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► **To cite this version:**

Marina Grimaldi, Abdelhay Boulahtouf, Lucia Toporova, Patrick Balaguer. Functional profiling of bisphenols for nuclear receptors. *Toxicology*, 2019, 420, pp.39-45. 10.1016/j.tox.2019.04.003 . hal-02420939

HAL Id: hal-02420939

<https://hal.umontpellier.fr/hal-02420939v1>

Submitted on 22 Oct 2021

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Functional profiling of bisphenols for nuclear receptors

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Running title:

Nuclear receptors, the targets of bisphenols

Abstract

Bisphenol-A (BPA) is one of the most abundant chemicals produced worldwide. Exposure to BPA has been associated with various physiological dysregulations, involving reproduction, development, metabolism, as well as genesis and progression of hormone-dependent cancers. It has been well published that BPA along with its analogs bind and activate estrogen receptors (ER) α and β , estrogen related receptor (ERR) γ and pregnan X receptor (PXR). BPA has been also characterized as an inhibitor of the androgen (AR) and progesterone (PR) receptor. Thus, the need for safer alternatives to BPA among bisphenols is rising. In this regard, we used reporter cell lines to analyze

the effects of 24 bisphenols on the selected nuclear receptors (NRs), known and potential targets of BPA. We showed that bisphenols differently modulated the activities of NRs. ERs, ERR γ and PXR were generally activated by bisphenols, whereas many compounds of this family acted as AR, PR, GR and MR antagonists. On the other hand, some bisphenols such as BPA, BPC and BPE modulated the activity of several NRs, but others lacked the activity of other NRs. Altogether, these data provide the guidelines for development of safer BPA substitutes with reduced hormonal activity.

Key Words: bisphenols, nuclear receptors, reporter cell lines

Highlights

Using in vitro transactivation assays, we showed that bisphenols act essentially as ERs, ERR γ and PXR activators and AR, GR, PR and MR antagonists.

We also showed that some bisphenols can bind to several NRs whereas other bisphenols interact with fewer NRs.

1. Introduction

Human nuclear receptors (NRs) represent a family of 48 transcription factors, mainly activated by ligands. NRs regulate cognate gene networks involved in key physiological processes such as cell growth and differentiation, development, homeostasis, or metabolism (Germain et al, 2006; Gronemeyer et al, 2004). Inappropriate exposure to environmental pollutants as potent ligands of NRs, consequently leads to proliferative, reproductive and metabolic diseases, including hormonal cancers, infertility, obesity or diabetes. NRs are modular proteins composed of several domains, a N-terminal domain harboring ligand-independent activation function, a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) hosting ligand-dependent transcriptional activation function (Gronemeyer et al, 2004). In absence of ligand, some NRs are located in the nucleus bound to DNA response elements of their target genes, where they recruit corepressors. Others are located in the cytoplasm as inactive complex with chaperones. Ligand binding induces major structural alterations of the receptor LBD resulting in destabilization of corepressor or chaperone interfaces. LBD is subsequently exposed to localization signals needed for nuclear translocation, DNA binding (in case of cytoplasmic receptors), recruitment of coactivators triggering chromatin remodeling and activation of general transcription machinery.

Bisphenols form a large family of chemical molecules used in various consumer products. The most common member of this family, bisphenol A (BPA), has been widely used for decades (Huang et al, 2012). Several authors have proposed that many health defects, including obesity, attention deficit, hyperactivity, infertility and genital tract abnormalities increased incidence of breast and prostate cancers may be associated with BPA exposure (Giulivo et al, 2016; Rochester, 2013; Tse et al, 2017; Weber Lozada and Keri, 2011). Finally, perinatal exposure to BPA alters the development of mammary gland, predisposing mice to tumorigenesis and increasing the sensibility to carcinogenic

events (Jenkins et al, 2009).

With the concerns raised about the use of BPA manifesting as adverse effects on wildlife and human health and following the consequent restrictions of its use in many countries, some applications have shifted to use BPA analogs. Among them, there are the bisphenol-S (BPS), the bisphenol-F (BPF) and the bisphenol-AF (BPAF). BPS is used in plastics, food packaging, cosmetics, baby bottles and in BPA-free paper (Wu et al, 2018). BPF is used in epoxy resins, food packaging, soda cans, cosmetics and hygiene products, whereas BPAF is mainly used in electronic materials, high temperature copolymers and gas permeable membranes (Liao et al, 2012). Nowadays, BPA analogs become environmental pollutants, as some of them are now detected in various environment media and in human samples (Duan et al, 2018; Wu et al, 2018).

Finally, halogenated derivatives of BPA, that feature bromine (TBBPA) or chlorine (TCBPA) substituents on the phenolic rings, are used as flame retardants. Tetrabromobisphenol-A (TBBPA) is mainly used as a flame retardant protecting computer motherboards and other electronic equipment and its presence has been reported in environment (de Wit et al, 2010) and in wildlife (Darnerud, 2003). Tetrachlorobisphenol-A (TCBPA) has also been reported as a component of flame retardants, but in much lower quantities than TBBPA (Chu et al, 2005). Contrary to TBBPA, and given the low levels of production of TCBPA, the origin of majority chlorinated-BPA in the environment is more likely the result of chlorination of BPA than dechlorination of TCBPA.

Bisphenols have been shown to target several NRs including steroid estrogen ($ER\alpha$, $ER\beta$), androgen (AR), and progesterone (PR) receptors, orphan estrogen related receptor γ ($ERR\gamma$) and pregnane X receptor (PXR) (Acconcia et al, 2015; Delfosse et al, 2012; Matsushima et al, 2007; Molina Molina et al, 2013; Okada et al, 2008; Rehan et al, 2015; Sui et al, 2012). Among them, BPC chlorinated and BPAF were the most potent (Delfosse et al, 2012; 2014). BPA is considered as a weak

environmental antiandrogen, because of its relatively low affinity to AR (NR3C4) (Delfosse et al, 2012; Li et al, 2010; Paris et al, 2002; Stroheker et al, 2004). The estrogen-related receptor γ (ERR γ , NR3B3) is a constitutively active NR which could act acting as a mediator of low-dose effects of some bisphenols like BPA and BPE (Matsushima et al, 2007). Finally, BPA and some of its analogs were shown to activate PXR (NR1I2) in the 1-100 μ M concentration range (Jacobs et al, 2005; Mnif et al, 2007; Sui et al, 2012; Takeshita et al, 2001; Xue et al, 2007).

