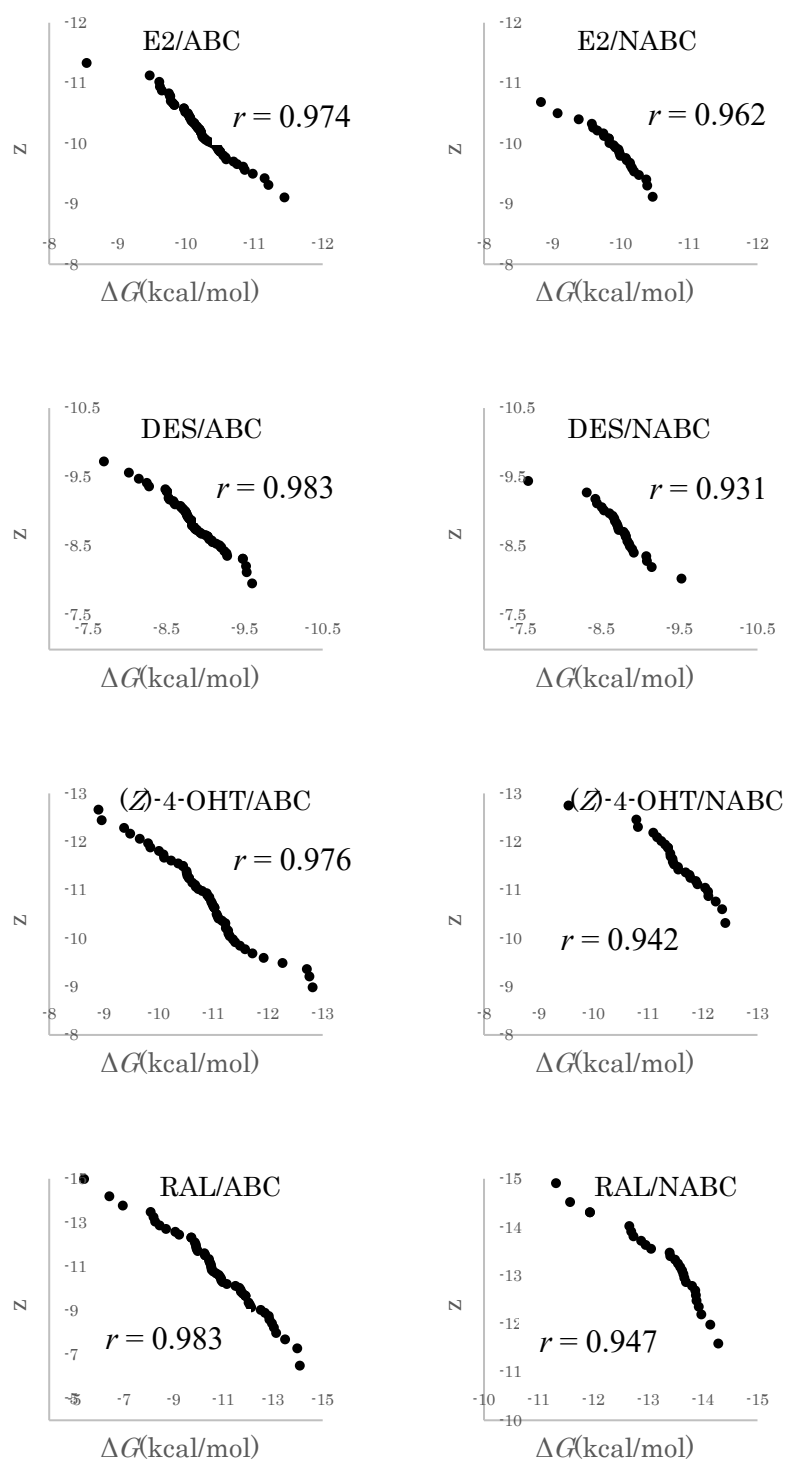


Figure 3. Q-Q plot for estimation of average binding energy ΔG (1/3).

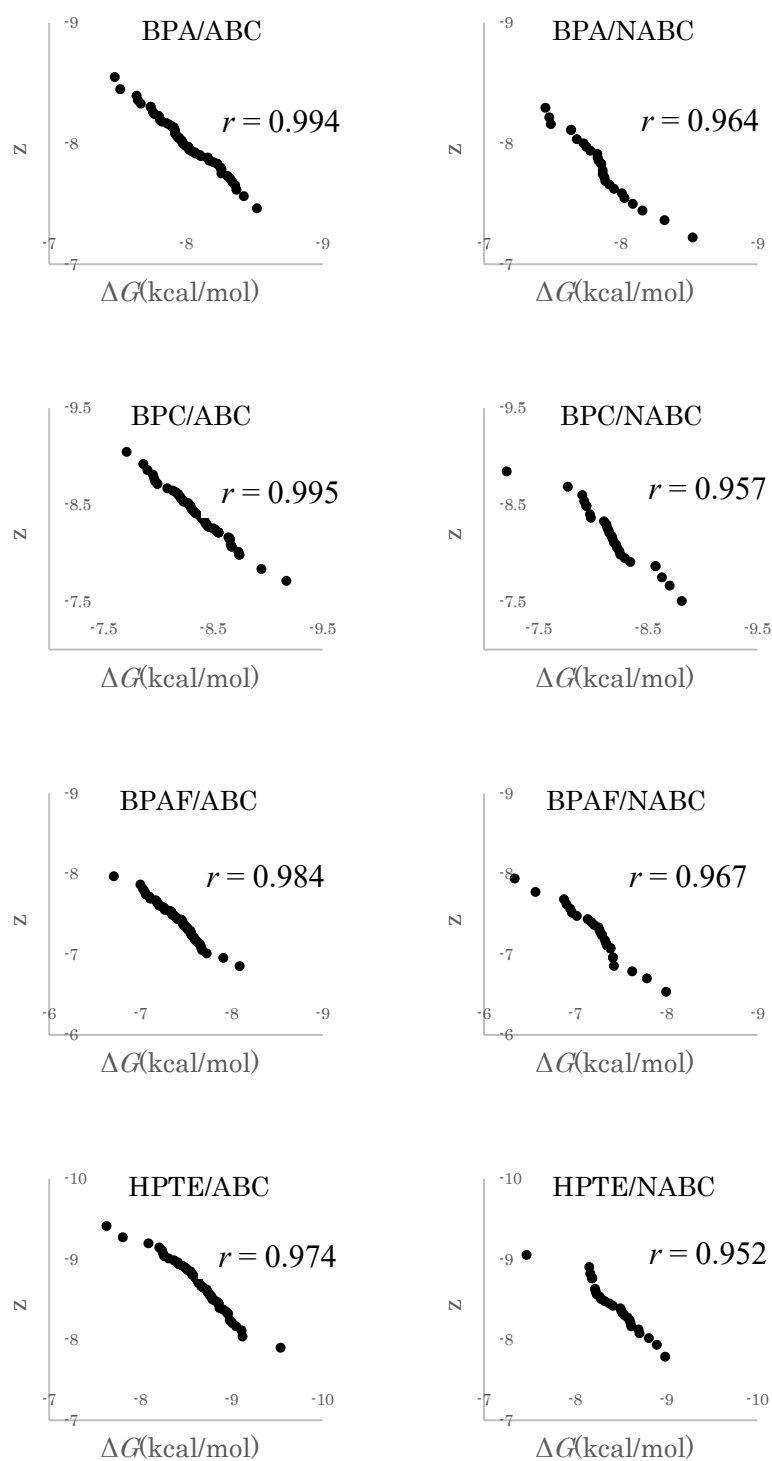


Figure 3. Q-Q plot for estimation of average binding energy ΔG (2/3).

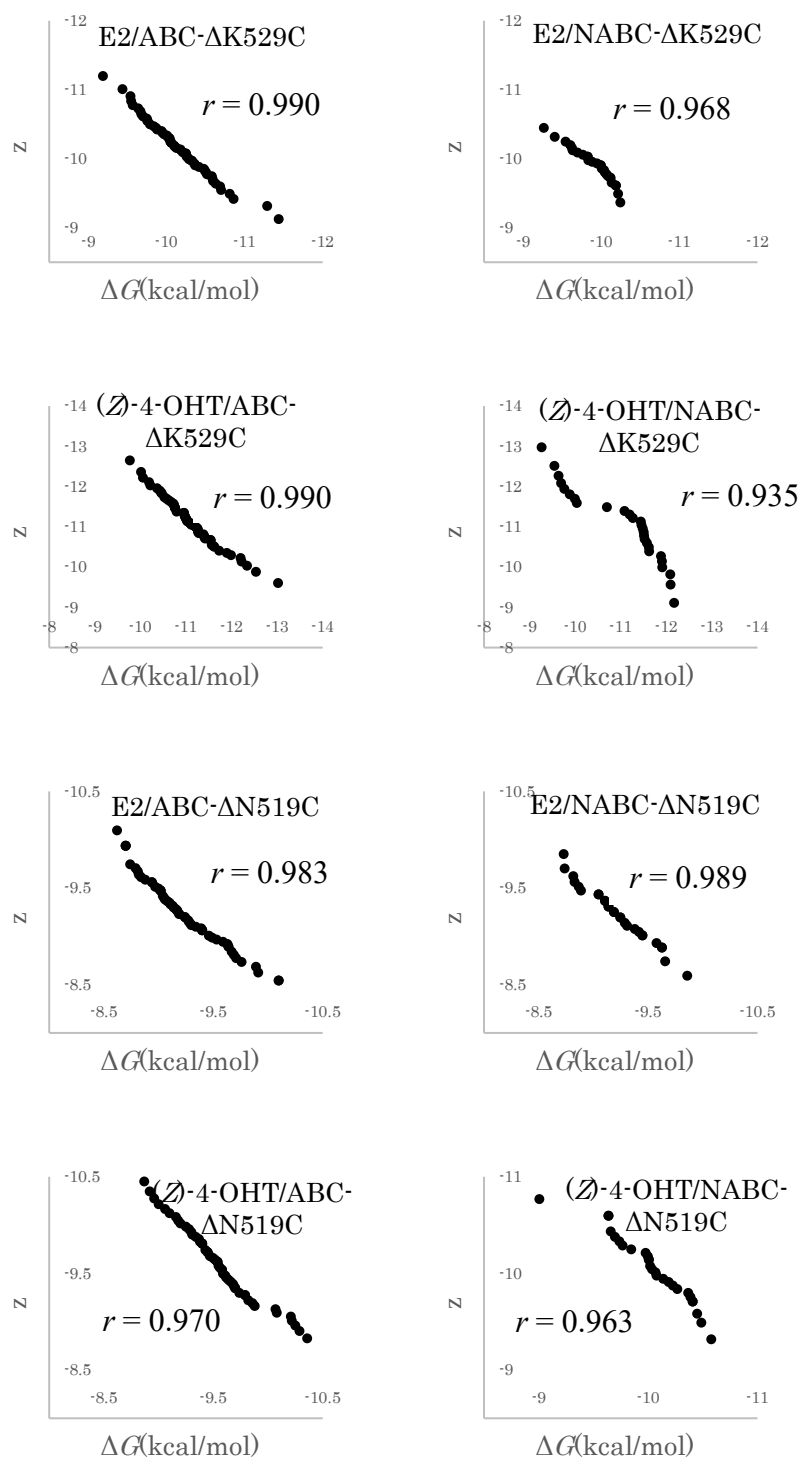


Figure 3. Q-Q plot for estimation of average binding energy ΔG (3/3).

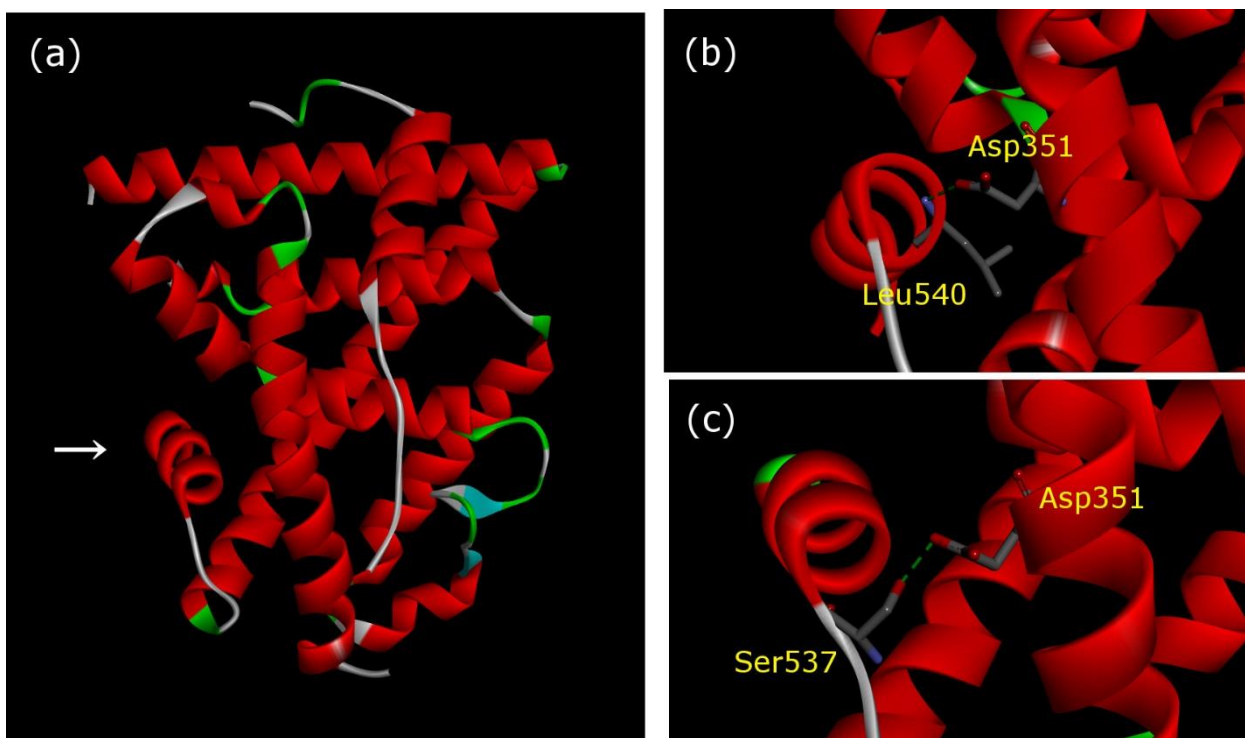


Figure 4. Representative position of H12 in agonist-bound conformation (ABC).

(a) H12 of the ER α -LBD (PDB: 1ERE) was indicated by an arrow. (b) Hydrogen bonds between Leu540 and Asp351 was formed in the ABC of wild type ER α -LBD. (c) Ser537 and Asp351 in the Y537S mutant formed hydrogen bond.

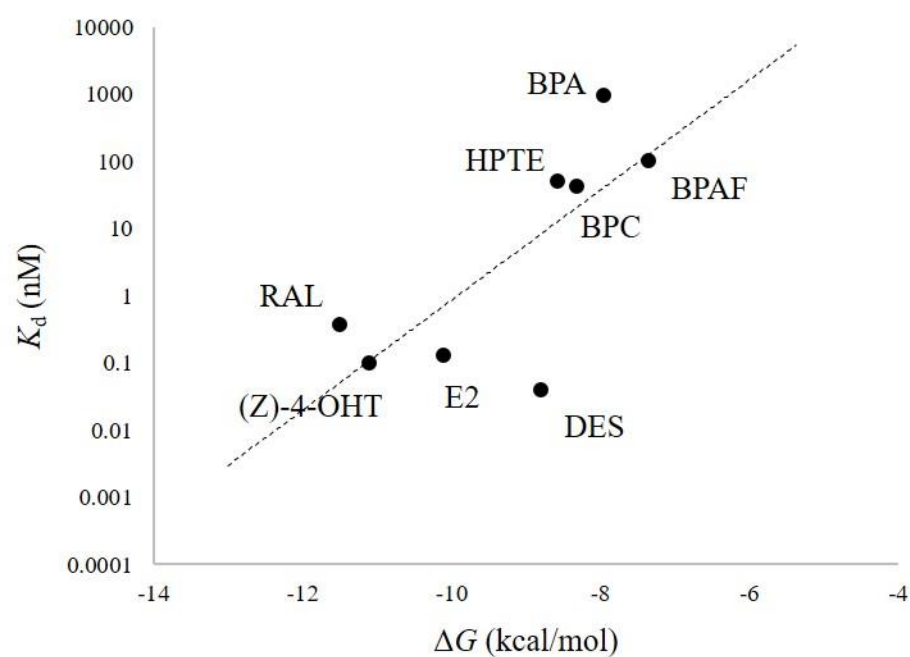


Figure 5. Pearson's product-moment correlation between calculated ΔG and reported K_d value.

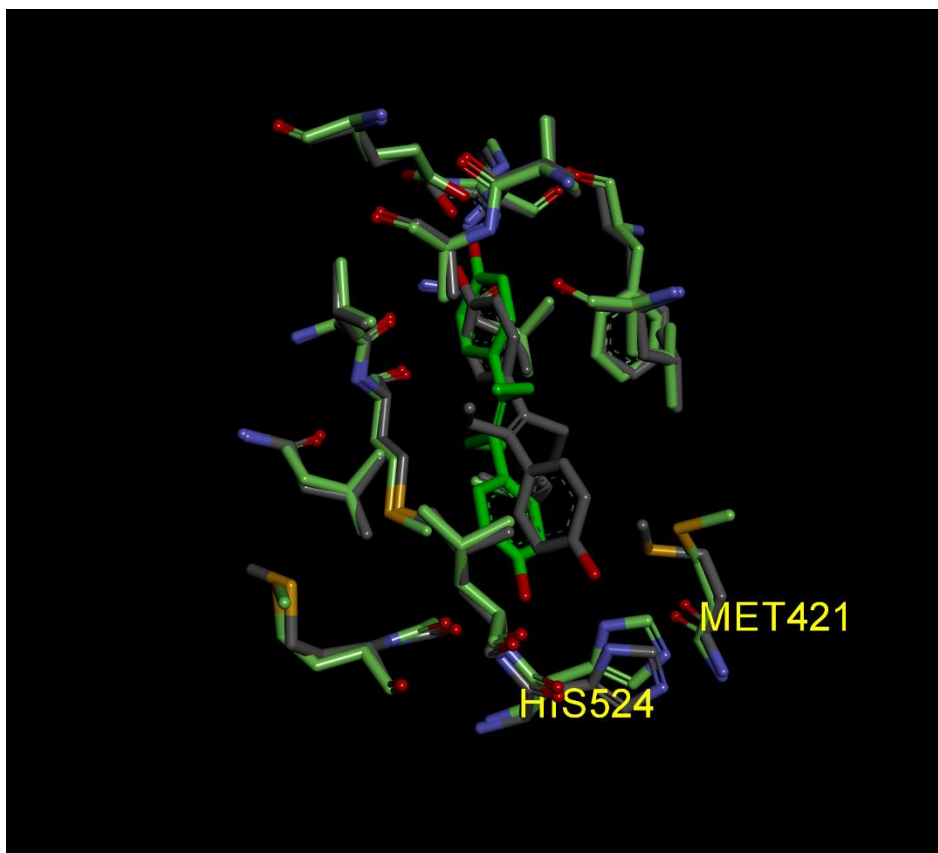


Figure 6. Comparison of the structures between calculated and X-ray crystallographically determined DES-docked ER α complexes.

