Biochemical and Biophysical Research Communications 426 (2012) 215–220

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Molecular docking of bisphenol A and its nitrated and chlorinated metabolites onto human estrogen-related receptor-gamma

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ARTICLE INFO

Article history:
Received 14 August 2012
Available online 23 August 2012

Keywords:
Bisphenol A
Metabolites of bisphenol A
Xenoestrogens
Estrogen receptors
Human estrogen-related receptor
AutoDock 4.2 software

ABSTRACT

A xenoestrogen and known endocrine disruptor, bisphenol A (BPA) binds the human estrogen-related receptor-gamma (ERRγ) with high affinity (Kd ≈ 5.5 nM). It is likely that BPA undergoes oxidative biotransformation by hypochlorite/hypochlorous acid ($^{-}$OCl/HOCl) and peroxynitrite (PN) and the products formed in these reactions may serve as secondary estrogens and contribute to the toxicodynamics of BPA. Therefore, in the present study we have examined the formation of chlorinated and nitrated BPA in reactions of BPA with $^{-}$OCl/HOCl and PN(+CO_2) performed around the neutral pH. We have identified four major products in these reactions and they include 3-chloro-BPA (CBPA), 3,3′-dichloro-BPA (DCBPA), 3-nitro-BPA (NBPA) and 3,3′-dinitro-BPA (DNBPA). Towards understanding the toxicodynamics and estrogenic activity of BPA in biological systems, we have performed molecular docking of BPA, CBPA, DCBPA, DNBPA and NBPA onto the ERRγ using AutoDock 4.2 software and compared the binding energies with those of estradiol, the natural ligand. Based on the genetic algorithm, the three best conformations were selected and averaged for each ligand and a detailed analysis of molecular interactions based on free energies of binding (kcal/mol) was computed. The results indicate the following rank order of binding to ERRγ: BPA (−8.78 ± 0.06) > CBPA (−8.53 ± 0.41) > NBPA (−7.36 ± 0.74) > DCBPA (−5.24 ± 0.17) > DNBPA (−4.95 ± 0.78) > estradiol (−4.94 ± 1.04). The docking studies revealed that the OH group of one of the phenyl rings forms a hydrogen bond with Glu275/Arg316, while the OH group of other phenyl ring was bound to Asp346. These results suggest that both BPA and its putative chlorinated and nitrated metabolites have strong binding affinity compared to estradiol.

1. Introduction

Bisphenol A (BPA; Fig. 1), a xenoestrogen and semi-persistent organic pollutant in urban environments, adversely affects the endocrine system even at low doses in experimental animals and humans [1–3]. Recent studies show that BPA binds strongly to the human estrogen-related receptor-gamma (ERRγ; Kd ≈ 5.5 nM) as compared to the estrogen receptor (ER) itself [4,5]. ERRγ belongs to the group orphan nuclear receptors, and these are closely related to ER. As on date, three different ERR proteins (α, β, γ) have been identified [5]. The function of ERRγ is not well known, however, high levels of this receptor are expressed in the fetal brain and in other tissues of adult rodents and humans [6,7].

Approximately 20–25% of BPA is known to undergo metabolic transformation by enzymes of cytochrome P450 system [8,9] and, possibly, neutrophil and macrophage derived oxidants, viz., hypochlorite/hypochlorous acid ($^{-}$OCl/HOCl) and peroxynitrite (PN). The direct reaction of BPA with $^{-}$OCl/HOCl has been studied in the context of bioremediation and treatment of waste water and industrial effluents [10,11]. These reactions mainly produce chlorinated BPA. Interestingly, the reaction of BPA with PN has never been studied; however, an analogy of reactions of PN (±CO_2) with most phenolic compounds [12–14] suggests nitrated BPA could be the major products. Studies have shown that chlorinated and nitrated BPA exhibit greater toxicity than native BPA itself and elaborates mutagenic and/or genotoxic effects [15–17]. Therefore, it is likely that these chlorinated and nitrated BPA if formed in biological systems can serve as secondary estrogens and relay, at least, in part, the toxic effects of BPA.