In this study, we have tested the effect of BPA and 24 analogs for their ability to modulate the activity of ERs, AR and PR, but also glucocorticoid and mineralocorticoid receptors (GR and MR) since several environmental antiandrogens were shown to interact with other steroid receptors (Molina Molina et al, 2006; Rehan et al, 2015; Zhang et al, 2018). Interestingly, we demonstrate that NRs were differently impacted by bisphenols. Some NRs had their activity modulated by several bisphenols (ERs, AR, PR and MR) whereas other NRs (GR, ERR γ and PXR) bound a smaller number of bisphenols. Moreover, ERs, ERR γ and PXR, were essentially activated, but AR, PR, GR and MR were always antagonized by bisphenols.

2. Materials and methods

2.1 Chemicals

Individual bisphenols tested in this study are listed in supplementary table 1 and their structures are showed in figure 1. 4,4'-oxydiphenol was purchased from Clinisciences (Nanterre, France). Monochloro2BPA (mono2CBPA), 2,2'-dichloroBPA (2,2'-diCBPA), 2,6-dichloroBPA (2,6diCBPA), 2,2,6-trichloroBPA (2,2',6triCBPA) were purchased from Artmolecule (Poitiers, France). The other bisphenols and aldosterone were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). 4-hydroxytamoxifen (OHT), dexametasone (DEX) and SR12813 were purchased

from bio-technique (Lille, France). R1181 (methyltrienolone) was purchased from Perkin Elmer (Courtaboeuf, France). R5020 (promegestone) is a kind gift from Sanofi (Aramon, France). Purity of the compounds is between 95% (aldosterone) and 99% (bisphenol C). All compound stock solutions were prepared at 10 mM in dimethyl sulfoxide (DMSO). The final DMSO concentrations during treatment did not exceed 0.1% (v/v) of the culture medium.

2.2 Cell lines

To characterize the bisphenol-induced ER α , ER, AR, MR, ERR γ and PXR activities, we used already established HELN ER α , HELN ER β , HELN AR, HG5LN MR, HG5LN ERR γ and HG5LN PXR reporter cell lines (Bellet et al, 2012; Delfosse et al, 2012; 2015). Briefly, HELN ER α , HELN ER β , HELN AR cells were obtained by stable expression of ER α , ER β and AR with ER α DNA binding domain in HELN (HeLa ERE-luciferase) cells whereas HG5LN MR, HG5LN ERR γ and HG5LN PXR cells were obtained by stable expression of individual ligand binding domains fused to GAL4 DNA binding domain in HG5LN (HeLa GAL4REx5-luciferase) cells.

To characterize the bisphenol-induced PR and GR activity, we established new reporter cell lines. Briefly, we stably expressed PR with ER α DNA binding domain in HELN cells (HELN PR cell line). Finally, HeLa cells were stably co-transfected with a glucocorticoid responsive gene, MMTV-Luc-SV-Neo, and a glucocorticoid receptor expressing plasmid.

HG5LN and HELN cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing phenol red and 1 g/l glucose and supplemented with 5% fetal bovine serum, 100 units/ml of penicillin, 100 μ g/ml of streptomycin and 1 mg/ml geneticin in a 5% CO₂ humidified atmosphere at 37°C. HELN AR, HELN PR, HELN GR, HG5LN MR, HG5LN ERR γ

and HG5LN PXR cells were cultured in the same medium supplemented with 0.5 µg/ml puromycin. HELN ER α and HELN ER β cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) without phenol red and 1 g/l glucose and supplemented with 5% stripped fetal bovine serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 0.5 µg/ml puromycin and 1 mg/ml geneticin in a 5% CO₂ humidified atmosphere at 37°C.

2.3 Transactivations experiments

Reporter cell lines were seeded at a density of 25,000 cells per well in 96-well white opaque tissue culture plates (Greiner CellStar) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) without phenol red and 1 g/l glucose and supplemented with 5% stripped fetal bovine serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin (test medium). Compounds to be tested were added 24h later, and cells were incubated at 37°C for 16h. At the end of the incubation period, culture medium was replaced with test medium containing 0.3 mM luciferin. Luciferase activity was measured for 2s in intact living cells using a MicroBeta Wallac luminometer (PerkinElmer). Tests were performed in quadruplicate in at least 3 independent experiments. Data were expressed as % of the maximal activity obtained with E2 10 nM (for ER α and ER β), R1181 10 nM (for AR), R5020 10 nM (for PR), dexamethasone 100 nM (for GR), aldosterone 10 nM (for MR) and SR12813 3 µM (for PXR). For ERR γ reporter cells, data were expressed as % of the basal activity.

Agonistic activities of the different NRs were tested in presence of increasing concentrations of bisphenols (0.001-10 µM). For each compound, the potency corresponding to the concentration yielding half-maximal luciferase activity (EC₅₀ value) was calculated using GraphPad Prism (GraphPad Software Inc).

Antagonistic assays were performed using a concentration approximately 80% of maximal luciferase activity (excepted for AR and GR which the activity was respectively 60 and 40%). The antagonistic activity of bisphenols (tested at 0.001-10 μ M) were determined by coincubation with E2 0.1 and 0.3 nM (for ER α and ER β , respectively), R1181 1 nM (for AR), R5020 1 nM (for PR), dexamethasone 5 nM (for GR), aldosterone 1 nM (for MR) and SR12813 1 μ M (for PXR). Due to the strong basal activity of ERR γ , the antagonistic activity of bisphenols on this receptor were measured in absence of agonist ligand. For each compound, the potency corresponding to the concentration yielding half-maximal inhibitory concentration (IC₅₀ value) was calculated using GraphPad Prism (GraphPad Software Inc).

Bisphenols were also tested for non-specific modulation of luciferase expression on the HELN and HG5LN parental cell lines. HELN cells which contain the same reporter gene as HELN ER α , ER β AR and PR. HG5LN cells which contain the same reporter gene as HG5LN MR, ERR γ and PXR. The bisphenols do not show non-specific inhibition or activation of luciferase expression at concentrations lower than 10 μ M.

3 Results

3.1 ERs activity of bisphenols

The agonistic potential of bisphenols (Fig. 1, 2) was monitored on ER α (NR3A1) and ER β (NR3A2) transcriptional activities using HeLa reporter cells stably expressing human ER α and ER β (HELN ER α and ER β cell lines) as it was previously reported for some bisphenols and xenoestrogens (Delfosse et al, 2012, 2014). In these cells, the EC₅₀ value of E2 for ER α and ER β is 19.3 and 94

pM, respectively (Supplemental Fig. S1). Representative bisphenols dose-response curves are shown in Figures 2A and 2B. EC₅₀ values as well as the maximal activities in comparison with E₂ for all bisphenols are summarized in Table 1. Chlorinated BPC (BPCcl) was the most potent bisphenol with an EC₅₀ of 30 and 46 nM for ER α and ER β , respectively. BPAF, BPZ, BPB, BPC, BPA, BPE and BPAP were also potent bisphenols with EC₅₀ values between 0.068 and 1 μ M. BPF, 4,4'-oxydiphenol, chlorinated BPAs and BPS exhibited EC₅₀ in the range 1-28 μ M. Bisphenols devoid of agonistic activity were also tested for their antagonistic activity in presence of E₂ 0,1 nM and 0.3 nM for ER α and ER β , respectively. Among them, BPP, BPM, BPBP and BPFL antagonized E₂-induced luciferase expression in both HELN ER α and ER β cell lines (Fig. 3 and Table 2). Finally, PBPH, BADGE, bis(2hydroxyphenyl)methane and TBBPA were not effective.