Black: DES-ER α complex as 3ERD in PDB (38). Green: DES-ER α complex calculated in this study using 1GWR as a template.

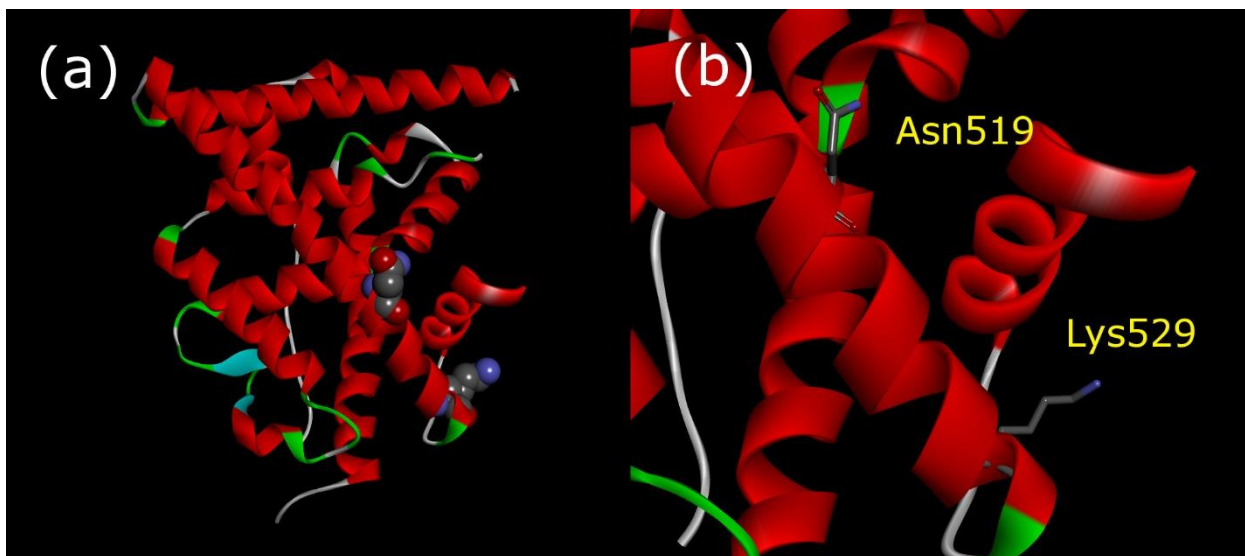


Figure 7. Spatial positioning of Asn519 and Lys529 on ER α -LBD (PDB: 1GWR).

(a) Whole ER α -LBD structure is shown. Asn519 and Lys529 are shown by CPK models.

(b) Enlarged view around H11 and H12 is shown. Asn519 and Lys529 are shown by stick models. N519 was located on H11, whereas K529 was located on the loop between H11 and H12.

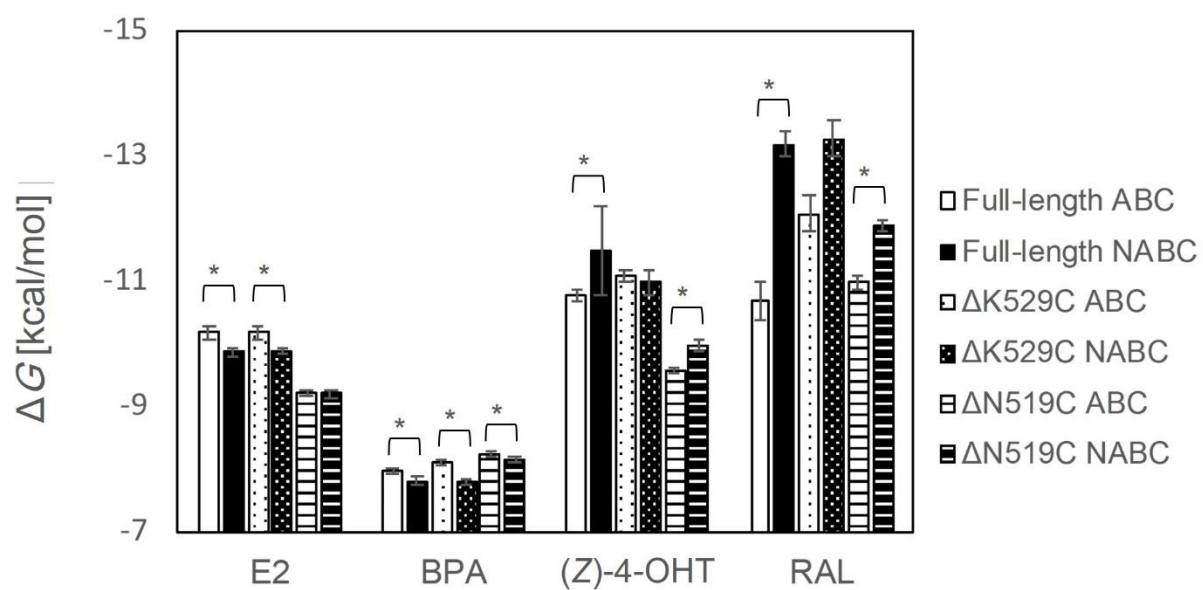


Figure 8. Comparison of binding energies of the ligands for the full-length LBD, ΔK529C, and ΔN519C.

Ligands indicated are: E2, BPA, (Z)-4-OHT, and RAL.

* $P < 0.05$ in Welch's t -test between ΔG values of ABC and NABC.

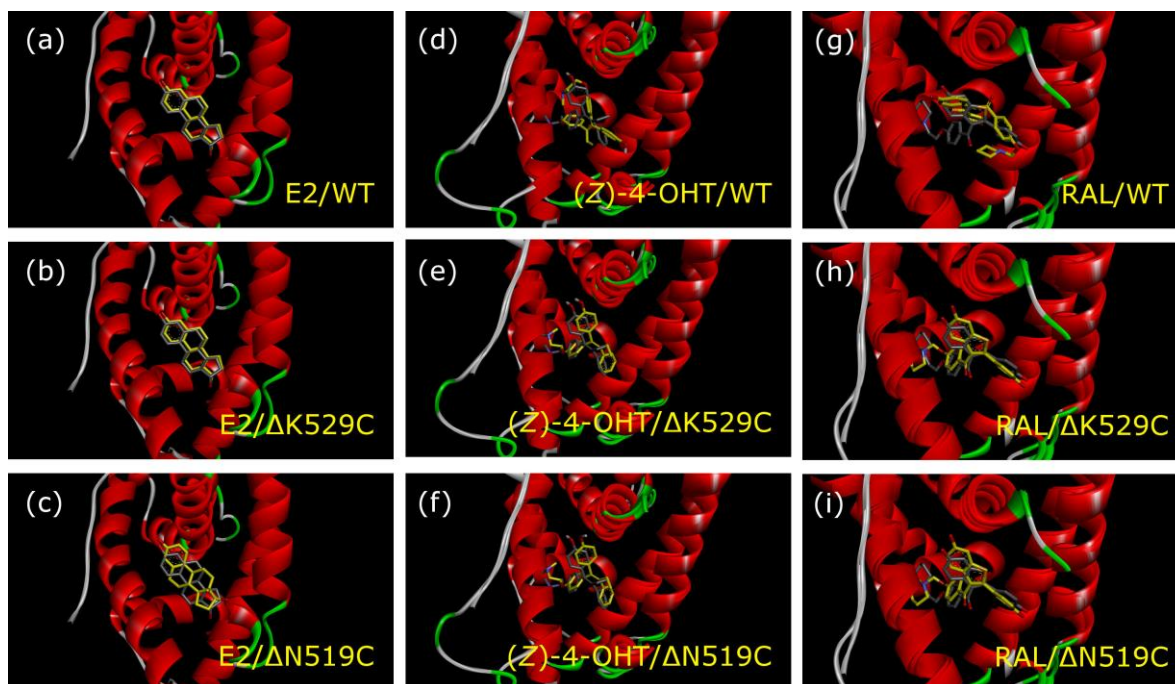


Figure 9. Predicted binding conformation of E2, (Z)-4-OHT, and RAL to the ABC.

Predicted conformations of E2 with (a) full-length, (b) $\Delta K529C$, and (c) $\Delta N519C$ of 1GWR-LBD were shown. Ligand conformation from PDB is colored black (PDB: 1GWR). Predicted conformations of (Z)-4-OHT with (d) full-length, (e) $\Delta K529C$, and (f) $\Delta N519C$ of 1GWR-LBD were shown. Ligand conformation from PDB is colored black (PDB: 3ERT). Predicted conformations of RAL docked with (g) full-length, (h) $\Delta K529C$, and (i) $\Delta N519C$ 1GWR-LBD were shown. Predicted conformation is colored yellow, and ligand conformation from PDB is colored black (PDB: 1ERR).

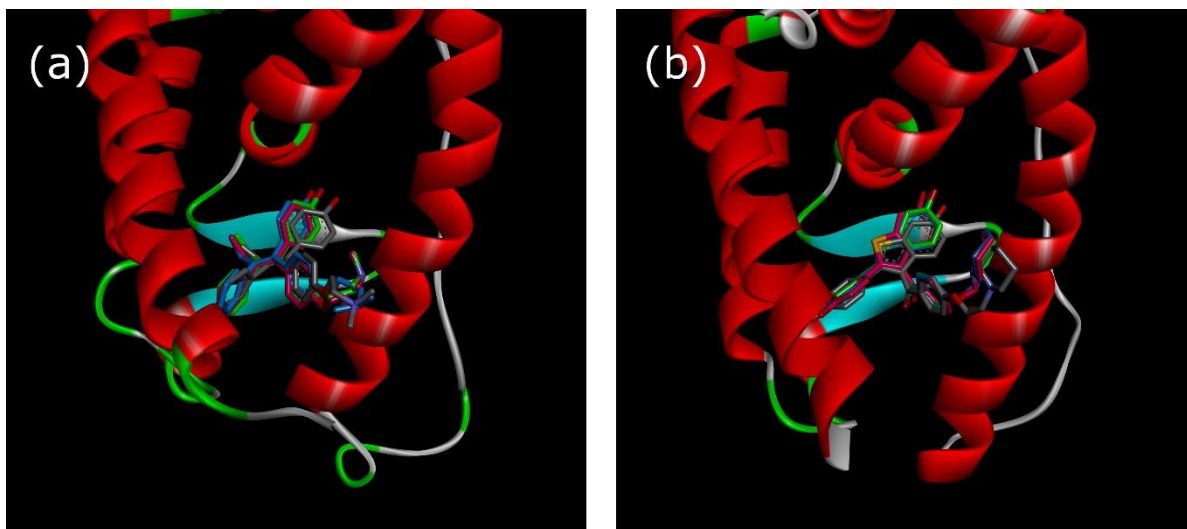


Figure 10. Predicted binding conformation of (Z)-4-OHT and RAL to the NABC.

(a) Predicted conformation of (Z)-4-OHT docked with full-length (magenta), $\Delta K529C$ (cyan), and $\Delta N519C$ (green) of 3ERT-LBD were shown. Ligand conformation from PDB is colored black.

(b) Predicted conformation of RAL docked with full-length (magenta), $\Delta K529C$ (cyan), and $\Delta N519C$ (green) of 1ERR were shown. Ligand conformation from PDB is colored black.

CHAPTER 2

Evaluation of the influence of halogenation on the binding affinity and transcriptional activity of bisphenol A to the estrogen receptors

Abstract

As alternatives to bisphenol A (BPA), a variety of halogenated bisphenol analogs are developed as base materials and used for many types of consumer products. Although there are so many opportunities for the BPA analogs to contact living systems, the available information on the potential toxicity of halogenated BPA analogs is still very limited. Therefore, assessment of the latent toxicity of halogenated BPA analogs is required. In the present study, the binding and transcriptional activities of halogenated BPA analogs to the estrogen receptors (ERs), a nuclear receptor known as representative target of endocrine disrupting chemicals including BPA were examined by *in vitro* methods. The halogenation slightly increased the ER α -binding affinity of BPA. Contrastingly, tri- or tetra-fluorinated BPA analogs showed strong binding affinity to ER β although other halogenated BPA analogs with large and multiple halogen substituents indicated binding affinity equivalent to or weaker than that of BPA. Furthermore, the fluorinated BPA analogs had stronger agonistic activity than BPA on both subtypes of ERs. Thus, it was suggested that the fluorination of BPA may have a potential to induce endocrine disruption via ERs. These results suggest that it is necessary to conduct extensive investigations on the risk of halogenation of chemicals involving halogenation of BPA analogs, due to their unanticipated effects on binding and activation for various receptor molecules including the ERs.

1. Introduction

Bisphenol A (4,4'-(propane-2,2-diyl)diphenol: BPA) is an organic compound consisting of two phenol rings and two methyl groups connected by a methylene bridge. BPA has been used in polycarbonate plastics and epoxy resins for products such as baby bottles

and food storage containers¹⁻³, even though BPA is recognized as an endocrine disrupting chemical (EDC)^{2,4}. EDCs have been concerned to cause adverse effects on nerves⁵, sexual glands⁶, and other endocrine components^{7,8} by binding to a variety of nuclear receptors (NRs). Therefore, several NRs are considered to be major target of EDCs. Although BPA is referred to as a xenoestrogen, it shows only modest binding activity to estrogen receptors (ERs). On the other hand, it is reported that BPA strongly binds to estrogen-related receptor γ (ERR γ)⁹, which is an NR closely related to ERs and contributes to the growth of nerves and sexual glands of the mammals. ERR γ is one of the constitutively active receptors that activate signal transduction without binding of agonists. Interestingly, BPA does not change the transcriptional activity of ERR γ , despite showing significantly strong binding affinity to the ERR γ . Thus, specific mechanism of endocrine disruption by BPA via NRs (including ERs and ERR γ) has still been under investigation. In addition, previous studies also demonstrated that BPA has not only estrogenic activity, but also several other types of biological toxicity; for example, BPA can induce mitochondrial injury¹⁰ and apoptotic death in several types of cells^{5,11-13}. In this context, the use of BPA is currently regulated in the countries, including the United States, Canada, the EU and the People 's Republic of China¹⁴.

Owing to the current restrictions on the use of BPA, several bisphenol analogs, known as “next-generation bisphenols,” have been developed and used as alternatives to BPA¹⁵. Most of the next-generation bisphenols share a backbone structure similar to BPA with various additional functional groups in the central part of the molecule or on the phenolic moieties (Figure 1). Halogenated BPA analogs, possessing halogen atoms on their aromatic rings or methyl groups, are included in next-generation bisphenols that applied to manufacture variety of consumer products. For instance, tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA), the representative halogenated BPA analogs, used mainly to produce flame retardants (Figure 1). Thus, these halogenated BPA analogs come to be found ubiquitously in the environment^{16,17}. Recently, these halogenated BPA analogs including TBBPA began to attract much attention, as it was emerged that these BPA analogs showed higher estrogenic activity or toxicity than BPA itself. As an example of toxicity, it was previously reported that TBBPA and TCBPA bound to thyroid hormone receptors and

suppress triiodothyronine (T₃)-induced tail shortening of tadpoles *in vivo*¹⁶. It was also reported that TBBPA binds to peroxisome proliferator-activated receptor γ (PPAR γ) and is able to act as a partial agonist¹⁷. These studies suggested that the halogenated BPAs may lead to endocrine disruption as same as BPA. In addition, TCBPA exhibited estrogenic activity equivalent to or higher than that of BPA^{18,19}. This result indicated that halogenated BPA analogs act as strong xenoestrogens, which bind to estrogen receptor subtypes and disrupt transcriptional activities via these receptors. However, the available information related to the endocrine disruption and potential toxicity of the halogenated BPA analogs is much scarcer than that of original BPA.

(Figure 1)

In the present study, a series of halogenated BPA analogs was synthesized and investigated ERs-binding activities to elucidate the effects of the halogenation of BPA at the aromatic rings on the activities. The transcriptional activity of the ERs induced by the synthetic BPA analogs was also examined by using a reporter gene assay.

2. Material and Methods

2. 1. Chemicals

2,2-Bis(4-hydroxyphenyl)propane (bisphenol A), *N*-fluorobenzenesulfonimide, trichloroisocyanuric acid, *N*-bromosuccinimide, and *N*-iodosuccinimide were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). *N,N*-dimethylformamide (DMF) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents and solvents were purchased from commercial suppliers and used without further purification. All organic reactions were carried out in oven-dried glassware with magnetic stirring. Column chromatography was performed using silica gel (Silica Gel 60 N, 0.063-0.210 mm, Kanto Chemical (Tokyo, Japan)). ¹H-NMR spectra were measured on JEOL JNM-ECA600 spectrometer and JEOL JNM-AL400 spectrometer (JEOL Ltd., Tokyo, Japan). Tetramethylsilane (TMS) served as the internal standard for ¹H-NMR (0 ppm) and ¹³C-NMR (0 ppm). (Trifluoromethyl)benzene served as the internal standard for ¹⁹F-NMR (-63.72 ppm).

2. 2. Synthesis of halogenated BPA analogs

Synthesis of the halogenated BPA analogs was carried out by aromatic electrophilic substitution reaction of BPA. All reactions were carried out in oven-dried glassware with magnetic stirring. Purity and molecular structure of each BPA analog were confirmed by NMR. ^1H -NMR spectra were measured on JEOL JNM-ECA600 spectrometer and JEOL JNM-AL400 spectrometer (JEOL Ltd.). Tetramethylsilane (TMS) was used as the internal standard for ^1H -NMR (0 ppm) and ^{13}C -NMR (0 ppm). (Trifluoromethyl)benzene was used as the internal standard for ^{19}F -NMR (-63.72 ppm).

2-Fluoro-4-[1-(4-hydroxyphenyl)-1-methylethyl]phenol (BPA-monoF).

N-fluoro-benzenesulfonimide (1.00 mmol, 0.323 g) was added to a solution of bisphenol A (1.00 mmol, 0.232 g) in DMF (0.99 mL)²⁰. The resulting mixture was stirred overnight at 100°C. The mixture was extracted with ethyl acetate. The organic phase was washed with water and brine, and dried over Na_2SO_4 . After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed solvent of petroleum ether and ethyl acetate (3.5:1, v/v). Removal of the solvent from appropriate fractions gave BPA-monoF.

