— Regular Article —

Estrogenic/Antiestrogenic Activities of Quinoid Polycyclic Aromatic Hydrocarbons

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Estrogenic and antiestrogenic activities of 19 quinoid polycyclic aromatic hydrocarbons (PAHQs) and 9 ketone PAHs were evaluated by the yeast two-hybrid assay using yeast cells expressing estrogen receptor-α (ERα). Binding affinity of PAHQs to ERα was assayed by the polarized fluorescence method using Fluormone^{TM} ES2. Ten PAHQs having 3–5 rings showed antiestrogenic activities. The most strongly antiestrogenic PAHQs were 1,4-chrysenequinone and 5,6-chrysenequinone. On the other hand, benzo[*a*]pyrene-3,6-quinone showed the strongest estrogenic activity. However, the other compounds tested did not show so strong estrogenic/antiestrogenic activities. Binding affinity to ER was required but not sufficient for estrogenic/antiestrogenic activities of PAHQs. The length-to-breadth ratios of the rectangular planes surrounding the ring molecules and the distances between the oxygen atom of the carbonyl group and farthest hydrogen atom of estrogenic/antiestrogenic PAHQs were in narrow ranges, suggesting a structure-activity relationship. As interactions between active PAHQ and ER, hydrogen bonding between carbonyl groups and amino acid residues and van der Waals forces were considered.

Key words —— polycyclic aromatic hydrocarbon, quinone, antiestrogenic activity, yeast two-hybrid, structure-activity relationship

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. In recent years there has been increasing interest in endocrine disruptors which may cause dysfunction of human and wildlife endocrine systems leading to cancers, reproductive system abnormalities and immune system deficiencies.[1,2] Several screening tests have been developed to evaluate the endocrine-disrupting activities of chemicals. As for PAHs, antiestrogenic activity was observed in a yeast assay system[3] and estrogenic activity was found in MCF-7 cells.[4] The estrogenic activities of 517 chemicals were evaluated by using a yeast two-hybrid assay system based on the ligand-dependent interaction of estrogen receptor (ER) and its co-activators.[5]

Several studies have evaluated the estrogenic and antiestrogenic activities of hydroxy PAHs (OHPAHs) such as hydroxybenzo[*a*]pyrene (OHBaP).[4,6,7] Several other OHPAHs and *n*-propyl *p*-hydroxybenzoate were identified as estrogenic compounds in cigarette smoke condensate.[8] We previously evaluated estrogenic/antiestrogenic activities of 14 PAHs and 63 OHPAHs having 2–6 rings by yeast two-hybrid assay and found that PAHs did not show any estrogenic/antiestrogenic activity, but several OHPAHs having 3–5 rings showed activity. Especially, OHPAHs having 4 rings such as 3-, 4- and 10-hydroxybenz[*a*]anthracenes (3-, 4- and 10-OHBaAs) and 2-hydroxychrysene (2-OHCh) showed strong estrogenic activity. Several other OHPAHs having 4 rings such as 2- and 3-hydroxybenzo[*c*]phenanthrenes (2- and 3-OHBcPh), 2-OHBaA and 3-OHCh showed strong antiestrogenic activity. Both length-to-breadth (L/B) ratios of the

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rectangular van der Waals planes surrounding the ring molecules and distances between the oxygen atom of the phenol group and farthest hydrogen atom (O-H distance) of the estrogenic OHPAHs were in narrower ranges than those of antiestrogenic OHPAHs. The similarity of the values of OHPAHs to those of estradiol (E_2) and diethylstilbestrol (DES) suggested that OHPAHs interact with the ER through hydrogen bonding between the phenol groups of OHPAH and amino acid residues of ER and van der Waals fitting between benzene rings.[9)]

PAHs are oxidized to OHPAHs, PAH quinones (PAHQs) and ketones *etc*. in the presence of cytochrome P450 enzymes (CYPs), epoxide hydrase and/or aldo-keto reductase in human and animals.[10)] These metabolites are also secondarily formed from mother PAHs in the atmosphere.[11)] Both biological and chemical formations of OHPAHs or PAHQs suggest that the concentrations of these oxidized derivatives of PAHs increase easily in animal bodies. Recently, we have found that several PAHQs show oxidative damage to cellular components and DNA by producing reactive oxygen species (ROS) in the redox cycling.[12)] However, it is unclear whether PAHQs have endocrine-disrupting activities. The purpose of this study was to estimate estrogenic/antiestrogenic activities by the yeast two-hybrid assay and binding affinity of PAHQs by polarized fluorescence detection. Based on the results, we discuss the relationship between structures and activities of quinoid PAHs.

