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P450 aromatase inhibition assay using a competitive ELISA

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Abstract

P450 aromatase (P450arom) is a well known target by anti-cancer drugs and toxic chemicals and efficient and convenient analytical tools are desired for. We established a convenient assay for P450arom inhibition based on an enzyme-linked immunosorbent assay (ELISA). The first step of the assay consists of a P450arom reaction, which converts a testosterone to a 17β-estradiol using a recombinant human P450arom and a NADPH regenerating system. The second step of the assay consists of an ELISA system using a highly specific and sensitive anti-estradiol monoclonal antibody in conjunction with estradiol-3-CMO-horseradish peroxidase (E_2-3-CMO-HRP). This system has advantages over other P450arom assays because it does not use radioactive ligands and because it is not subject to interference from self-fluorescing test compounds. We could successfully estimate some types of P450arom inhibitors reported before. This assay should be very useful for high throughput screening of drug candidates and endocrine disrupting chemicals via P450arom.

Keywords: Aromatase; P450; Inhibition; ELISA; Endocrine disruptors; Drug discovery; High throughput screening

1. Introduction

P450 aromatase (P450arom) is a member of the well-known P450 enzyme family, which mediate steroid hormone biosynthesis. Specifically, P450arom converts androgens to estrogens. The estrogens and androgens regulate many proteins, which, in turn, control development, sexual differentiation and sexual activity, as well as estrogen receptor and an androgen receptor expression [1–6]. Inhibition of P450arom can reduce the production of estrogens, and thereby reduce the growth of estrogen-dependent tumors. Therefore, P450arom has been well studied as a drug target, and several effective inhibitors have been discovered. However, there is need for even more effective and selective P450arom inhibitors. In addition, many drugs, including cancer drugs, have a potential to interact with P450arom, and thus disrupt hormone homeostasis. Also, various environmental pollutants have the potential for influencing P450arom activity of shellfish and other animals. For example, some organic tins (like tributyl tin, etc.) inhibit shellfish P450arom [7,8] and may cause a disruption of their sexual organs [9,10], although this is somewhat controversial [11]. Because of the interest in this enzyme for drug discovery and for studying the effects of endocrine-disrupting chemicals, recombinant human P450arom [12] and P450arom from other species [13–16] have been generated. This enzyme can be produced in a variety of expression systems, including mammalian cells [17], yeast [18], bacteria [19] and insect cells [20]. Currently, P450arom activity is measured by several methods, including a radioisotope assay [21,22], HPLC [23], mammalian cell bioassay [24], fluorescence substrate assay [25], and in vivo tests using transgenic AROM+ male mice [26]. The most well known assay is the “tritium water assay”, which uses tritium-labeled androsten-di-one. In this assay the radioactive tritium is transferred to a water molecule (H_2O) from androsten-di-one [11]. Each of these methods has different advantages and disadvantages. The fluorescent methods have an extremely high throughput but they are susceptible to interference from self-fluorescing test compounds. The cell-based bioassays

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have differential uptake of test compounds depending on the cell type. The AROM+ bioassay requires a long time for animal preparations and for compound evaluations. Finally, the tritium water assay requires specialized equipment and methods for measuring and disposing of radioisotope as well as specialized training for users. Currently, there is no single method to satisfy all requirements for the rapid, reliable, user-friendly and effective evaluation of P450arom activity. We expected that a competitive enzyme-linked immunosorbent assay (ELISA) might be the solution to this problem because this type of assay is easy to perform and does not require the use of radioisotopes or specialized equipment. In this report, we describe the successful development of a competitive ELISA system for measuring P450arom activity that can be carried out using either endogenous testosterones or androsten-di-one as a substrate.

2. Materials and methods

2.1. Reagents

Human recombinant P450arom was purchased from BD science via Daiichi Pure Chemical, Japan. Anti-estradiol-3 monoclonal antibody was purchased from Fitzgerald Inc., USA. Estradiol-3-CMO-horseradish peroxidase (E_2-3-CMO-HRP) was purchased from Cosmo Bio, Japan. Testosterone, α-naphtoflavone (αNF), bovine serum albumin (BSA) and other chemicals are purchased from Nakarai tesque, Japan; Kanto chemicals, Japan; and Sigma, Japan.