2,2-Bis(3-fluoro-4-hydroxyphenyl)propane (BPA-diF).

Selectfluor (4.43 mmol, 1.57 g) was added to a solution of bisphenol A (2.01 mmol, 0.460 g) in a mixed solvent of methanol and acetonitrile (2:1, v/v, 60 mL). The resulting mixture was stirred for 4 h at 70°C. The reaction solvent was removed under reduced pressure. The crude reaction mixture was redissolved in 50 mL of dichloromethane. After removal of the insoluble matter by filtration, the filtrate was washed with water and brine, and dried over Na_2SO_4 . After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed solvent of hexane and ethyl acetate (5:1, v/v). Removal of the solvent from appropriate fractions gave BPA-diF.

2,6-Difluoro-4-[1-(3-fluoro-4-hydroxyphenyl)-1-methylethyl]phenol (BPA-triF).

To a solution of BPA-triBr-MOM (described later) (0.805 g, 1.46 mmol) in THF (10 mL) at -78°C was slowly added *n*-butyllithium (7.4 mL of 2.5 M in hexanes)²¹. The resulting solution was stirred at -78°C for 0.5 h and a solution of *N*-fluorobenzenesulfonimide (2.296 g, 7.28 mmol) in THF (15 mL) was added. After an additional 2 h at -78°C, the reaction mixture was warmed to room temperature, poured into water, and extracted with ethyl acetate. The organic phase was washed with water and brine, and dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The residual product was then added to a mixed solvent of 12 M of HCl and THF (1:1, v/v, 10 mL) and stirred at 25°C for 24 h to cleave MOM ether. The resulting mixture was concentrated under reduced pressure. The obtained crude product was purified by silica gel column chromatography using a mixed solvent of hexane and ethyl acetate (15:1, v/v). Removal of the solvent from appropriate fractions gave BPA-triF.

2,2-Bis(3,5-difluoro-4-hydroxyphenyl)propane (BPA-tetraF).

BPA-tetraF was synthesized and purified by the same procedure as BPA-triF synthesis except for usage of BPA-tetraBr-MOM (described later) as a starting material.

2-Chloro-4-[1-(4-hydroxyphenyl)-1-methylethyl]phenol (BPA-monoCl).

Pyridine (0.200 mmol, 16.0 µL) was added to a solution of bisphenol A (2.00 mmol, 0.455 g) in a mixed solvent of dichloromethane and ethyl acetate (10:1, v/v, 11 mL) was added pyridine (0.200 mmol, 16.0 µL). The mixture was slowly added trichloroisocyanuric acid (4.00 mmol, 0.925 g)²². The resulting mixture was stirred overnight at reflux. The mixture was extracted with ethyl acetate. The organic phase was washed with water and brine, and dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed solvent of hexane and ethyl acetate (4:1, v/v). Removal of the solvent from appropriate fractions gave BPA-monoCl.

2,2-Bis(3-chloro-4-hydroxyphenyl)propane (BPA-diCl) and 2,6-dichloro-4-[2-(3-chloro-4-hydroxyphenyl)propan-2-yl]phenol (BPA-triCl).

Pyridine (0.200 mmol, 16.0 μ L) was added to a solution of bisphenol A (2.01 mmol, 0.460 g) in a mixed solvent of dichloromethane and ethyl acetate (5:1, v/v, 12 mL). The mixture was slowly added trichloroisocyanulic acid (1.35 mmol, 0.314 g) at 0°C. The resulting mixture was stirred for 4 h at room temperature. The mixture was extracted with ethyl acetate. The organic phase was washed with water and brine, and then dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The obtained crude product, which including BPA-diCl and BPA-triCl, was purified by silica gel column chromatography using a mixed solvent of hexane and ethyl acetate (5:1, v/v). Removal of the solvent from appropriate fractions gave BPA-diCl and BPA-triCl separately.

2-Bromo-4-[1-(4-hydroxyphenyl)-1-methylethyl]phenol (BPA-monoBr).

N-bromosuccinimide (2.06 mmol, 0.366 g) and methanesulfonic acid (2.00 mmol, 130 μ L) was added to a solution of bisphenol A (6.07 mmol, 1.39 g) in acetonitrile (5.0 mL)²³. The resulting mixture was stirred for 22 h at room temperature. The mixture was extracted with ethyl acetate. The organic phase was washed with water and brine, and then dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed solvent of petroleum ether and ethyl acetate (6:1, v/v). Removal of the solvent from appropriate fractions gave BPA-monoBr.

2,2-Bis(3-bromo-4-hydroxyphenyl)propane (BPA-diBr).

N-bromosuccinimide (4.06 mmol, 0.721 g) and methanesulfonic acid (2.00 mmol, 130 μ L) was added to a solution of bisphenol A (2.01 mmol, 0.460 g) in acetonitrile (5.0 mL). The resulting mixture was stirred for 24 h at room temperature. The mixture was extracted with ethyl acetate. The organic phase was washed with water and brine, and then dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed

solvent of petroleum ether and ethyl acetate (8:1, v/v). Removal of the solvent from appropriate fractions gave BPA-diBr.

2,6-Dibromo-4-[1-(3-bromo-4-hydroxyphenyl)-1-methylethyl]phenol (BPA-triBr).

N-bromosuccinimide (12.1 mmol, 2.148 g) was added to a solution of bisphenol A (4.01 mmol, 0.916 g) in acetonitrile (15 mL), followed by methanesulfonic acid (6.01 mmol, 390 μ L). The resulting mixture was stirred for 24 h at room temperature. The mixture was extracted with ethyl acetate. The organic phase was washed with water and brine, and then dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed solvent of hexane and ethyl acetate (8:1, v/v). Removal of the solvent from appropriate fractions gave BPA-triBr.

1,3-Dibromo-5-{2-[3-bromo-4-(methoxymethoxy)phenyl]propan-2-yl}-2-(methoxymethoxy) benzene (BPA-triBr-MOM)²⁴.

Sodium hydride (60% oil, 0.625 g, 15.6 mmol) was suspended in THF (7.5 mL) at 0°C under N₂. A solution of BPA-triBr (0.893 g, 1.92 mmol) in THF (7.5 mL) was slowly added to NaH suspension. The resulting mixture was stirred for 2 h at 25°C. Chloromethyl methyl ether (1.0 mL, 13.2 mmol) was slowly added to the mixture at 0°C. The reaction mixture was stirred for 20 h at 25°C and extracted with ethyl acetate. The organic phase was washed with water and brine, and dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The obtained crude product was purified by silica gel column chromatography using a mixed solvent of hexane and ethyl acetate (15:1, v/v). Removal of the solvent from appropriate fractions gave BPA-triBr-MOM.

5,5'-(Propane-2,2-diyl)bis[1,3-dibromo-2-(methoxymethoxy)benzene] (BPA-tetraBr - MOM).

Sodium hydride (60% oil, 0.962 g, 24.1 mmol) was suspended in THF (10 mL) at 0°C under N₂. A solution of BPA-tetraBr (1.64 g, 3.01 mmol, purchased from Tokyo

Chemical Industry Co. Ltd.) in THF (10 mL) was slowly added to NaH suspension. The resulting mixture was stirred for 2 h at 25°C. Chloromethyl methyl ether (1.40 mL, 18.4 mmol) was slowly added to the mixture at 0°C. The reaction mixture was stirred for 20 h at 25°C and extracted with ethyl acetate. The organic phase was washed with water and brine, and dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The obtained crude product was purified by silica gel column chromatography using a mixed solvent of hexane and ethyl acetate (20:1, v/v). Removal of the solvent from appropriate fractions gave BPA-tetraBr-MOM.

2-Iodo-4-[1-(4-hydroxyphenyl)-1-methylethyl]phenol (BPA-monoI).

N-iodosuccinimide (2.00 mmol, 0.451 g) and methanesulfonic acid (2.00 mmol, 130 µL) was added to a solution of Bisphenol A (6.00 mmol, 1.37 g) in acetonitrile (4.5 mL)²⁵. The resulting mixture was stirred overnight at room temperature. The mixture was extracted with ethyl acetate. The organic phase was washed with water and brine, and dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed solvent of dichloromethane and methanol (100:1, v/v). Removal of the solvent from appropriate fractions gave BPA-monoI.

2,2-Bis(3-iodo-4-hydroxyphenyl)propane (BPA-diI).

N-iodosuccinimide (2.00 mmol, 0.465 g) and methanesulfonic acid (1.00 mmol, 65.0 µL) was added to a solution of bisphenol A (1.00 mmol, 0.229 g) in acetonitrile (3.0 mL)²⁵. The resulting mixture was stirred overnight at room temperature. The mixture was extracted with ethyl acetate. The organic phase was washed with water and brine, and dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed solvent of hexane and ethyl acetate (8:1, v/v). Removal of the solvent from appropriate fractions gave BPA-diI.

2,6-Diiodo-4-[1-(3-iodo-4-hydroxyphenyl)-1-methylethyl]phenol (BPA-triI) and 2,2-bis(3,5-dichloro-4-hydroxyphenyl)propane (BPA-tetraI).

Sulfuric acid (2.0 mL) was slowly added to a solution of bisphenol A (2.00 mmol, 0.457 g) in ethanol (15 mL) at 0°C, followed by 1,3-diiodo-5,5-dimethylhydantoin (4.206 mmol, 1.598 g). The resulting mixture was stirred for 1 h at room temperature. The mixture was extracted with dichloromethane. The organic phase was washed with 5% NaHCO₃ aq., 5% Na₂S₂O₃ aq., water and brine, and then dried over Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure and the obtained crude product was dissolved by a mixed solvent of dichloromethane and hexane (5:1, v/v, 60 mL). After standing solution for 24 h, the precipitated solid was filtrated and washed with dichloromethane and methanol to give a pure BPA-tetraI. The filtrate was concentrated under reduced pressure and the obtained crude product was purified by silica gel column chromatography using a mixed solvent of hexane and dichloromethane (1:1, v/v). Removal of the solvent from appropriate fractions gave BPA-triI.

Yield and NMR data

BPA-monoF. 15.0% yield (0.150 mmol, 37.0 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.05–7.09 (m, 2H, ArH), 6.86–6.94 (m, 3H, ArH), 6.73–6.76 (m, 2H, ArH), 5.00 (d, 1H, OH), 4.690 (s, 1H, OH) 1.61 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 153.4 (Ar-COH), 150.5 (d, Ar-CF), 144.3 (Ar-CC), 142.6 (Ar-COH), 141.1 (Ar-CC), 127.9 (Ar-CH), 122.8 (Ar-CH), 116.4 (Ar-CH), 114.8 (Ar-CH), 114.1 (Ar-CH), 41.8 (C(CH₃)₂), 30.9 (CCH₃). ¹⁹F NMR (600 MHz, CDCl₃): δ -142.0 (ArF).

BPA-diF. 5.93% yield (0.119 mmol, 31.5 mg). ¹H-NMR (600 MHz, CDCl₃): δ 6.86–6.93 (m, 6H, ArH), 4.99–5.00 (d, 2H, OH), 1.60 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 150.6 (d, Ar-CF), 143.6 (Ar-COH), 141.3 (Ar-CC), 122.8 (Ar-CH), 116.6 (Ar-CH), 114.1 (Ar-CH), 41.9 (C(CH₃)₂), 30.8 (CCH₃). ¹⁹F NMR (600 MHz, CDCl₃): δ -141.7 (ArF).

BPA-triF. 11.1% yield (0.163 mmol, 46.1 mg) ¹H-NMR (600 MHz, CDCl₃): δ 6.83–6.93 (m, 3H, ArH), 6.71–6.77 (m, 2H, ArH), 5.08 (br, 2H, OH), 1.59 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 151.4 (d, Ar-CF), 150.7 (d, Ar-CF), 142.7–142.8 (m, Ar-CC, Ar-COH),

141.6 (Ar-CC), 130.6 (Ar-COH), 122.8 (Ar-CH), 116.9 (Ar-CH), 114.1 (Ar-CH), 42.1 (C(CH₃)₂), 30.7 (CCH₃). ¹⁹F NMR (600 MHz, CDCl₃): δ -141.5 (ArF), -136.5 (ArF). MS (FAB+) *m/z*, calculated for C₁₅H₁₃F₃O₂: 282.0868 ([M]⁺), found 282.0839.

BPA-tetraF. 23.0% yield (0.232 mmol, 69.6 mg). ¹H-NMR (600 MHz, CDCl₃): δ 6.70–6.76 (m, 4H, ArH), 5.20 (br, 2H, OH), 1.57 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 151.4 (d, Ar-CF), 141.9 (Ar-CC), 130.8 (Ar-COH), 110.1 (Ar-CH), 42.2 (C(CH₃)₂), 30.5 (CCH₃). ¹⁹F NMR (600 MHz, CDCl₃): δ -136.7 (ArF).

BPA-monoCl. 4.55% yield (0.0910 mmol, 23.0 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.16–7.18 (d, 1H, ArH), 7.06–7.09 (m, 2H, ArH), 6.99–7.02 (m, 1H, ArH), 6.89–6.91 (d, 1H, ArH), 6.72–6.75 (m, 2H, ArH), 5.42 (s, 1H, OH), 4.76 (s, 1H, OH), 1.61 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 153.4 (Ar-COH), 149.4 (Ar-COH), 144.5 (Ar-CC), 142.5 (Ar-CC), 127.9 (Ar-CH), 127.1 (Ar-CH), 127.0 (Ar-CH), 119.3 (Ar-CCl), 115.6 (Ar-CH), 114.8 (Ar-CH), 41.8 (C(CH₃)₂), 30.9 (CCH₃).

BPA-diCl. *R_F* = 0.324 using a mixed solvent of hexane and ethyl acetate (4:1, v/v). 47.0% yield (0.946 mmol, 281 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.15–7.17 (m, 2H, ArH), 6.98–7.01 (m, 2H, ArH), 6.90–6.93 (m, 2H, ArH), 5.42 (s, 2H, OH), 1.60 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 149.3 (d, Ar-COH), 143.7 (Ar-CC), 127.0 (Ar-CH), 126.9 (Ar-CH), 119.4 (Ar-CCl), 115.8 (Ar-CH), 41.9 (C(CH₃)₂), 30.8 (CCH₃).

BPA-triCl. *R_F* = 0.378 using a mixed solvent of hexane and ethyl acetate (4:1, v/v). 13.9% yield (0.28 mmol, 93.2 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.15–7.16 (m, 1H, ArH), 7.08 (s, 2H, ArH), 6.97–7.00 (m, 1H, ArH), 6.92–6.94 (d, 1H, ArH), 5.74 (s, 1H, OH), 5.46 (s, 1H, OH), 1.60 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 149.5 (Ar-COH), 145.8 (Ar-COH), 143.9 (Ar-CC), 142.8 (Ar-CC), 127.0 (Ar-CH), 126.9 (Ar-CH), 126.7 (Ar-CH), 120.6 (Ar-CCl), 119.5 (Ar-CCl), 116.0 (Ar-CH), 42.0 (C(CH₃)₂), 30.7 (CCH₃).

BPA-monoBr. 80.4% yield (1.65 mmol, 508 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.31–7.32 (d, 1H, ArH), 7.06–7.09 (m, 2H, ArH), 7.03–7.05 (m, 1H, ArH), 6.89–6.92 (d, 1H, ArH), 6.72–6.76 (m, 2H, ArH), 5.38 (s, 1H, OH), 4.70 (s, 1H, OH), 1.61 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 153.5 (Ar-COH), 150.0 (Ar-COH), 145.0 (Ar-CC), 142.5 (Ar-CC),

130.0 (Ar-CH), 127.9 (Ar-CH), 127.9 (Ar-CH), 115.5 (Ar-CH), 114.9 (Ar-CH), 109.8 (Ar-CBr), 41.8 (C(CH₃)₂), 31.0 (CCH₃).

BPA-diBr. 68.6% yield (1.38 mmol, 534 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.29–7.32 (m, 2H, ArH), 7.01–7.04 (m, 2H, ArH), 6.90–6.93 (m, 2H, ArH), 5.42 (s, 2H, OH), 1.60 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 150.2 (d, Ar-COH), 144.1 (Ar-CC), 130.0 (Ar-CH), 127.8 (Ar-CH), 115.7 (Ar-CH), 110.0 (Ar-CBr), 41.8 (C(CH₃)₂), 30.9 (CCH₃).

BPA-triBr. 49.9% yield (2.000 mmol, 930 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.29–7.32 (d, 2H, ArH), 7.262 (s, 2H, ArH), 7.00–7.02 (m, 1H, ArH), 6.92–6.94 (d, 1H, ArH), 5.79 (s, 1H, OH), 5.46 (s, 1H, OH), 1.59 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 150.4 (Ar-COH), 147.4 (Ar-COH), 145.0 (Ar-CC), 143.3 (Ar-CC), 130.4 (Ar-CH), 129.8 (Ar-CH), 127.7 (Ar-CH), 115.8 (Ar-CH), 110.0 (Ar-CBr), 109.6 (Ar-CBr), 41.9 (C(CH₃)₂), 30.8 (CCH₃).

BPA-triBr-MOM. 75.8% yield (1.46 mmol, 805 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.387–7.402 (m, 1H, ArH), 7.332 (s, 2H, ArH), 7.048–7.072 (m, 1H, ArH), 7.014–7.040 (m, 2H, ArH), 5.230 (s, 2H, CH₂), 5.157 (s, 4H, CH₂), 3.719 (s, 3H, CH₃), 3.525 (s, 3H, CH₃), 1.600 (s, 6H, CH₃).

BPA-tetraBr-MOM. 87.4% yield (2.64 mmol, 1668 mg). ¹H-NMR (600 MHz, CDCl₃): δ 7.32 (s, 4H, ArH), 5.17 (s, 4H, CH₂), 3.73 (s, 6H, CH₃), 1.59 (s, 6H, CH₃).

BPA-monoI. 58.0% yield (1.20 mmol, 0.410 g). ¹H-NMR (600 MHz, CDCl₃): δ 7.50–7.51 (d, 1H, ArH), 7.04–7.09 (m, 3H, ArH), 6.86–6.88 (d, 1H, ArH), 6.72–6.75 (m, 2H, ArH), 5.19 (s, 1H, OH), 4.76 (s, 1H, OH), 1.60 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 153.5 (Ar-COH), 152.6 (Ar-COH), 145.4 (Ar-CC), 142.5 (Ar-CC), 136.0 (Ar-CH), 129.1 (Ar-CH), 127.9 (Ar-CH), 114.8 (Ar-CH), 114.5 (Ar-CH), 85.5 (Ar-Cl), 41.6 (C(CH₃)₂), 31.0 (CCH₃).

