Identification of endocrine disrupting chemicals acting on human aromatase

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IDENTIFICATION OF ENDOCRINE DISRUPTING CHEMICALS

ACTING ON HUMAN AROMATASE

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ABSTRACT

Human aromatase is the cytochrome P450 catalysing the conversion of androgens into estrogens playing a key role in the endocrine system. Due to this role, it is likely to be a target of the so-called endocrine disrupting chemicals, a series of compounds able to interfere with the hormone system with toxic effects. If on one side the toxicity of some compounds such as bisphenol A is well known, on the other side the toxic concentrations of such compounds as well as the effect of the many other molecules that are in contact with us in everyday life still need a deep investigation. The availability of biological assays able to detect the interaction of chemicals with key molecular targets of the endocrine system represent a possible solution to identify potential endocrine disrupting chemicals.

Here the so-called alkali assay previously developed in our laboratory is applied to test the effect of different compounds on the activity of human aromatase. The assay is based on the detection of the alkali product that forms upon strong alkali treatment of the $NADP^+$ released upon enzyme turnover. Here it is applied on human aromatase and validated using anastrozole and sildenafil as known aromatase inhibitors. Out of the small library of compounds tested, resveratrol and ketoconazole resulted to inhibit aromatase activity, while bisphenol A and nicotine were found to exert an inhibitory effect at relatively high concentrations (100 μM), and other molecules such as lindane and four plasticizers did not show any significant effect. These data are confirmed by quantification of the product estrone in the same reaction mixtures through ELISA.

Overall, the results show that the alkali assay is suitable to screen for molecules that interfere with aromatase activity. As a consequence it can also be applied to other molecular targets of EDCs that use NAD(P)H for catalysis in a high throughput format for the fast screening of many different compounds as endocrine disrupting chemicals.

Keywords: human aromatase, alkali assay, endocrine disrupting chemicals.

1. INTRODUCTION

During the last decades various human activities have contributed to increase the number of chemicals introduced in air, water and soil, with a consequent increase of harmful compounds present in the environment. A lot of concern is about the so-called "emerging pollutants", a series of compounds such as pesticides, cosmetics, personal and household care products, pharmaceuticals that have been recently detected for example in urban rivers and that are potentially toxic for human health and environment [1]. However, their toxicity, together with their concentrations and persistence in the environment, still need to be assessed through a combination of chemical and biological assays. In general, the toxicity of new chemical entities should be checked before their introduction in the environment. Thus, research and innovation should aim at the development of methods that allow to verify the toxicity of such compounds that are used in everyday life. Such methods should include high throughput assays that allow the screening of several chemicals on key molecular targets in order to quickly identify the ones that are potentially harmful.

Among others, the endocrine system is a well-known target for chemicals that are toxic for human health. The so-called endocrine disrupting chemicals (EDCs) are a series of compounds able to interact with the hormone system by influencing hormone metabolism [2]. They can disrupt the synthesis, secretion, transport, binding, action or elimination of hormones, including the ones that are responsible for behaviour and fertility, resulting always in severe consequences for the reproductive and endocrine systems [3]. EDCs include a huge variety of different molecules, from pesticides to plasticizers, pharmaceuticals, personal care products and dietary components, which have caught the attention of scientists in the last twenty years because of their widespread diffusion and

incidence on human health. They are widely used for several daily applications, like medical devices, children toys as well as food and beverage packaging [4]. They are raising much concern because of their possible solubilisation and thus contamination for example of food products due to the non-covalent interactions between plastic materials and plasticizers [5].

The molecular targets of such compounds are different proteins, including enzymes involved in hormones biosynthesis and hormone receptors. Among them, different cytochromes P450 are involved in steroidogenesis, including human aromatase that catalyses the conversion of androgens into estrogens, a step that can be crucial for the development of estrogen-dependent pathologies such as breast cancer [6-9]. The enzyme is also expressed in several regions of the brain, where estrogens are responsible for neurite growth and migration [10], protection against neurodegenerative pathologies like Alzheimer's and Parkinson's diseases [11-13]. Thus, considering the central role human aromatase plays at the endocrine and neuroendocrine levels, a method for the fast and reliable screening of aromatase potential inhibitors can represent a step forward for the identification of EDCs.

Up to now the most widely used aromatase activity assay in cell lines as well as on the purified protein is based on radiolabelled androstenedione [14-17]. Such a methodology implies the use of a radiolabelled substrate that is an expensive molecule that needs adequately equipped laboratories to be handled. Moreover, this method cannot be adapted to a high throughput format for the rapid screening of different molecules. Another possibility is the use of ELISA that quantifies estrogens, but again these commercially available kits are quite expensive.

Here the so-called alkali assay [18] is proposed as a method to be applied directly on the purified recombinant enzyme sharing the same structural and functional features of the

wild-type enzyme [19-21] using its physiological substrate androstenedione. The assay that was already successfully applied on the cytochrome P450 BM3 for the screening of libraries of substrates [18, 22], is based on the detection of the so-called alkali product that forms upon strong alkali treatment of the $NADP^+$ released during enzyme turnover. More specifically, in the presence of NaOH the $NADP^+$ is firstly converted into an unstable pseudobase, a para-substituted $NADP^+$ derivative that absorbs at 347 nm and decays in 1.5 hours. Upon condensation of the pyridinium and the ribose rings of the pyridinic coenzyme a stable product, the alkali product, is formed, absorbing at 360 nm and exhibiting a maximum fluorescence emission at 455 nm [23, 24].

