

1 **A stable fish reporter cell line to study estrogen receptor transactivation by environmental**
2 **(xeno)estrogens**

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7
8 **Abstract :** Cross-species differences between human and fish estrogen receptor (ER) binding by
9 environmental chemicals have been reported. To study ER transactivation in a fish cellular
10 context, we stably co-transfected the PLHC-1 fish hepatoma cell line with a rainbow trout
11 estrogen receptor (rtER) and the luciferase reporter gene driven by an estrogen response element
12 (ERE). This new cell model, called PELN-rtER (for PLHC-1-ERE-Luciferase-Neomycin),
13 responded to 17 β -estradiol (E2) in a both concentration- and temperature-dependent manner, as
14 well as to environmental ER ligands from different chemical classes: natural and synthetic
15 estrogens, zearalenone metabolites, genistein, alkyphenoles and benzophenone derivatives. The
16 comparison with other *in vitro* models, i.e. human reporter cell lines (HELN-rtER, MELN) and
17 vitellogenin induction in primary cultures of rainbow trout hepatocytes, showed an overall higher
18 sensitivity of the human cells for a majority of ligands, except for benzophenone derivatives
19 which were active at similar or lower concentrations in fish cells, suggesting species-specificity
20 for these substances. Correlation analyses suggest that the fish cell line is closer to the trout
21 hepatocyte than to the human cell context, and could serve as a relevant mechanistic tool to study
22 ER activation in fish hepatic cellular context.

23

24 **Introduction**

25 The widespread presence of endocrine disrupter compounds (EDCs) in the aquatic environment
26 has become a very important issue of environmental concern over the past few decades, as these
27 natural or man-made chemicals may cause adverse effects on wildlife (Sumpter, 2005). Given
28 the complexity of the endocrine system as well as the diversity of chemicals and their modes of
29 action, tiered approaches have been proposed for the screening (Tier 1) and testing (Tier 2) of
30 EDCs (reviewed by Hotchkiss et al., 2008). Tier 1 includes both *in vitro* and short term *in vivo*
31 assays. In this context, the evaluation of non mammalian *in vitro* screening assays has been
32 clearly identified as an important need to be addressed in EDC testing strategies (Hotchkiss et
33 al., 2008). However, compared to mammalian species, fewer non mammalian *in vitro* screening
34 assays have been developed. The lack of species specific screening assay may represent an
35 important gap in risk assessment of EDCs for aquatic organisms, and for fish in particular, since
36 cross-species differences have been identified with regard to the molecular mode of hormone
37 action (i.e. receptor binding affinities) or xenobiotic metabolism (Matthews et al., 2000; Wilson
38 et al., 2007; Hotchkiss et al., 2008).

39 One important mechanism in EDC action is mediated by the modulation of estrogen receptor
40 (ER) activation. Different assays exist to assess estrogenic activity of chemicals in fish. Among
41 them, the most widely used is based on vitellogenin (VTG) induction in isolated fish hepatocytes
42 (e.g. Pelissero et al., 1993, Smeet et al., 1999). Such *in vitro* assay is toxicologically relevant
43 because it measures natural gene response in cultured cells derived from a main target organ of
44 EDC (i.e. liver) and because it retains metabolic properties close to the *in vivo* situation.
45 However, it has also some limitations for screening purpose since it is relatively time-consuming
46 and may be the subject of inter-assay variability (reviewed by Navas and Segner, 2006). On the

47 other hand, the use of receptor mediated expression of stable reporter gene system using
48 established cell lines serves as rapid, reproducible and specific assay. However to our
49 knowledge, only few stable reporter gene assays using fish cell lines have been described
50 (Ackermann et al., 2002), and none in hepatic cell context.

51 In this study, we describe the development of a new stable reporter gene assay for the assessment
52 of ER activation by chemicals in fish cellular context, by using the PLHC-1 hepatoma fish cell
53 line (Ryan and Hightower, 1994). In this model, kinetics of luciferase transactivation by estradiol
54 as function of exposure duration and temperature were determined, as well as its activation by
55 various ER ligands representative of different chemical classes. Finally, the comparison of this
56 new *in vitro* model with other well established assays for estrogenicity assessment, namely VTG
57 induction in isolated rainbow trout hepatocytes and human reporter cell lines derived from HeLa
58 (HELN-rtER) and MCF-7 (MELN) cells, highlighted some response specificity, possibly linked
59 to the fish receptor and/or fish cell context.