3.2 AR activity of bisphenols

In this study, we tested the different bisphenols to reveal their (anti)androgenic activities using HELN cells expressing a chimeric AR, where the DNA binding domain was replaced by that of ER α (Delfosse et al, 2012). In these cells, the EC₅₀ value of R1881 for AR is 0.57 nM (Supplemental Fig. S1). None of the tested bisphenols exhibited AR agonistic activity. In the same cells, we tested the ability of bisphenols to inhibit the R1881-induced luciferase expression. As previously observed (Delfosse et al, 2012), the most potent compound is BPCcl (IC₅₀ value of 171 nM) (Fig 4A). It is noteworthy, that this bisphenol revealed the same potency as the most potent xenoantiandrogen, vinclozolin metabolite M2 (Kelce et al, 1997; Molina Molina et al, 2006). Other bisphenols, BPE, BPAF, BPC, BPB, BPZ, BPA and the most of chlorinated BPAs had IC₅₀ values in 1-10 μ M range

(Fig 4A and table 3). BPP, TCBPA, BPAP, BPM, BPBP showed less activity with individual IC₅₀ varied between 10 and 22 μM. Only BPS, BPFL, BPPH, BADGE and TBBPA had no effect on AR.

3.3 PR, GR and MR activity of bisphenols

We also tested bisphenols for their (anti)progestative, glucocorticoid and mineralocorticoid activities. At first, we tested the PR (NR3C3) activity on HELN cells expressing a chimeric PR, where DBD was replaced by ER α . In these cells, the EC₅₀ value of R5020 for PR is 0.38 nM (Supplemental Fig. S1). Bisphenols did not show agonistic activities, but the majority of them antagonized R5020-induced luciferase expression. Their IC₅₀ values varied between 5.6 and 29 μ M, while the most potent were BPM, 2, 2'-diCIBBPA, 2,6diCIBBPA, BPP and BPZ (Fig 4B and table 3). Finally, some bisphenols including BPA did not display measurable antiprogestative activity using our reporter cell line.

Further, we tested their GR (NR3C1) agonistic activity on a reporter cell line expressing GR and the steroid-responsive gene MMTV-luciferase. In these cells, the EC₅₀ value of dexametasone for GR is 5 nM (Supplemental Fig. S1). Similarly to AR and PR, bisphenols did not exhibit agonistic activity on GR. On the contrary, BPP, BPPH and BPBP antagonized dexamethasone-induced activity at concentrations ranging between 15 and 23 μ M (Fig 5A and table 3).

The activity of bisphenols was then tested on MR (NR3C2) using a reporter cell line expressing the luciferase gene under the control of yeast GAL4 transcription factor (HG5LN cells) and the chimeric construct of GAL4 DBD was fused to MR LBD (Bellet et al, 2012). In these cells, the EC₅₀ value of alodsterone for MR is 1 nM (Supplemental Fig. S1). Similar to AR, PR and GR, several bisphenols did not present agonistic mineralocorticoid activity. Vice versa, the most of them displayed antagonistic activities on MR. IC₅₀ values were ranging between 1 and 22 μ M. The most potent bisphenols were BPAF, BPCcl, BPC, 2monoCBPA, 2,2-dichloroBPA, BPB, BPM and BPA (Fig 5B and table 3). Only BPF, 4,4'-dioxydiphenol, BPS, BPFL and BADGE were completely lacking

antimineralocorticoid activity.

3.4 ERR γ activity of bisphenols

To characterize the ability of bisphenols to modulate ERR γ activity, we used HG5LN cells expressing GAL4(DBD)-ERR γ (LBD) chimeric protein. In these cells, ERR γ manifested high constitutive basal activity that could be antagonized in a dose-dependent manner to maximum of about 50% by ERR γ and ERs antagonist, 4-hydroxytamoxifen (OHT). Contrary, BPA slightly increased luciferase activity to a maximum of 125% (Delfosse et al, 2012). None of tested bisphenols was able to decrease luciferase expression, vice versa some of them slightly increased it (data not shown). When cells were co-treated with OHT (10^{-6} M), the bisphenols successfully reversed the effect of antagonist (Fig 6A and table 1). In this study, we confirmed that BPE and BPA display the highest potency for ERR γ (Delfosse et al, 2012; Li et al, 2010; Okada et al, 2008). BPCcl, BPB, 2-monoCBPA, BPZ, BPF, 2,6-diCBPA and BPAF were also active, but other bisphenols were devoid of ERR γ activity (table 1).

3.5 PXR activity of bisphenols

To test the PXR activity of different bisphenols, we used HG5LN cells expressing GAL4(DBD)-PXR(LBD) chimeric protein (Delfosse et al, 2015). In this reporter cell line, the EC₅₀ value of SR12813 for PXR is 380 nM (Supplemental Fig. S1). In these cells, we demonstrated that several bisphenols (BPAF, BPZ, BPB, BPC, 2,2-diCBPA, BPM, BPP, 2,6-diBPA, TCBPA, BPPH and BPBP) activated PXR with EC₅₀ values ranging between 7.5 and 92 μ M. On the contrary, others (BPCcl, BPE, BPF, 4,4'-oxydiphenol, 2monoCBPA, BPS, BADGE, Bis(2hydroxyphenyl)methane

did not modulate PXR activity (table1).

4. Discussion

Bisphenols are widely in the industry of various consumer products. BPA, the most common bisphenol, is suspected to cause various health effects in humans. However, the mechanism of BPA action still remains controversial. A fundamental question is: How low doses of BPA could influence endocrine functions ? The most commonly used BPA alternatives are BPS, BPF and BPAF. Ideally, the substituents used to replace molecule of concern should be inert, or even less toxic than the original molecule. In spite of this effort, there are few studies reporting the risk of BPA analogs on human health if compared to BPA. Interestingly, BPA bind to different NRs. This simultaneous action on several nuclear receptors possibly contributes to the endocrine disrupting mechanism exerted by BPA. Based on these facts, we studied the action of BPA and its analogs on several NRs (ERs, AR, PR, GR, MR, ERR γ and PXR).

