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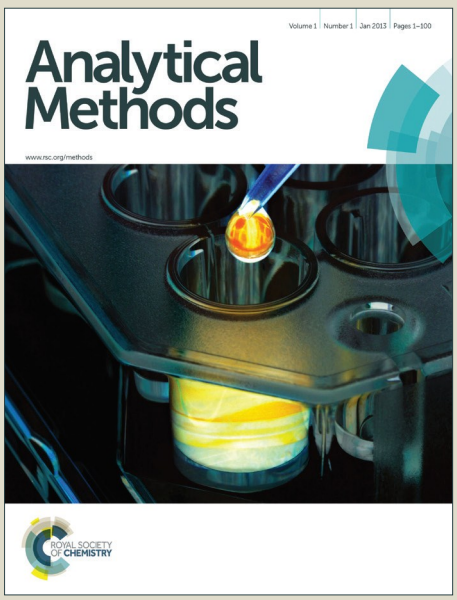

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ARTICLE

Characterization of the binding between phthalate esters and mouse PPARα for the development of a fluorescence polarization-based competitive binding assay

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The binding of phthalate esters (PAEs) to peroxisome proliferator-activated receptor α (PPARα) has been investigated by using fluorescence polarization (FP) in combination with molecular modeling techniques. The FP competitive binding assay is based on mouse-derived recombinant PPARα ligand binding domain (LBD) and a fluorescent-labeled fatty acid (C4-BODIPY-C9). A soluble mPPARα-LBD protein derivative, named mPPARα-LBD*, was expressed and purified. By using C4-BODIPY-C9 as a probe, 10 common PAEs with different carbon chain length and functional groups were assessed for their binding affinities with mPPARα-LBD*, respectively. PAEs displace the probe from the C4-BODIPY-C9-mPPARα-LBD* complex, resulting in lower polarization values. FP assay showed that PAEs compete for the C4-BODIPY-C9 binding sites in a concentration-dependent manner, and the potency of the tested PAEs increase with increasing side chain length. Molecular docking suggested that the length and hydrophobicity of the side chain of PAEs have contributed a lot to the ligand-receptor binding, and there are four prominent interactions observed to stabilize the PAEs-mPPARα-LBD* binding. In addition, comparison of docking scores vs experimental binding affinities yielded a good correlation (R^2=0.948). The most active DEHP (K_d = 19.6 ± 1.7 μM) have the lowest ranking on docking score. The fluorescence polarization-based competitive binding assay can potentially be used for high-throughput screening of PAEs, which may serve as an assistant of chromatographic techniques.

Introduction

Phthalate esters (PAEs) have been widely used over the past few decades as plasticizers. Since PAEs are not chemically bound to plastic, they can leak, migrate or evaporate from polyvinylchloride-containing products to the environment.[1-3] Nowadays, PAEs are environmental endocrine-disrupting compounds (EDCs)[4-6] to which humans are exposed through multiple routes, such as dermal, inhalation and oral intake.[7, 8] PAEs and their metabolites are known to cause adverse effects in the liver and reproductive organs of test animals and cause hepatomegaly, osteoporosis, peroxisome proliferation, feminization in boys, reduction in body weight, breast cancer, etc.[9]

The conventional methods for the determination of PAEs include gas chromatography (GC), high-performance liquid chromatography (HPLC), and mass spectrometry (MS). Although these techniques provide a low level of detection for PAEs, they are expensive, time-consuming, and unable to analyze many samples simultaneously. Immunoassay is a cost-effective and time-saving method for identifying and quantitating PAEs in samples. Based on the specific interaction between antigen and antibody, an enzyme-linked immunosorbent assay (ELISA) has been developed for detection of PAEs.[10-14] Nevertheless, polyclonal antibody is restricted by immunized animals and cannot be produced unlimitedly.[15] Furthermore, it is difficult to produce a broad spectrum antibody that can recognize the whole group of PAEs. Hence, a fast and multi-residue method using relatively inexpensive equipment is desired for the high throughput screening (HTS) of PAEs.