In order to understand the molecular targets for BPA and its putative chlorinated and nitrated products and the likely disruption of endocrine function, in the current study, we carried out the reactions of $^{-}$OCl/HOCl and PN (+CO_2) with BPA around the neutral pH. We identified the major products formed in these

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http://dx.doi.org/10.1016/j.bbrc.2012.08.065

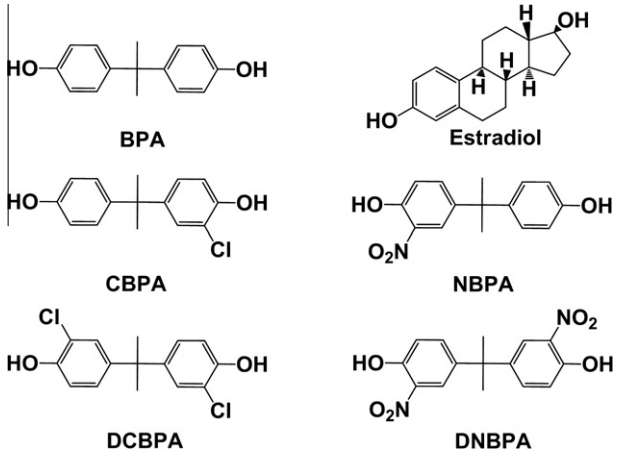

Fig. 1. Structures of estradiol, BPA, nitrated BPA (NBPA and DNBPA), and chlorinated BPA (CBPA and DCBPA).

reactions, and investigated their estrogenic activities along with BPA based on molecular docking onto ERRγ.

2. Materials and methods

2.1. Materials

Chemicals and reagents were obtained as follows: bisphenol A, H_2O_2 (30%), isoamyl nitrite (96%), dichloromethane (DCM), DMSO-d_6, MnO_2 (granular), 1-methylimidazolium hydrogen sulfate ($[MIM]^+$ HSO_4^-), $NaHCO_3$, $NaNO_3$, potassium phosphate monobasic, sodium phosphate dibasic, and sodium hypochlorite (chlorine content: *ca.* 5%) from Sigma (St. Louis, MO); diethylenetriaminepentaacetic acid (DTPA) from ACROS Organics (New Jersey, NJ); HNO_3 (70%) and trifluroacetic acid (TFA) from Fisher Scientific (Fair Lawn, NJ); and acetonitrile and acetone from Mallinckrodt (Phillipsburg, NJ). Peroxynitrite (PN) and 3,3′-dinitro-BPA (DNBPA) were synthesized as described in our previous publications [18,19]. Water used in all experiments was deionized to a final resistance of 18.0 MΩ/cm or higher.

The concentration of $^-$OCl and H_2O_2 in stock solutions was determined using $\varepsilon = 350\ M^{-1}\ cm^{-1}$ at 292 nm [20] and $\varepsilon = 41\ M^{-1}\ cm^{-1}$ at 240 nm [21], respectively. Working standards of PN and $^-$OCl/HOCl (2 or 5 mM) were prepared by dilution of the respective stock solutions with water and used immediately (usually within 5 min). Stock solutions of BPA (10 mM) were prepared in 0.01 M NaOH and stored in aliquots of 1 mL each at −20 °C until use.

2.2. Reactions of BPA with PN and $^-$OCl/HOCl

BPA (200 μM) was allowed to react with 0 to 500 μM of PN (or $^-$OCl/HOCl) in 2 mL of 0.08 M phosphate buffer, pH 7.0 that also contained 0.1 mM DTPA. In reactions performed with PN, $NaHCO_3$ was included in the assay at a final concentration of 10 mM [22]. The reaction in all cases was initiated by the addition of PN (or $^-$OCl/HOCl). Throughout the course of addition of PN (or $^-$OCl/HOCl), which typically took 5 s, the contents were constantly stirred and stirring continued for an addition 10 s. Before further analysis by reversed phase HPLC (see below), the reaction mixtures were left at room temperature (25 ± 1 °C) for 5–15 min.