The alkali assay is here applied to different molecules and the data validated through ELISA quantification of the product formed.

2. MATERIALS AND METHODS

All chemicals used were purchased from Sigma Aldrich and were analytical grade. Human cytochrome P450-redictase (hCPR) was purchased from Life Technologies. NADPH was purchased from Millipore.

2.1. rArom heterologous expression and purification

Recombinant human aromatase (rArom) was expressed and purified as previously described [19, 21]. Briefly transformed *E. coli* DH5a competent cells were grown in Terrific Broth (TB). Expression was induced by 0.5 mM IPTG and cells were let grown 48 hours at 28°C in the presence of the heme precursor δ-aminolevulinic acid. Cells were harvested and re-suspended in a 100 mM KPi pH 7.4, 20% glycerol, 0.1% Tween-20 and 1mM β-mercaptoethanol buffer supplemented with 1 mg/mL lysozyme, 1% v/v Tween-20 and 1 mM PMSF at 4°C, disrupted by sonication and ultra-centrifuged for 25 minutes at 4°C and 40,000 rpm in a Beckman Coulter ultracentrifuge. rArom was purified by loading the supernatant on a diethylaminoethyl ion-exchange column (DEAE-Sepharose Fast-Flow, GE Healthcare) followed by a Nickel-ion affinity column (Chelating-Sepharose Fast-Flow, GE Healthcare). The protein was eluted applying a linear histidine gradient from 1 to 40 mM. The fractions containing the enzyme were pooled and histidine was removed in Amicon Ultra 30,000 MWCO devices (Millipore).

2.2. Binding studies

Binding studies were performed in an Agilent 8453 UV-Vis spectrophotometer (diode array) at the controlled temperature of 25°C (Peltier Agilent 89090 A).

The rArom protein (1 µM) was incubated with 100 and 200 µM of each EDC in 100 mM KPi pH 7.4 containing 20% glycerol, 0.1 % Tween-20 and 1 mM β-mercaptoethanol and the spectrum recorded at different times (up to 10 minutes) from the addition.

2.3. NADPH consumption assay

rArom (250 nM) was placed in a quartz cuvette and mixed with 250 nM human cytochrome P450 reductase (hCPR) and 20 µM androstenedione up to 400 µL in a 100 mM KPi pH 7.4, 20% glycerol, 0.1% Tween-20 and 1 mM β-mercaptoethanol buffer. Reactions were started by adding 200 µM NADPH and were carried out for 30 minutes at 30°C (Agilent 89090 A Peltier) monitoring the absorbance at 340 nm in an Agilent 8453 UV-vis spectrophotometer (diode array). The amount of consumed NADPH was calculated using a molar extinction coefficient (ε) of 6,220 $M^{-1}cm^{-1}$. After this time 120 µL of the reaction were collected, heat inactivated for 10 minutes at 90°C and centrifuged for 5 minutes at 11,000g. 85 µL of the supernatant were collected and injected into the HPLC system.

2.4. HPLC analysis and quantification of the reaction products

Reaction products formed by rArom were analysed in a 1200 series HPLC apparatus (Agilent Technologies) using a ZORBAX Eclipse Plus C18 reverse phase column (Agilent Technologies). The analytes were eluted applying an acetonitrile HPLC grade (Sigma Aldrich) linear gradient (5–100%) mixed to filtered and degased MilliQ water at the flow rate of 0.5 mL/min. The final product estrone (E) and the intermediates 19-hydroxyandrostenedione (19-OH AD) and 19-oxoandrostenedione (19-OXO AD) were detected by a diode array detector set at the wavelengths of 280 nm and 237 nm, respectively. Different concentrations of 19-OH AD (0.5–100 µM), 19-OXO AD (0.5–

300 µM) and E (0.2–10 µM) were dissolved in a 100 mM KPi pH 7.4, 20% glycerol, 0.1% Tween-20 and 1 mM β-mercaptoethanol buffer to be injected into the HPLC system and build calibration curves. The peaks were integrated and the corresponding areas plotted as a function of the standard concentration resulting in a linear regression curve used for the quantification of aromatase reaction products.

HPLC was performed to calculate the IC_{50} of anastrozole on recombinant human aromatase expressed and purified in our conditions. Reactions were set up by mixing 250 nM rArom, 250 nM hCPR, 10 μ M androstenedione and anastrozole at different concentrations (from 10 nM to 5 µM) and started by adding 0.5 mM NAPDH in a 100 mM KPi pH 7.4, 20 % glycerol, 0.1 % Tween-20 and 1 mM β-mercaptoethanol buffer. Reactions performed in the presence of previously heat-inactivated rArom were used as control. Reactions were carried out 10 minutes at 30°C, heat inactivated for 10 minutes at 90°C and centrifuged 5 minutes at 11,000*g*. 85 µL of the supernatant were collected and injected into a 1200 series HPLC apparatus (Agilent Technologies) and analysed using a ZORBAX Eclipse Plus C18 reverse phase column (Agilent Technologies). Analytes were eluted applying a linear HPLC grade acetonitrile gradient (5-100%) mixed to filtered and degased MilliQ water at the flow rate of 0.5 mL/min.