This work aims to develop a fluorescence polarization competitive binding assay for PAEs, based on ligand-receptor interaction. Fluorescence polarization has been used to develop high throughput screening assays for nuclear receptor-ligand displacement and kinase inhibition.[16] On the other hand, receptor binding assay has been widely used in screening of multiple residues due to the advantages of broad spectrum property. It's easier to prepare large scale receptor proteins than antibodies. Therefore, it is suitable for monitoring multiple PAEs simultaneously by using receptor.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily. A variety of studies conformed that PAEs toxicity may be mediated by PPARs,[17-20] which consist of three isoforms, namely PPARα, PPARβ(δ), and PPARγ and exhibit significant species differences in response to ligand activation.[21] PPARα has been

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reported to bind to an even wider range of ligands than either PPARγ or PPARβ,[22] and the mouse PPARα generally being activated at lower concentrations of PAEs than human PPARα.[21] The binding efficiencies of PAEs to PPARs could be predicted by molecular docking. In general, natural ligands of PPARs showed less binding efficiencies than PAEs.[9, 23-25]

In the present work, the binding of PAEs to mPPARα-LBD* was investigated. In order to achieve high-level soluble expression of mPPARα-LBD, the deletion of C-terminal amino acids from 202 to 266 were conducted to produce a new soluble protein named mPPARα-LBD*. Fluorescence polarization assay and C4-BODIPY-C9 (fluorescent-labeled fatty acid) were utilized to determine the IC_{50} and K_d of mPPARα-LBD* for 10 common PAEs with different carbon chain length and functional groups. Furthermore, molecular docking approach was performed to explore the probable binding modes between PAEs and mPPARα-LBD* for better understanding their interactions, and the docking scores were calculated to correlate with the experimental data.

Meterials and methods

Meterials

5-Butyl-4,4-Difluoro-4-Bora-3a,4a-Diaza-s-Indacene-3-Nonanoic Acid (C4-BODIPY-C9) was purchased from Invitrogen Molecular Probes (Eugene, OR, USA). Dimethyl phthalate (DMP), Diethyl phthalate (DEP), Diallyl phthalate (DAP), Dipropyl phthalate (DPrP), Diisobutyl phthalate (DIBP), Dibutyl phthalate (DBP), Bis(2-methoxyethyl) phthalate (DMEP), Dicyclohexyl phthalate (DCHP), Bis(2-ethylhexyl) phthalate (DEHP), Diphenyl phthalate (DPhP) were purchased from Aladdin (Shanghai, China). All other reagents used were of analytical grade. Anti-His mouse monoclonal antibody was purchased from TransGen Biotech (Beijing, China) and peroxidase-labeled rabbit anti-mouse IgG (whole molecule) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cloning, expression, purification and identification of mPPARα-LBD*

The mPPARα-LBD* (amino acids 267-468) cDNA fragment was amplified from cDNA generated from mouse liver mRNA by reverse transcription-PCR (RT-PCR) using primer set (5'-ATTATATTATCCATGGAGGCAGAGGTCCGATTC-3'/5'-ATTATATTATCTCGAGTCCTCCGTACATGTCTCTGTAGATCTC-3'), and then cloned by restriction enzymes (Nco I and Xho I) into pET28a vector, a $(His)_6$-tagged bacterial expression vector. The pET28a-mPPARα-LBD* plasmids was transformed into *Escherichia coli* strain Rosetta (DE3). Expression of the His-tagged mPPARα-LBD* protein was induced with 0.5 mM IPTG at 20 °C for 20 h. Bacteria were collected, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), then disrupted by sonication. In order to purify the recombinant protein, the supernatant was applied to a Ni-NTA column, washed with 10 column volumes of loading buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, pH 8.0), buffer 3 (50 mM NaH_2PO_4, 300 mM NaCl, 100 mM imidazole, pH 8.0), buffer 4 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0), and then eluted with buffer 5 (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0). The protein solutions were concentrated using Amicon Ultra-15 centrifugal filter devices (10 K MWCO; Millipore, USA), and the concentration was measured by BCA assay. Proteins were resolved in SDS-PAGE, stained with Coomassie blue, and characterized using immunoblotting.

Fluorescence polarization assay of C4-BODIPY-C9 binding to mPPARα-LBD*

Binding of C4-BODIPY-C9 to mPPARα-LBD* was monitored by measuring the enhanced FP of the probe upon titration of the protein. Fluorescence polarization assay was carried out by using a method slightly modified from Zhang *et al*.[26] Fluorescence polarization was measured with fluorescence spectrophotometer (F-7000, Hitachi, Tokyo, Japan) with excitation at 488 nm and emission at 535 nm[27-29] through a pair of polarizers. C4-BODIPY-C9 was added into 400 μL Tris-HCl buffer to a final concentration of 40 nM. Different concentrations of mPPARα-LBD* were added into the solution, and the increase of FP values was monitored. The K_d value for C4-BODIPY-C9 was obtained by nonlinear curve fitting. Data analysis was performed using GraphPad Prism 5 (GraphPad Software, USA).