2.3. RP-HPLC analysis of BPA and its oxidation products

The reaction mixtures of PN-BPA and $^-$OCl/HOCl-BPA were analyzed using a Hypersil Gold LC18 column (150 × 3.6 mm) and a mobile phase consisting of 0.1% TFA and 50% acetonitrile in water. The flow of the mobile phase was set at 1 mL/min and the eluent was monitored at 292 nm. A Thermo Scientific Spectra System SCM 1000 equipped with a Spectra System P4000 (quaternary pump) and a Spectra System UV2000 (dual wavelength detector) was used for the purpose of sample analysis.

2.4. Synthesis and characterization of NBPA

Nitration of BPA was performed using a combination of sodium nitrate and an acidic ionic liquid, $[MIM]^+$ HSO_4^- [23]. BPA (3.32 g; 20 mmol) and $[MIM]^+$ HSO_4^- (5.72 g; 20 mmol) were dissolved in 80 mL of acetonitrile. To this mixture, 1.7 g (20 mmol) of $NaNO_3$ was added and stirred overnight at 25 ± 1 °C. At the end, the mixture was filtered and the filtrate was evaporated under reduced pressure at *ca.* 50 °C. The thick brown liquid-like residue was extracted with hot hexane, which was then evaporated under reduced pressure.

The yellow liquid residue, dissolved in dichloromethane, was analyzed by GC/MS/EI using an Agilent Technologies 7890A GC equipped with the Agilent Technologies 5975 C V L triple-axis MSD and a HP-5MS capillary column (length: 30 m; internal diameter: 0.25 mm; and film thickness: 0.25 μm). Helium was used as the carrier gas (total flow: 3 mL/min; split ratio: 1:50) with temperature programming as follows: 40 °C for 2 min (isothermal); 20 °C/min up to 150 °C (ramp 1), 150 °C for 3 min (isothermal); 20 °C/min up to 300 °C (ramp 2), and 300 °C for 2 min (isothermal) (total run time: 20 min; temperature of the inlet port: 270 °C).

When analyzed by GC/MS/EI, the nitro product resolved as a single peak at 17.34 min. The ion chromatogram of this product showed a molecular ion ($M^{+\cdot}$) at *m/z* 273 and a daughter ion at *m/z* 258 (100%; base peak; $[M-15]^+$), confirming that the chemical identity of the product is 3-nitro-BPA.

2.5. Synthesis and characterization of CBPA, DCBPA and TCBPA

BPA (1.37 g; 6 mmol) was allowed to react with excess $^-$OCl/HOCl (*ca.* 25 mmol) in 20 mL of 50% methanol in water, as described by Fukazawa et al. [10]. Following an overnight stirring, aliquots (200 μL each) were subjected to RP-HPLC analysis using an Econosphere C8-column (250 × 10 mm; particle size: 10 μ) and a mobile consisting of 50% acetonitrile in water that also contained 0.1% TFA (flow: 3 mL/min). The eluent was monitored at 292 nm. A Lab Alliance series II/III liquid chromatograph equipped with a Lab Alliance model 500 UV–Vis detector was used. Peaks with significant absorbance were collected and pooled individually from several HPLC runs and concentrated by flash evaporation.