2.2. ELISA plates

Antibody-coated were prepared as follows. Anti- E_2-3 antibody (100 μl of 0.5–10 μg/ml) was mixed with ice-cold 20 mM carbonate buffer, pH 9.6 and was added to each well of a 96 polystyrene microtiter plate. The plates were incubated overnight at 4 °C after which the wells were washed three times with 200 μl 10 mM phosphate-buffered saline (10 mM PBS: 10 mM phosphate + 150 mM NaCl), pH 7.2 containing 0.1% Tween20. Blocking solution, consisting of 1% BSA and 5% sucrose, 10 mM PBS (200 μl) was added to each well and the plates were incubated overnight at 4 °C. After removing the blocking solution, plates were dried under vacuum, and packed in plastic bags and refrigerated (2–9 °C). The concentration of E_2-3-CMO-HRP was selected to give an $A_{450\,nm/650\,nm}$ between 1.5 and 2.5 enough to obtain sensitivity and dynamic range.

2.3. Reagents for aromatase reaction

R1 solution consisted of 0.1% BSA, 50 mM phosphate buffer (PB), pH 7.2, 3.3 mM NADP-2Na, 0.8 μM glucose-6-phosphate and 62.5 nM testosterone. R2 solution consisted of 0.1% BSA, 50 mM PB, pH 7.2, 8.3 mM magnesium chloride and 1 U/mL glucose-6-phosphate dehydrogenase (from *Leuconostoc*. sp.). Concentration of testosterone that is a substrate of P450arom was decided by considering a cross-reactivity of the antibody immobilized on the plate. The antibody has high specificity to E_2 and can detect pmol/L level of E_2. It has very low cross-reactivity to E_2 (the ratio E2/testosterone is less than 0.001%). As a substrate of P450arom we typically use μM–mM level. Too much substrate will effect on the ELISA signals because of the cross-reactivity, so we must have set the concentration of testosterone at 62.5 nM (in R1 sol.).

Diluent-1 for chemical samples and concentrated P450arom consisted of 0.1% BSA, 50 mM PB, pH 7.2. Diluent-2 for E_2-3-CMO-HRP consisted of 0.2% BSA, 10 mM PBS, pH 7.2. Concentrated human P450arom (1 ng/ml) was diluted to 1:1000 in Diluent-2 just prior to starting the P450arom reaction. Stopping solution for P450arom consisted of 500 μM αNF, 0.1% BSA and 50 mM PB, pH 7.2.

2.4. Aromatase reaction

Test chemicals and positive control (αNF) were dissolved in dimethylsulfoxide (DMSO) and then diluted 1:10 in Diluent-1. Sample (10 μl) followed by 50 μl ice cold R1 solution was added to wells of a polypropylene 96-well plate on ice (sample plate). A second polypropylene 96 well plate (P450arom reaction plate), also kept on ice, and received 10 μl of diluted P450arom solution in each well. After preparing the P450arom working solution, 50 μl R2 solution was added to each well of the sample plate. The contents of the wells were mixed by repetitive (2–3 times) and very gentle pipeting. A 90 μl portion from each well of the sample plate was transferred to the P450arom reaction plate and incubated for 20 min at 37 °C. The reaction was terminated with 10 μl of stopping solution.

2.5. Immuno reaction

After completion of the P450arom reaction, 50 μl of each final mixture was transferred to wells of the ELISA plate. The immuno reaction was initiated by adding 50 μl diluted E_2-3-CMO-HRP solution to each well, followed by incubation at 4 °C for 1 h. The wells were washed three times with 200 μl of PBS containing 0.1% Tween 20. Next, 100 μl of TMB solution (BioFx Laboratories, Inc., USA) was dispensed into each well and incubated for 20 min at 37 °C. Color development was stopped with 100 μl of 1 N sulfuric acid. The $A_{450\,nm/650\,nm}$ was measured using a Spectra Classic microplate reader (Tecan Austria Gesellschaft, M.B.H., Austria).

2.6. Test chemicals

The following compounds were tested in our ELISA system: αNF, aminoglutethimide (AG), chrysin, triphenyl tin (TPT), tributyl tin (TBT), androsten-di-one (AG),