In our work, we demonstrated that bisphenols have different effects depending on individual NRs. In case of ER α and β , the majority of bisphenols were able to modulate NRs activities. Among the most potent bisphenols were BPCl, BPAF, BPZ and BPB, whereas PBPH, BADGE, bis(2hydroxyphenyl)methane and TBBPA were devoid of ERs activities. We also observed differences in bisphenols efficacies. BPF was the most efficacious on both ER α and ER β (92 and 100% of activity for ER α and ER β , respectively). On the contrary, BPCl and BPAP displayed the lowest efficacy with maximal activity ranking between 35 and 51%. These data suggest that bulky groups presented on the central C atom could be counterproductive in terms of efficacy. Finally, BPP, BPM, BPBP and BPFL fully antagonized E2-induced luciferase expression. Thus, bisphenols

should be considered as selective estrogen receptor modulators (SERMs) with variable potency and efficacy.

On $ERR\gamma$, only 9 among 24 bisphenols were able to modulate receptor activity. All of them acted as agonists. The most active were BPE and BPA (EC_{50} of 0.69 and 0.97 μ M for BPE and BPA respectively). None of bisphenols acted as PXR antagonists, whereas 16 of them exhibited agonistic activity. The most potent of them were BPM, BPBP, BPZ and BPCCl.

Regarding the oxo-steroid receptors AR, PR, GR and MR, bisphenols behaved as full antagonists. AR activity was inhibited by almost all bisphenols. Interestingly, the most potent AR inhibitors corresponded to the most potent ERs activators, while BPCCl revealed the highest potency (IC_{50} of 171 nM for AR, table 2). PR activity was inhibited by several bisphenols. The most potent PR antagonists, BPM, BPP and BPBP acted also as ERs antagonists. GR activity was inhibited only by 4 of tested bisphenols. Interestingly, 3 of them (2,2'-diCBPA, BPP and BPBP) were also AR and PR antagonists. Finally, MR activity was inhibited by the most of bisphenols.

Altogether, our results showed that bisphenols are able to modulate the activities of several NRs, therefore it should be limitation on their use. As an example, BPCCl is one of the most potent ERs and $ERR\gamma$ agonist, as well as AR and MR antagonist. BPZ and BPB modulate the activity of all tested receptors, except GR. On the contrary, some bisphenols had few NRs interactions. BADGE did not modulate NRs activity and BPS modulated only ERs activity.

Noteworthy, bisphenol-related effects on different NRs could have synergistic potential. The simultaneous action on several NRs correspond to the mechanism by which bisphenols exert their endocrine disrupting effects. Further, bisphenols could also exert their cellular effects through alterations of other signaling pathways. Bisphenols were shown to bind to membrane receptors, such as GPR30 (Cao et al, 2017) and activate intracellular signaling pathways (Castillo Sanchez et al,

2016; Dong et al, 2011; Goodson et al, 2011; Li et al, 2014; Sauer et al, 2017). Thus, bisphenols could possibly act in a synergistic way through simultaneous action on NRs and membrane receptors. Despite the fact that some molecular targets of bisphenols have been described, their exact mechanism of action as well as their specific signaling pathways by which they exert their effects remain largely unknown. In this study, we identified some bisphenols with fewer NR-mediated side effects which might serve as substitutes for BPA.

Acknowledgements

This work was supported by the Agence Nationale de Recherches (projects BISCOT 2010, NEWPLAST 2013 and TOXSYN 2013) and by ANSES (project EVALPE).

Figure captions

Figure 1. **Chemical structure of the bisphenols used in this study.** BPA, 2,2-Bis(4-hydroxyphenyl)propane, cas number 80-05-7. BPCl, Bis(4-hydroxyphenyl)-2,2-dichloroethylene, cas number 14868-03-2. BPAF, 2,2-Bis(4-hydroxyphenyl)hexafluoropropane, cas number 1478-61-1. BPZ, 4,4'-Cyclohexylidenebisphenol, cas number 843-550. BPB, 2,2-Bis(4-hydroxyphenyl)butane, cas number 77-40-7. BPC, 2,2-Bis(4-hydroxy-3-methylphenyl)propane, cas number 79-97-0. BPE, 1,1-Bis(4-hydroxyphenyl)ethane, cas number 2081-08-05. BPAP, 4,4'-(1-Phenylethylidene)bisphenol, cas number 151-75-1). BPF, 4,4'-Methylenediphenol, cas number : 620-92-8. BPS, 4,4'-Sulfonyldiphenol, 4-Hydroxyphenyl sulfone, cas number 80-09-1. 4,4'-oxydiphenol, cas number : 1965-09-9. Bis(2-hydroxyphenyl)methane, cas number 79-94-0.

BPM, 4,4'-(1,3-Phenylenediisopropylidene)bisphenol, cas number 13595-25-0. BPP, 4,4'-(1,4-Phenylenediisopropylidene)bisphenol, cas number 1844-01-5. BPBP, 1,1-Bis(4-hydroxyphenyl)-1,1-diphenylmethane, cas number : 1844-01-5. BPFL, 4,4'-(9-Fluorenylidene)diphenol, cas number : 3236-1-3. BPPH, 2,2-Bis(2-hydroxy-5-biphenyl)propane, cas number 24038-68-4. BADGE, 2,2-Bis[4-(glycidyloxy)phenyl]propane, cas number : 1675-54-3. TBBPA, 4,4'-Isopropylidenebis(2,6-dibromophenol), cas number 620-92-8. 2monoCBPA, 2-Chlorobisphenol A, cas number 74129-35-1. 2,2'diCBPA, 2,2'-Dichloro-4,4'-isopropylidenediphenol A, cas number 79-98-1. 2,6'diCBPA, 2,6-dichloro-4,4'-isopropylidenediphenol, cas number : 14151-65-6. 2,2',6'diCBPA, 2,2',6'trichloro-4,4'-isopropylidenediphenol, cas number : 40346-65-6. TCBPA, 2,2',6,6'-Tetrachloro-4,4'-isopropylidenediphenol, cas number 79-95-8.

Figure 2. **Bisphenols were ER α and ER β partial agonists.** Dose response curves of bisphenols in HELN ER α (A) and ER β (B) cells. Cells were incubated for 16h with various concentrations of BPC, BPAF, BPZ, BPCnc, BPA and BPF.

Figure 3. **Some bisphenols were ER α and ER β full antagonists.** Dose response curves of bisphenols with antagonist activity in HELN ER α (A) and ER β (B) cells. Cells were incubated for 16h with various concentrations of BPM, BPP, BPBP and BPFL in presence of E2 100 and 300 pM for ER α and ER β , respectively.