GC/MS/EI analysis of chlorinated BPA was performed using a Hewlett Packard-6890 GC equipped with HP-5973 MS and a RTX-5MS capillary column (length, 30 m; internal diameter, 0.25 mm; and film thickness, 0.25 μm). Helium was used as the carrier gas (total flow: 1 mL/min; splitless) with temperature programming as follows, 150 °C for 2 min (isothermal); 30 °C/min up to 270 °C (ramp), and held at 270 °C for 6 min (isothermal) (total run time: 12 min; temperature of the inlet port was 260 °C). All three products resolved as single peaks. Each of them showed $M^{+\cdot}$ corresponding to the predicted molecular weight of 262 for NBPA, 296 for DCBPA, and 330 for TCBPA. In each case, the daughter fragment corresponded to $[M-15]^+$.

2.6. Preparation of receptor structure

The structure of ligand binding domain of ERRγ was obtained from Research Collaboratory for Structural Bioinformatics (RCSB)-Protein Data Bank (PDB). The PDB entry 2E2R was selected for

structural analysis based on its high resolution (1.6 Å) and specificity of binding to bisphenol Z [5]. The ligand binding domain comprises of 232–458 amino acid range (chain length of 226 amino acids) was co-crystallized with BPA compound as the ligand. For docking studies, the pdb file was refined by removing the ligand and water molecules from the protein X-ray structure. The initial refinement was done by utilizing various tools present in Discovery Studio ViewerPro program. The final refinement was made with AutoDock Tools 4.2 [24; The Scripps Research Institute, La Jolla, CA] by adding missing hydrogens and adding partial charges on all the atoms based on Kollman charges. The active site was defined by a grid box of $52 \times 52 \times 52$ points and spacing of 0.375 Å with the ligand binding site as the center. The final structure was then saved in *.pdbqt* format.

2.7. Ligand structure

BPA and its putative metabolites (*viz.*, CBPA, DCBPA, NBPA and DNBPA) were selected for the docking studies against ERRγ. Estradiol, the natural ligand, was also used to compare the docking and binding efficiency. The *.pdb* format of DNBPA was obtained using the crystal structure reported earlier (*.cif* format) from our laboratory [19]. The structural coordinates of BPA, NBPA, CBPA, DCBPA and estradiol were obtained from ChemDraw and converted to *.pdb* format by Mercury software. The 3D conformations of all the ligands were generated by DS viewerpro program and minimized using 500 steps of steepest descent algorithm. These *.pdb* files were imported to AutoDock 4.2 program and minimized further by adding kollman charges and setting the charged residues on the active site to be flexible. The multiconformation library of all the four ligands was generated by exploring the torsional space of the ligands using AutoDock program. The final ligand structures were saved in *.pdbqt* format.

2.8. Docking protocol

A grid box was generated using the parameters described in protein preparation section. The genetic algorithm was used to find the probable fit for each ligand to receptor. The docking was done with Lamarckian genetic algorithm with population size of 150.

3. Results and discussion

3.1. Reaction of PN (+CO_2) with BPA forms NBPA and DNBPA as major products

Earlier, it has been shown that nitrated BPA, in particular NBPA and DNBPA, can be formed in reactions of nitrite with BPA under simulated acidic conditions of the stomach [16]. We reasoned that similar nitrated BPA could also be formed predominantly in the cellular environment as a result of BPA reaction with PN (+CO_2). Both NBPA and DNBPA are strongly mutagenic either with or without a metabolic activation system (S9 mix) and also have been shown to be clastogenic [15,16]. Given these important properties of nitrated BPA, in the present study, we explored the oxidative biotransformation of BPA by PN under biologically reverent conditions of pH and carbonate concentration. Using a combination of several synthetic and analytical techniques, we identified that both NBPA and DNBPA in reactions of PN with BPA (see below).