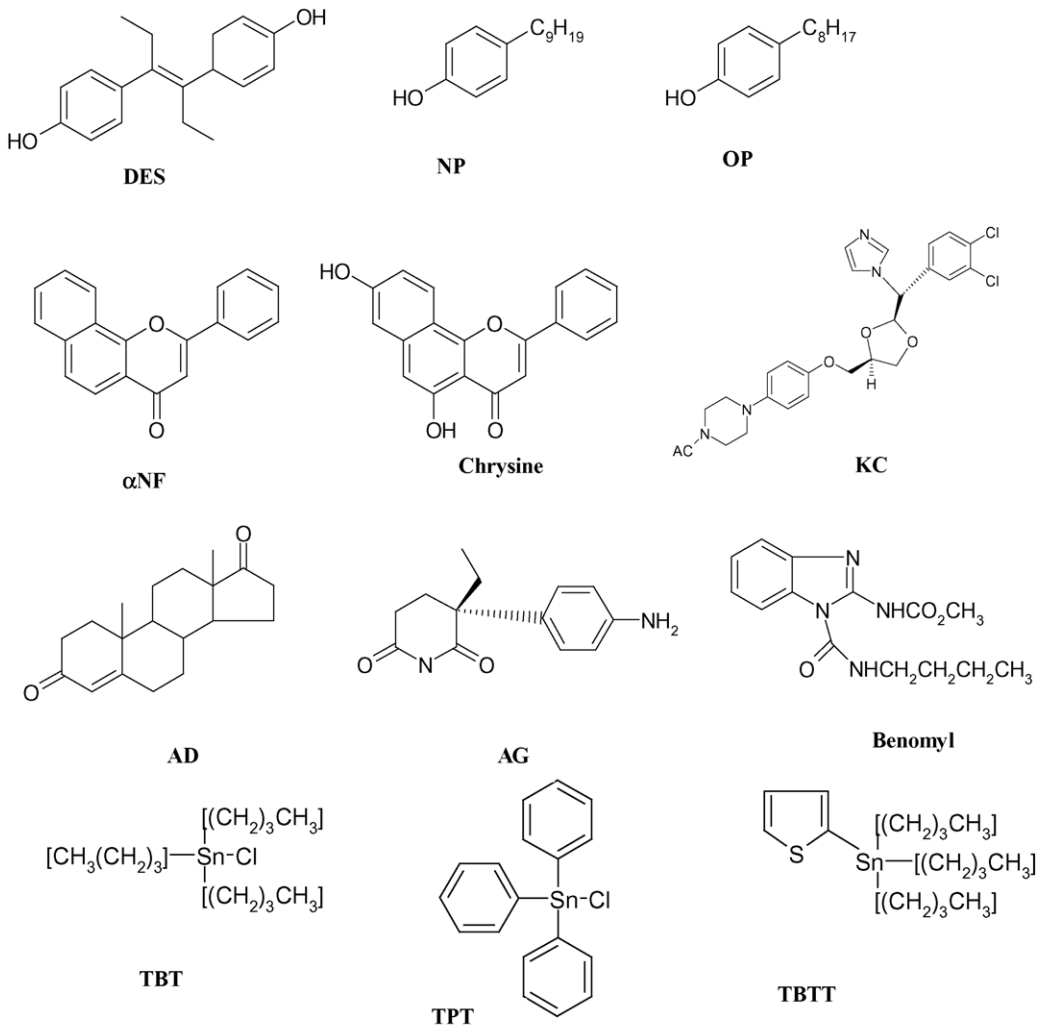

Fig. 1. Chemical structures of evaluated chemicals. Benomyl, *n*-nonyl phenol (NP), *n*-octyl phenol (OP), *n*-diethylstibestrol (DES), α-naphtoflavone (αNF), chrysin, aminoglutethimide (AG), ketoconazole (KC), androsten-di-one (AD), tri-butyl tin (TBT), tri-phenyl tin (TPT), tri-*n*-butyl (2-thienyl) tin (TBTT).

ketoconazole (KC), benomyl, diethylstibestrol (DES), *n*-octyl phenol (OP), *n*-nonyl phenol (NP), tri-*n*-butyl (2-thienyl) tin (TBTT). All compounds were dissolved in DMSO. Chemical structures are shown in Fig. 1.

3. Results and discussion

3.1. Principle of the P450arom inhibition assay by competitive ELISA

When R1 solution and R2 solution are mixed together and incubated at 37 °C, NADP is converted to NADPH by G6PDH. The R1/R2 mixture includes a NADPH regenerating system. P450arom converts testosterone in the R1/R2 mixture into E_2, which can be detected by the competitive ELISA system. Strong inhibitors of P450arom can decrease the amount of E_2 produced, and this is detected as an increase in the color production. Fig. 2a shows an illustration of detection curve of typical competitive ELISA assay. A maximal signal is obtained in the ELISA if there is no targeted chemical (E_2). Fig. 2b shows a model of dose–response curve for P450arom inhibitor. Fig. 2c is a typical dose–response curve of αNF.