MATERIALS AND METHODS

Chemicals —— 1,2-Naphthoquinone (1,2-NQ), acenaphthoquinone (AcQ), 1,4-phenanthrenequinone (1,4-PhQ), 9,10-PhQ, 1,4-anthraquinone (1,4-AQ), 9,10-AQ, 1,4-chrysenequinone (1,4-ChQ), 1,2-benzanthraquinone (1,2-BAQ), benzo[*c*]phenanthrene[1,4]quinine (BcPh-1,4-Q), BcPh-5,6-Q, benzo[*a*]pyrene[1,6]quinone (BaP-1,6-Q), BaP-3,6-Q, BaP-4,5-Q, BaP-6,12-Q, BaP-7,8-Q, BaP-7,10-Q, BaP-11,12-Q, 5,6,8,9-tetrahydrobenz[*a*]anthracen-11[10 H]-one (BaA-11-one), benzo(*a*)fluoren-11-one (BaFl-11-one), benzo[*b*]fluoren-11-one (BbFl-11-one) and 1-hydroxy-9-fluorenone (1-OH-Fl-9-one) were from Chiron AS (Trondheim, Norway). 3,4-Dihydrobenz[*a*]anthracen-1[2 H]-one (BaA-1-one) and 9,10-dihydrobenzo[*a*]pyrene-7[8 H]-one (BaP-7-one) were from Sigma-Aldrich (St. Louis, MO, U.S.A.). 9-Fluorenone, anthrone and benzanthrone were purchased from Tokyo Chemical (Tokyo, Japan). Structures and abbreviations of test compounds are shown in Figs. 1 and 2. Test compounds were dissolved in ethanol and stored at −20°C until use. All other chemicals were of the highest quality available from commercial sources.