Displacement assay to determine PAEs-mPPARα-LBD* binding

Analysis and comparison of the PAEs binding affinities for mPPARα-LBD* were carried out by using C4-BODIPY-C9 as a fluorescence probe. Displacement of C4-BODIPY-C9, which has high polarization when bound to mPPARα-LBD* and low polarization when not bound, was assessed by measuring loss of FP. In competitive binding assays, mPPARα-LBD* (80 nM) was preincubated with C4-BODIPY-C9 (40 nM) in a total volume of 200 μL, then fixed aliquots with increasing concentrations of PAEs dissolved in DMSO were added into the system. The total DMSO concentration never exceeded 0.5% (v/v), which did not produce any significant changes in the observed fluorescence polarization. The decrease of FP values upon addition of competing ligand was monitored and plotted as a function of the concentration of PAE. The IC_{50} values (concentrations required to reduce effect by 50%) were achieved by nonlinear curve fitting. Dissociation constants for PAEs ($K_{d,ligand}$) were derived from the measured IC_{50} values, the dissociation constant $K_{d,probe}$, and the concentration of added C4-BODIPY-C9 by the relationship IC_{50} / [C4-BODIPY-C9] = $K_{d,ligand}$ / $K_{d,probe}$.

Computational estimation of PAEs binding to mPPARα-LBD*

The initial structures of 10 PAEs were constructed by Gaussview and then optimized with Gaussian 09 using the B3LYP/6-31G(d) method. Homology modeling and molecular dynamics simulation were used to predict and refine the mPPARα-LBD* model. Automated ligand-receptor docking calculations were performed with AutoDock Vina to explore

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the interaction modes of mPPARα-LBD* and different PAEs. AutoDock Tools (ADT) was utilized to set the size and the center of the grid box, and to prepare the input .pdbqt file. The predicted binding affinity (kcal/mol) was calculated based on the scoring function used in AutoDock Vina. Pymol program was used to analyze the molecular interaction between mPPARα-LBD* and PAEs.

Results and discussion

Expression and identification of mPPARα-LBD*

It has been reported that expression of soluble fusion protein of the mouse PPARα was not as efficient as for PPARβ and PPARγ.[30] In this work, mPPARα-LBD was expressed predominantly as insoluble inclusion bodies. In order to produce soluble protein, the C-terminal amino acids from 202 to 266 of mPPARα-LBD were deleted. Given that the derivative mPPARα-LBD* (amino acids 267-468) contains the main construct of LBD, it is expected to show ligand binding properties identical to mPPARα-LBD.

The mPPARα-LBD* can be obtained in high amounts as a recombinant soluble protein in the present work. His-tagged mPPARα-LBD* recombinant protein was over-expressed in *Escherichia coli* and purified by Ni^{2+} affinity chromatography. As shown in Fig. 1a, a high level of expression of an induced protein of about 24.4 kDa has been achieved, which is consistent with His-tagged mPPARα-LBD*. Immunoblotting confirmed the predominant protein band corresponded to His-tagged mPPARα-LBD* (Fig. 1b).

Performance of fluorescence polarization assay

Fluorescence polarization binding assays can be used quantitatively to analyse binding of any small soluble fluorescent molecule (and any soluble ligand that competes with it) to a larger soluble protein.[31] A key factor in the performance of fluorescence polarization assays is the extent to which the biological activity of the tracer is perturbed by the dye modification. BODIPY dyes are more useful than most other long-wavelength dyes, including fluoresceins and carbocyanines, for assays that measure fluorescence polarization.[32] A HTS FP assay was developed for VEGF receptor by using a BODIPY-conjugated peptide, which retained constant FP signal for 8 h.[33]

To examine the ligand-binding properties of mPPARα-LBD*, the fluorescent fatty acid analogue C4-BODIPY-C9 was used. C4-BODIPY-C9 tumbles quickly in solution and produces a low polarization value. However, if C4-BODIPY-C9 is bound to mPPARα-LBD*, thereby increasing its molecular volume, it tumbles slowly and produces a high polarization value. In this work, the FP values of the probe increased from 16 mP to 262 mP once the protein was added, reflecting the binding of C4-BODIPY-C9 to mPPARα-LBD*. As shown in Fig. 2, C4-BODIPY-C9 binds to mPPARα-LBD* in saturable manner with a dissociation constant of 67.08 ± 17.54 nM.