2.5. EDCs preparation

Bisphenol A (BPA), lindane and nicotine were dissolved in absolute methanol and then diluted in 100 mM potassium phosphate buffer (KPi) pH 7.4.

Plasticizers diethylene glycol dibenzoate (DEGB), diisodecyl phthalate (DIDP), diisononyl phthalate (DINP) and tri-m-cresyl phosphate (TMCP) were diluted into 100 mM KPi pH 7.4.

Sildenafil was solved in absolute dimethyl sulfoxide and then diluted in 100 mM KPi pH 7.4. Resveratrol and ketoconazole were dissolved in warm absolute ethanol and diluted in 100 mM KPi pH 7.4

2.6. Alkali assay applied to rArom

The effect of known and potential EDCs on the activity of human aromatase was evaluated by mixing different concentrations of the selected molecules (20 μM, 50 μM and 100 μM) with 100 nM rArom, 25 nM hCPR and 20 μM androstenedione up to 100 μL in 100 mM KPi pH 7.4. Reactions were started by adding 200 μM NADPH and were carried out for 30 minutes at the controlled temperature of 30°C in a 96-wells microplate. 80 μl of each reaction were transferred into a new 96-wells microplate and incubated for 15 minutes at room temperature in the presence of 80 μl of 0.3 M HCl to lower the pH to a value ranging from 1.0-2.0 and thus remove all the not consumed NADPH. 80 μl of the solution were then transferred into a new 96-wells microplate and mixed with 270 μl of 10 M NaOH to increase the pH to 14.8. The mixture was left in incubation in the dark for 2 hours at the controlled temperature of 30°C and continuous shacking to promote the formation of the alkali product. After this time imidazole solved in 100 mM KPi pH 7.4 was added at the final concentration of 10 mM to stabilise the time- and light-sensitive alkali product [25]. Reactions performed in the presence of 1 μM anastrozole were used as reference for an inhibited rArom reaction. Reactions performed in the absence of androstenedione were performed to ascertain whether the screened molecules could be somehow metabolised by rArom. Since the amount of alkali product formed is directly proportional to the concentration of $NADP^+$ released during aromatase catalysis, increasing concentrations of $NADP^+$ (10-160 μM) dissolved in 100 mM KPi pH 7.4 were treated as described above to build a calibration curve in fluorescence spectroscopy by

monitoring the maximum fluorescence emission at 455 nm upon excitation at 360 nm [24]. The results are expressed as relative amount and are the mean of four independent experiments. Error bars represent the standard deviation.

2.7. Estrone-ELISA

The quantification of aromatase final reaction product estrone is essential to confirm the effect of known or suspected EDCs on the activity of rArom. For this purpose a direct competitive estrone-ELISA (Diagnostic Biochem. Canada Inc.) was performed immediately after the alkali assay. The remaining 20 µL of each reaction set up to perform the alkali assay were diluted 1:53.3 in KPi pH 7.4, heat inactivated for 10 minutes at 90°C and centrifuged for 5 minutes at 11,000g. 7 µL of the supernatant were collected and diluted 1:8 in calibrator A (purchased with the kit) to then be used to perform ELISA following strictly manufacturer recommendations. The results are the mean of four independent experiments and error bars represent the standard deviation.

2.8. Molecular docking

The crystal structure of rArom (PDB ID 4KQ8) was used as target for molecular docking with the same compounds used for the alkali assay and estrone ELISA. To this end, the substrate androstenedione was manually removed and AutoDock V4 algorithm [26] embedded in the YASARA package [27] was used to predict protein-ligand interactions. A simulation cell (17 × 17 × 17 Å) was built around the heme iron atom and 999 runs of local docking were performed allowing ligand flexibility. The binding energies were predicted using the scoring function included in AutoDock.

3. RESULTS AND DISCUSSION

3.1 Binding of EDCs to rArom

The binding of the selected EDCs to aromatase was first monitored through visible spectroscopy by following possible transitions of the Soret peak.

Most of EDCs tested did not show any spin shift and the Soret peak was found at 419 nm, indicating that they are not able to displace the water molecule coordinating the heme iron and they do not coordinate heme iron themselves.

Bisphenol A induced a little shift of the Soret peak from 418 toward shorter wavelengths, whereas ketoconazole induced a shift from 418 to 421 nm, behaving as a Type II ligand (Figure 1). In the case of bisphenol A, the difference spectrum shows a peak at 410 nm and a trough at 430 nm. Such behaviour is known as reverse Type IIb and it has been reported before for other cytochromes P450 [28]. In the case of keteoconazole, the difference spectrum shows a peak at 435 and a trough at 408 nm, typical of Type IIb ligands.

Since it has previously been observed that substrates and inhibitors in cytochromes P450 are not always detectable by visible spectroscopy because they do not induce any spin shift, activity assays are necessary to ultimately establish if a compound affects the enzyme activity.

3.2 Study of the uncoupling of rArom

The coupling between the NADPH consumption and the product formation in cytochromes P450 can range from 1% to 95% [29]. This is due to the formation of oxygen species (super oxide anion radical, hydrogen peroxide and water), in the so-called uncoupling reactions. Thus, an assay based on NADPH consumption can fail to detect

inhibition from a compound when the product formed is very low compared to the amount of NADPH used by the enzyme in the uncoupling reactions.