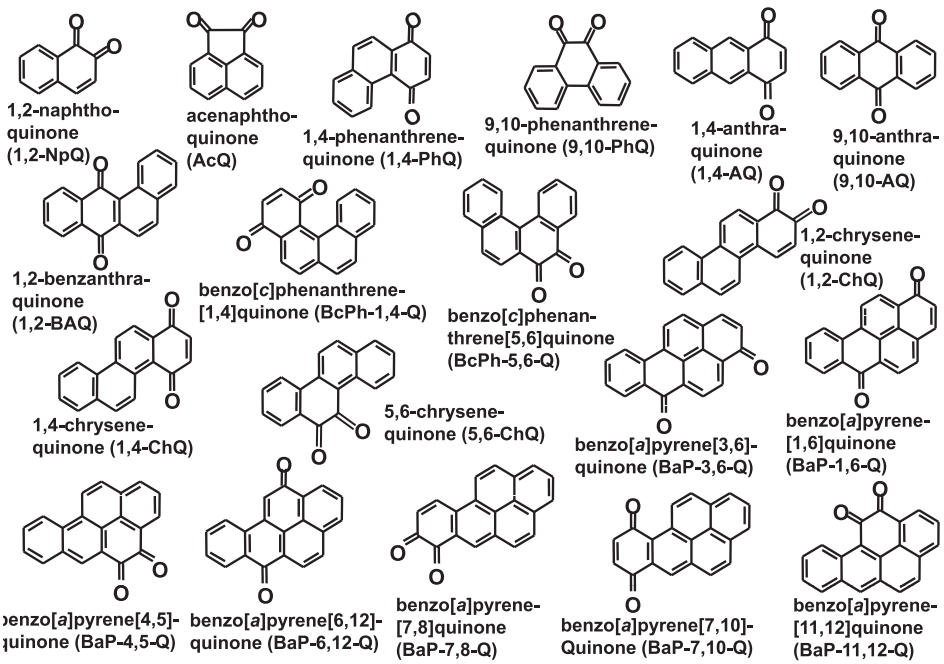

Fig. 1. Structures and Abbreviations of PAHQs

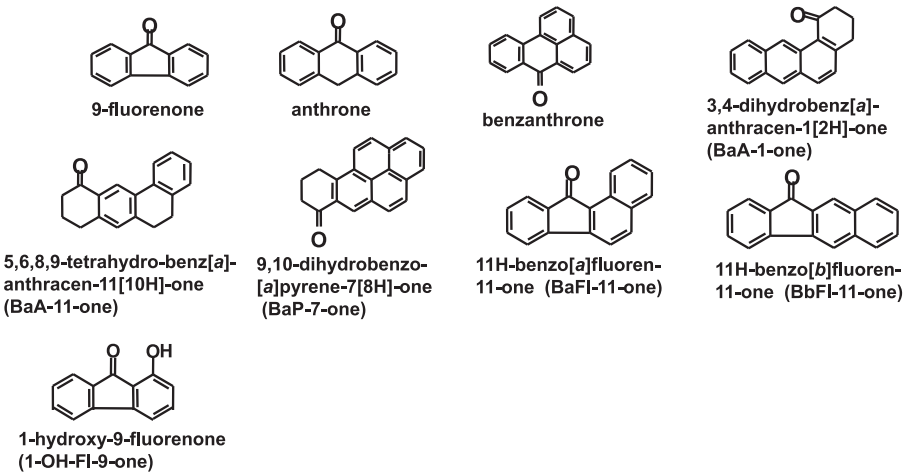

Fig. 2. Structures and Abbreviations of Ketone PAHs

Assay of Estrogenic and Antiestrogenic Activities —— Estrogenic and antiestrogenic activities of PAH derivatives were evaluated by the yeast two-hybrid assay method using yeast cells expressing human ERα according to our previous reports.[7,9] To examine the agonistic activity, the yeast cells were grown overnight at 30°C with shaking in synthetic defined medium free from tryptophan and leucine, and treated with each test compound in the concentration range from 1×10^{-9} M to 1×10^{-6} M at 30°C for 4 hr. After the incubation, the treated cells were collected and enzymatically digested with 1 mg/ml Zymolyase 20 T at 37°C for 30 min. 2-Nitrophenyl-β-D-galactoside was added to the lysate to a final concentration of 4 mg/ml. After incubation at 30°C for 45 min, the reaction was stopped by the addition of 1 M Na_2CO_3. The yeast debris was removed by centrifugation and β-galactosidase activity was assayed by measuring the absorbance of supernatant at 415 nm. The data were representative of three independent experiments. Relative effective potency of estrogenic activity (REP_E) was calculated as the inverse value of the relative concentration of the test compound that gave the same activity of E_2. Relative effective potency of antiestrogenic activity (REP_{AE}) was calculated as the inverse value of the relative concentration of the test compound that gave the same activity of 4-hydroxytamoxifen (4-OHT) in the presence of 1 nM E_2.

Receptor Binding Assay —— The assay was performed by using a PanVera P2698 ERα Competition Assay, Green (Takara Bio, Otsu, Japan). Fourteen μl of Fluormone^{TM} ES2 (FES2) and 14 μl of ERα were mixed with 2672 μl of screening buffer (SB) in a glass tube. The mixture was settled for 1 hr at room temperature to form FES2-ERα complex. Then, 50 μl of the complex solution was transferred from the tube into a 96-well plate. Forty-eight μl of SB and 2 μl of test solution were successively added to the complex solution in the well and the contents in the well were mixed. After settling the plate for 1 hr at room temperature, the polarized fluorescence intensity of the solution was monitored by a Fluplo monitor (Takara Bio). The control solution was a mixture of 98 μl of SB and 2 μl of demethyl sulfoxide (DMSO). Positive control solution was a mixture of 48 μl of SB, 50 μl of the complex solution and 2 μl of DMSO. Negative control solution was a mixture of 48 μl of SB, 50 μl of the complex solution and 2 μl high-concentrated ($\geq 1 \times 10^{-5}$ M) E_2 solution, respectively. Relative binding affinity (RBA) was calculated as the inverse value of the relative concentration of the test compound that gave the same competition activity of E_2 in the presence of FES2.