All of the 10 PAEs exhibited dose-dependent binding to mPPARα-LBD* (Fig. 3). The IC_{50} together with K_d of PAEs, which achieved as described above, were summarized in Table 1. The IC_{50} values observed were in the order: DMP (37.8) > DAP (25.9) > DEP (23.8) > DIBP (21.9) > DPrP (21.3) > DMEP (20.5) > DBP (19.1) > DCHP (18.6) > DPhP (16.8) > DEHP (11.7). The data presented herein show that the binding affinities of the tested PAEs increase with increasing side chain length, which is consistent with previous reports.[9, 21, 34]

Computational estimation of PAEs binding to mPPARα-LBD*

Molecular docking simulations are widely used in structure-based drug design, where they provide useful information about key ligand-receptor interactions for known ligands as well as for putative ligands for which there may be little or no structural data.[23] To better understand the ligand-receptor interactions, the 10 PAEs described in Table 1 were docked using AutoDock Vina to explore the probable binding mechanism between PAEs and mPPARα-LBD*.

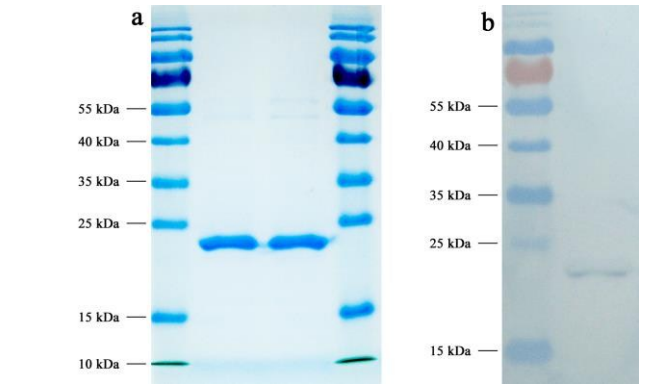

Fig. 1 Expression and identification of his-tagged mPPARα-LBD*. (a) Proteins were resolved in SDS-PAGE and stained with Coomassie blue. (b) Western blot analysis using anti-His antibody.

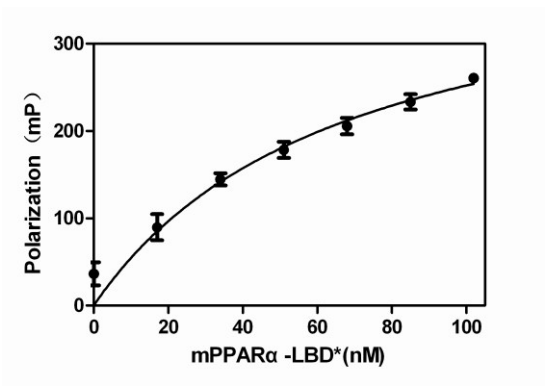

Fig. 2 Nonlinear fitting of data obtained from saturation experiments for binding of C4-BODIPY-C9 to mPPARα-LBD*. Results are given as means ± SEM of three independent experiments.