For this reason, the uncoupling level in rArom reaction was first minimized and calculated as the ratio between the amount of products formed, measured by HPLC quantification, and the amount of NADPH consumed in the same reaction mixture (Table 1). In particular, since the electron transfer rate can affect the coupling level [30-32], different rArom:CPR ratios (from 1:1 to 1:5) were tested and the coupling level was calculated. It was found that, using a 1:1 rArom:CPR ratio, the coupling level was as high as 87.0% whereas the production of H_2O_2, one of the products of uncoupling reactions, was estimated to be 17.3%. The coupling efficiency was also calculated using the alkali assay to measure the amount of $NADP^+$ formed and the ELISA assay to quantify the estrone formed in the same reaction mixture. In this case, rArom showed a coupling level of 79.3% and a production of H_2O_2 of 15.4% (Table 1). The lower coupling efficiency calculated in this way can be attributed to the fact that the ELISA assay quantifies only the final aromatase reaction product, whereas with HPLC it is possible to take into account also of the contribution of the two reaction intermediates (19-hydroxy- and 19-oxoandrostenedione) that are present in the reaction mixture.

In any case, the high coupling level found in rArom in our conditions suggests that the alkali assay can be suitable for the detection of EDCs as aromatase inhibitors.

3.3 Validation of the alkali assay using known aromatase inhibitors

In order to check whether the alkali assay is able to detect aromatase reaction and inhibition, the reaction with the substrate androstenedione was carried out using the heat-inactivated enzyme as a control before NADPH addition. Figure 2A shows that the alkali product is more than 10 times higher in the reaction mixture compared to the control. A

strong (anastrozole) and a partial (sildenafil) aromatase inhibitor were then selected. Anastrozole is a non-steroidal third generation aromatase inhibitor used for the treatment of postmenopausal estrogen-dependent breast cancers [8, 33], while sildenafil, a drug used for the treatment of erectile dysfunction, was recently demonstrated to act as a partial inhibitor both for the recombinant purified and the native enzyme [34].

The alkali assay was performed in the presence of 1 µM of anastrozole (Figure 2B) or 20 µM of sildenafil and showed 67.3% and 26.2% inhibition on aromatase activity, respectively.

HPLC product quantification in the presence of of 1 µM anastrozole showed an inhibition of aromatse activity by 84.2% (Figure 2D), whereas estrone-ELISA resulted in an inhibiton by 76.9% (Figure 2C). The lower inhibition detected by alkali assay can be attributed to rArom uncoupling that was previously calculated to span between 13.0% and 20.7%. However, form a qualitative point of view, the alkali assay resulted to be able to detect inhibition using the strong inhibitor anastrozole.

As for the partial inhibitor sildenafil, the alkali assay was able to detect 26.2% inhibition on aromatase activity, lower by 9% when compared to the value detected by HPLC quantification and ELISA and reported to be 35% [34]. Nevertheless, even in this case the alkali assay was able to detect aromatase inhibition by sildenafil.

3.4 Screening of EDCs on aromatase activity by alkali assay and ELISA.

A series of compounds known to be be introduced in the environment from different sources such as food, smoking or plastic material [35, 36], were then chosen and tested on rArom using both alkali assay and ELISA (Table 2). They include EDCs known to inhibit aromatase activity (resveratrol and ketoconazole), EDCs with controversial data on their

action on aromatase activity (bisphenol A, lindane and nicotine) and EDCs with unknown effect on aromatase activity (plasticizers).

Both alkali assay and ELISA showed that resveratrol and ketoconazole significantly affect the activity of human aromatase to a different extent. In the case of resveratrol, the formation of alkali product and thus the consumption of NAPDH were increasingly inhibited in a concentration-dependent manner to a maximum of 78 % (Figure 3A). These data were validated by ELISA that showed an almost complete abolishment of aromatase turnover, since in the presence of 100 μM resveratrol the enzyme retained only 6.0 % of its activity compared to control reactions (Figure 3A), in line with what was previously observed by Wang and Leung [37]. These results suggest that some compounds may act also by disrupting the coupling between NADPH consumed and product formed. Moreover, it should be always taken into account that ELISA quantifies only the final product estrone and does not take into account the reaction intermediates that require NADPH as well to form.

In the case of ketoconazole (100 μM), the alkali product formation was decreased by 68 % (Figure 3B). Estrone quantification by ELISA showed an even higher (97.4%) inhibition on aromatase activity when used at a final concentration of 100 μM (Figure 3B). These data confirm that both resveratrol and ketoconazole are potent aromatase inhibitors and, as a consequence, they can exert a strong endocrine disrupting activity by decreasing the concentration of the estrogen sexual hormones. Resveratrol, a polyphenol naturally present in grape peel, was proven to inhibit aromatase activity with an IC_{50} of 25 μM in MCF-7 breast cancer cells and 25 μM in JEG-3 cells [16, 37]. Also ketoconazole, an imidazole showing antifungal activity, was found to inhibit human aromatase by 50% at the concentration of 30 μM in H295R cells, even though the molecule is currently used for the treatment of mycosis also in pregnant women [38].