60 **Materials and methods**

61 Chemicals, materials and reagents

62 17 β -estradiol (E2, CAS#50-28-2), 17 α -Ethinylestradiol (EE2, CAS#57-63-6), Estrone (E1,
63 CAS#53-16-7), Estriol (E3, CAS#50-27-1), 2,4-dihydroxybenzophenone (BP1, CAS#131-56-6),
64 2,2',4,4'-tetrahydroxybenzophenone (BP2, CAS#131-55-5), 2-hydroxy-4-methoxybenzophenone
65 (BP3, CAS#131-57-7), 2,4,4'-trihydroxybenzophenone (THB, CAS#1470-79-7),
66 diethylstilbestrol (DES, CAS#56-53-1), Hexestrol (Hex, CAS#84-16-2), genistein (Gen,
67 CAS#446-72-0), 4-*tert*-octylphenol (4OP, CAS#140-66-9), 4-nonylphenol (4NP, CAS#54181-
68 64-5), bisphenol A (BPA, CAS#80-05-7), α -zearalenol (α -ZEE, CAS#36455-72-8), β -zearalenol
69 (β -ZEE, CAS#71030-11-0) and α -zearalanol (α -ZEA, CAS#26538-44-3) were purchased from

70 Sigma-Aldrich (France). All compounds were of purity higher than 98 %. Stock solutions of
71 chemicals were prepared in dimethyl sulfoxide (DMSO) at 10 mM and stored at -20°C . Fresh
72 dilutions of test chemicals were prepared before each experiment. Culture medium and additives
73 were purchased from Gibco (France), fetal calf serum (FCS) and D-luciferin from Sigma-Aldrich
74 (Quentin Fallavier, France). Cell culture plastics were obtained from BD Bioscience (France),
75 except 96-well plates which were purchased from Greiner (France).

76 Plasmids

77 The construction of ERE- β Glob-Luc-SVNeo and pSG5-rtER_S-puro plasmids that encode
78 respectively for the luciferase reporter gene and the rainbow trout estrogen receptor short form
79 (rtER α), has been described previously by Balaguer et al. (1999) and Molina-Molina et al.
80 (2008), respectively.

81 PLHC-1 cell line : culture conditions and stable transfection

82 The PLHC-1 cell line, obtained from the American Type Culture Collection (ATCC CRL 2406),
83 is derived from the hepatocellular carcinoma of the topminnow *Poeciliopsis lucida* (Ryan and
84 Hightower, 1994). PLHC-1 cells were routinely cultured at 30°C in minimum essential medium
85 with Earle's salts (E-MEM) supplemented with 10% v/v decompemented fetal calf serum
86 (FCS), 1% v/v non-essential amino acids, 1% v/v of sodium pyruvate, 50 U/ml of penicillin and
87 streptomycin antibiotics in a 5% CO₂ humidified atmosphere. For stable transfection
88 experiments, PLHC-1 cells were plated onto 100 mm diameter Petri dishes in complete E-MEM
89 without antibiotics. Twenty four hours after plating, confluent cells were co-transfected with the
90 two plasmids described above by using the Lipofectamine 2000TM reagent (Gibco, France),
91 according to the manufacturer's instructions. After three hours, transfection reagent was removed
92 and cells were allowed to recover for 24 hours before addition of 3 mg/ml G418 and 0.5 $\mu\text{g}/\text{ml}$

93 puromycin as selecting agents. Medium was renewed every two days during one month before
94 first clones were isolated and amplified. Only few resistant clones (about forty clones in three
95 transfection dishes) were developed on the plates after one month of selection treatment with
96 antibiotics. Nevertheless twenty clones could be isolated and tested for luciferase induction by
97 E2. Among them, the clone 1.1 showed the highest induction of luciferase activity by E2. This
98 clone was chosen for further experiments and called PELN-rtER for PLHC-1 ERE-Luciferase
99 Neomycin-rtER.