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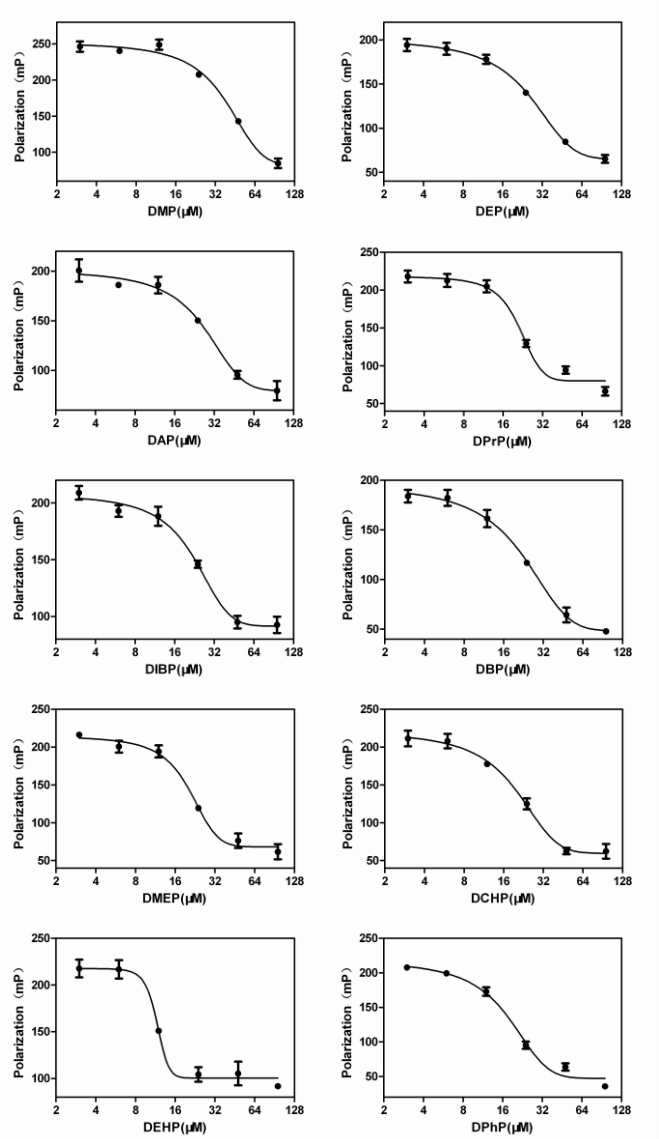
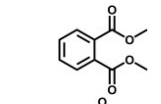
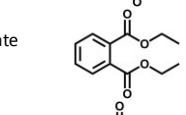
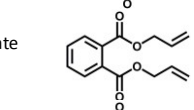
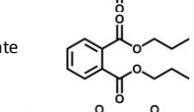
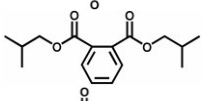
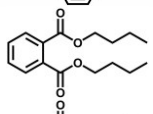
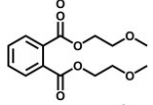
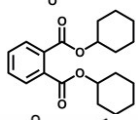
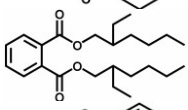
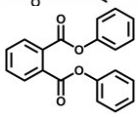


Fig. 3 Nonlinear fitting of data obtained from competition experiments for binding of 10 PAEs to mPPARα-LBD*. Results are given as means ± SEM of three independent experiments.

As shown in Fig. 4, the molecular docking scores are in good agreement with the K_d values of PAEs (R^2=0.948). The most active DEHP (K_d = 19.6 ± 1.7 μM) have the lowest ranking on docking score. DPhP and DCHP are the second and third most active compounds among these compounds. The substitution of long-chain alkyl group with large hydrophobic cyclohexane or phenyl rings causes a relatively low ranking of the two compounds on docking score. This may be due to these two side chain groups are not exactly proportional to the length and shape of the channel located in the active site of mPPARα-LBD*, resulting in a slightly decreased binding efficiency. Investigation of a series of short-chain alkyl substitutes further supports this hypothesis that the length and hydrophobicity of the side chain of PAEs have contributed a lot to the ligand-receptor binding. With the decrease of the alkyl chain length from 4 (DBP) to 1 (DMP), the binding efficiency decreases progressively. As shown in Fig. 5a, the hydrophobic pocket in mPPARα-LBD* is large enough to accommodate DBP. There are four prominent interactions observed to stabilize the DBP-mPPARα-LBD* binding. Residue His249 can form hydrogen bonds with the carbonyl oxygen of both ester groups of DBP, while Ser89 makes a hydrogen bond interaction with the

Table 1 IC_{50} values, dissociation constants (K_d), and G scores for 10 PAEs

Compound Name	Structure	IC_{50}(μM)	K_d(μM)	G score
Dimethyl phthalate (DMP)		37.8 ± 3.4	63.4 ± 5.7	-5.9
Diethyl phthalate (DEP)		23.8 ± 3.4	39.9 ± 5.7	-6.6
Diallyl phthalate (DAP)		25.9 ± 4.3	43.4 ± 7.2	-6.5
Dipropyl phthalate (DPrP)		21.3 ± 1.7	35.7 ± 2.9	-6.7
Diisobutyl phthalate (DIBP)		21.9 ± 2.9	36.7 ± 4.9	-6.7
Dibutyl phthalate (DBP)		19.1 ± 5.2	32.0 ± 8.7	-6.9
Bis(2-methoxyethyl) phthalate (DMEP)		20.5 ± 1.9	34.4 ± 3.2	-6.8
Dicyclohexyl phthalate (DCHP)		18.6 ± 3.8	31.2 ± 6.4	-7.0
Bis(2-ethylhexyl) phthalate (DEHP)		11.7 ± 1.0	19.6 ± 1.7	-7.4
Diphenyl phthalate (DPhP)		16.8 ± 2.5	28.2 ± 4.2	-7.3