The alkali assay was then applied on the purified enzyme in the presence of increasing concentrations of known EDCs acting on different targets. Bisphenol A (BPA, a ubiquitous environmental contaminant used for the production of epoxy resins), lindane (the main component of shampoos against lice) and nicotine (Table 2). BPA was found to decrease the amount of estrone formation in a concentration-dependent manner (Figure 3C). Lindane showed some inhibition on human aromatase both by alkali assay and ELISA, that however was not significant (Figure 3D) whereas nicotine shows a concentration-dependent inhibition that resulted to become significant at a concentration of 100 µM, where a 20% and 35% inhibition was detected by alkali assay and ELISA, respectively (Figure 3E).

Even though all the three compounds have been classified as EDCs, it exists some controversy regarding their relationship with human aromatase, since it is not clear weather these molecules act on aromatase expression levels or if and at which concentration they play some inhibitory effect on enzyme activity. Lindane and BPA were previously proven to modulate aromatase activity in a dose-dependent manner in JEG-3 cells and transfected E293 cells [39]. Nicotine, indeed, was reported to increase the serum concentration of testosterone in female foetus and new-borns [40]. According to our data, bisphenol A and nicotine are aromatase inhibitor whereas lindane does not exert an inhibitory effect on the enzyme.

The last molecules that were tested were four compounds commonly used as plasticizers (Table 1). They are defined as suspected endocrine disrupting chemicals for the concern they are nowadays raising because of their possible solubilisation and thus contamination of food and beverages.

The four phtalates were found not to decrease the activity of human aromatase (Figure 3F, 3G and 3I), exception made for diisodecyl phthalate (DIDP) that, only at the final concentration of 100 µM produced a 24.2% inhibition on enzyme catalysis (Figure 3H).

3.5 Docking of rArom with EDCs

The *in silico* approach was first validated by docking the substrate androstenedione into the crystal structure of rArom (PDB ID 4KQ8). Figure 4A shows an almost complete superimposition of the substrate in the crystal structure and in the best pose resulted form the docking simulation. Then, the docking was performed on the compounds previously tested by alkali assay and ELISA and the results are shown in Figure 4. The binding energy values show that all the compounds tested can fit into the catalytic pocket of human aromatase with ketoconazole having the highest binding energy (Table 3). This compound was found to induce a small shift toward longer wavelength, suggesting a Type II spectrum, involving the direct coordination of a nitrogen atom to the heme iron. The best docking pose shows that N2 of the diazole moiety is the closest atom to the heme iron. These data indicate that the compounds tested can accommodate into the active site of the protein and can potentially inhibit aromatase activity. However, due to the high affinity of the enzyme for its physiological androgen substrates (nM-low µM range) [21, 41] and to the fact that the binding of these compounds is weak as suggested by the absence or the presence of poor spin shift, most of them can be easily displaced from the active site of the protein exerting no inhibition or only a partial inhibitory effect, exception made for ketoconazole.

4. CONCLUSIONS

The alkali assay was successfully applied on the recombinant purified form of human aromatase for the identification of compounds acting as enzyme inhibitors and, more in general, as potential endocrine disrupting chemicals.

The results show that the method can be used as a technique to be applied in a microtiter plate format for the rapid fluorometric detection of $NADP^+$ released during enzyme turnover. Despite the alkali assay is an indirect measurement of the aromatase activity, it is a cheap method that can be applied to other NAD(P)H dependent enzymes that are potential molecular targets of EDCs, including the other cytochromes P450 involved in steroidogenesis.

Moreover, the assay uses the physiological substrate androstenedione that exhibits K_M values reported to be in the nM-low μM range [21, 41], difficult to reach by non-natural substrates that have been already used for fluorimetric assays to detect aromatase inhibition in high throughput formats.

The assay represent a first test to identify potentially toxic compounds since their endocrine disrupting activity should then be confirmed by *in vivo* experiments. It has also to be taken into account that aromatase-inhibiting EDCs can act by altering aromatase expression due to the lack of the negative feedback exerted by estrogens through the hypothalamus-pituitary-gonadal axis [42]. For this reason, the assay should be combined with other techniques detecting possible EDCs-induced changes in aromatase levels.

However, the alkali assay can represent a step forward in the development of reliable methods that are nowadays required to assess the endocrine disrupting activity of environmental chemicals.

Acknowledgements

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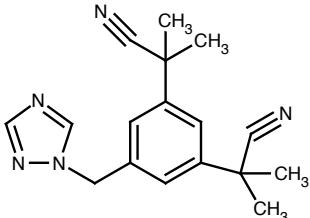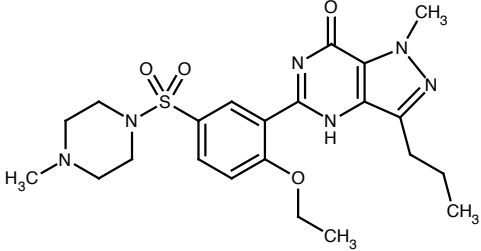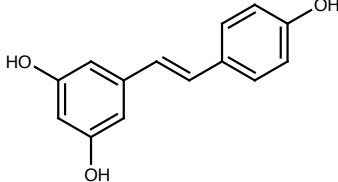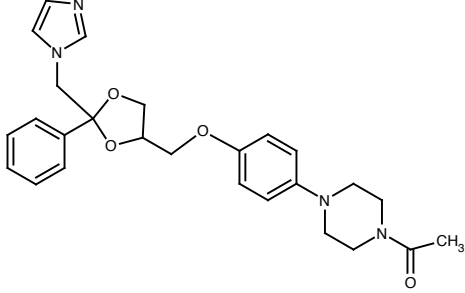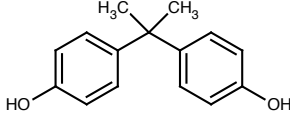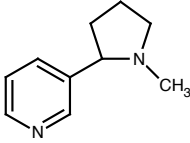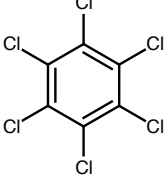
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Table 1. Coupling efficiency calculated for rArom.