Calculation of Physical Parameters —— In order to estimate the structural characteristics of PAH derivatives used, the following physical parameters were used. The L/B ratio of the rectangular van der Waals plane surrounding each mother PAH molecule was calculated with a molecular modeling program (CAChe Worksystem ver.4.1.1 for Apple Macintosh, Fujitsu Co. Ltd., Chiba, Japan) using the L and B values obtained from the Polycyclic Aromatic Hydrocarbon Structure Index.[13] The O-H distance, the distance between the oxygen atom of the carbonyl group and the hydrogen atom located

farthest from the carbonyl group was calculated by using a computer with a molecular modeling program. Other conditions for calculating physical parameters were the same as in our previous report.[9)]

RESULTS

Estrogenic Activity

An increase of β-galactosidase activity was observed for several PAHQs such as BaP-3,6-Q ($REP_E = 2.3 \times 10^{-4}$), 1,2-ChQ ($REP_E = 0.5 \times 10^{-4}$) and BaP-7,8-Q ($REP_E = 0.2 \times 10^{-4}$) (Table 1). However, the activities of these quinines were much weaker than those of 4-OHBaA ($REP_E = 7.5 \times 10^{-3}$) which showed the strongest estrogenic activity among OHPAHs.[9)] The other PAHQs did not show estrogenic activity. None of the ketone PAHs showed estrogenic activity at concentrations between 1×10^{-9} M and 1×10^{-6} M.

Antiestrogenic Activity

Significant decreases of β-galactosidase activity were observed for 1,4-PhQ, BcPh-5,6-Q, 1,2-ChQ, 1,4-ChQ, 5,6-ChQ, BaP-1,6-Q, BaP-4,5-Q, BaP-7,8-Q, BaP-7,10-Q and BaP-11,12-Q (Table 1). Among them, 1,4-ChQ and 5,6-ChQ exhibited the strongest antagonistic effect ($REP_{AE} = 0.97$). Their activity of these two quinines was almost as strong as that of 4-OHT and about a half of 3-OHBcPh ($REP_{AE} = 1.9$) which showed an antagonistic effect in our previous report.[9)] None of the ketone PAHs showed antiestrogenic activity at concentrations between 1×10^{-9} M and 1×10^{-6} M.

Table 1. Estrogenic/Antiestrogenic Activities and Receptor Binding Affinities

Type[a)]	Abbreviation	REP_E[b)], $\times 10^{-4}$	REP_{AE}[c)]	RBA[d)]
Q-2	1,2-NQ	< 0.01	< 0.01	0.94
Q-3	AcQ	< 0.01	< 0.01	0.11
	1,4- PhQ	< 0.01	0.65	0.78
	9,10-PhQ	< 0.01	< 0.01	0.01
	1,4- AQ	< 0.01	< 0.01	0.72
	AQ	< 0.01	< 0.01	0.14
Q-4	1,2-BAQ	< 0.01	< 0.01	0.13
	BcPh-1,4-Q	< 0.01	< 0.01	1.07
	BcPh-5,6-Q	< 0.01	0.76	0.33
	1,2-ChQ	0.5	0.50	0.93
	1,4-ChQ	< 0.01	0.97	1.03
	5,6-ChQ	< 0.01	0.97	0.81
Q-5	BaP-1,6-Q	< 0.01	0.65	0.56
	BaP-3,6-Q	2.3	< 0.01	0.84
	BaP-4,5-Q	< 0.01	0.69	0.11
	BaP-6,12-Q	< 0.01	< 0.01	—
	BaP-7,8-Q	0.2	0.60	0.96
	BaP-7,10-Q	< 0.01	0.50	0.85
	BaP-11,12-Q	< 0.01	0.42	0.78
K-3	9-Fluorenone	< 0.01	< 0.01	0.29
	Anthrone	< 0.01	< 0.01	0.26
	1-OH-Fl-9-one	< 0.01	< 0.01	—
K-4	Benzanthrone	< 0.01	< 0.01	0.25
	BaA-1-one	< 0.01	< 0.01	0.48
	BaA-11-one	< 0.01	< 0.01	—
K-5	BaP-7-one	< 0.01	< 0.01	0.02
	BaF-11-one	< 0.01	< 0.01	0.17
	BbF-11-one	< 0.01	< 0.01	0.33