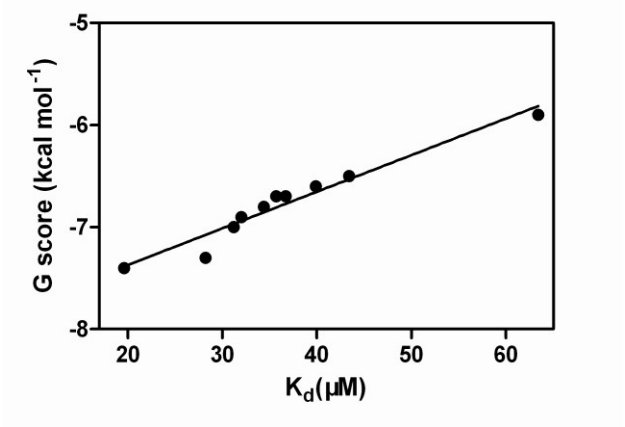


Fig. 4 Correlation between G scores and K_d values of PAEs.

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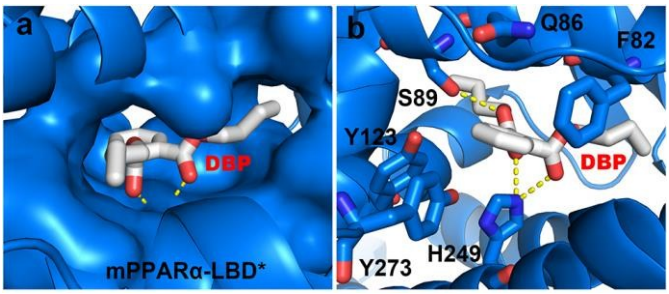

Fig. 5 Molecular docking result of the ligand-receptor binding. (a) The hydrophobic binding pocket of mPPARα-LBD* to stabilize DBP. (b) The detailed interactions between DBP and the key residues in mPPARα-LBD*.

alkoxyl oxygen of one ester group of DBP (Fig. 5b). Moreover, Tyr123, Tyr273, and Phe82 also provide the aromatic stacking interactions with the phenyl moiety of DBP. The fourth interaction was found between the hydrophobic channel and the alkyl chain of DBP. These data will provide valuable information for prediction of the binding efficiencies of novel PAEs for a fluorescence polarization-based competitive binding assay.

Conclusions

In this work, the binding of PAEs to mPPARα-LBD* was investigated by using fluorescence polarization competitive binding assay. FP assay showed that PAEs compete for the C4-BODIPY-C9 binding sites in a concentration-dependent manner with IC_{50} values from 11.7 to 37.8 μM, and the binding affinities of the tested PAEs increase with the length of their side chain. Molecular docking suggested that the length and hydrophobicity of the side chain of PAEs have contributed a lot to the ligand-receptor binding, and there are four prominent interactions observed to stabilize the PAEs-mPPARα-LBD* binding. In addition, the molecular docking scores correlate well with the experimentally determined dissociation constants of PAEs, resulting in an R-squared value of 0.948. These results indicated that FP-based competitive binding assay could be a potential screening method for PAEs.

Acknowledgements

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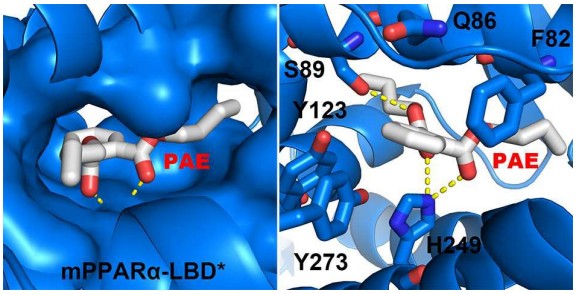

The binding between PPAR and PAEs was investigated for developing an efficient fluorescence polarization-based competitive binding assay.

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