NADPH consumed/ μM	Reaction products/ μM	H_2O_2/ μM	Coupling	Alkali product/ μM	Estrone product/ μM	H_2O_2/ μM	Coupling
Absorbance decrease at 340 nm	HPLC quantification of products	Amplex Reagent Kit		Alkali assay	Estrone-ELISA	Amplex Reagent Kit	
138.6 ± 5.5	120.6 ± 2.9	24.0 ± 2.0	87.0%	22.7 ± 2.0	18.0 ± 2.9	3.5 ± 0.5	79.3%

Table 2. Compounds tested by alkali assay.

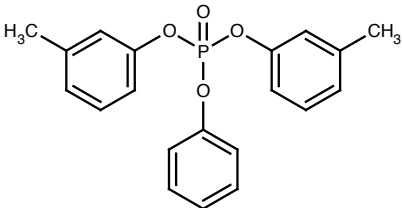| Molecule	Chemical structure	Use/Source
Anastrozole		Breast cancer therapy in post-menopausal women
Sildenafil		Pulmonary hypertension and erectile dysfunction treatment
Resveratrol		Plant natural phenol present in wine
Ketoconazole		Synthetic imidazole antifungal drug
Bisphenol A		Epoxy resins and polycarbonate plastics production
Nicotine		Tobacco
Lindane		Agricultural insecticide and as a pharmaceutical treatment for lice and scabies
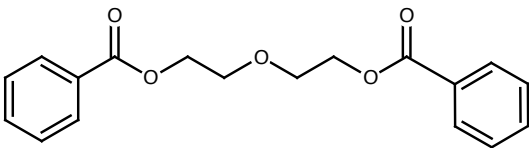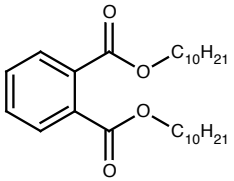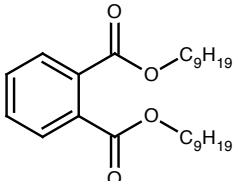
Diethylene glycol dibenzoate (DEGB)		Polyethylene terephthalate and polyvinyl chloride production
Diisodecyl phthalate (DIDP)		Coating pills, lubricants and gelling agents production
Diisononyl phthalate (DINP)		Polyethylene terephthalate and polyvinyl chloride production
Tri-m-cresyl phosphate (TMCP)		Flame retardant in rubbers and plastics

Table 3. Results from docking simulation of EDCs into the active site of rArom.

Compound	Binding energy (kcal/mol)	K_D (nM)
Androstenedione	12.42	0.8
Bisphenol A	9.11	210
DEGB	10.24	31
DINP	11.35	4.8
Ketoconazole	11.74	2.5
Lindane	8.08	1200
Nicotine	5.13	174300
Resveratrol	10.60	17
TMCP	10.19	33.7

Figures and legends.

Figure 1. Spectral transition of the heme Soret peak induced by A) bisphenol A and B) ketoconazole. The black line represents the spectrum of ligand-free rArom and the grey line represents the spectrum recorded upon addition of 100 μM of ligand. Insets: difference spectra of A) bisphenol A-bound minus ligand-free rArom and B) ketoconazole-bound minus ligand-free rArom.
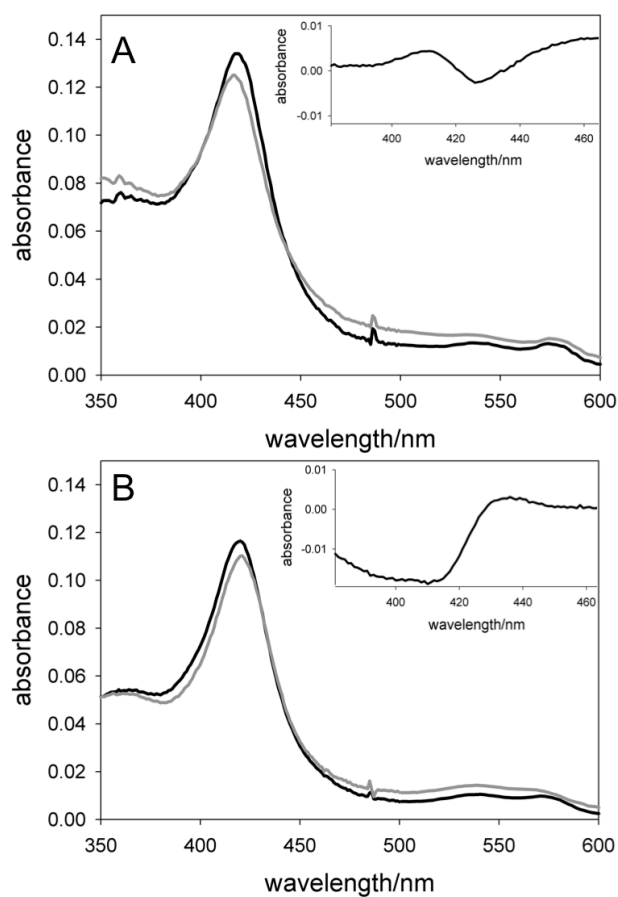