When BPA (200 μM) was allowed to react with equimolar PN at pH 7.0 in the presence of 10 mM carbonated species, there was marked oxidation of BPA (Fig. 2). In this particular assay, the concentration of residual and unreacted BPA was analyzed along with the putative nitration products by utilizing the RP-HPLC. Based on the decrease in the peak areas corresponding to BPA (see A and B),

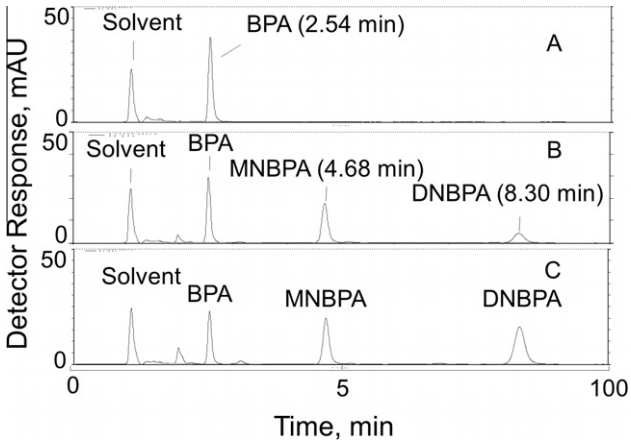

Fig. 2. Typical RP-HPLC chromatograms of the reaction mixtures in which BPA (initial concentration: 200 μM) was allowed to react with (A) 0, (B) 200, or (C) 500 μM peroxynitrite in 2.0 mL of 0.08 M phosphate buffer, pH 7.0 that also contained 0.1 mM DTPA and 0.01 M $NaHCO_3$. Aliquots (20 μL each) of the reaction mixture were analyzed as described in Section 2.

the extent of BPA oxidation was calculated as 20 (±0.5) mol% (i.e., relative to the initial concentration of PN employed in the reaction). Similar lower yields of oxidation have been reported to be typical of PN (+CO_2)-mediated reactions of phenols [12–14,18,22], which typically follow zero-order kinetics. Thus, it appeared that the PN (±CO_2) reaction with BPA was mediated mainly by the free radical oxidants including $^{\cdot}NO_2$ and $CO_3^{\cdot-}$ rather than PN itself.

The reaction of PN (+CO_2) with BPA (Fig. 2) resulted in the formation of two major products, NBPA (retention time: 4.68 min) and DNBPA (retention time: 8.30 min). These products were identified based on a comparison of retention times with authentic samples. As expected, the yields of NBPA appeared to be higher than that of DNBPA at the concentration of PN of 200 μM (A and B). This, however, was not the case when higher concentrations of PN (500 μM) was employed in the reaction. There was more of DNBPA formed and the yields of BPA oxidation on a mole-to-mole basis with PN (±CO_2) was somewhat lowered (15 mol%) (A and C). Even in these reactions, there was significant amount of BPA (125 μM) that was left unreacted. These results were consistent with higher yields of DNBPA which required two consecutive nitrations on the same BPA molecule by PN (+CO_2). Hence, this observation suggested that in the biological systems where PN could be truly a limiting reactant, NBPA would be the most predominant nitroproduct of BPA.

3.2. Reactions of $^{-}$OCl/HOCl with BPA forms mainly CBPA and DCBPA with small yields of TCBPA

Often, the chlorinated BPA species have been studied in connection with bioremediation and advanced oxidations [10,11]. It has been shown that chlorinated BPA products are frequently found in waste and runoff water [10] and they display greater affinities of binding to the estrogen receptor [25]. Thus, it appears that similar to nitrated BPA products, the chlorinated BPA species can serve as potential mediators of toxicity and/or estrogenic activity of BPA.

Our current focus on the $^{-}$OCl/HOCl-mediated oxidation of BPA stems from the reasoning that there could be a likelihood of formation of the chlorinated BPA products under biologically relevant conditions, mainly at the inflammatory sites due to excessive production of $^{-}$OCl/HOCl by the neutrophils and macrophages. Also, as mentioned earlier (see Section 1), the $^{-}$OCl/HOCl-mediated oxidations could partly contribute to the Phase I biotransformation of BPA.