Figure 4. **Some bisphenols were AR and PR full antagonists.** Dose response curves of bisphenols with antagonist activity on HELN AR (A) and HELN PR (B) cells. Cells were incubated for 16h with various concentrations of bisphenols in presence of R1881 1 nM (A) and R5020 1 nM (B).

Figure 5. **Some bisphenols were GR and MR full antagonists.** Dose response curves of bisphenols with antagonist activity on HMLN GR (C) and HG5LN MR (D) cells. Cells were incubated for 16h with various concentrations of bisphenols in presence of Dexamethasone 5 nM (C) and aldosterone 1 nM (B).

Figure 6. **Some bisphenols were ERR γ and PXR agonists.** Dose response curves of OHT, BPA and BPF in HG5LN-ERR γ cells (A). Cells were incubated for 16h with various concentrations of OHT or BPA and BPE in presence of OHT 1 μ M. Dose response curves of bisphenols in HG5LN-PXR cells (B). Cells were incubated for 16h with various concentrations of bisphenols.

Table 1. Functional characteristics of bisphenols for ER α , ER β , ERR γ and PXR. EC₅₀: half maximal effective concentration.

Bisphenol	ER α EC ₅₀ \pm SD (μ M) (% max act)	ER β EC ₅₀ \pm SD (μ M) (max act)	ERR γ EC ₅₀ \pm SD (μ M) (max act)	PXR EC ₅₀ \pm SD (μ M) (max act)
BPCcl	0.030 \pm 0,005 (51)	0.046 \pm 0.017 (35)	2.55 \pm 0.2 (120)	NA
BPAF	0.109 \pm 0.037 (63)	0.068 \pm 0.01 (71)	24.9 \pm 8 (76)	34.4 \pm 6.4 (39)
BPZ	0.124 \pm 0.30 (45)	0.088 \pm 0.03 (53)	5.24 \pm 0.84 (115)	10.2 \pm 0.98 (58)
BPB	0.204 \pm 0.039(61)	0.128 \pm 0.014 (60)	3.22 \pm 0.39 (131)	22.1 \pm 8.8(46)
BPC	0.294 \pm 0.036 (72)	0.559 \pm 0.214 (31)	NA	36.4 \pm 4.6 (37)
BPA	0.457 \pm 0.064 (68)	0.384 \pm 0.66 (92)	0.97 \pm 0.26 (131)	93.7 \pm 27.6 (28)
BPAP	0.761 \pm 0.151 (40)	0.61 \pm 0.28 (40)	NA	65.9 \pm 15(30)
BPE	0.843 \pm 113 (72)	0.69 \pm 0.088 (87)	0.69 \pm 0.28 (131)	NA
4,4'oxydiphenol	1 \pm 0.25 (69)	3,06 \pm 0.92 (72)	NA	NA
2monoCBPA	0.8 \pm 0.19 (70)	1.33 \pm 0.17 (73)	3.7 \pm 0.82 (119)	NA
2,2'diCBPA	1.15 \pm 0.2 (75)	2.21 \pm 0.06 (66)	NA	9.85 \pm 0.62 (59)
2,6diCBPA	1.23 \pm 0.05 (68)	4.59 \pm 1.81 (61)	9.14 \pm 0.56 (93)	16.95 \pm 10 (52)
BPF	1.37 \pm 0.11 (92)	0.952 \pm 0.039 (100)	5.36 \pm 1.7 (121)	NA
2,2',6triCBPA	1.67 \pm 0.12 (70)	17.2 \pm 3.8 (42)	NA	10.66 \pm 0.44 (56)
BPS	4.47 \pm 0.59 (74)	1.72 \pm 0.11 (100)	NA	NA
TCBPA	11.7 \pm 1.7 (70)	68 \pm 17 (21)	NA	24.1 \pm 1.5 (35)
BPM	Antagonist	Antagonist	NA	7.51 \pm 2.38 (66)
BPP	Antagonist	Antagonist	NA	19 \pm 2.3 (44)
BPBP	Antagonist	Antagonist	NA	8.61 \pm 1.25 (63)
BPFL	Antagonist	Antagonist	NA	54.3 \pm 14.1 (65)
BPPH	NA	NA	NA	8.23 \pm 0.69 (32)
BADGE	NA	NA	NA	NA
Bis(2hydroxyphenyl)methane	NA	NA	NA	NA
TBBPA	NA	NA	NA	123 \pm 49.4 (27)

NA: non active. The maximal activity (% max act) was taken as 100% in presence of E2 10 nM for ER α and ER β , DMSO alone for ERR γ and SR12813 3 μ M for PXR.

Table 2. IC₅₀ (half maximal inhibitory concentration) of bisphenols for ER α , ER β .

Bisphenol	ER α IC ₅₀ \pm SD (μ M)	ER β IC ₅₀ \pm SD (μ M)
BPM	* 1.83 \pm 0.46	* 1.22 \pm 0.46
BPP	* 1.91 \pm 0.69	* 1.81 \pm 0.52
BPBP	* 5.49 \pm 1.58	* 3.99 \pm 1.13
BPFL	* 5.91 \pm 1.92	* 5.53 \pm 0.6

The IC₅₀ was determined in presence of E2 0.1 nM.

Table 3. Functional characteristics of bisphenols for AR, PR, GR and MR. IC₅₀: half maximal inhibitory concentration.

Bisphenol	AR IC₅₀ ± SD (µM)	PR IC₅₀ ± SD (µM)	GR IC₅₀ ± SD (µM)	MR IC₅₀ ± SD (µM)
BPCcl	0.171 ± 0.052	20.2 ± 10.7	NA	1.76 ± 0.32
BPAF	1.54 ± 0.63	28.5 ± 12.2	NA	1.59 ± 0.32
BPZ	3.58 ± 1.78	7.64 ± 3.22	NA	6.61 ± 2.05
BPB	2.15 ± 1.01	12.1 ± 3.28	NA	2.74 ± 0.24
BPC	2.03 ± 0.62	12.2 ± 4.25	NA	2.12 ± 0.31
BPA	4.32 ± 1.22	NA	NA	2.94 ± 0.94
BPAP	15.9 ± 3.3	23.1 ± 9.4	NA	9.81 ± 3.23
BPE	1.46 ± 0.68	NA	NA	10.9 ± 4.1
4,4' dioxydiphenol	5.97 ± 2.24	NA	NA	NA
2monoCBPA	3.5 ± 0.44	11.8 ± 5.1	NA	2.32 ± 0.4
2,2'diCBPA	4.3 ± 1.22	6.09 ± 3.95	19.6 ± 1.9	2.13 ± 0.23
2,6diCBPA	4 ± 1.48	7.03 ± 3.54	NA	6.12 ± 2.56
BPF	5.1 ± 0.74	NA	NA	NA
2,2',6triCBPA	7.22 ± 2.94	NA	NA	5.02 ± 2.66
BPS	NA	NA	NA	NA
TCBPA	13.4 ± 3.7	NA	NA	6.74 ± 2.75
BPM	17.2 ± 4.1	5.63 ± 3.43	NA	2.25 ± 0.17
BPP	10.8 ± 6.5	7.18 ± 3.69	16.1 ± 5.7	6.52 ± 2.33
BPBP	22 ± 9.4	5.91 ± 1.64	15.7 ± 3.3	4.26 ± 2.64
BPFL	NA	NA	NA	NA
BPPH	NA	8.97 ± 4.78	22.8 ± 1.7	4.29 ± 1.65
BADGE	NA	NA	NA	NA
Bis(2-hydroxyphenyl)methane	3.06 ± 0.86	NA	NA	21.6 ± 5.5
TBBPA	NA	NA	NA	10.4 ± 3