BPA-diI. 47.0% yield (0.470 mmol, 0.230 g). ¹H-NMR (600 MHz, CDCl₃): δ 7.49–7.50 (d, 2H, ArH), 7.02–7.05 (m, 2H, ArH), 6.86–6.90 (d, 2H, ArH), 5.25 (s, 2H, OH), 1.58 (s, 6H, CH₃). ¹³C NMR (600 MHz, CDCl₃): δ 152.9 (d, Ar-COH), 144.6 (Ar-CC), 136.0 (Ar-CH), 130.0 (Ar-CH), 114.6 (Ar-CH), 85.6 (Ar-Cl), 41.5 (C(CH₃)₂), 30.9 (CCH₃).

BPA-triI. 6.53% yield (0.131 mmol, 79.2 mg).

^1H -NMR (600 MHz, CDCl_3): δ 7.47–7.49 (d, 1H, ArH), 7.47 (s, 2H, ArH), 7.00–7.03 (m, 1H, ArH), 6.88–6.91 (d, 1H, ArH), 5.63 (br, 1H, OH), 5.20 (br, 1H, OH), 1.57 (s, 6H, CH_3).
 ^{13}C NMR (600 MHz, CDCl_3): δ 153.0 (Ar-COH), 151.6 (Ar-COH), 146.5 (Ar-CC), 143.9 (Ar-CC), 137.6 (Ar-CH), 135.8 (Ar-CH), 129.0 (Ar-CH), 114.8 (Ar-CH), 85.7 (Ar-CI) 114.5 (Ar-CH), 85.5 (Ar-CI), 82.1 (Ar-CI), 41.3 ($\text{C}(\text{CH}_3)_2$), 30.9 (CCH_3).

BPA-tetraI. 17.8% yield (0.357 mmol, 261 mg).

^1H -NMR (600 MHz, DMSO-d_6): δ 9.39 (br, 2H, OH), 7.46 (s, 4H, ArH), 1.48 (s, 6H, CH_3).
 ^{13}C NMR (600 MHz, DMSO-d_6): δ 154.0 (d, Ar-COH), 146.2 (Ar-CC), 137.6 (Ar-CH), 87.7 (Ar-CI), 41.0 ($\text{C}(\text{CH}_3)_2$), 30.7 (CCH_3).

2. 3. Preparation of receptor protein GST-fused ER-LBDs

Recombinant proteins of the ligand-binding domain (LBD) of human $\text{ER}\alpha$ and $\text{ER}\beta$, namely, $\text{ER}\alpha$ -LBD and $\text{ER}\beta$ -LBD, were expressed in a glutathione-S-transferase (GST)-fused form. The LBD was ligated into a prokaryotic expression vector pGEX-4T1 (Amersham Pharmacia Biotech, Bucks, UK) at the *Bam*HI and *Not*I sites. *E. coli* BL21 transformed with the expression plasmid was cultured in 1 L of L-broth containing 0.2 mg/ml of ampicillin, and protein expression was induced by isopropyl 1-thio- β -D-galactoside (Nacalai Tesque, Kyoto). The cells were harvested by centrifugation (2300 $\times g$, 30 min, 4°C) and resuspended in 4 mL of STE buffer (10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT)), and 150 $\mu\text{g}/\text{mL}$ lysozyme. After sonication and centrifugation (14,000 $\times g$, 30 min, 4°C), a soluble fraction was loaded on 500 μL affinity column of glutathione-Sepharose 4B (15 $\times$ 10 mm; GE Healthcare BioSciences Co., Piscataway, NJ, USA). After incubation for 1 h at 4°C, the column was washed three times with PBS containing 0.5% (v/v) Triton X-100. Protein was eluted out with 1 M Tris-HCl (pH 8.0) containing 20 mM reduced glutathione, and the eluate was applied to Amicon ultra-4 (Merck KGaA, Darmstadt, Germany) to remove glutathione by ultrafiltration. The purity of expressed protein was confirmed by SDS-PAGE (12.5% polyacrylamide gel) stained by Coomassie brilliant blue R-250 (CBB R-250, Nacalai Tesque). The protein concentrations were determined by the Bradford method ²⁶.

2. 4. Competitive binding assay for ERs

Binding affinities between the halogenated BPA analogs and ERs were determined by the competitive binding assay according to the previous studies²⁷. Briefly, the halogenated BPA analogs were examined for their ability to inhibit the binding of [³H]E2 (radioisotope-labeled ligand: 0.5 nM in the final solution) to GST-ER α -LBD or GST-ER β -LBD (2 nM in the final solution). BPA and its analogs were dissolved in the binding buffer containing 0.3–1.0% dimethylsulfoxide. The reaction mixtures were incubated for 1 h at 25°C and receptor-unbound radioisotope-labeled ligands were removed by 0.4% dextran-coated charcoal. Radioactivity of receptor-bound radioisotope-labeled ligands was determined on a liquid scintillation counter (MicroBeta; PerkinElmer, Waltham, MA, USA). The IC₅₀ values (the concentrations for the half-maximal inhibition) were calculated from the dose-response curves obtained using the nonlinear analysis program GraphPad Prism 7 (GraphPad Software Inc., CA, USA). Each assay was performed in duplicate and repeated at least three times.

2. 5. Cell culture

HeLa cell line was obtained from JCRB (JCRB9004, JCRB cell bank, NIBIO, Osaka, Japan). HeLa cells were used between P6-30 and were maintained in Eagle's MEM (EMEM) (Nissui Pharma Cosmetics Co., Ltd., Tokyo, Japan) containing 10% FBS (treated with dextran-coated charcoal), 2 mM L-Glutamine and 0.18% sodium bicarbonate at 37°C, in an atmosphere of 5% CO₂ and 95% air.

2. 6. Reporter gene assay

Transcriptional activities of the halogenated BPA analogs via ERs were determined by reporter gene assay using luciferase as an expression vector on HeLa cells. For luciferase assays, HeLa cells were seeded at 1.0×10^6 cells per 6-cm dish for 24 h and then transfected with 3 μ g reporter gene (pGL3/3xERE) and 1 μ g of ER α or ER β expression plasmid (pcDNA3.1/ERs) by Lipofectamine Plus reagent (Invitrogen Japan, Tokyo, Japan) according to the manufacturer's protocol. Approximately 24 h after transfection, cells were harvested

and plated into 96-well plates at 1.0×10^5 cells/well. The cells were treated with varying doses of chemicals diluted with 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS, w/w). To measure the agonistic activity, each BPA analog (10^{-5} M to 10^{-11} M in the final solution) was added to the plate. Also, to measure the antagonistic activity, a fixed concentration of E2 (10 nM in the final solution) and the halogenated BPAs (10^{-5} M to 10^{-11} M in the final solution) were added. After 24 h, luciferase activity was measured with the appropriate reagent using Britelite Plus (PerkinElmer, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Light emissions were measured using LuminoskanTM Ascent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells treated with 1% BSA/PBS were used as a vehicle control²⁷. The dose-response curves were obtained using the nonlinear analysis program GraphPad Prism 7 (GraphPad). Each assay was performed in duplicate and repeated at least three times.

3. Results and Discussion

3. 1. Synthesis of BPA analogs

All halogenated BPA analogs were synthesized by aromatic electrophilic substitution of BPA with electrophilic halogenation agents and their structural formulae are illustrated in Figure 2. Fluorination of BPA was achieved by using *N*-fluoronebenzensulfonimide or selectfluor as halogenation reagents. The reaction yield of BPA-monoF and BPA-diF were slightly low (15.0% and 5.93%, respectively). To make matters worse, BPA-triF and BPA-tetraF could not be directly synthesized from BPA by the halogenation. Therefore, these multi-fluorinated BPA analogs were synthesized from the corresponding brominated BPA analogs²¹. To the best of our knowledge, the synthesis of BPA-triF, possessing three fluorine substituents at the phenolic rings of BPA, has not been reported previously. Therefore, its chemical structure was confirmed by NMR, and, furthermore, elemental analysis and molecular weight measurement. Chlorination reactions of BPA readily proceeded by using trichloroisocyanuric acid. Both brominated BPA analogs and iodinated BPA analogs were successfully synthesized by using *N*-bromosuccinimide and *N*-iodosuccinimide as the synthetic agents, respectively.

(Figure 2)

Among the synthesized BPA analogs, the reaction yields of fluorination of BPAs were relatively low. It was suggested that the low reactivity in the halogenation on phenolic moieties of BPA with fluorine atom(s) was probably due to a low reactivity or an undesired side reaction such as oxidation of BPA induced by the fluorination agents used in this study. Furthermore, the tri- and the tetra-fluorination of BPA analogs could not be achieved conventionally by the fluorination agents. Therefore, these multi-fluorinated BPA analogs were converted from the corresponding brominated BPA analogs via lithiation of ortho-position of the hydroxyl group. By contrast, the chlorinated BPA analogs were readily obtained by using trichloroisocyanuric acid. Under the reaction condition used in the present study, it was identified that BPA-diCl, BPA-triCl, and BPA-tetraCl (TCBPA) were generated concurrently. Therefore, to improve reaction yields of the objective chlorinated BPA analog, it was considered to be necessary to regulate the amount of the chlorination agent. Bromination and iodination of BPA were successfully achieved by using *N*-bromo- or *N*-iodosuccinimide, respectively.

3. 2. Binding affinity of the halogenated BPA analogs to ERs

In order to assess halogenation effects on ERs-binding activity of BPA, binding affinity of the halogenated BPA analogs to ERs was determined by the competitive binding assay using [³H]E2 as a tracer. In this study, E2 was used as a positive control of the receptor binding and its IC₅₀ value was calculated to be 5.631 nM for ER α and 4.579 nM for ER β , respectively. Subsequently, binding affinity of each halogenated BPA analog was evaluated by the binding assay. IC₅₀ values of each analog were shown in Table 1 and Table 2. In case of ER α , binding affinities of the all halogenated BPA analogs were slightly stronger than that of BPA (Figure 3). However, the IC₅₀ values of each halogenated BPA analogs for ER α were on the order of approximately 100 to 500 nM. Thus, binding affinity of BPA were not enhanced significantly by the halogenation (Table 1). On the contrary, trend of the binding affinity of halogenated BPAs to ER β was slightly different from that to ER α . That is BPA-triF and BPA-tetraF clearly showed strong binding affinity to ER β , whereas the other analogs

possessing large halogen substituents did not show strong binding affinity to ER β (Figure 4). IC₅₀ values of BPA-triF and BPA-tetraF were ranged in 10 - 100 nM (Table 2). These results indicated that the halogenation at the aromatic ring(s) of BPA did not increased the binding affinity to ERs significantly, except for binding potency of BPA-triF and BPA-tetraF to ER β .

(Tables 1 and 2, and Figures 3 and 4)

3. 3. Transcriptional activity of the halogenated BPA analogs via ERs

In the receptor binding assay, some BPA analogs showed relatively strong binding potency to ERs. To determine whether the BPA analogs that can bind ERs act as agonist or antagonist, transcriptional activity of the halogenated BPA analogs via ERs were evaluated by the reporter gene assay using HeLa cells. Since most of the nuclear receptors are known to show ligand-dependent transcription activation²⁸, agonistic activity of various chemicals has been investigated using reporter gene assay. At first, transcriptional activity of the halogenated BPAs was evaluated to identify whether these analogs showed agonistic activity for ERs or not. The results of the reporter gene assay showed that E2, a representative agonist of ERs, exhibited strong agonistic activity, whereas BPA showed approximately 50 to 60% (ER α) or 40 to 50% (ER β) of the transcription activity compared to E2, respectively. Although BPA showed dose-dependent transcription activation, it was observed that BPA exerted toxic effects at the maximum concentration (10^{-5} M) (Table 3 and Table 4). Subsequently, transcriptional activity of the halogenated BPA analogs was also examined for ERs. As shown in Table 3 and Figure 5, BPA-diF and BPA-triF showed stronger agonistic activity than BPA, although they did not show full-agonistic activity for ER α . The other halogenated BPA analogs showed weaker agonistic activity for ER α than BPA and most of the analogs reached less than 50% relative activity to E2 (Table 3). These results indicated that the halogenated BPA analogs were categorized as partial agonists of ER α . With regard to ER α -transcriptional activity of the halogenated BPA analogs, when the larger halogen atom(s) were introduced to BPA as substituents, resulted compounds showed weaker agonistic activities than BPA. On the other hand, all fluorinated BPA analogs showed almost similar or stronger agonistic activity for ER β than BPA (Table 4 and Figure 6). Other

halogenated BPA analogs showed weaker ER β -agonistic activity than BPA. Unlike ER α , no correlation was found between the size or the number of halogen atoms and agonistic activity via ER β .

(Tables 3 and 4, and Figures 5 and 6)

Subsequently, reporter gene assay was carried out in the presence of E2 to investigate the antagonistic activity of BPA and the halogenated BPA analogs. Under the tested concentrations, BPA did not show antagonistic activity. Similarly, all halogenated BPA analogs also showed no antagonistic activity (Figures 7 and 8). These results indicated that the halogen substitution affected the transcriptional activities of BPA but did not induce antagonistic activity. Previously, it has been reported that bisphenol AF (BPAF) and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Figure 1), which are also halogen containing BPA relating chemicals, show agonistic activity for ER α and antagonistic activity to ER β ^{27,29}. However, the halogenated BPA analogs used in this study showed agonistic activity rather than antagonistic activity for both ER subtypes. This difference in the activity between HPTE and the aromatic-halogenated BPA analogs indicated that introduction of halogen atoms into the aromatic ring(s) do not affect the transcriptional activity of either subtypes of ER. Furthermore, it was suggested that positions of halogen substituents on the BPA scaffold differently affected transcriptional activity of ERs, especially that of ER β .

(Figures 7 and 8)

3. 4. Influence of the introduced halogen atoms on binding affinity and transcriptional activity of BPA

In this study, synthetic fluorinated BPA analogs showed stronger ER-binding and higher transcriptional activity for ER than other halogenated analogs. In regard to binding properties of the BPA analogs, it is considered that the size of the ligand binding pocket on ER β -LBD contributes greatly. It was reported that the ligand binding pocket of ER α was approximately 20% larger than that of ER β ³⁰. Therefore, it was suggested that ER α -LBD could accept larger ligands than ER β -LBD. The calculated molecule volume of BPA, BPA-tetraF, BPA-tetraCl, BPA-tetraBr, and BPA-tetraI were 226.3 Å³, 250.2 Å³, 283.4 Å³, 302.7

$\text{\AA}^3$, and $327.6 \text{ \AA}^3$, respectively. Notably, there is a difference of almost $100 \text{ \AA}^3$ in the volume of molecules between BPA and BPA-tetraI. Thus, large halogenated BPA molecules, which possesses large and multiple halogen substituent(s), could be captured in the ligand binding pocket of $\text{ER}\alpha$, but could not easily enter into the relatively narrow ligand binding pocket of $\text{ER}\beta$. Due to the greater ligand accessibility of $\text{ER}\alpha$ -LBD than that of $\text{ER}\beta$ -LBD, there was no correlation between the size or number of halogen atoms and the binding properties for $\text{ER}\alpha$, whereas the halogenation at the aromatic ring(s) of BPA decreased its binding potency to $\text{ER}\beta$ -LBD depending on the size or number of halogen atoms.

With regard to the physiological activity of ERs, the displacement of helix 11, which is one of the α -helices consisting $\text{ER}\alpha$ -LBD, is thought to be greatly related to the transcriptional activity of ERs. It was reported that the inner displacement of helix 11 was involved in the agonist activity of prenylated flavonoids and isoflavonoids³¹. That is, helix 11 was deformed to inner side of the LBD in accordance with binding of the flavonoids acted as agonists. When H11 was oriented outside of the binding pocket, agonistic activity of the ligands was decreased³¹. In fact, it was reported that BPA actually interacted with His 524 on H11 in the crystal structure of BPA and $\text{ER}\alpha$ complex³². Furthermore, it was observed that a chlorine atom of the bound TCBPBPA was facing and close to H11 in the crystal structure of TCBPBPA (represented as BPA-tetraCl in this study) and $\text{ER}\alpha$ complex (PDB: 4MGB; Figure 9)³³. Because of the proximity of the chloride atom to H11, the BPA analogs with large halogen atoms might disturb the interaction that was observed between BPA and histidine residue (His524 of $\text{ER}\alpha$ or His475 of $\text{ER}\beta$) causing the deformation of H11. Therefore, due to the disturbance, the agonistic activity of the BPA analogs was considered to be lower than that of BPA. On the other hand, owing to the small atomic volume of fluorine, it was considered that the introduced fluorine atom(s) did not affect the agonistic orientation of H11. It was reported that the fluorine atom of perfluorinated compounds (PFCs) formed a hydrophobic interaction with residues Met343, Leu346, Phe404, Met421, and His524 of $\text{ER}\alpha$ -LBD with the number of interaction similar to E2 in molecular dynamics study³⁴. Likewise, it was considered that the fluorinated BPA induced agonistic activity of ERs by chiefly interacting with ERs by the hydrophobic interaction similar to E2. To elucidate further

detailed intermolecular interactions, it is necessary to investigate interaction between the BPA analogs and ERs by structural analyses such as X-ray crystallography, NMR, and *in silico* docking calculation.

(Figure 9)

4. Conclusion

In this study, a series of halogenated BPA analogs was successfully synthesized and the effects of the halogenation on binding potency and transcriptional activity of BPA on ERs were examined. Competitive binding assays revealed that the halogenation slightly increased the binding affinity between all BPA analogs and ER α . Contrastingly, tri- or tetra-fluorinated BPA analogs showed strong binding affinity to ER β , although other halogenated BPA analogs with large halogen substituents exhibited binding affinity equivalent to or weaker than that of BPA. These results suggested that the potency of endocrine disruption by the halogenated BPA analogs via ER β could be correlated with the size of the substituent(s) on the phenol ring(s) of BPA. Furthermore, the results of the reporter gene assay indicated that the fluorinated BPA had relatively stronger agonistic activity than BPA. Thus, it was suggested that the fluorination of BPA may have a potential to induce endocrine disruption via ERs. Furthermore, it have been reported that some PFCs are very slowly eliminated from the human body³⁵. Therefore, due to the slow elimination of PFCs, it was suggested that the fluorinated BPAs can give relatively serious adverse effects in comparison with BPA. In conclusion, halogenation, is a simple chemical modification method widely used for the development of various new materials. Therefore, it is necessary to conduct extensive investigations on the risk of halogenation of chemical substances involving halogenation of BPA analogs, due to their unanticipated effects on binding and activation for various receptor molecules including the ERs.