3.2. Evaluation

The absorbance values were converted to P450arom inhibition ratios (AI ratios) as follows:

$$[\text{AI ratio}] = \frac{\{A_{450\,\text{nm}/650\,\text{nm}}(\text{blank}) - A_{450\,\text{nm}/650\,\text{nm}}(\text{evaluated chemicals})\}}{\{A_{450\,\text{nm}/650\,\text{nm}}(\text{blank}) - A_{450\,\text{nm}/650\,\text{nm}}(\text{positive control : } \alpha\text{NF})\}} \quad (1)$$

Calculated AI ratios are apparent values as inhibition ratios compared to full inhibition by the positive control (αNF). AI ratio is an original scale proposed by us and used

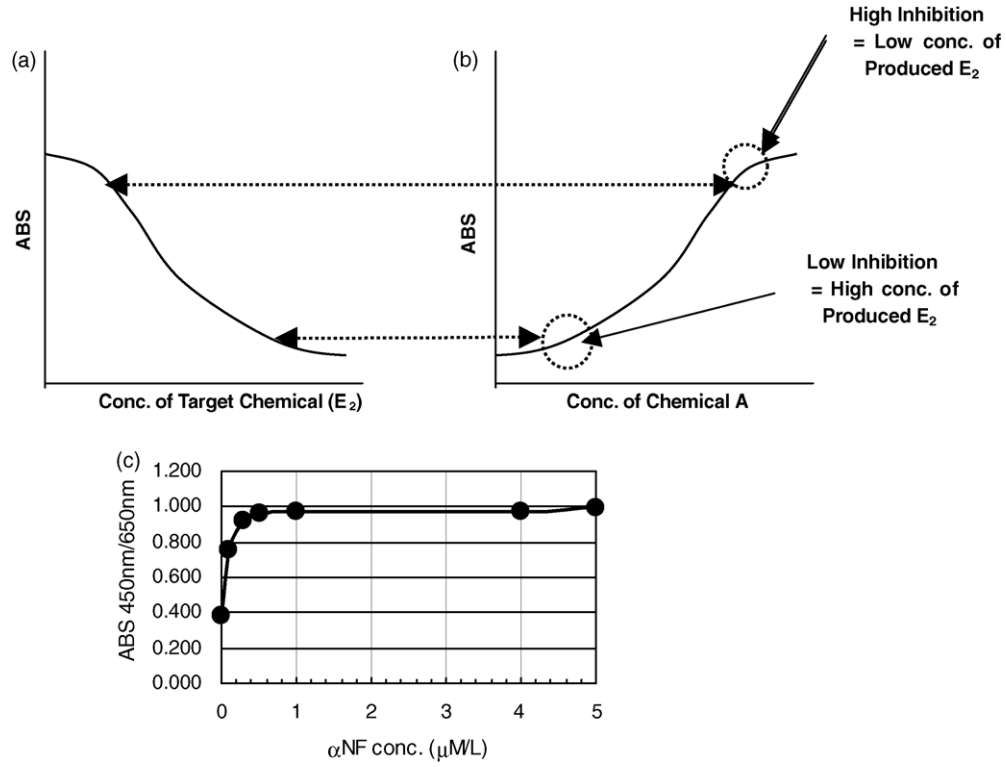

Fig. 2. The principle of P450arom inhibition ELISA. (a) A model curve for a detection of target chemical on a competitive ELISA. (b) Dose–response curve for a P450arom inhibitor chemical A. (c) Typical dose–response curve for αNF.

for calculating approximate marker to comparison. After determining AI ratios for each concentration of an evaluated chemical, the P450arom inhibition index (AI index) can be calculated from the dose responsive curve. AI index (50) or AI index (30) indicates 50% or 30% inhibition, respectively. In these experiments, we used αNF at 0.4 μM as a positive control for P450arom inhibition (final concentration). Formula (2) was used to determine the relative inhibition activity (RIA) for the various tested compounds:

$$\text{Relative inhibition activity at 50\% inhibition (RIA50)} = \frac{[\text{AI ratio for an evaluated chemical (50)}]}{[\text{AI ratio for } \alpha\text{NF (50)}]}. \quad (2)$$

3.3. Determination of the testosterone concentration to be used in the assay

Testosterone is utilized in the assay as a substrate. Its concentration must be carefully selected because there is some weak cross-reactivity with the anti- E_2-3 antibody, and too much could cause interference in the ELISA reaction. On the other hand, too little testosterone will give too low of a signal in the ELISA reaction. We found that the appropriate concentration of testosterone in the P450arom reaction mixture is between 0.05 and 0.8 μM.