NA : non active. The IC₅₀ was determined in presence of R1881 1 nM for AR, R5020 1 nM for PR, dexamethasone 1 nM for GR and aldosterone 1 nM for MR.

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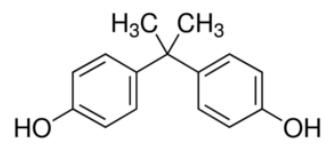
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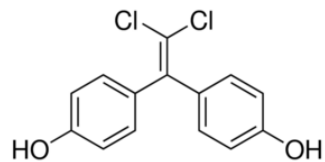
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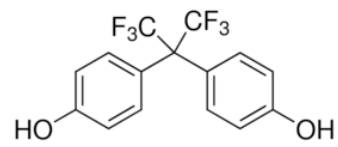
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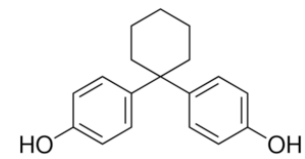
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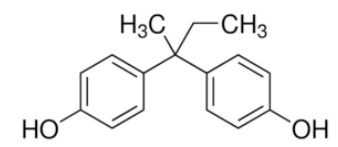
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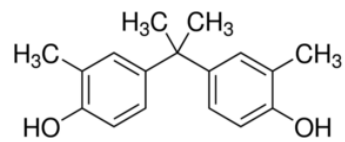
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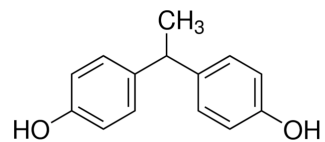
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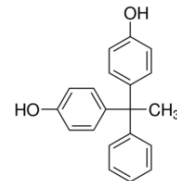
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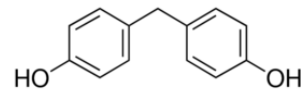
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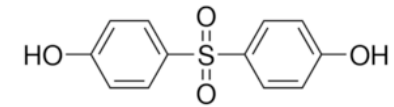
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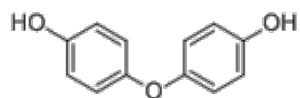
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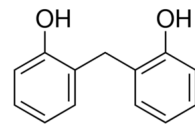
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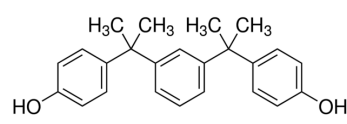
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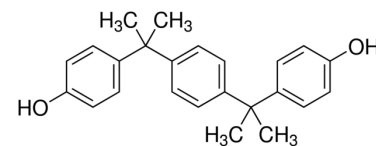
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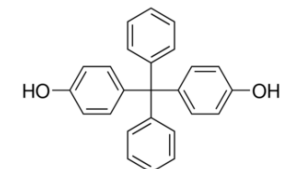
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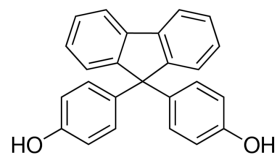
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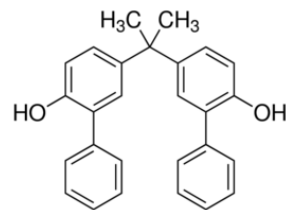
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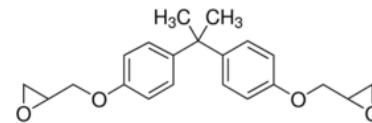
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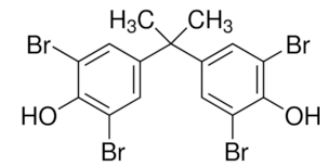
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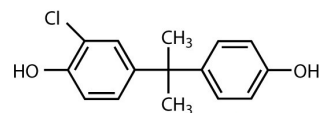
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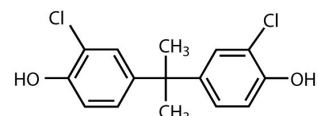
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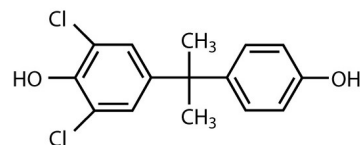
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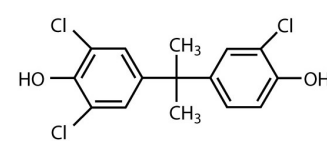
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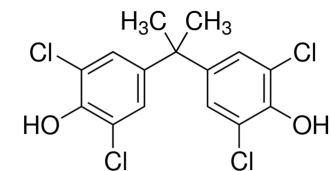
2,2'-diCBPA