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Table 1. IC₅₀ value of BPA analogs for ER α .

Chemical	IC ₅₀ [nM]		
E2	5.631	$\pm$	1.219
BPA	576.3	$\pm$	76.6
BPA-monoF	366.0	$\pm$	50.3
BPA-diF	137.3	$\pm$	41.9
BPA-triF	140.4	$\pm$	7.9
BPA-tetraF	139.7	$\pm$	15.5
BPA-monoCl	467.2	$\pm$	20.4
BPA-diCl	359.1	$\pm$	22.6
BPA-triCl	135.0	$\pm$	11.0
BPA-tetraCl	177.2	$\pm$	18.1
BPA-monoBr	372.7	$\pm$	34.0
BPA-diBr	400.5	$\pm$	19.6
BPA-triBr	113.5	$\pm$	10.9
BPA-tetraBr	215.5	$\pm$	31.5
BPA-monoI	344.1	$\pm$	18.4
BPA-diI	431.9	$\pm$	21.1
BPA-triI	326.8	$\pm$	34.7
BPA-tetraI	191.0	$\pm$	20.5

Assays were repeated three times and the IC₅₀ values were shown with the mean S.E.

Table 2. IC₅₀ value of BPA analogs for ER β .

Chemical	IC ₅₀ [nM]		
E2	4.579	$\pm$	1.738
BPA	342.9	$\pm$	43.5
BPA-monoF	456.0	$\pm$	48.0
BPA-diF	216.2	$\pm$	54.5
BPA-triF	79.76	$\pm$	8.20
BPA-tetraF	97.02	$\pm$	1.78
BPA-monoCl	390.2	$\pm$	23.7
BPA-diCl	351.7	$\pm$	43.3
BPA-triCl	366.0	$\pm$	58.7
BPA-tetraCl	304.9	$\pm$	14.2
BPA-monoBr	340.0	$\pm$	34.7
BPA-diBr	608.3	$\pm$	30.5
BPA-triBr	500.3	$\pm$	15.2
BPA-tetraBr	418.7	$\pm$	4.5
BPA-monoI	249.5	$\pm$	14.7
BPA-diI	842.0	$\pm$	93.0
BPA-triI	557.4	$\pm$	27.1
BPA-tetraI	499.8	$\pm$	58.0

Assays were repeated three times and the IC₅₀ values were shown with the mean S.E.

Table 3. Relative transcription activity of BPA analogs for ER α .

Chemical	Relative activity [%]		
E2	100		
BPA	57.22	$\pm$	5.12
BPA-monoF	47.73	$\pm$	4.59
BPA-diF	61.58	$\pm$	5.57
BPA-triF	60.73	$\pm$	3.31
BPA-tetraF	51.46	$\pm$	7.13
BPA-monoCl	51.17	$\pm$	2.13
BPA-diCl	35.12	$\pm$	2.50
BPA-triCl	33.50	$\pm$	3.95
BPA-tetraCl	29.14	$\pm$	0.82
BPA-monoBr	39.28	$\pm$	3.65
BPA-diBr	27.15	$\pm$	0.53
BPA-triBr	20.11	$\pm$	3.19
BPA-tetraBr	18.78	$\pm$	3.22
BPA-monoI	32.09	$\pm$	3.86
BPA-diI	18.30	$\pm$	2.42
BPA-triI	25.84	$\pm$	5.39
BPA-tetraI	19.16	$\pm$	0.02

Assays were repeated three times and the values were shown with the mean $\pm$ S.E.

Table 4. Relative transcription activity of BPA analogs for ER β .

Chemical	Relative activity [%]		
E2	100		
BPA	45.88	$\pm$	3.47
BPA-monoF	60.80	$\pm$	6.01
BPA-diF	63.77	$\pm$	2.01
BPA-triF	47.49	$\pm$	7.90
BPA-tetraF	61.55	$\pm$	11.31
BPA-monoCl	42.90	$\pm$	1.53
BPA-diCl	29.13	$\pm$	2.42
BPA-triCl	37.46	$\pm$	5.00
BPA-tetraCl	34.41	$\pm$	8.50
BPA-monoBr	35.37	$\pm$	2.30
BPA-diBr	24.49	$\pm$	3.51
BPA-triBr	34.07	$\pm$	7.52
BPA-tetraBr	31.26	$\pm$	9.34
BPA-monoI	27.56	$\pm$	0.09
BPA-diI	18.05	$\pm$	2.72
BPA-triI	31.16	$\pm$	7.21
BPA-tetraI	30.52	$\pm$	9.66

Assays were repeated three times and the values were shown with the mean $\pm$ S.E.

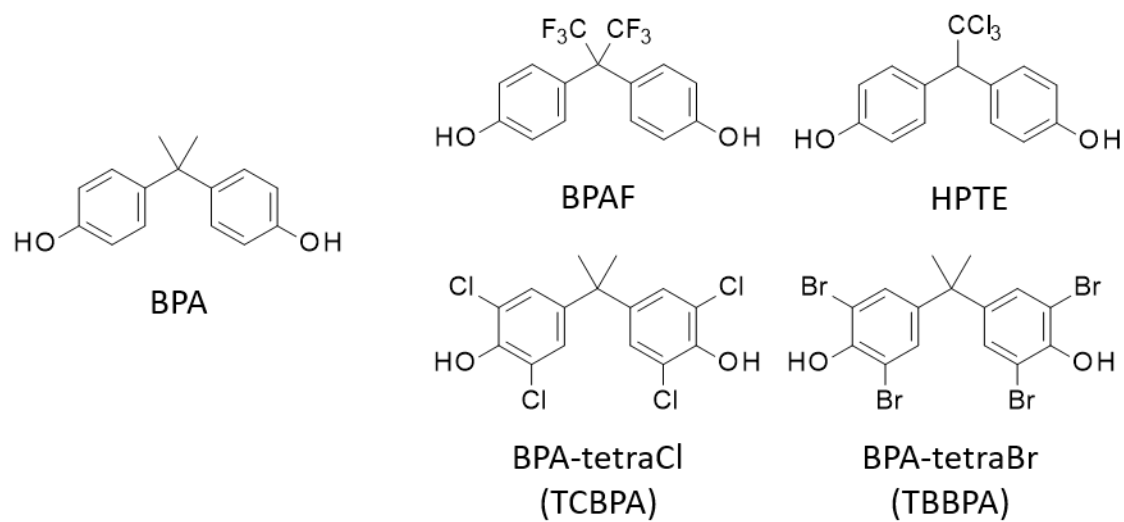


Figure 1. Chemical structures of representative next-generation BPAs.

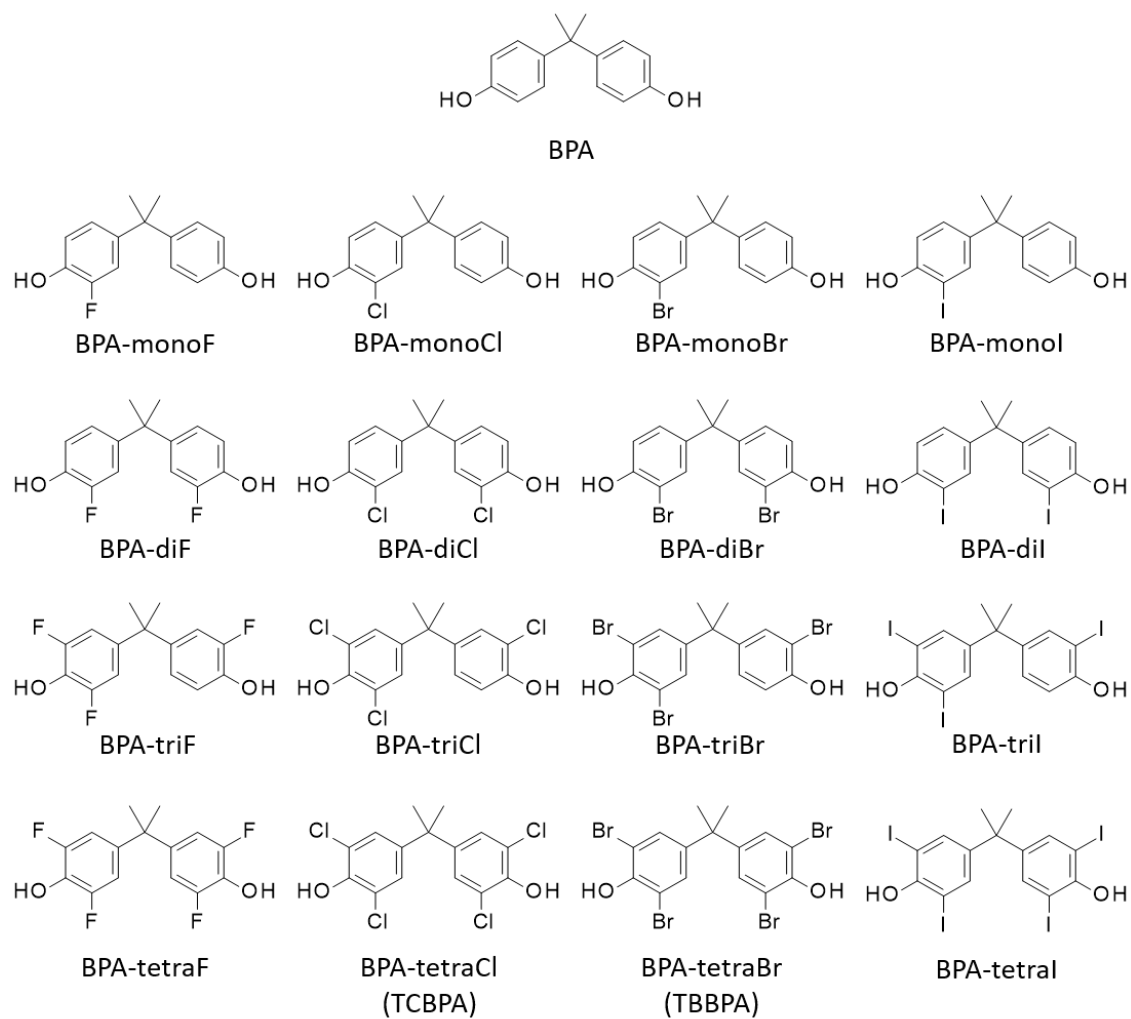


Figure 2. Chemical structures of BPA and the halogenated BPA analogs used in this study.

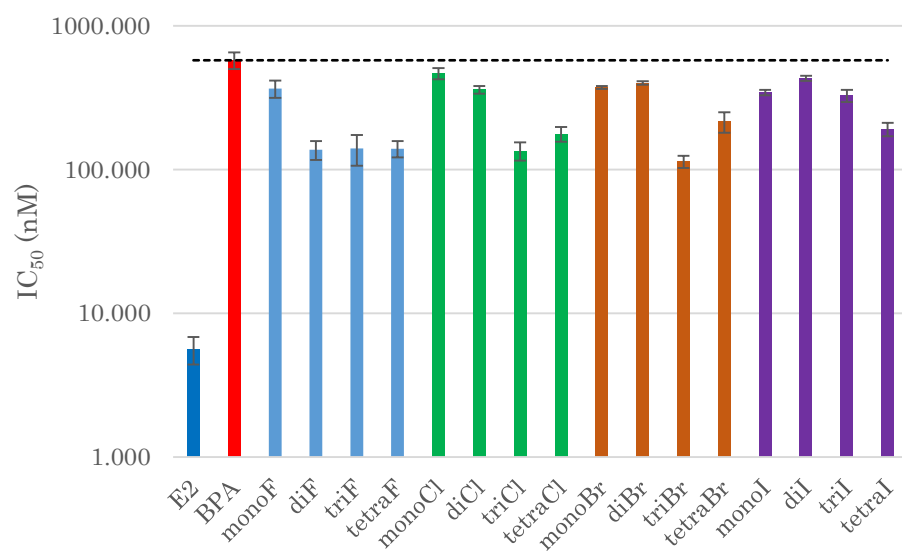


Figure 3. Comparison of the IC_{50} values of the BPA analogs for $ER\alpha$.

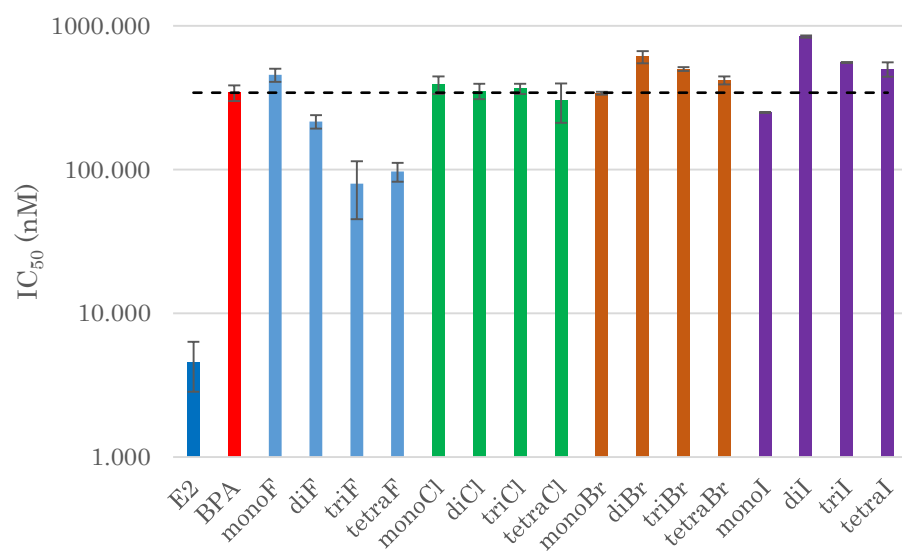


Figure 4. Comparison of the IC_{50} value of the BPA analogs for $ER\beta$.

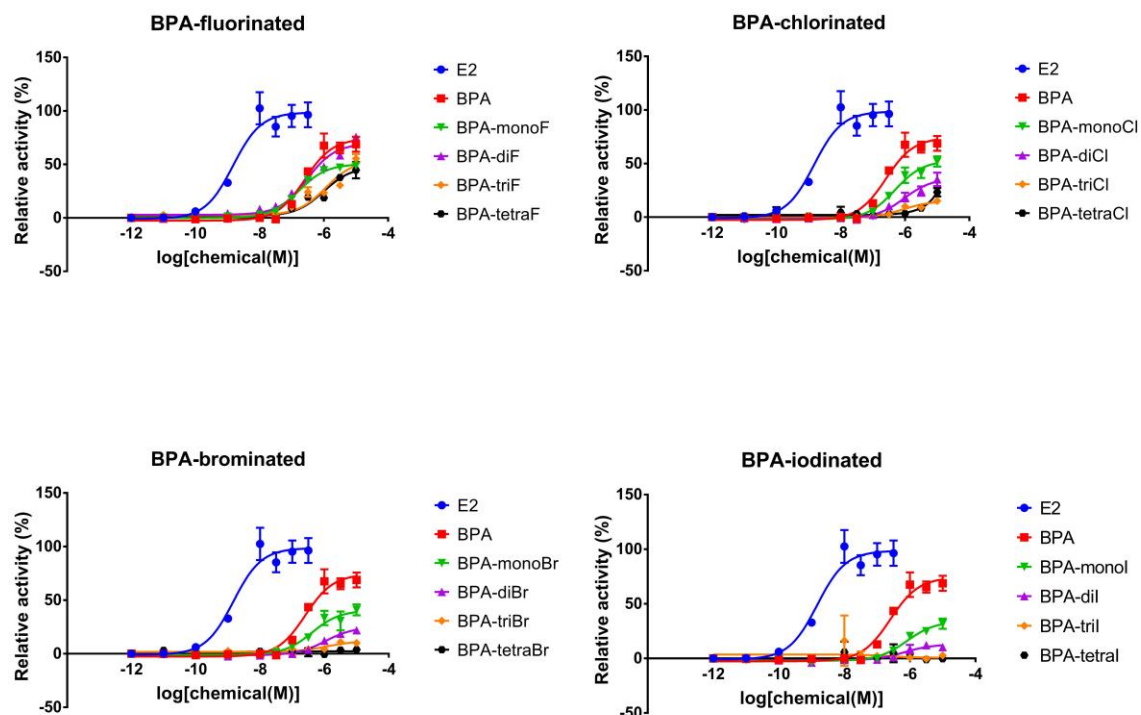


Figure 5. Transcriptional activity of the BPA analogs for ER α .

The representative data of relative ER α transcriptional activities induced by (a) fluorinated BPA analogs, (b) chlorinated BPA analogs, (c) brominated BPA analogs, and (d) iodinated BPA analogs are shown. In addition, the relative ER α transcriptional activities induced by E2 and BPA are shown as references. The maximum activity of E2 was taken as 100%.

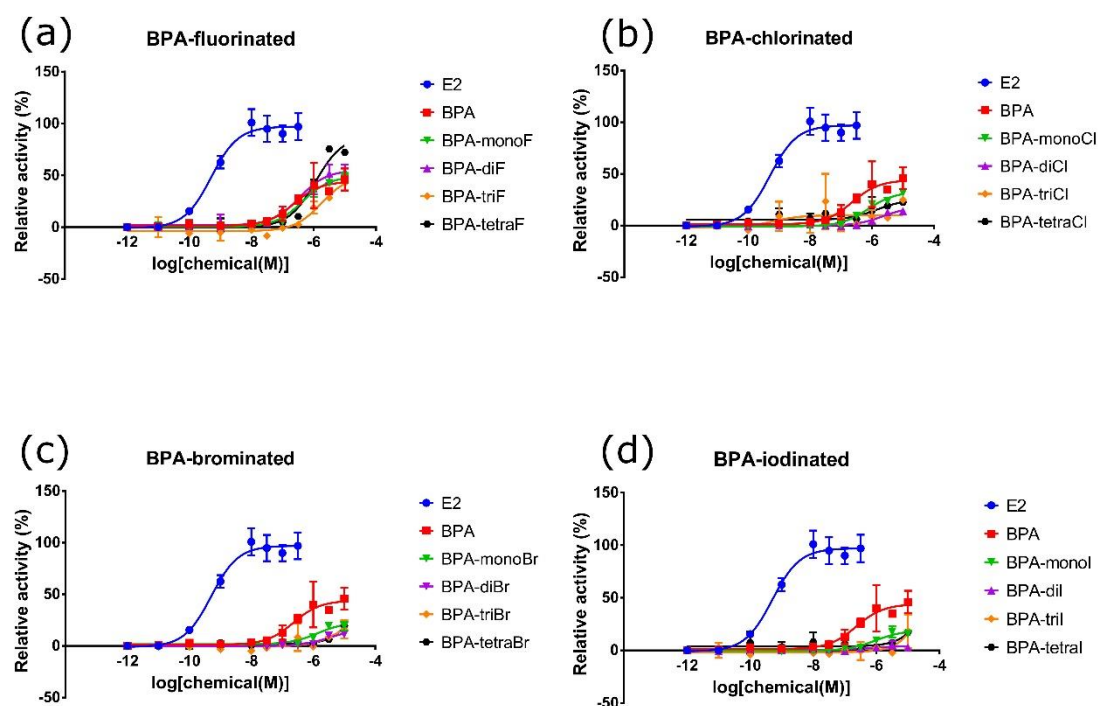


Figure 6. Transcriptional activity of the BPA analogs for ER β .