3.4. Determination of the amount of P450arom to be used in the assay

Fig. 3 shows the relationship between P450arom concentration and the ELISA signal. If the P450arom concentration is above 0.4 pg/ml (dilution ratio: 1:250) in the reaction mixture, inhibition cannot be detected. We found that a concentration of P450arom between 0.05 and 0.1 pg/ml (dilution ratio 1:1000–1:2000) provided sensitive detection.

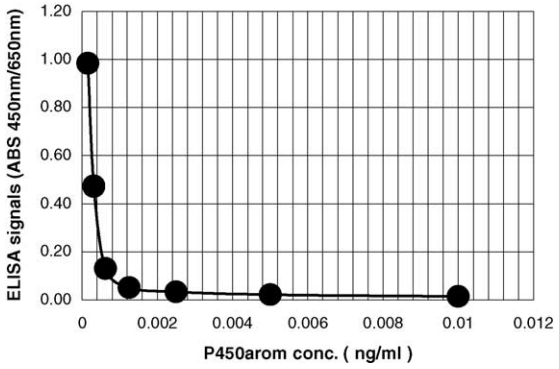

Fig. 3. The relationship between concentrations of human P450 in P450arom assay mixture and signals from ELISA.

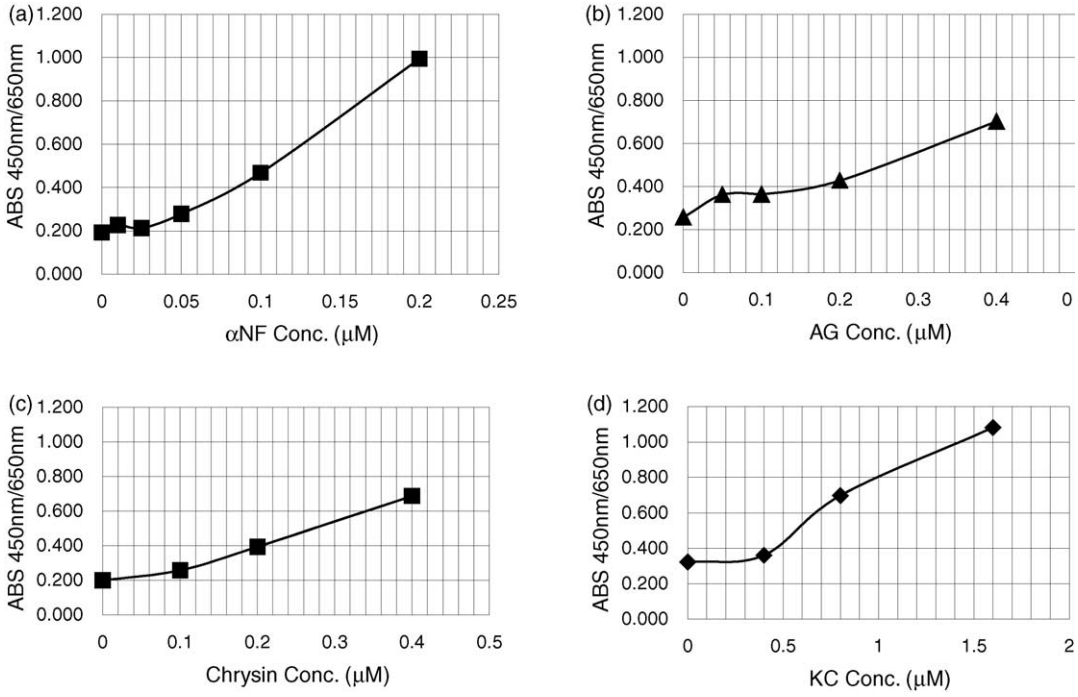

Fig. 4. Dose–response curves for various kinds of evaluated chemicals. (a) αNF, (b) AG, (c) chrysin, and (d) KC. All points of data were plotted as average values ($n = 2$–4).