2,6'-diCBPA



2,2,2'6'-triCBPA



TCBPA

Fig 1

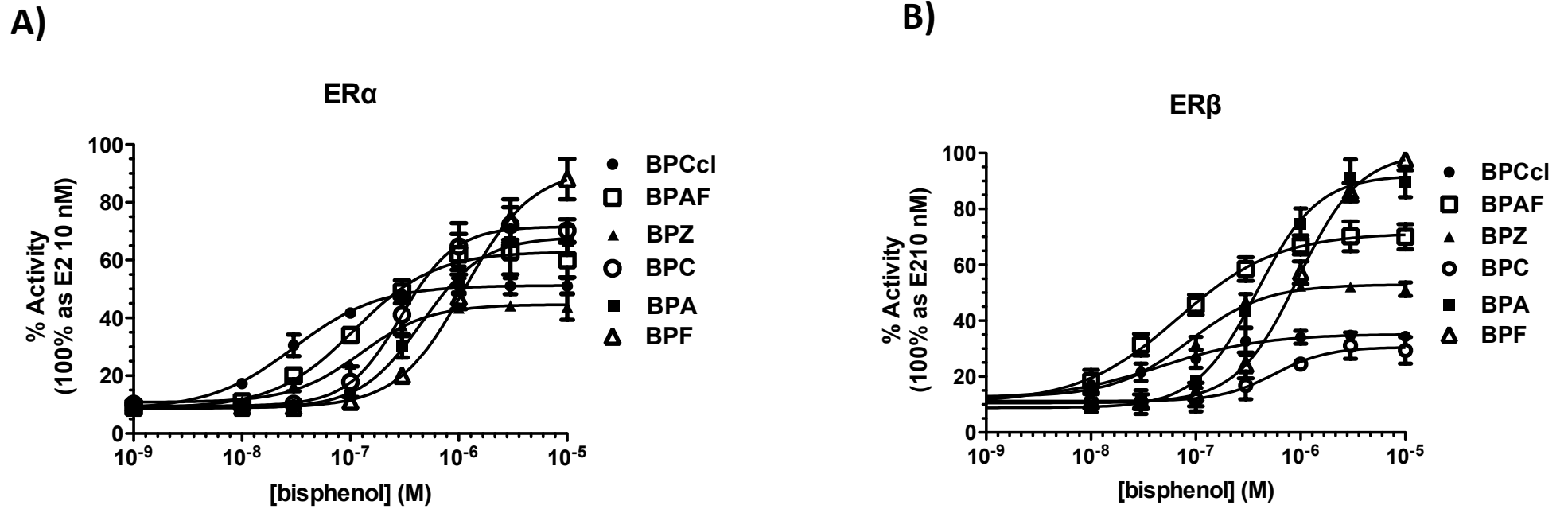
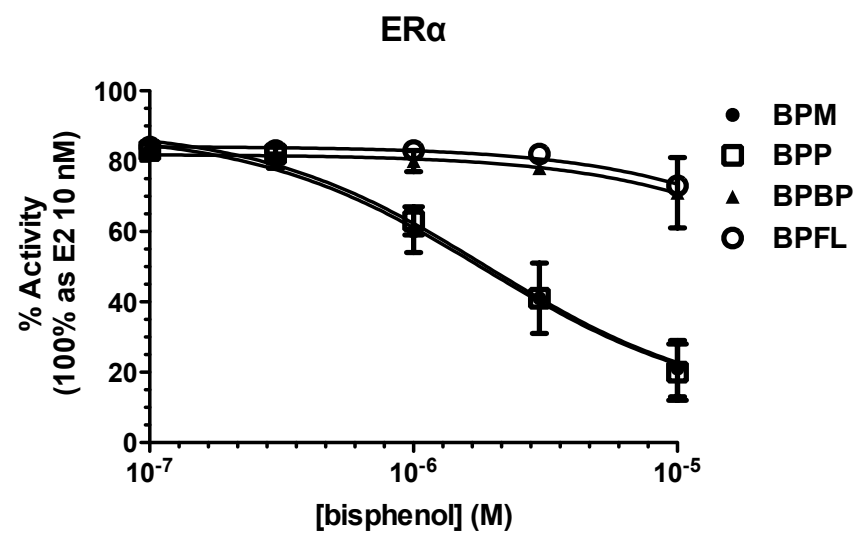


Fig 2

A)



B)