The representative data of relative ER β transcriptional activities induced by (a) fluorinated BPA analogs, (b) chlorinated BPA analogs, (c) brominated BPA analogs, and (d) iodinated BPA analogs are shown. In addition, the relative ER β transcriptional activities induced by E2 and BPA are shown as references. The maximum activity of E2 was taken as 100%.

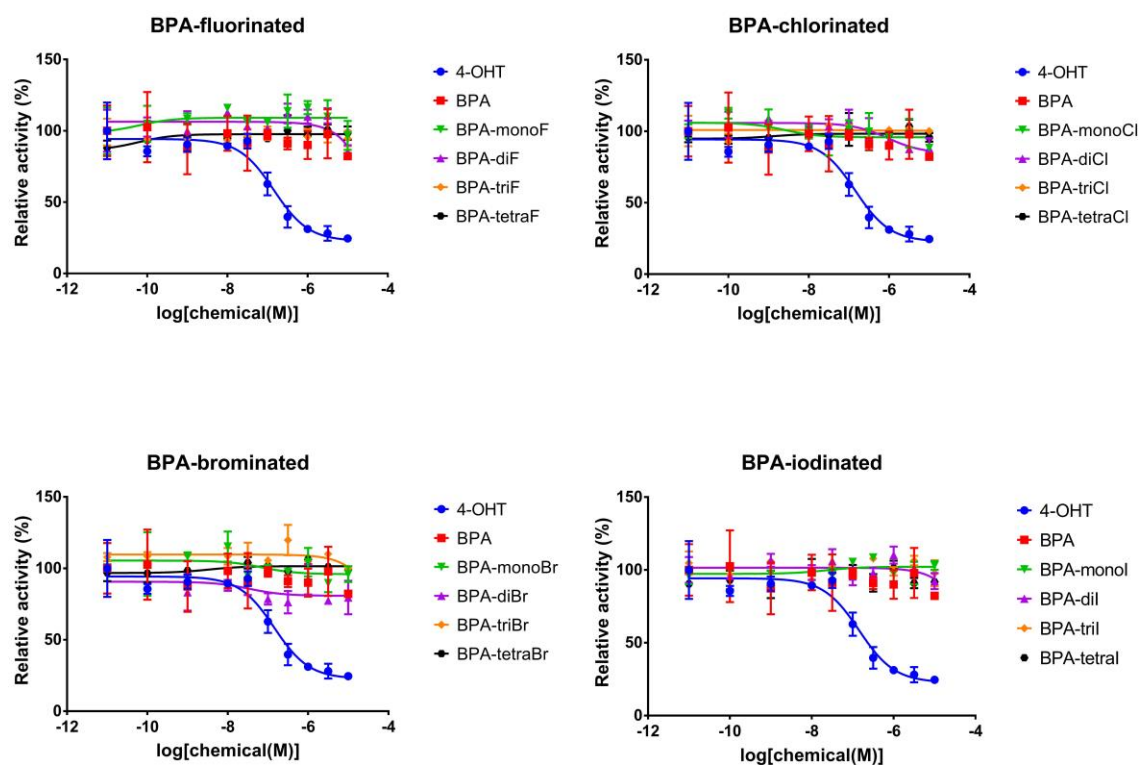


Figure 7. Antagonistic activity of the BPA analogs for ER α .

The representative data of relative ER α transcriptional inhibition induced by (a) fluorinated BPA analogs, (b) chlorinated BPA analogs, (c) brominated BPA analogs, and (d) iodinated BPA analogs are shown. In addition, the relative ER α transcriptional activities inhibited by (Z)-4-OHT are shown as references. The maximum activity of E2 was taken as 100%.

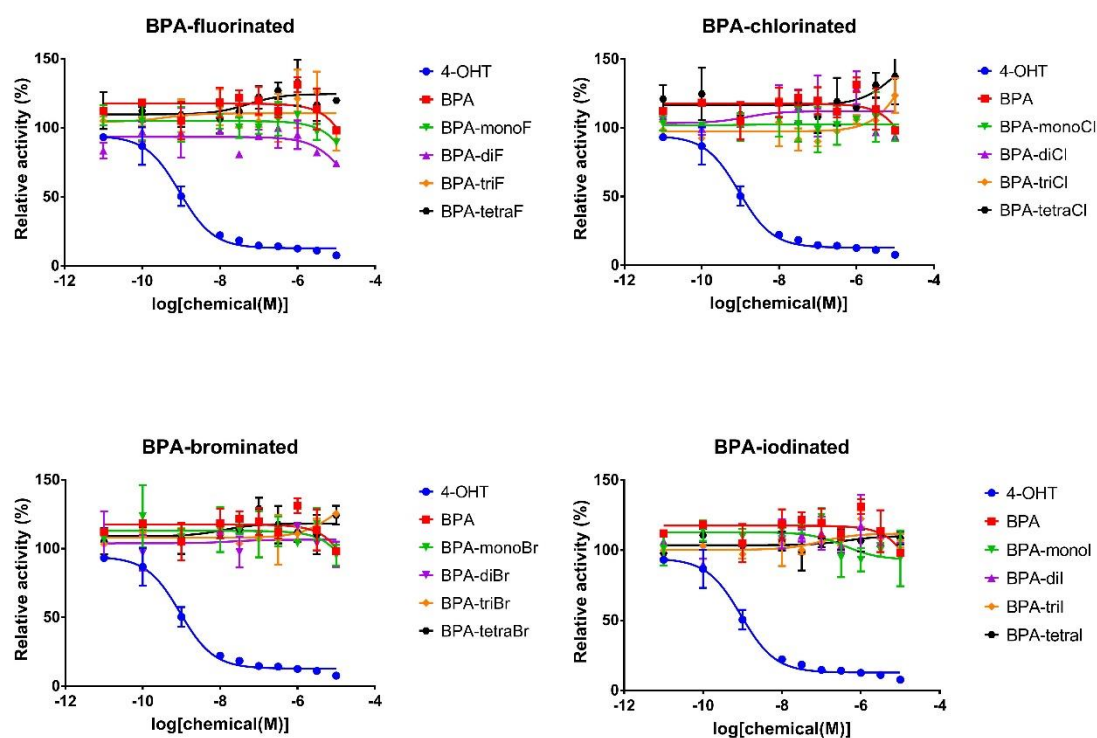


Figure 8. Antagonistic activity of the BPA analogs for ER β .

The representative data of relative ER β transcriptional inhibition induced by (a) fluorinated BPA analogs, (b) chlorinated BPA analogs, (c) brominated BPA analogs, and (d) iodinated BPA analogs are shown. In addition, the relative ER β transcriptional activities inhibited by (Z)-4-OHT are shown as references. The maximum activity of E2 was taken as 100%.

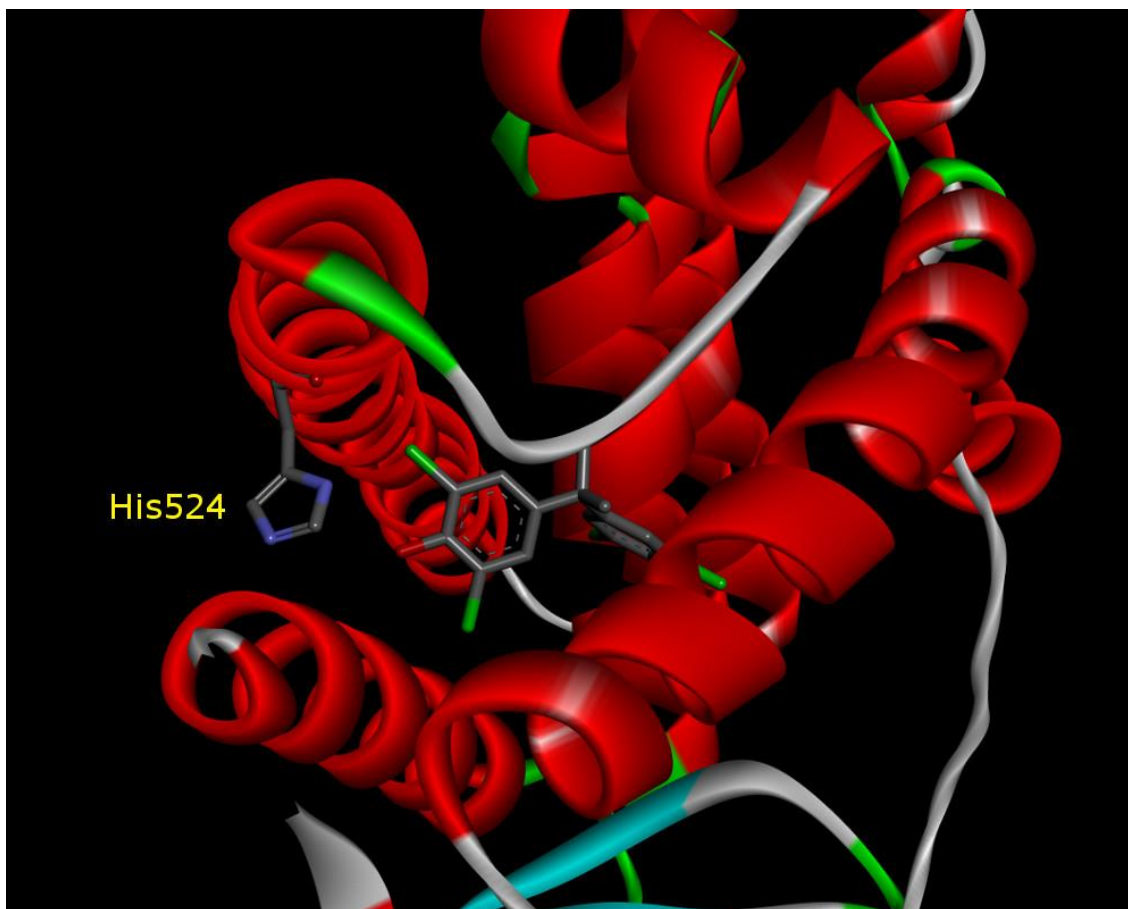


Figure 9. Crystal structure of ER α -LBD and TCBPA complex.

X-ray crystal structure of TCBPA with ER α -LBD (PDB: 4MGB) were shown. His524 which is represented as stick located close to TCBPA.

CHAPTER 3

Effects of halogenation of bisphenol A analogs on cytotoxicity for HeLa cells: di-halogenated BPA analogs inducing different types of cell death depending on halogen atom species

Abstract

Bisphenol A (BPA) and several bisphenol analogs known as “next-generation bisphenols” have been extensively used to manufacture various consumer products. However, it has been concerned that these BPA analogs can bind to human NRs and disrupt gene transcription. In addition, previous studies demonstrated that BPA shows not only endocrine disrupting activities but also biological toxicities including induction of mitochondrial injury and apoptotic death in several types of cells. However, available information regarding the potential cytotoxicity of the BPA analogs with halogenation at phenolic group(s) is still much scarcer. To evaluate the potential cytotoxic effects of the BPA analogs, we synthesized halogenated BPA analogs that possessing one to four halogen atoms as substituents on its aromatic rings and investigated *in vitro* cytotoxicity of those analogs for HeLa cells by MTT assay and LDH release assay. It was indicated that the aromatic-substituted BPA exhibited significantly stronger cytotoxicity for HeLa cells than BPA itself. Especially, it was revealed that the di-halogenated BPA analogs, which possess two halogen atoms, exhibited the strongest cytotoxicity among the halogenated BPA analogs. In addition, the result of fluorescent spectroscopy observation indicated that the halogenated BPA induced different types of cell death depending on induced halogen atom species.

1. Introduction

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane; BPA) is one of the most produced chemicals more than 3 million tons/year worldwide¹⁻⁴. BPA has been used to manufacture

polycarbonate plastics and epoxy resins that are raw materials to product baby bottles and food storage containers⁵⁻⁷, despite BPA is recognized as one of the representative endocrine disrupting chemicals (EDCs)^{4,7}. Due to leakage of BPA from consumer products consisting of polycarbonate plastics or epoxy resins, BPA has been detected ubiquitously in water, air, soil, and even living organisms including human⁸⁻¹¹. Over the last decade, many *in vitro* and *in vivo* experimental procedures were performed to evaluate adverse effects of BPA on reproduction, development, neural networks, cardiovascular, metabolic, and immune systems¹²⁻¹⁸. Furthermore, it has been hypothesized that exposure of BPA especially to fetuses or infants could induce adverse roles in the onset of obesity and other metabolic syndromes¹⁹. Previously, such adverse effects of BPA were considered to be via activation of estrogen receptors (ERs), which are members of the nuclear receptors (NRs)^{20,21}. Although it is thought that BPA is able to exert adverse effects by binding to ERs, BPA exhibits 10,000–100,000-fold weaker binding affinity to ERs and only modest estrogenic activity than 17 β -estradiol (E2) that is a specific and endogenous agonist for ERs^{4,8,14}. By contrast, it was also reported that BPA strongly bound to another NR, estrogen-related receptor γ (ERR γ)²², which is one of the constitutively active NRs. ERR γ is expressed in the mammalian brain and contributes to growth of nerves and sexual glands. It was surprisingly revealed that BPA hardly changed transcriptional activity of ERR γ , even though BPA strongly binds to ERR γ ²². Therefore, the specific mechanisms of adverse effects that were induced by BPA via NRs, have still been controversial. On the other hand, previous studies also demonstrated that BPA shows not only estrogenic activity but also several other types of biological toxicity. For example, induction of mitochondrial injury²³ and apoptotic death in several types of cells^{16,24-26} were previously reported as biological toxicities induced by BPA. Although the risk of BPA has not been completely elucidated yet, the usage of BPA has come to be regulated globally in consideration of various concerns about widespread exposure of BPAs and associated their adverse effects on human and wild animals²⁷.

Since the usage of BPA itself has become restricted, several bisphenol analogs such as bisphenol F, bisphenol C, and bisphenol S, etc. (Figure 1) have been developed as “new generation bisphenols” to avoid BPA’s adverse effects for human health. Accordingly, the

environmental occurrence, human exposure, and toxicity of these bisphenol analogs began to gain considerable attention¹¹. Previously, it was reported that these BPA analogs exhibited weak estrogenic activity and toxicity^{28–30}. On the other hand, BPA analogs with halogenation at the phenolic moieties have been receiving a lot of attention, because of their higher estrogenic potency or toxicity than BPA itself. In this regard, there are some reports that tetrachlorobisphenol A (TCBPA) and tetrabromobisphenol A (TBBPA), which are mainly used as a flame retardant, exhibit disruption of signaling pathways via thyroid hormone receptors³¹ and peroxisome proliferator-activated receptor γ ^{2,29}. They also show more toxic effect on aquatic organisms and rat hepatocytes than BPA^{32–34}. In addition, estrogenic activity of lower-chlorinated BPA analogs (BPA-monoCl, BPA-diCl, and BPA-triCl) were almost equal to or higher than that of BPA^{29,31,35,36}. However, available information regarding the potential toxicity of the BPA analogs with halogenation at phenolic group(s) is still much scarcer, although BPA itself and the BPA analogs that modified at the central carbon atom have been studied well as endocrine disruptors. In addition, their toxicological behaviors have been widely reviewed in the literature^{11,37–41}. On the other hand, there are still few of the studies regarding the cytotoxic effects by the BPA analogs with halogenation at phenolic group(s) compared to the endocrine disrupting effects. Thus, the further investigation of the potential toxicity of the BPA analogs with halogenation at phenolic group(s) is urgently required as well as the BPA analogs that modified at the central carbon atom in order to assess the safety of these chemicals as alternatives to BPA.

In the present study, we explored the potential cytotoxic effects of 16 BPA analogs possessing one to four halogen atoms as substituents on its aromatic ring(s). To achieve this, we synthesized the BPA analogs by aromatic electrophilic substitution reaction of BPA. The cytotoxic effects on HeLa cell lines of the synthesized BPA derivatives were investigated by MTT assay and LDH release assay. Furthermore, fluorescent spectroscopic analysis was carried out to detect apoptotic and necrotic cells after exposure to the halogenated BPA analogs.

(Figure 1)

2. Materials and Methods

2. 1. Chemicals

2,2-Bis(4-hydroxyphenyl)propane (bisphenol A), tetrachlorobisphenol A (TCBPA or BPA-tetraCl), *N*-fluorobenzenesulfonimide, trichloroisocyanuric acid, *N*-bromosuccinimide, and *N*-iodosuccinimide were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Tetrabromobisphenol A (TBBPA or BPA-tetraBr), selectfluor, and *N,N*-dimethylformamide (DMF) were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-(4,5-Dimethyl-2-thiazolyl)- 2,5-diphenyltetrazolium bromide (MTT) and 2-propanol were purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS), L-glutamine, sodium bicarbonate solution, and 0.5% trypsin-EDTA were purchased from Thermo Fisher Scientific Inc. (MA, USA). Other reagents and solvents were purchased from commercial suppliers and used without further purification. All organic reactions were carried out in oven-dried glassware with magnetic stirring. Column chromatography was performed using silica gel (Silica Gel 60 N, 0.063-0.210 mm, Kanto Chemical Co., Inc. (Tokyo, Japan)).

2. 2. Synthesis of halogenated BPA analogs

Synthesis of halogenated BPA analogs was carried out by aromatic electrophilic substitution reaction on phenolic rings of BPA with electrophilic halogenation agents. Synthesized BPA analogs are shown in Figure 2. Further detailed synthesis is described in Chapter 2.

(Figure 2)

2. 3. Cell culture

HeLa cell line was obtained from JCRB (JCRB9004, JCRB cell bank, NIBIO, Osaka, Japan). HeLa cells were used from P6 to 14 and were maintained in Eagle's MEM (EMEM) (Nissui Pharma Cosmetics Co., Ltd., Tokyo, Japan) containing 10% FBS (treated with dextran-coated charcoal), 2 mM L-glutamine and 0.15% sodium bicarbonate at 37°C, in an atmosphere of 5% CO₂ and 95% air.