a) Q, quinoid; K, ketone; numbers indicate number of rings. *b*) Estrogenic activity of each test compound was measured by the yeast-two hybrid assay. REP_E was calculated from the value of E_2 as a positive control. *c*) Antiestrogenic activity of each test compound was measured by the yeast-two hybrid assay. REP_{AE} was calculated from the value of 4-OHT as a positive control. *d*) Receptor binding affinity was monitored by the polarized fluorescence method. RBA was calculated as the inverse value of the relative concentration of the test compound that gave the same competition activity of E_2 in the presence of FES2.

Among OHPAHs tested in our previous work, hydroxylated derivatives of BaA, BcPh and BaP, which have 4 and 5 rings, respectively, showed antiestrogenic activity. However, PAHQs showed a different profile. Quinoid derivatives of Ph, BcPh, Ch and BaP having 3–5 rings showed antiestrogenic activity.

Binding Affinity

Strong binding affinity (RBA ≥ 0.56) was observed for 1,2-NQ, 1,4-PhQ, 1,4-AQ, BcPh-1,4-Q, 1,2-ChQ, 1,4-ChQ, 5,6-ChQ, BaP-1,6-Q, BaP-3,6-Q, BaP-7,8-Q, BaP-7,10-Q and BaP-11,12-Q. Among them, BcPh-1,4-Q and 1,4-ChQ showed the strongest affinities which were comparable to that of E_2. It should be noted that all PAHQs which showed estrogenic/antiestrogenic activities had RBA ≥ 0.18. The RBA values of ketone PAHs tested were not strong. The RBA values were in the range from 0.17 to 0.48. RBA of each test compound is given in Table 1.

DISCUSSION

Among 28 PAH derivatives having 2–5 rings, only three quinones, BaP-3,6-Q , 1,2-ChQ and BaP-7,8-Q showed estrogenic activity. However, their activities were much weaker than those of estrogenic OHPAHs. Therefore, in the following discussion, only BaP-3,6-Q was used as an estrogenic PAHQ. On the other hand, 10 PAHQs having 3–5 rings exhibited antiestrogenic activity (REP_{AE} ≥ 0.42, Table 1). The activities of 1,4-ChQ and 5,6-ChQ were about 1/2 of the activity of 3OHBcPh (REP_{AE} = 1.9) but comparable to the activity of 2-OHBcPh (REP_{AE} = 0.69).[9] Importantly, the data in Table 1 show that phenol group was not always necessary to exhibit estrogenic/antiestrogenic activities. However, ketone PAHs did not show estrogenic/antiestrogenic activities.

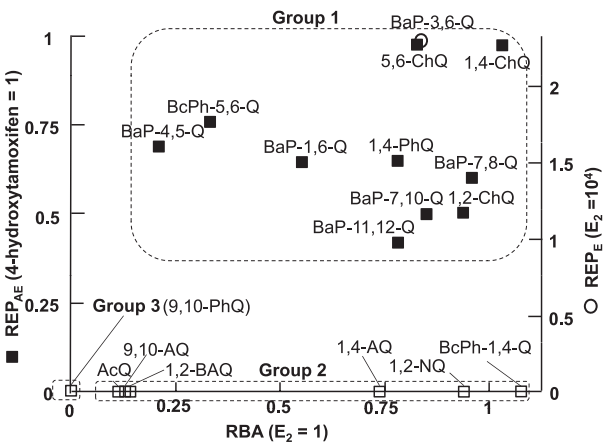

Fig. 3. Relationship between RBA and REP_{AE} or REP_E of PAHQs

■, antiestrogenic; ○, estrogenic; □, inactive.