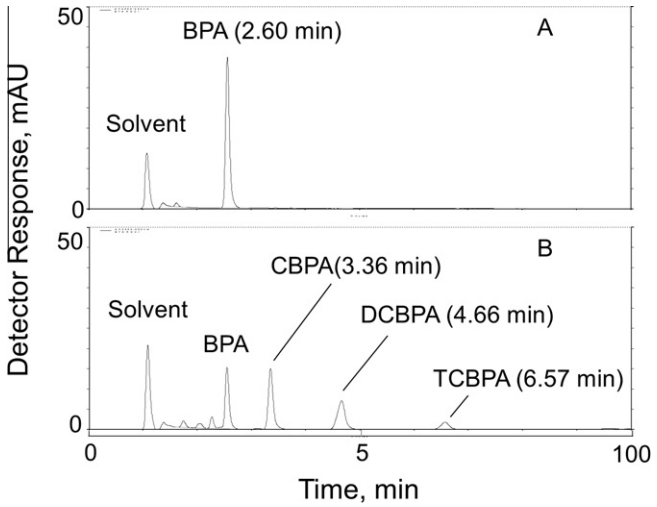

Fig. 3. Typical RP-HPLC chromatograms of the reaction mixtures in which BPA (initial concentration: 200 μM) was allowed to react with (A) 0 or (B) 200 μM hypochlorite in 2.0 mL of 0.08 M phosphate buffer, pH 7.0 that also contained 0.1 mM DTPA. Aliquots (20 μL each) of the reaction mixture were analyzed as described in Section 2.

The current study also revealed that $^{-}$OCl/HOCl could mediate the oxidation of BPA under biologically relevant conditions of pH. When $^{-}$OCl/HOCl (200 μM) was allowed to react with equimolar BPA, three major products of BPA were formed (Fig. 3). These included CBPA (retention time: 3.36 min), DCBPA (retention time: 4.66 min), and TCBPA (6.57 min). All these chlorinated BPA products were identified based on a comparison of retention times with authentic samples and by co-chromatography. Unlike in the case of PN (+CO_2), the $^{-}$OCl/HOCl gave rise to higher yields of BPA oxidation products (52 mol%; compare the peak area of BPA in A and B). Among the three chlorinated BPA products, the relative yields were as follows: CBPA (51%), DCBPA (39%) and TCBPA (10%) (Fig. 3B).

3.3. Active site in human ERRγ

Primarily, the active site was analyzed in detail to understand the nature of interaction of BPA and its metabolites with the ERRγ protein. Based on the ligand–protein interactions, the active site was mainly composed of hydrophobic core and polar charged amino acids. The ends of the active site contains charged residues lining outside the active site, providing hydrogen bonding affinity to the alcohol group of the ligand, while the cleft in the middle regions comprises of aliphatic amino acids interacting with the non-polar part of the BPA ligands. In general, three pharmacophores were identified,

(1) Core hydrophobic moiety making van der Waal interactions provided by Tyr326, Phe435, and Leu256, 271, 346
(2) Terminal charged pocket formed by Arg316, Glu275, and
(3) Terminal Asn346.

In addition to the scores and ranking obtained from AutoDock, the interaction provided by three pharmacophores was mainly selected as criteria for identifying the best possible fit for protein.

3.4. Biding energies reveal a rank order in which estradiol is the least favorable ligand

A total of 150 independent docking trials were performed using Lamarckian genetic algorithm for each of the target ligands. Out of which three best molecules were selected based on the scoring method and averaged. Table 1 shows the results of binding affinities of various ligands. The average binding energies were BPA, CBPA, NBPA, DCBPA, DNBPA and estradiol were −8.78 (±0.06), −8.53 (±0.41), −7.36 (±0.74) −5.24 (±0.17), −4.95 (±0.78), and −4.94 (±1.04) kcal/mol, respectively. The highest binding affinity was exhibited by BPA whereas the lowest affinity was exhibited by estradiol.