2. 4. MTT assays for HeLa cells

HeLa cells were seeded in a 96-cell plate at a density of 1.00×10^4 cells/well in 100 μL of medium, cultured for 24 h, and then treated with different concentrations of the halogenated BPA analogs in 10 μL PBS for 24 h. At the end of treatment, 10 μL of MTT (5 mg/mL in PBS) was added and incubated for an additional 160 min. Then formazan crystals were dissolved in a mixed solvent of 4 M HCl and 2-propanol (1:100, v/v, 200 μL). After dissolution, cell viability was assessed by measuring the absorbance at 590 nm by using ImmunoMini NJ-2300 colorimeter (BIOTEC Co Ltd., Tokyo, Japan). The data are the average of three independent experiments. Comparison of the means and standard errors of the mean was performed using Welch's *t*-test⁴².

2. 5. LDH release assays for HeLa cells

LDH release assay was carried out by using Cytotoxicity LDH Assay Kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) on HeLa cells. HeLa cells were seeded in a 96-cell plate at a density of 1.00×10^4 cells/well in 100 μL of medium and cultured for 24 h. Then, 10 μL different concentration of the halogenated BPA analogs dissolved in PBS was added to the cells and incubated at 37°C for 24 h in a CO₂ incubator. Working solution (100 μL , included in the kits) was added to each well and the plate was incubated for 30 min under a light-shielded condition at 25°C. Finally, 50 μL of the stop solution (included in the kits) was added to each well. Then, 50 μL of final mixture was diluted to 200 μL with pure water and measured the absorbance at 490 nm by ImmunoMini NJ-2300 colorimeter (BIOTEC Co Ltd.). For positive control, HeLa cells were added 10 μL of the Lysis Buffer (included in the kits), incubated at 37°C for 30 min in a CO₂ incubator, and measured the absorbance in the same manner. For negative control, the cells were treated only with medium. The relative LDH release was calculated by the following formula.

$$\text{Relative LDH release (\%)} = (T-N)/(P-N) \times 100$$

(*T* is the absorbance of the cell culture at 490 nm treated with test compounds, *P* is the absorbance of positive control, and *N* is the absorbance of negative control)

2. 6. Fluorescent spectroscopy

Detection of apoptotic and necrotic cells was carried out by using Apoptotic & Necrotic Cell Detection Kits (Promocell GmbH, Heidelberg, Germany) on HeLa cells. HeLa cells were harvested and concentrated to approximately 2.00×10^6 cells/well suspension by centrifugation, and then treated with different concentrations of the halogenated BPA analogs. Simultaneously, cells were stained by using an assay kit following the manufacturer's instruction to stain suspension cells. The fluorescent observation was performed using BIOREVO BZ-9000 (KEYENCE Co., Osaka, Japan), and the magnification was 4x [equipped with PlanApo VC 60x oil lens (Nikon Co., Tokyo, Japan)]. The measurement time of 0 hours (0 h) that was shown in Figures 5 to 10, was actually 30 minutes, that is unavoidable time to sample preparation, after adding each test reagent.

3. Results and discussion

3. 1. Cell viability determination by MTT assay

Cytotoxic effects of the halogenated BPA were evaluated by MTT assay on cultured HeLa cells. BPA and the halogenated BPA analogs were examined for their effect on the viability of HeLa cells after 24 hours treatment with different concentrations of them. It was revealed that the mono- and the di-halogen-substituted BPA analogs exhibited evident toxicity for HeLa cells in the concentration-dependent manner (Figure 3). The cell viability treated with 10^{-4} M of BPA-monoF, monoCl, monoBr, and monoI was apparently decreased to 70.03%, 53.20%, 26.84%, and 11.83%, respectively (Table 1). Therefore, it was suggested that BPA analogs possessing halogen substituent(s) on its aromatic ring(s) exhibited significantly stronger cytotoxicity than BPA itself. The cytotoxicity of the mono- and the di-halogenated BPA analogs increased with the increment of the size of introduced halogen atom(s) ($F < Cl < Br < I$). Furthermore, the di- halogenated BPA analogs showed stronger toxicity than the other tested compounds in this study. Notably, HeLa cells hardly survived after treatment of 10^{-4} M of BPA-diBr or BPA-diI (less than 5% viability). Among tested compounds in this study, BPA-diI exhibited the strongest cytotoxicity (less than 4% viability).

By contrast, the tri- and the tetra-halogenated analogs showed weak cytotoxicity for HeLa cells (more than 76.5% viability). Thus, these results clearly indicate that the existence of two (not three or four) halogen substituents at the ortho-positions of hydroxyl group of each phenolic moiety were important for the strong cytotoxicity of the BPA analogs. In this study, it was observed that BPA increased the viability of HeLa cells in a concentration dependent manner at the concentration below 10^{-4} M. It was good agreement with the previous report that BPA facilitated cell proliferation of HeLa cells at the lower concentration⁴³. Among the halogenated BPA analogs, it was confirmed that BPA-triF and BPA-tetraF analogs exhibited more evident cell proliferation activities than BPA. At the present, the cause of the cell proliferation activities of the BPA analogs has not been elucidated.

(Figure 3)

In addition to the pathways via NRs, it has been reported that BPA can activate GPR30, a G-protein-coupled receptor for estrogen: BPA binds to GPR30, increasing cAMP level, and inducing cell proliferation via PKA pathway or ERK1/2 pathway in estrogen-sensitive cells^{38,44–46}. Thus, it was considered that viability of HeLa cells could be increased by exposure of BPA or BPA analogs including the halogenated BPA analogs. Actually, when HeLa cells were exposed to BPA, the cell proliferation was observed in a concentration-dependent manner and decreased to approximately the same level of control at the highest concentration of BPA (100 μ M). This reduction of viability at the high concentration of BPA was consistent with the previous study indicating that BPA induced apoptotic cell death when the concentration of BPA was higher than 10^{-4} M¹⁶. On the other hand, our data clearly demonstrated that the synthetic halogenated BPA analogs possessing halogen substituent(s) on its aromatic ring(s) exhibited relatively stronger cytotoxicity than BPA. As mentioned above, the cytotoxicity of the halogenated BPA analogs was increased with the increment of the size of introduced halogen atom, but was not related to the number of halogen atoms incorporated.

It should be noted that the di-substituted BPA analogs showed stronger cell toxicity than the tri- and the tetra-substituted analogs. Since lower-substituted BPA analogs, such as mono- and di-halogenated BPA analogs could be generated by dehalogenation from higher-

substituted tri- and tetra-halogenated BPA analogs, the risk of contact with these lower-substituted BPA analogs is thought to be high. For example, it was reported that TBBPA (described as BPA-tetraBr in this study) is debrominated in the environment into lower-brominated BPA analogs (mono-, di-, and tri-brominated BPA)^{47,48}. Tetra-brominated and chlorinated analogs of BPA, namely, TBBPA and TCBPA (BPA-tetraCl), are generally used to produce fire-proof epoxy resins and have been unambiguously detected in the environment^{49,50}. Therefore, it was concerned that dehalogenation reaction of these BPA-related chemicals following with leakage from various consumer products could cause serious health damage to the living organisms including human.

3. 2. Effects of the halogenated BPA analogs on LDH release of HeLa cells

The release of LDH from HeLa cells was examined as an indicator for cell membrane damage after exposure of the halogenated BPA analogs to the cells. As shown in Figure 4a, the exposure of the mono-halogenated BPA analogs caused moderately increase in LDH release in a concentration dependent manner. Treatment of the di-halogenated BPA analogs to HeLa cells resulted in a significant concentration-dependent increase in LDH release in the concentration range from $10^{-4.5}$ to 10^{-4} M (Figure 4b). In the results of the mono- and the di-halogenated BPA analogs exposure, the LDH release was increased with the size of halogen substituent became larger. On the other hand, the tri- and the tetra-halogenated BPA analogs did not obviously affect the LDH release. These data obtained from the LDH release assay were evidently consistent with the results from the MTT assay. Therefore, it was suggested that the cell membrane damage induced by the halogenated BPAs could be related to the viability of HeLa cells. In summary, the results of LDH release assay showed that treatment of the di-halogenated BPAs to HeLa cells resulted in a significantly dose-dependent increase in LDH release, suggesting that the cell membrane was damaged after exposure to highly cytotoxic di-halogenated BPA analogs.

(Figure 4)

3. 3. Apoptotic and necrotic cell detection

As mentioned above, the halogenated BPA analogs induced cell death by damaging cell membranes. To elucidate the mechanism of cell death induced by the BPA analogs, fluorescence microscopic analysis was carried out (Figures 5 to 10). In this analysis, apoptotic cells were distinguished from the dead cells including necrotic cells. In the present study, HeLa cells were exposed to 10^{-4} M of each di-halogenated BPA analog for 24 h and stained with hoechst 33342 (blue fluorescent dye), FITC-annexin V (green fluorescent dye), and ethidium homodimer III (EthD-III; red fluorescent dye). All cells displayed blue fluorescence by hoechst 33342 staining, the apoptotic cells displayed green fluorescence by FITC-annexin V staining, and the necrotic cells exhibited red fluorescence by ethidium homodimer III (Figures 5 to 10). As shown in Figure 9, BPA-diBr treated cells synchronously showed green and red fluorescence by FITC-annexin V and EthD-III at 8 hours. The number of co-stained cells was gradually increased with the lapse of time. Similar observations were confirmed in the cells treated by BPA-diF and BPA-diCl (Figures 7 and 8). These results indicated that di-halogenated BPA analogs induced apoptosis of HeLa cells and led to cell death almost simultaneously. Interestingly, treatment of BPA-diI, which showed toxicity as same as that of BPA-diBr in the MTT assay, showed a different staining pattern. As shown in Figure 10, cells that were stained immediately in red fluorescence by ethidium homodimer III, emerged after the treatment of BPA-diI. Thus, this result suggested that BPA-diI induced cell death via necrotic pathway. Thus, it was considered that the mechanism of cell death induced by the iodinated BPA analogs were different from that by other halogenated BPA analogs, although all of the halogenated BPA analogs showed stronger cytotoxicity than BPA with cell membrane damage to HeLa cells.

(Figures 5, to 10)

4. Conclusion

In this study, we evaluated the risk of BPA analogs from the view point of cytotoxic effects in addition to that of the conventional endocrine disrupting effects. We investigated the potential toxicity of the BPA analogs that possessed halogen substituents on the phenolic

moieties against HeLa cells. It was revealed that the mono- and the di-halogenated BPA analogs exhibited stronger cell toxicity than BPA itself. Cytotoxicity of the BPA analogs was increased with the increment of the size of introduced halogen substituent. Among the cytotoxic mono- and di-halogenated BPA analogs, the di-substituted BPA analogs showed relatively stronger toxicity than the mono-substituted analogs. The result of the LDH release assay indicated that cell membrane damage induced by the halogenated BPAs was closely related to the viability of HeLa cells. Furthermore, fluorescence microscopy revealed that the mechanisms of cytotoxicity were different from the halogen substituents; only the iodinated BPA analogs could cause necrotic cell death, whereas other halogenated BPAs could induce apoptosis of HeLa cells.

Our finding that the halogenated BPAs (especially the di-halogenated analogs) exhibited strong cytotoxicity for HeLa cells suggested that endocrine disrupting effects and potential health problems induced by the environmental chemicals including halogenated BPAs should be concerned more seriously. Since these new bisphenols have been developed and utilized as alternatives to BPA, it is necessary to carefully study the influence of these BPA analogs on organisms. It is noteworthy that the di-substituted BPA analogs showed stronger cell toxicity than the tri- and the tetra-substituted analogs. Since the lower-substituted BPA analogs could be generated by decomposition of the above-mentioned higher-substituted analogs, TBBPA and TCBPA, which have been used to produce high-functional plastic materials, further researches to clarify whether these halogenated BPA analogs exhibit potent cytotoxicity *in vivo* are urgently required. In addition, it should be noticed that the BPA analogs involving BPA-monoBr, BPA-diBr, BPA-monoI, and BPA-diI showed stronger cytotoxicity than BPA itself, whereas the tri- and tetra-fluorinated BPA analogs conversely showed more potent endocrine disruption than BPA. These results suggested a risk of environmental chemicals, that is, chemicals possessing a similar chemical structure each other could cause different adverse effects for living body via different and unexpected biological pathways, even if they do not show the activities via one pre-assumed pathway. Environmental chemicals including BPA analogs that are able to cause endocrine disruption and other hazardous effects has come to be detected ubiquitously in water, air, soil,

and even living organisms including human due to leakage from various consumer products. Therefore, various kinds of risk assessments from a broad perspectives ranging from molecular to environmental problems are necessary for the development and safety use of chemical substances including basic materials for manufacturing industrial products. To achieve this, continuous efforts should be made to further develop and improve various *in vitro*, *in vivo* and *in silico* methods to evaluate and predict endocrine disrupting activity, cytotoxicity, and physiological activity and toxicity of chemicals in high accuracy.

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Table 1. Cell viability of HeLa cells after incubation with 10^{-4} M of halogenated BPA analogs.

Chemical	Cell viability (%)		
BPA	121.5	±	3.40
BPA-monoF	70.03	±	1.57
BPA-monoCl	53.20	±	7.51
BPA-monoBr	26.84	±	6.82
BPA-monoI	11.83	±	4.24
BPA-diF	52.95	±	3.77
BPA-diCl	17.56	±	6.24
BPA-diBr	4.950	±	0.722
BPA-diI	3.942	±	0.793
BPA-triF	142.8	±	17.3
BPA-triCl	76.54	±	6.55
BPA-triBr	94.83	±	9.80
BPA-triI	94.29	±	10.56
BPA-tetraF	171.6	±	7.70
BPA-tetraCl	87.67	±	6.10
BPA-tetraBr	92.60	±	5.20
BPA-tetraI	85.09	±	3.95

Relative cell viability ratio compared to the control (0 M of each halogenated BPA analog) was determined by MTT assay. Values are Mean ± S. E. Duplicate assay were repeated at least three times for each BPA analog.

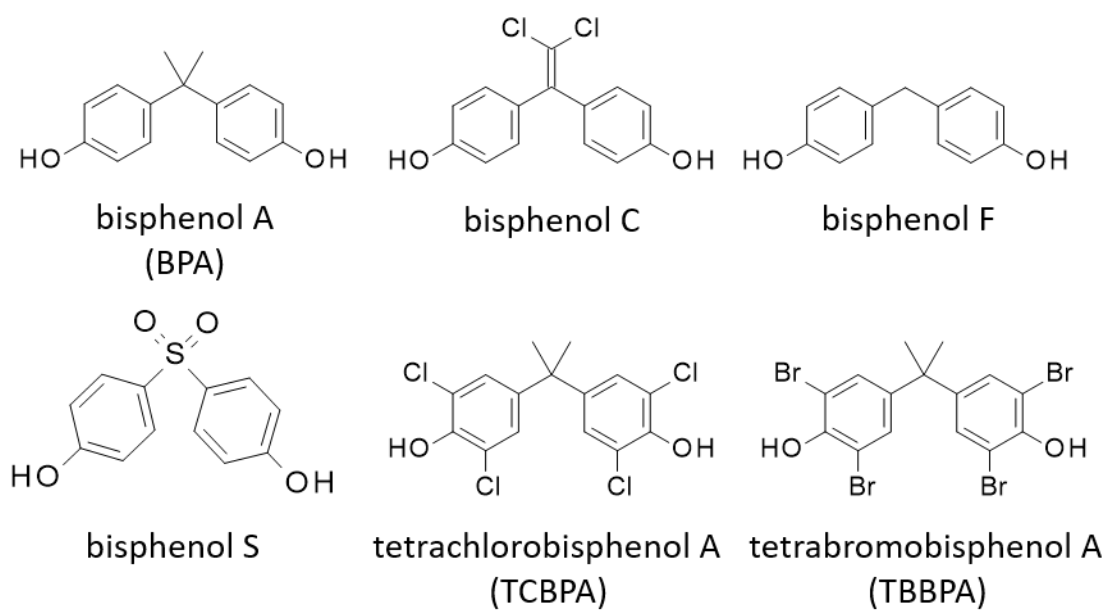


Figure 1. Chemical structures of BPA analogs known as “new generation bisphenols”.

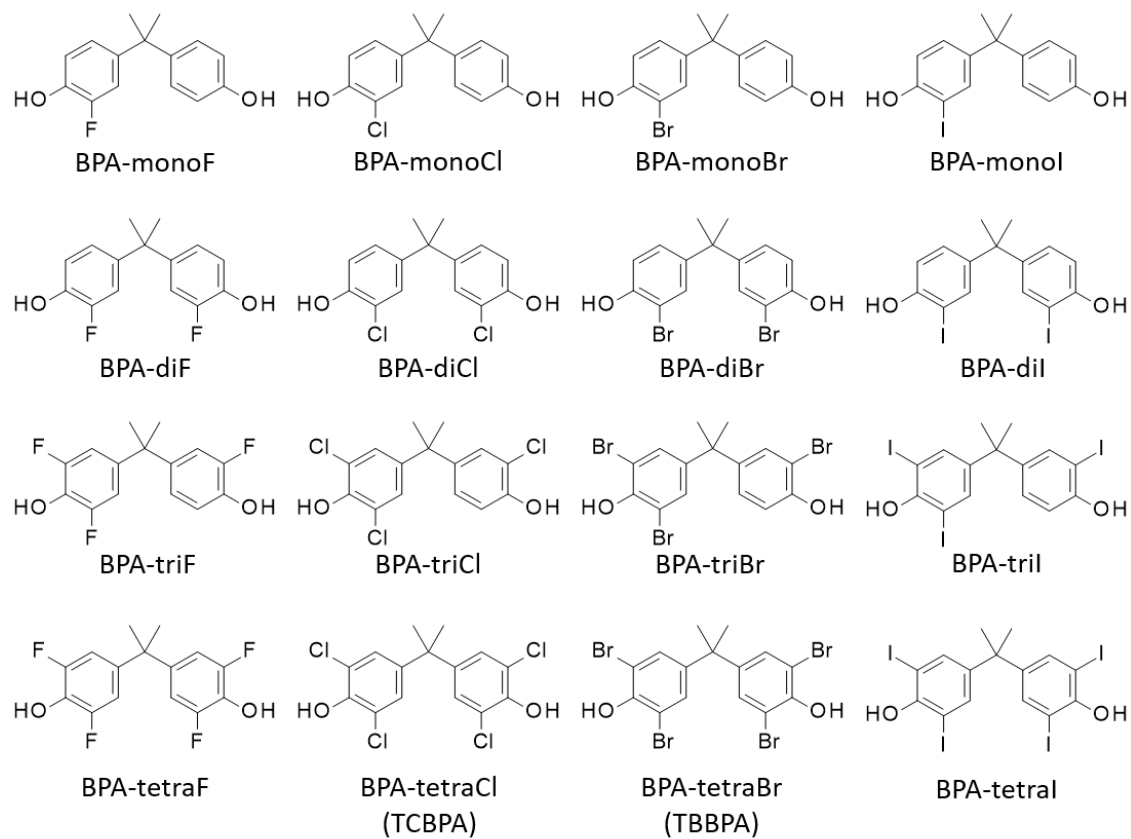


Figure 2. Synthesized halogenated BPA analogs used in this study.