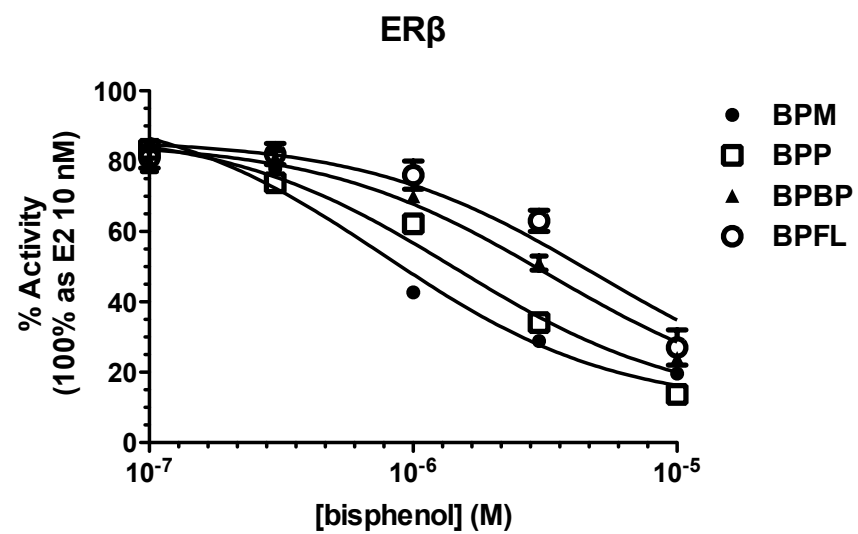


Fig 3

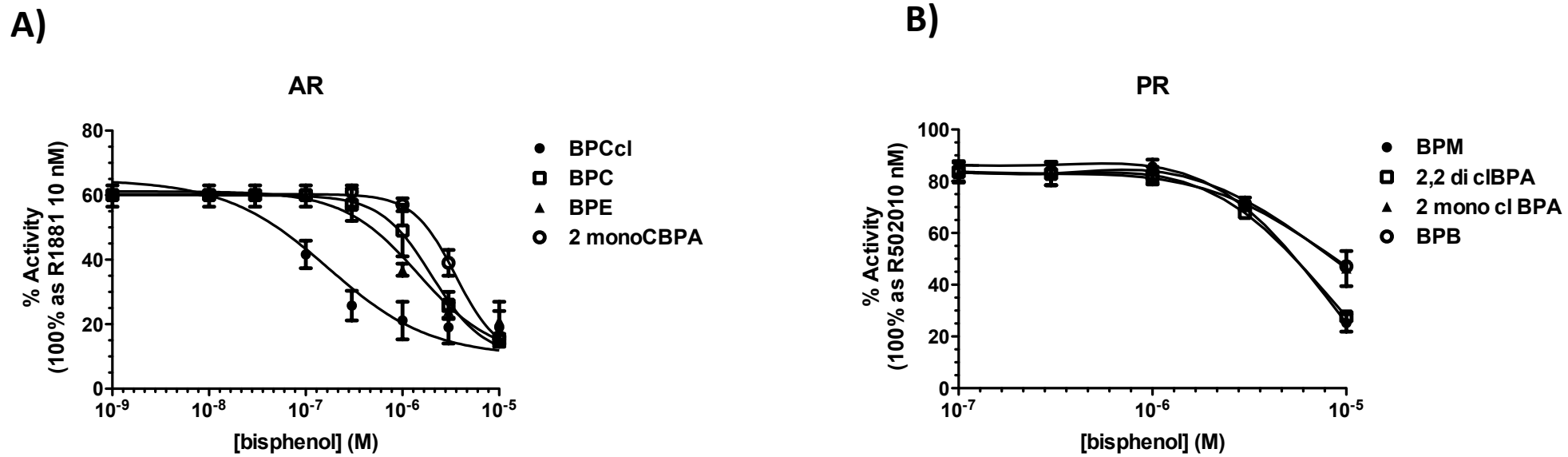


Fig 4

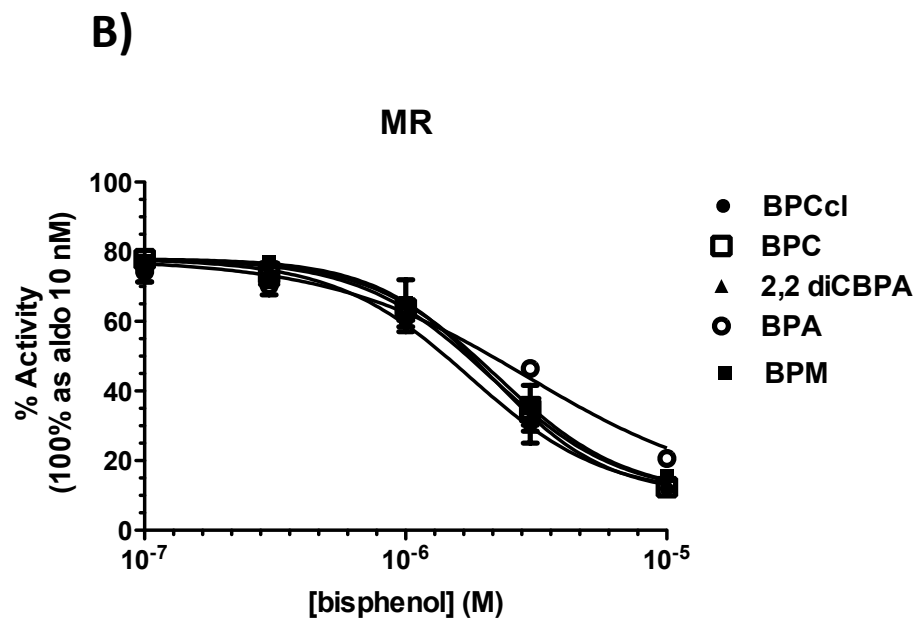
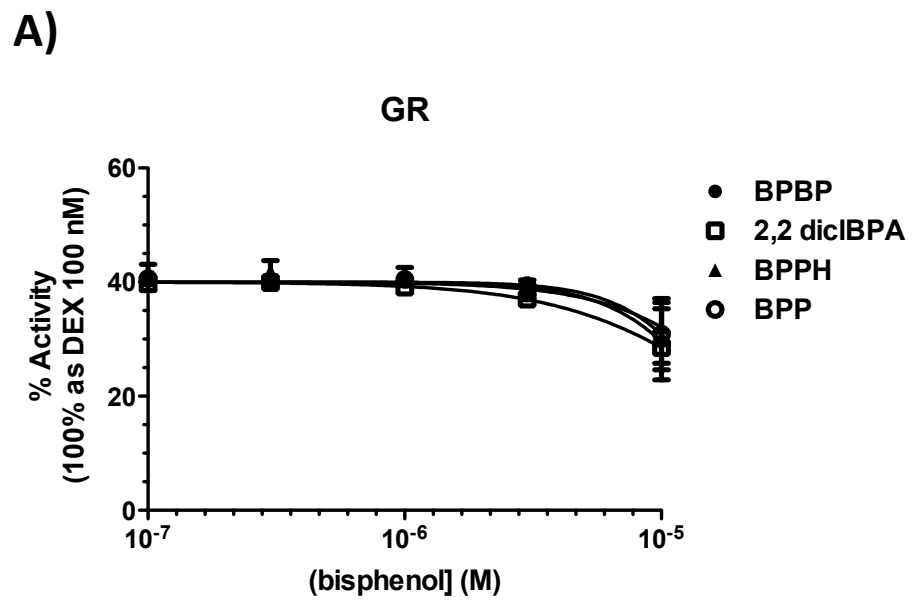


Fig 5

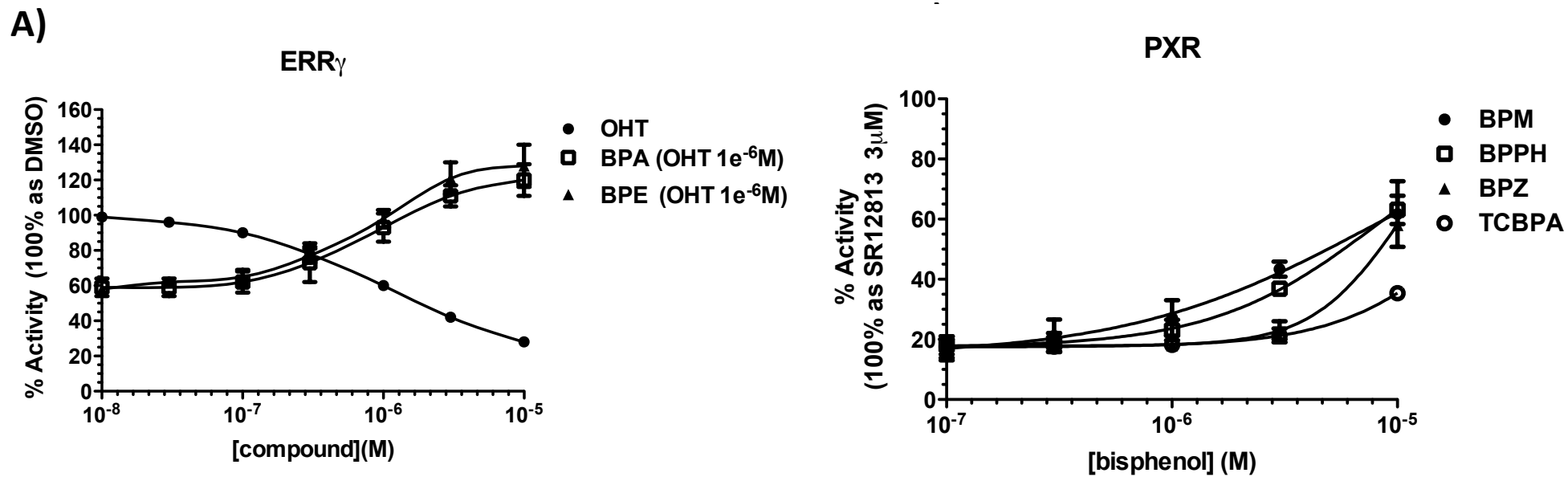


Fig 6