Table 1
Binding energies (kcal/mol) of BPA, CBPA, NBPA, DCBPA, DNBPA, and estradiol with ERRγ.

Conformation	BPA	CBPA	NBPA	DCBPA	DNBPA	Estradiol
1	−8.84	−8.98	−8.22	−5.36	−5.84	−5.94
2	−8.76	−8.43	−6.97	−5.32	−4.64	−5.03
3	−8.73	−8.19	−6.90	−5.04	−4.38	−3.85
Mean	−8.78	−8.53	−7.36	−5.24	−4.95	−4.94
SD	0.06	0.41	0.74	0.17	0.78	1.04

In general, in all the BPA compounds (Fig. 4), the hydroxyl group of one of the phenyl rings formed a hydrogen bond with Glu275 and/or Arg316, while the hydroxyl group of other phenyl ring was bound to Asp346. The lowest binding affinity for estradiol was evident because of the conjugate rings, which rendered it more inflexible and gave the undesired restrain energy to dock at the active site. Compared to the chloro- group, the nitro group was slightly bulkier, which made the phenol backbone less available for the hydrophobic interactions. The CBPA and NBPA was bound tight to the active site due to the electrostatic interaction provided by the functional group, which then formed hydrogen bond with Glu275/Arg316 at front end and with Asn346 at rear end. The hydrophobic region of chloro-ligand formed a T-shaped stacking with Tyr326 and π–π interaction with Phe435 and Leu346, 271, 256. Even though this π–π interaction was seen in other ligands, the stereo-chemical properties of chloro-ligand made it more available to the hydrophobic region. The lower affinity of binding of DNBPA as compared to DCBPA could be due to the formation of intra-molecular hydrogen bonding between the OH and NO_2 groups which prevented the interaction of phenolic OH groups with polar amino acid residues.

3.5. Summary and general implications

Bisphenol A is a high volume chemical used in the manufacture of polycarbonate plastics (PPs), epoxy resins and, to a limited extent, in thermal and carbonless paper. The extensive use of PPs in consumer and non-consumer products, and the likelihood that they contain small amounts of monomeric BPA [26], propelled studies of metabolic fate and toxicokinetics of BPA in rodents and humans [27–29]. Studies have shown that more than 75% of BPA is converted to much less toxic products, namely, BPA-glucuronide and BPA-sulfate, and are eliminated rapidly [29,30]. This probably is the reason for much less focus on metabolites of phase I biotransformation (20–25%), particularly those involving the cellular oxidants, peroxynitrite (+CO_2) and $^{-}$OCl/HOCl. We have observed for the first time that chlorinated and nitrated BPA could be formed in reactions of these oxidants under biologically relevant conditions, and these are reported in the present communication. Further, we observed that these putative products exhibit strong binding affinity to ERRγ much the same way as described for the native BPA. The studies of molecular docking described here can decipher ligand–protein interactions with respect to the participating functional groups. We anticipate that application of such studies to BPA isomers [2,2-bis(2-hydrophenyl)propane and 2-(2-hydroxyphenyl), 2-(4-hydroxyphenyl)propane] and other bisphenols [bisphenol B, bisphenol E, bisphenol F, bisphenol S