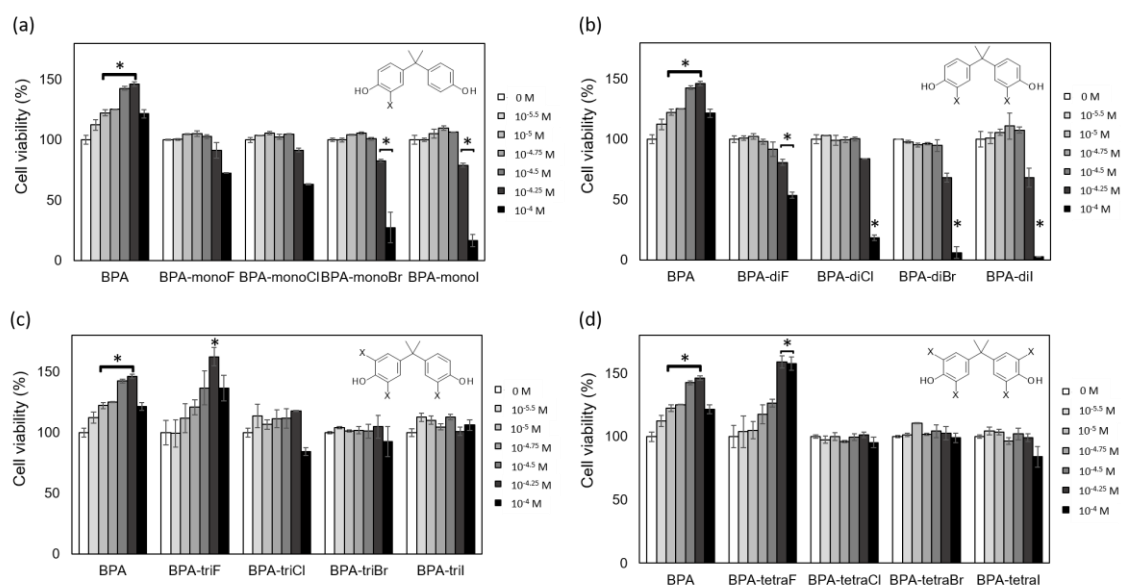


Figure 3. Effects of the BPA analogs on cell viability of HeLa cells.

BPA and the halogenated BPA analogs were examined their effect on the viability of HeLa cells (1.00×10^4 cells/well in 100 μ L of medium) after 24 hours of exposure. Cell viability is represented as relative absorbance compared to the control. Duplicate assays were repeated at least three times for each BPA analog.

* $P < 0.05$ in Welch's *t*-test compared with control (0 M).

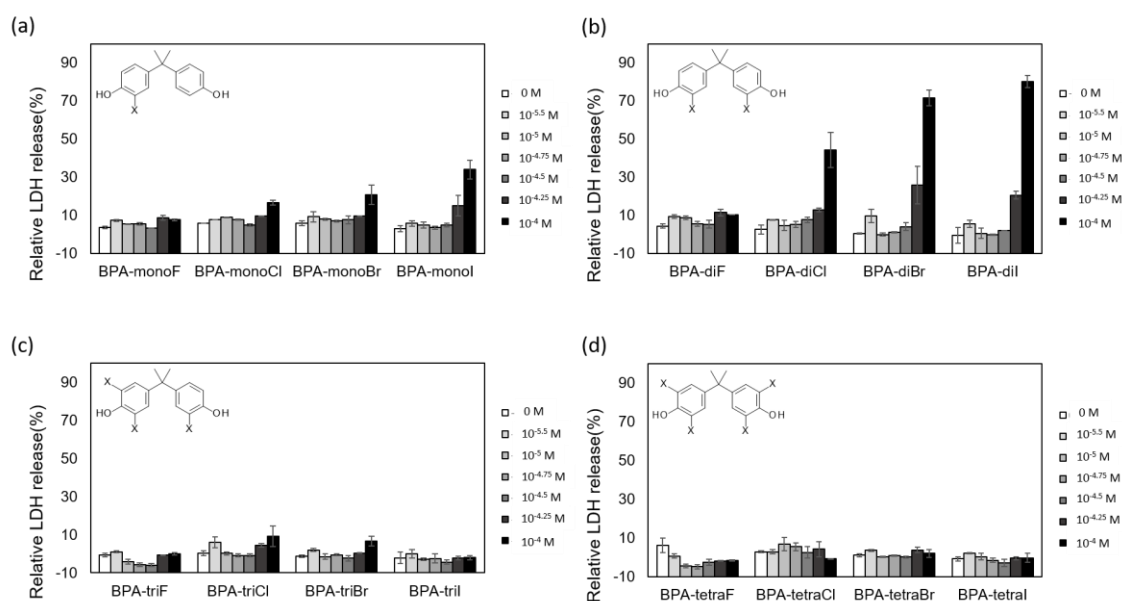


Figure 4. The effects of the halogenated BPA analogs on the LDH release in the culture medium of HeLa cells.

The effect of the halogenated BPA analogs on the LDH release of HeLa cells (1.00×10^4 cells/well in 100 μ L of medium) were examined after 24 hours of exposure. Duplicate assays were repeated at least three times for each BPA analog.

Control

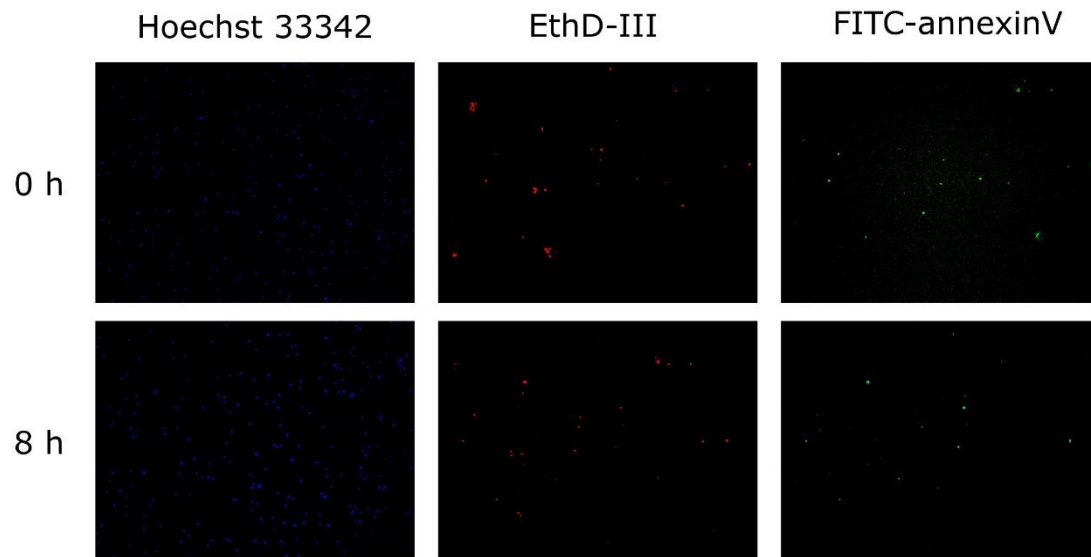


Figure 5. Fluorescence staining of HeLa cells after treatment of PBS (control).

HeLa cells treated with PBS were co-stained with Hoechst 33342 (blue), ethidium homodimer III (red), and FITC-annexin V (green). Fluorescence was visualized by a fluorescence microscope.

BPA

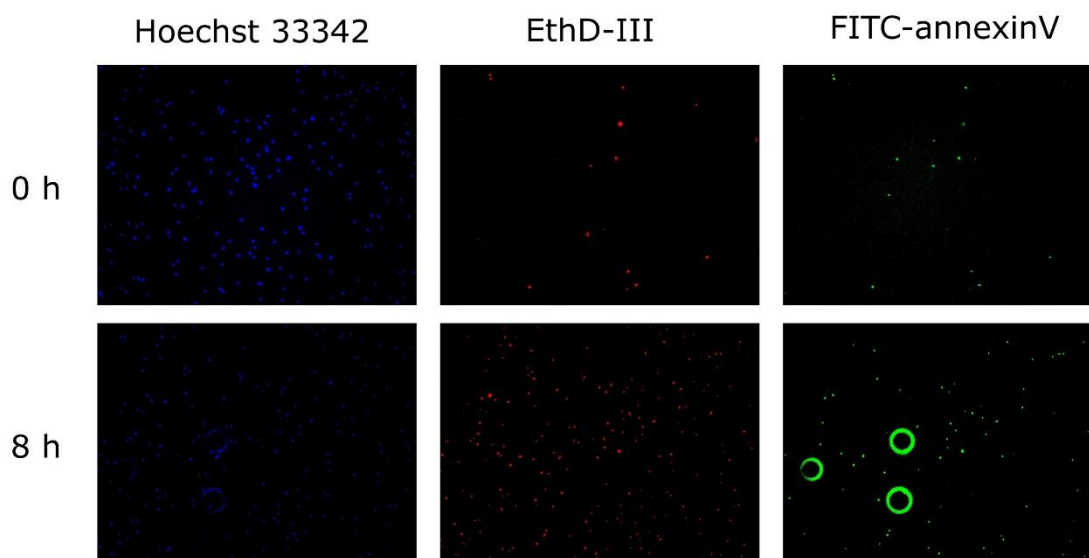


Figure 6. Fluorescence staining of HeLa cells after treatment of BPA.

HeLa cells treated with BPA were co-stained with Hoechst 33342 (blue), ethidium homodimer III (red), and FITC-annexin V (green). Fluorescence was visualized by a fluorescence microscope. Green circles were accidentally caused by air bubbles.

BPA-diF

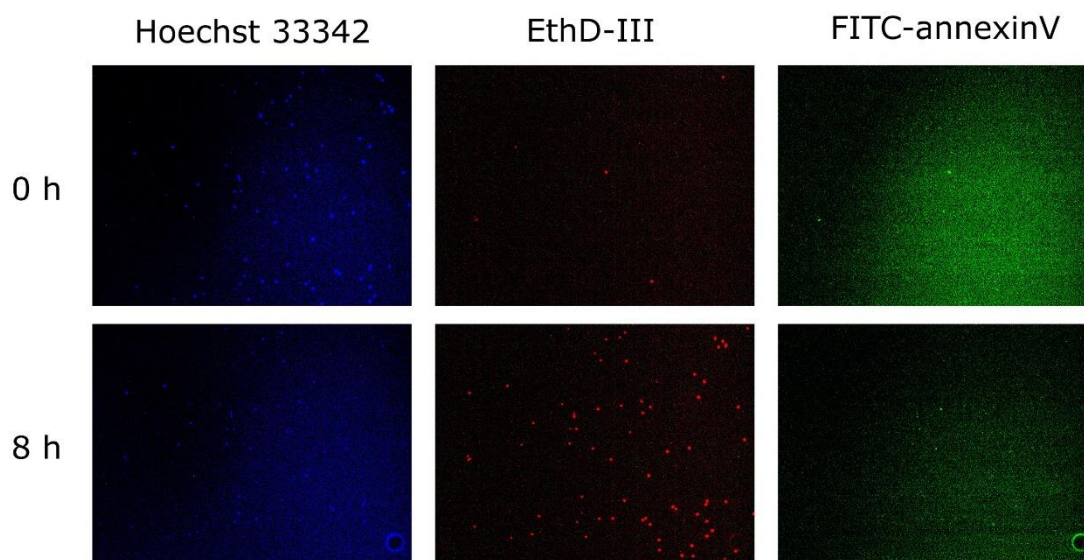


Figure 7. Fluorescence staining of HeLa cells after treatment of BPA-diF.

HeLa cells treated with BPA-diF were co-stained with Hoechst 33342 (blue), ethidium homodimer III (red), and FITC-annexin V (green). Fluorescence was visualized by a fluorescence microscope.

BPA-diCl

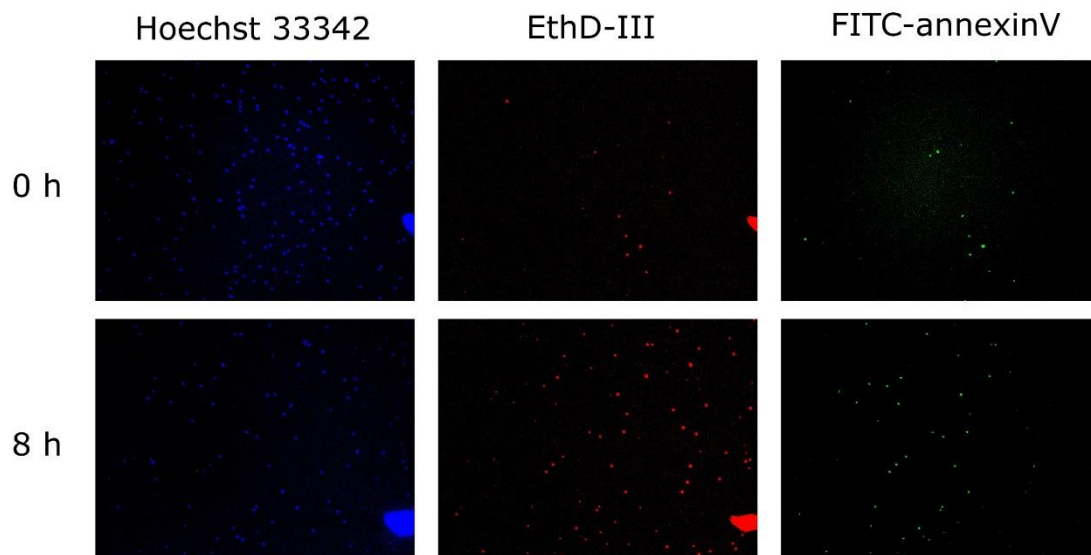


Figure 8. Fluorescence staining of HeLa cells after treatment of BPA-diCl.

HeLa cells treated with BPA-diCl were co-stained with Hoechst 33342 (blue), ethidium homodimer III (red), and FITC-annexin V (green). Fluorescence was visualized by a fluorescence microscope.

BPA-diBr

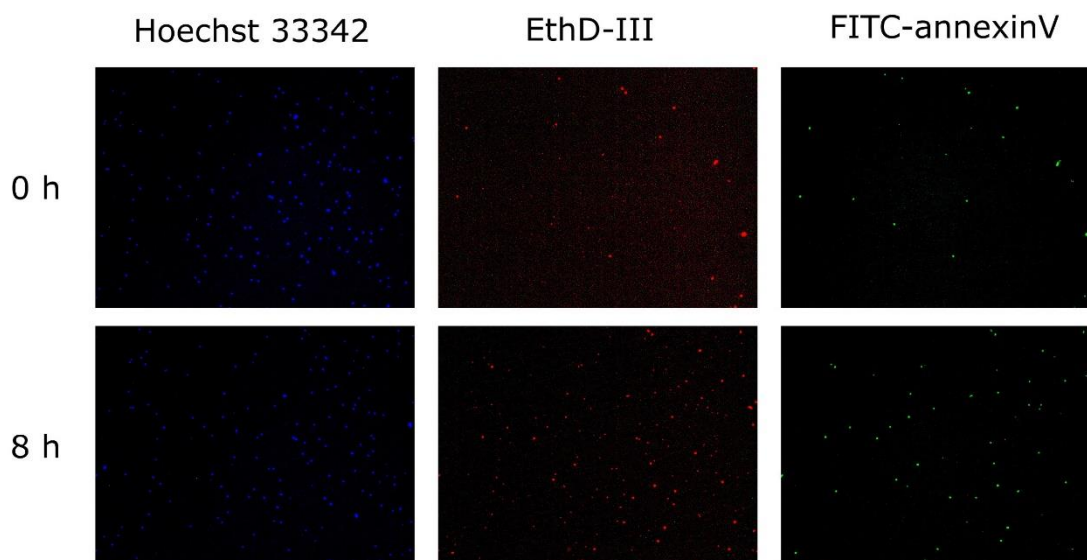


Figure 9. Fluorescence staining of HeLa cells after treatment of BPA-diBr.

HeLa cells treated with BPA-diBr were co-stained with Hoechst 33342 (blue), ethidium homodimer III (red), and FITC-annexin V (green). Fluorescence was visualized by a fluorescence microscope.

BPA-diI

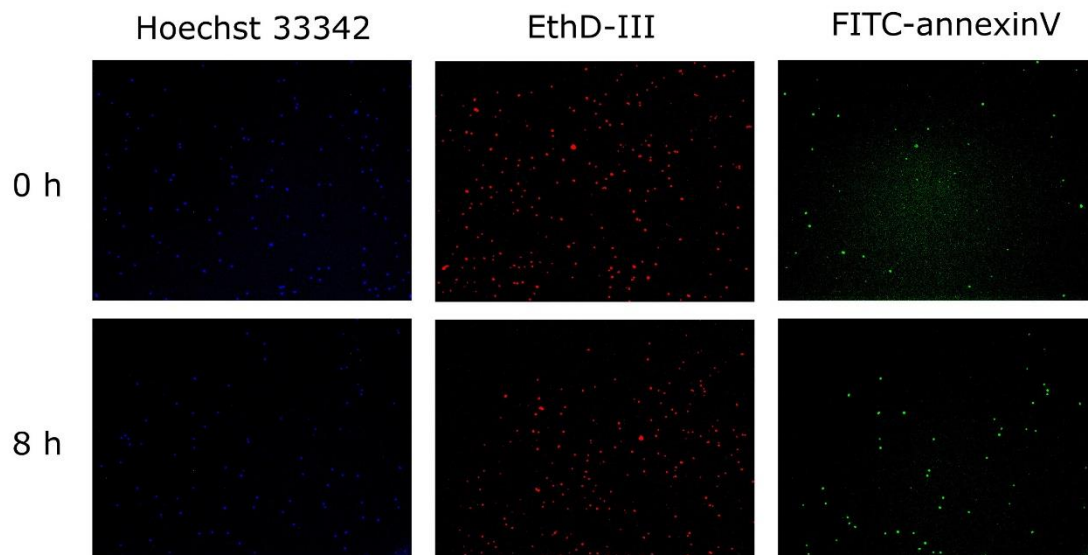


Figure 10. Fluorescence staining of HeLa cells after treatment of BPA-diI.

HeLa cells treated with BPA-diI were co-stained with Hoechst 33342 (blue), ethidium homodimer III (red), and FITC-annexin V (green). Fluorescence was visualized by a fluorescence microscope.

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