In order for OHPAHs to exhibit estrogenic/antiestrogenic activities, they must have an affinity for ER.[9] Furthermore, RBA is correlated with REP_E or REP_{AE}.[14] PAHQs fell into three groups (Fig. 3): RBA ≥ 0.18 and REP_{AE} ≥ 0.42 or REP_E = 2.3 such as 1,4-ChQ and BaP-7,8-Q, RBA ≥ 0.11 and REP_{AE} = 0 such as BcPh-1,4-Q and RBA = 0 and $REP_{AE} \fallingdotseq REP_E \fallingdotseq 0$ such as 9,10-PhQ. None of the PAHQs had RBA = 0 but REP_{AE} ≥ 0.42. Group 1 contained 10 PAHQs, although a parallel relationship was not observed between RBA and REP_{AE}. As an interaction of PAHQs in Group 2, such as 1,2-NQ, may interact with ER through covalent bonding to thiol groups of the protein. The data in Fig. 3 suggest that RBA is necessary but not sufficient for PAHQs to exhibit estrogenic/antiestrogenic activities. Several PAHQs such as 1,2-NQ, 1,4-AQ and 1,4-ChQ can covalently bind to macromolecules through reactive thiol residues.[15] Among these three PAHQs, both 1,2-NQ and 1,4-AQ were in Group 2 which did not show antiestrogenic activity. 1-4-ChQ only was in Group 1 which showed both strong binding affinity and strong antiestrogenic activity, suggesting that the covalent binding affinity of 1,4-ChQ may contribute to its strong antiestrogenic activity.

These results suggest that active PAHQs would pass through the cell membrane and bind to ER in the nucleus. Although the $P_{o/w}$ values of PAHQs having 3–5 rings are smaller than those of the corresponding OHPAHs (for example, the computer-calculated log P values of 1,4-ChQ, and 1-OHCh were 2.459 and 4.769) the hydrophobicity of PAHQs might be enough to pass through the cell membrane.

The data in Table 1 showed that 11 estrogenic or antiestrogenic PAHQs have 3–5 rings. We previously found that estrogenic OHPAHs had the narrow range of L/B ratios and that antiestrogenic OHPAHs had narrow range of OH distances which made it easer to bind to the active site of ER.[9] The computer-calculated L/B ratios and O-H distances of PAHQs tested are plotted in Fig. 4. The L/B ratios of active PAHQs are in the range from 1.27 (BaP-1,6-Q) to 1.41 (BaP-7,10-Q) and in the range from

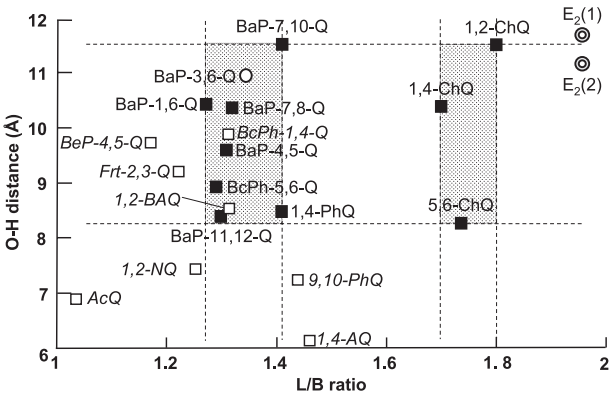

Fig. 4. Relationship between O-H Distance and L/B Ratio of PAHQs
■, antiestrogenic; ○, estrogenic; □, inactive, ◎, E2.