3.5. Inhibition dose–response curves for various compounds.

Several inhibition dose–response curves for various compounds (αNF, AG, chrysin, TPT, TBT, AD, KC and TBTT) are shown in Figs. 4–5. Average values ($n = 2$–4) were used to generate inhibition curves. The percent variance in control values (CV) of all experiments was between 2 and 10%. Table 1 shows the values of AI(40) and RIA(40) for some compounds. DES, OP, NP, TBTT displayed little inhibition of P450arom (up to 1 μM). These are consistent with previous findings that they do not inhibit P450arom. Also benomyl displayed little inhibition or little activation (up to 1 μM), although benomyl was reported as an aromatase inducer [27,28]. In addition, αNF, AG, chrysin, TPT, TBT, AD and KC inhibited P450arom. The rank order of potency

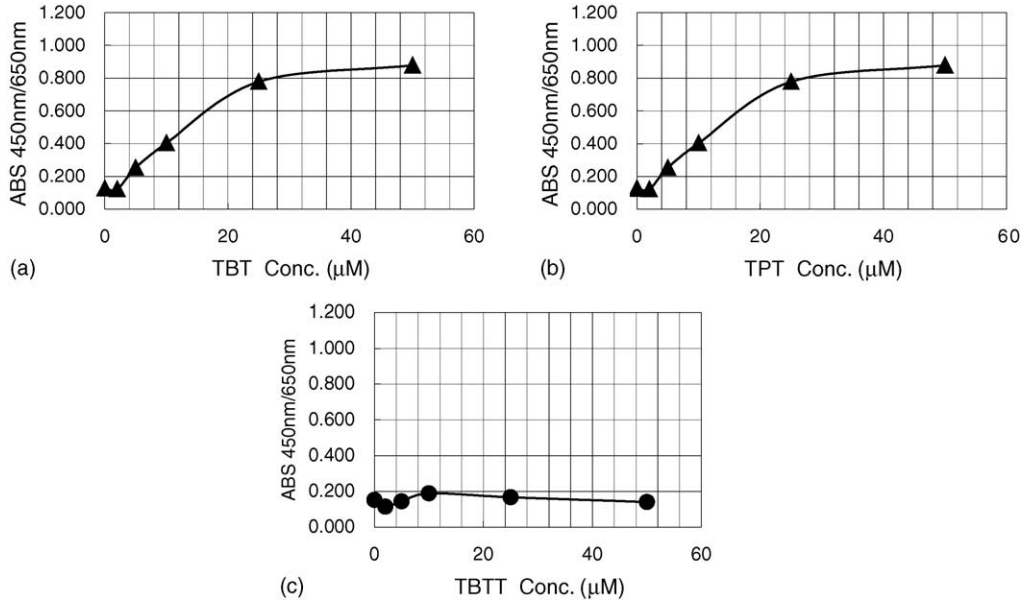

Fig. 5. Dose–response curves for various kinds of evaluated organic tins. (a) TBT, (b) TPT, and (c) TBTT. All points of data were plotted as average values ($n = 2$–4).

Table 1
Evaluations for some known aromatase inhibitors using AI (40) values and relative inhibition activities [RIA (40)]

	AI (40) (μM)	RIA (40)	Relative ratio αNF = 1.0 by fluorescence method [29]
αNF	0.11	1.0	1.0
Chrysin	0.34	3.0	3.9
KC	0.75	6.6	5.1
AG	0.35	3.1	4.3
AD	0.3	2.6	0.5
TPT	19	172.7	–

in the competitive ELISA system (αNF > chrysin, AG, AD > KC » TPT > TBT) supported by previous results [29]. Further, the dose–response curve for TBT suggests that it is a partial inhibitor of P450arom [8]. By separate experiments we could also confirm the inhibition activity by apigenin, hesperetin, naringenin, (+)-catechin, (−)-epicatechin which were reported as aromatase inhibitors by our method.

Our P450arom inhibition ELISA method requires 100 min except time for dispensing, washing and reading absorbance. This time can be reduced by shortening the length of the P450arom and/or immuno reactions. Further, our P450arom inhibition ELISA method has many advantages over other assays. For example, none of the compounds tested interfered with the assay. In addition, the assay is simple and suitable for high throughput screening and there is no need for radioactive compounds or specialized and expensive instruments.

4. Conclusion

Our P450arom inhibition ELISA method is suitable for screening drug candidates, endocrine-disrupting compounds. Our main purpose was not calculating kinetic values. Construction of a screening tool and approximate evaluation by a few assays seemed to be successful. For more thorough evaluations of potent chemicals we should conduct further experiments by other types of methods. We designed the method for screening mainly, however, the method can be adjusted and improved for a kinetic study with some changes in future.

Our method does not require extensive training, radioactivity or specialized equipment. Given the availability of recombinant protein, specific antibodies and substrates, this powerful assay system should also be applicable to other types of P450 which are also toxicologically and pharmaceutically important targets.

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