FIGURE 1

Figure 2. Validation of the alkali assay applied on rArom. A) Aromatase reaction detected by alkali assay. The control reaction was carried out using the heat-inactivated enzyme. Result from B) the alkali assay, C) estrone ELISA and D) HPLC quantification of the reaction products in the absence and presence of 1 µM of the known inhibitor anastrozole.
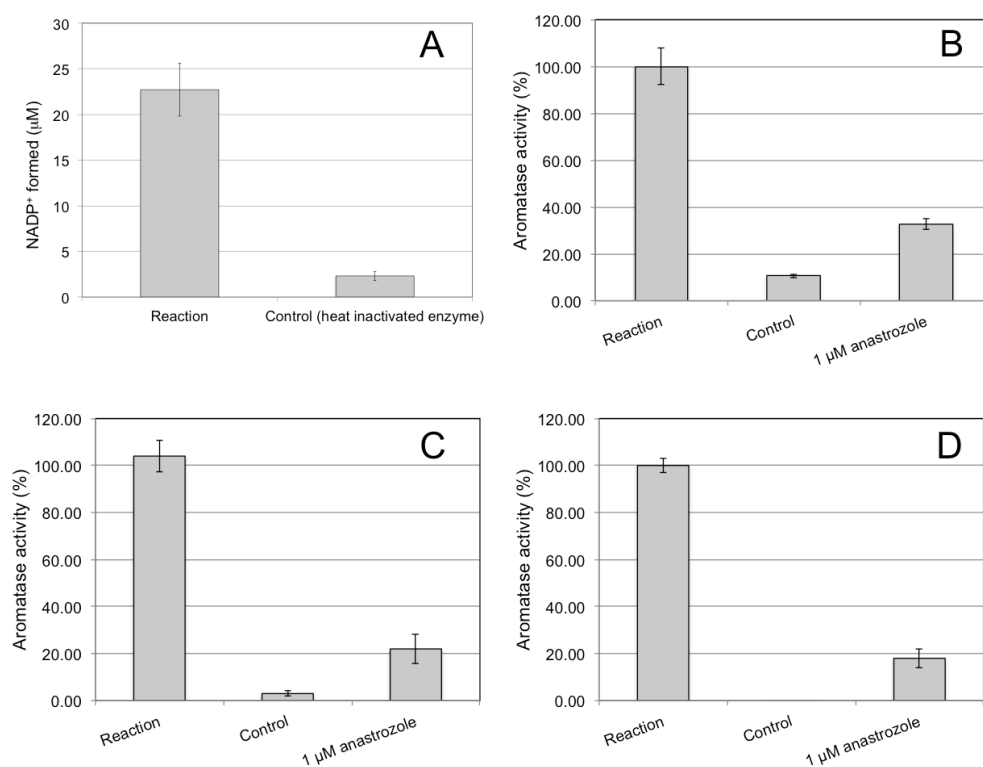