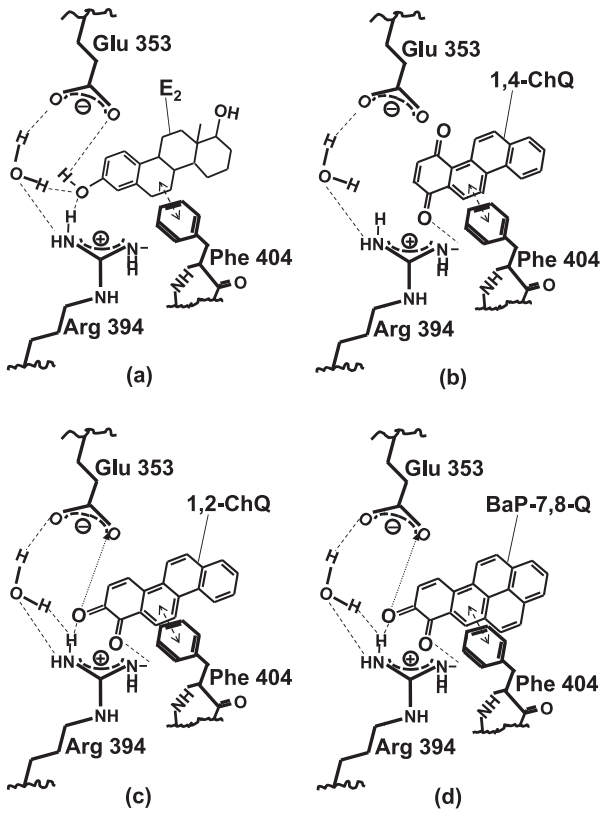

Fig. 5. Binding between E_2 and ER and Possible Bindings between PAHQ and ER
(a) E2; (b) 1,4-ChQ; (c) 1,2-ChQ.

1.7 (1,4-ChQ) to 1.8 (1,2-ChQ). The former range contains 8 quinoid derivatives of BaP, BcPh and Ph including estrogenic BaP-3,6-Q and the latter contains 3 quinoid derivatives of Ch. Considering that the L/B ratio of E_2 was 1.95 and PAHQs having 6 rings or more were not tested in this work, L/B = 1.8 might not be the upper limit. On the other hand, the O-H distances of estrogenic/antiestrogenic PAHQs were in the range from 8.2 Å (5,6-ChQ) and 11.5 Å (1,2-ChQ). This range was close to the values of O-O distances of E_2 (11.7 Å and 11.2 Å). The values of inactive PAHQs were outside these areas with two exceptions, 1,2-BAQ and BcPh-1,4-Q.

The phenol group (OH-3) of E_2 makes hydrogen bonds with glutamic acid (Glu)353 and arginine (Arg)394 of ER and H_2O and the alcohol group (OH-17) of E_2 has an affinity for the nitrogen atom of histidine 524 of ER. In these hydrogen bonds, the distance between Glu353 and the phenol group was the shortest (2.37 Å), which might be the strongest bond. On the other hand, there is a van der Waals interaction between E_2 and the binding site of ER.[16,17] Both the hydrogen bonding and van der Waals interaction were also necessary for OHPAHs to exhibit activity. Although PAHQs have no phenol groups, the carbonyl groups might play a role in the interaction with ER. Hydrogen bonds can be formed between one or two carbonyl groups of PAHQ and Glu353 and Arg394 of ER. According to the binding scheme of E_2 and ER [Fig. 5 (a)],[9] ER can bind to 1,4-ChQ, 1,2-ChQ and BaP-7,8-Q [Fig. 5 (b)–(d), respectively]. BcPh-1,4-Q and 1,2-BAQ were not antiestrogenic even though their L/B ratio and O-H distance were in the area of active PAHQs. This may be because the formation of hydrogen bond may be disturbed by aromatic rings surrounding the carbonyl groups of BcPh-1,4-Q and 1,2-BAQ.

Our results lead to three conclusions. Several PAHQs having 3–5 rings showed antiestrogenic activities. The most strongly antiestrogenic PAHQs tested were 1,4-ChQ and 5,6-ChQ, followed by BcPh-5,6-Q, BaP-4,5-Q, 1,4-PhQ, BaP-1,6-Q, BaP-7,8-Q, BaP-7,10-Q, BaP-11,12-Q and 1,2-ChQ. BaP-3,6-Q showed estrogenic activity. The other PAHQs and ketone PAHs did not show estrogenic/antiestrogenic activities. Several physical parameters such as L/B ratio and O-H distance of antiestrogenic/estrogenic PAHQs were in narrow ranges, suggesting a structure-activity relationship. The interaction between active PAHQs and ER may be due to hydrogen bonding between carbonyl groups and amino acid residues and van der Waals forces.

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