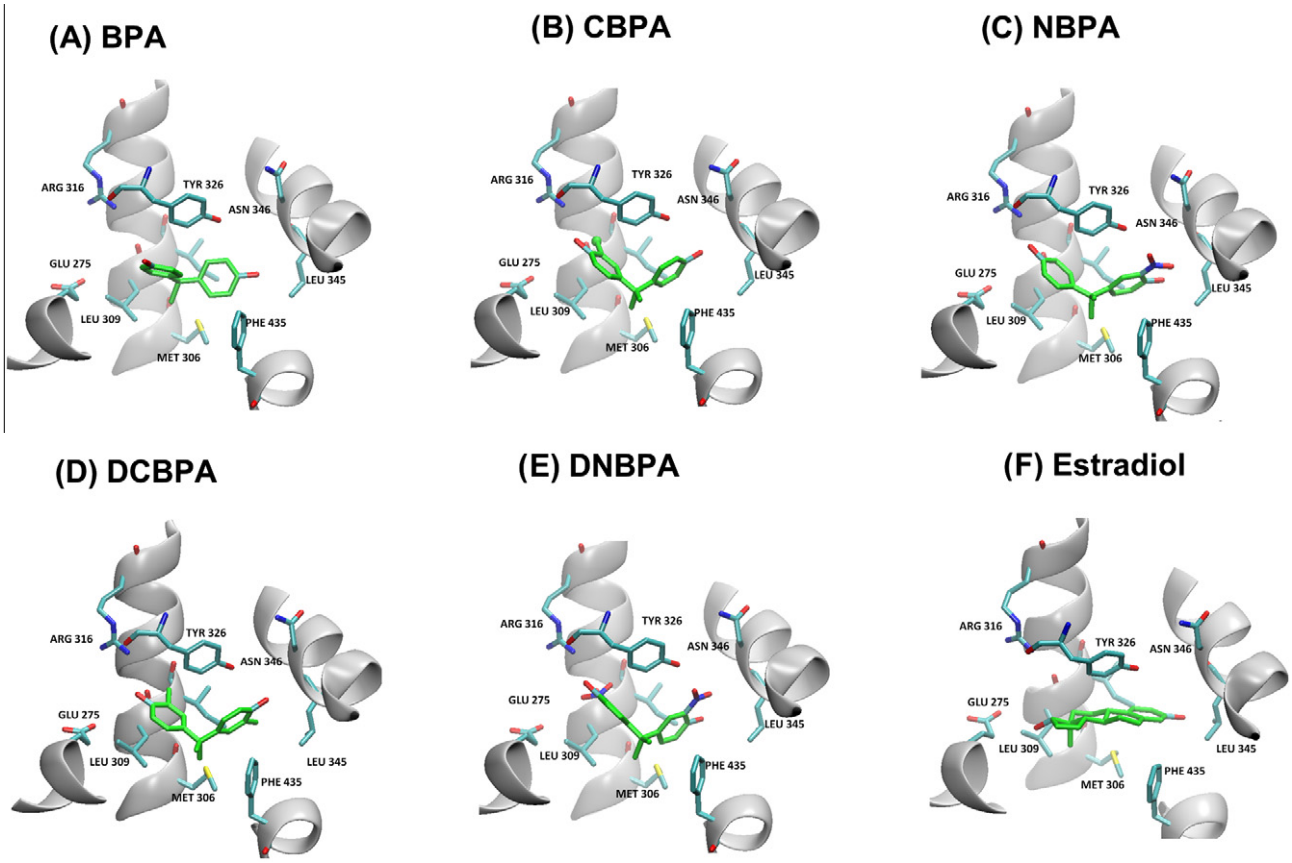

Fig. 4. Binding poses of (A) BPA, (B) CBPA, (C) NBPA, (D) DCBPA, (E) DNBPA, and (F) estradiol with ERRγ at its active site.

and bisphenol Z) and their metabolites could provide valuable information concerning the possible structure–activity relationships and furthering our understanding of the estrogenic activity.

Acknowledgments

This publication was made possible by National Institutes of Health (NIH) Grant P20 RR16456 (from the BRIN Program of the National Center for Research Resources), National Science Foundation (NSF) Grants and HRD1043316 (HBCU-UP ACE implementation program), and the US Department of Education (Title III, Part B – Strengthening Historically Black Graduate Institutions, HBGI; Grant number: PO31B040030). The authors acknowledge the year-long fellowship support from LONI-Institute for Sainath Babu and the use of high-performance computing (HPC) resources. The contents are solely the responsibility of authors and do not necessarily represent the official views of the NIH, NSF or the US Department of Education.

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