FIGURE 2

Figure 3. Comparison between the results obtained from alkali assay (grey bars) and estrone ELISA (black bars) in the presence of increasing amounts of A) resveratrol, B) ketoconazole, C) bisohenol A, D) lindane, E) nicotine, F) DEGB, G) DINP, H) DIDP and I) TMCP. * P-value < 0.05 by ANOVA test.
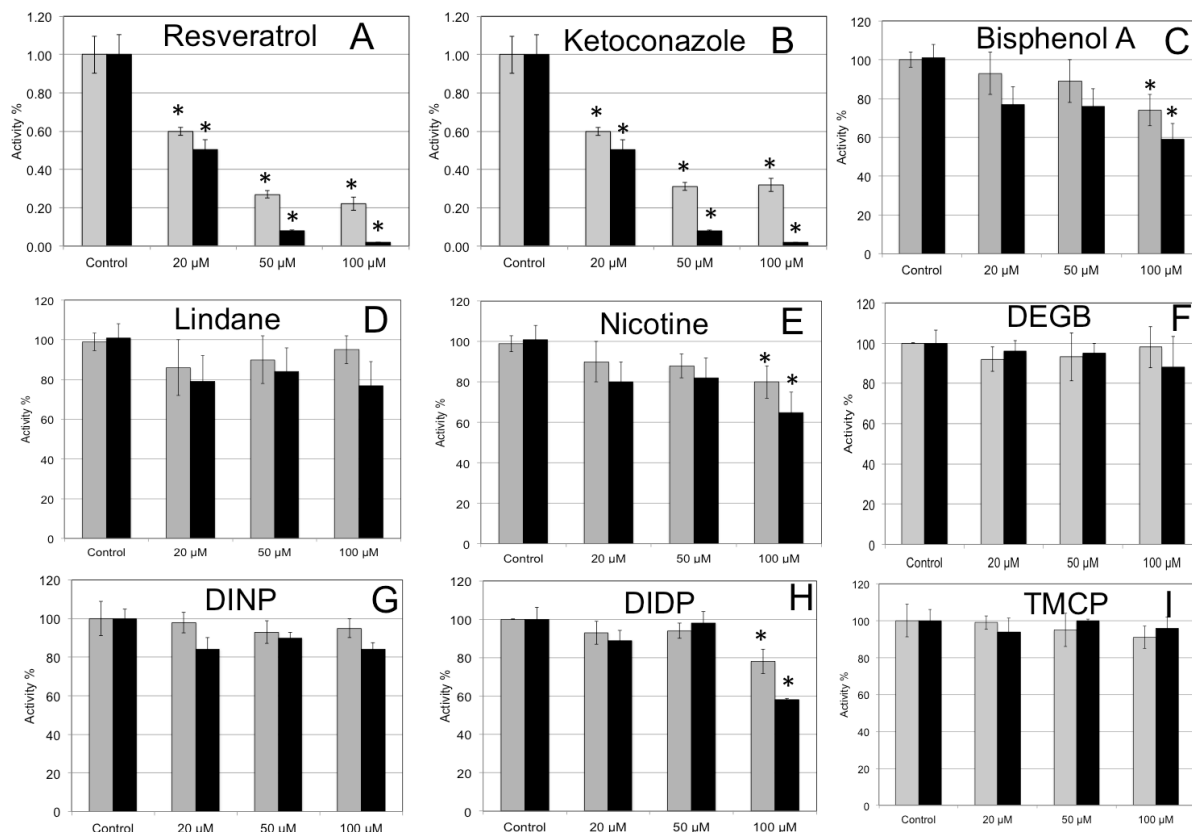

FIGURE 3

Figure 4. Results from the docking simulations of A) androstenedione, B) resveratrol, C) ketoconazole, D) bisphenol A, E) lindane, F) nicotine, G) DEGB, H) DINP and I) TMCP in the active site of rArom (PDB ID 4KQ8). In panel A, the androstenedione molecule from the crystal structure (green) is superimposed to the best pose obtained from molecular docking simulation (orange). The compounds used in plastic materials are coloured in light blue.
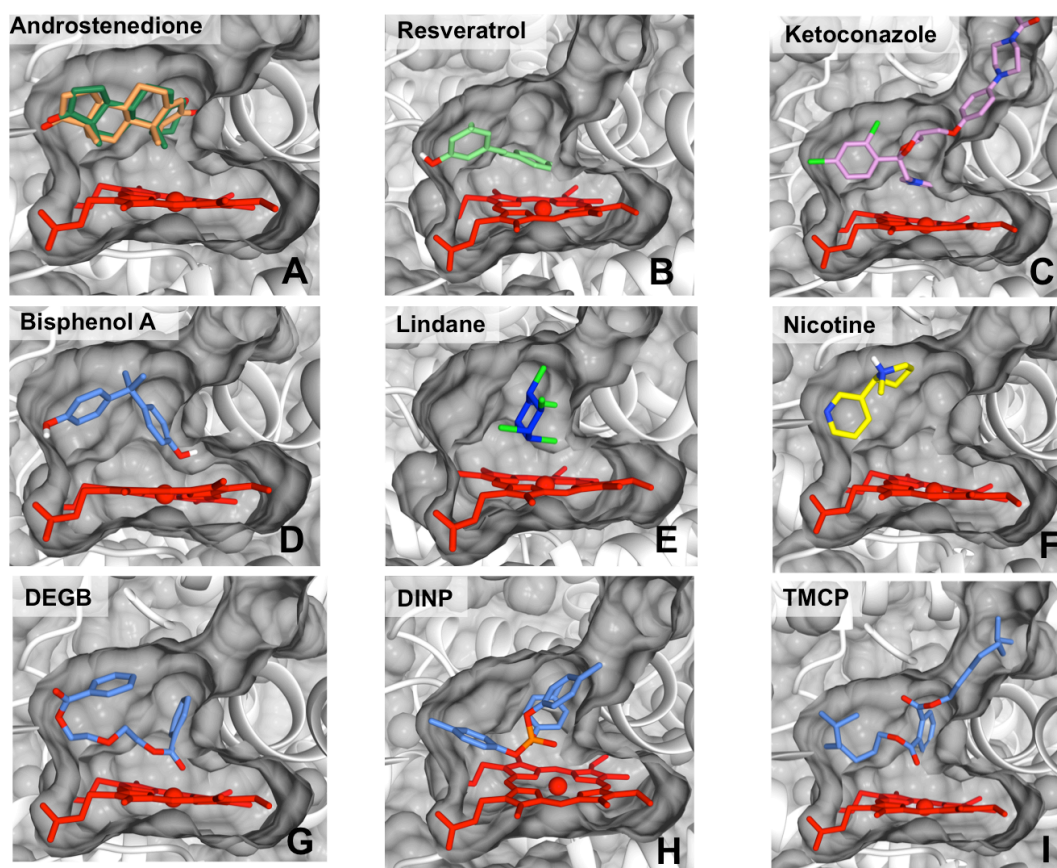

FIGURE 4