100 Luciferase induction assay

101 PELN-rtER cells were seeded on 96 well plates (50 000 cells per well) in phenol red free
102 medium supplemented with 3 % dextran-coated charcoal treated FCS to remove serum steroids
103 (DCC medium) and left to incubate for 24 h before chemical exposure. This medium was used to
104 avoid interference due to estrogenic activity of phenol red and serum steroids in the assay.
105 Solvent (DMSO) content did not exceed 0.1 % v/v in the culture medium. Cells were exposed to
106 test chemicals for 48 hours at 25°C. Luciferase activity was then determined in living cells as
107 follows. The culture medium was removed and replaced by 50 µl of D-luciferin 0.3 mM in DCC
108 medium. After 5 min allowing a stabilisation of the luminescent signal, luminescence counts
109 were determined in a microplate luminometer (µBeta, Wallac). Results were expressed as
110 percentage of maximal luciferase induced by E2, the reference ligand.

111 Vitellogenin assay in primary culture of rainbow trout hepatocytes (PRTH)

112 Adult male rainbow trout (*Onchorynchus mykiss*) were obtained from a local hatchery (INRA,
113 Gournay-sur-Aronde, France). Fish were kept in tanks with aerated charcoal filtered tap-water at
114 a temperature of 15 °C. Rainbow trout were fed with commercial fish food and acclimatized to
115 laboratory conditions for a minimum of 2 weeks before use in the experiments. Hepatocytes

116 were isolated as previously described (Laville et al., 2004) and seeded in 96 well Primaria™
117 microplates at a density of 5×10^5 cells per well and cultured at 15 °C in phenol red free
118 Leibovitz-15 medium (L-15) supplemented with 5% DCC serum, penicillin and streptomycin (50
119 U/mL each) and 10 mM HEPES. Cells were left to incubate for 24 h before exposure to
120 chemicals for 96 hours. Solvent content (DMSO) did not exceed 0.1 % v/v in the culture medium
121 and half of the medium was renewed after two days with fresh medium containing the test
122 chemical at the desired concentration. VTG quantification in extracellular culture medium was
123 performed using a competitive enzyme-linked immunosorbent assay (ELISA) according to the
124 method of Brion et al. (2002), using the AA-1 anti-salmon vitellogenin polyclonal antibodies
125 (Biosense, Norway) and home-made standard VTG purified from E2-induced male rainbow trout
126 (Brion et al., 2002).

127 Data analysis

128 A range of concentrations of chemical (0.01 nM to 1 µM for estrogens and zearalenone
129 metabolites and 1 nM to 10 µM for the other chemicals) were tested in triplicate in each
130 independent experiment. Data were expressed as mean value of relative luminescence units
131 (RLU) \pm standard deviation (SD). Dose–response curves were modeled by using the Regtox 7.5
132 Microsoft Excel™ macro (available at <http://eric.vindimian.9online.fr/>), which uses the Hill
133 equation model and allows calculation of EC₅₀. Relative estrogenic potencies (REP) were
134 determined as the ratio of 17β-estradiol EC₅₀ to EC₅₀ of the test chemical.

135 Results

136 Influence of temperature and exposure duration on luciferase induction by 17β-E2

137 The stable PELN reporter cell line was first examined for its ability to respond to the reference
138 ER ligand E2 under different assay conditions. Since the functionality of rtERα has been shown

139 to be sensitive to temperature (Matthews et al., 2002), we first tested effect of E2 at different
140 temperatures. As seen in Fig. 1a, the EC₅₀ value of E2 was slightly lower at 22 and 25 °C (5 nM)
141 than that observed at 30°C (7 nM) or 37°C (12 nM). In terms of fold induction, the luciferase
142 signal was also affected by the temperature as luciferase appeared to be less inducible at 22°C,
143 and to a lesser extent at 25°C, than at higher temperatures. Hence, by considering both the
144 affinity of E2 to the rtER α and fold induction of luciferase, we chose to perform assays at 25°C
145 in our experiments. Induction of luciferase was also dependent on exposure duration (Fig. 1b). In
146 our experiments, we thus determined that a 48 h exposure was appropriate to detect maximal
147 luciferase induction without affecting the EC₅₀ of E2.

148 Ability of different ER ligands to induce luciferase mediated by rtER α in PELN-rtER cells

149 The figure 2 presents the ability of known ER ligands from different chemicals classes to induce
150 luciferase in PELN-rtER cells. Overall, all examined compounds were able to induce luciferase
151 with various transactivation profiles, in terms of both EC₅₀ values (Table 1) and maximum
152 luciferase response achieved at the highest dose examined (Fig. 2). The natural steroid estrogens
153 E1 and E3 (Fig. 2a), as wells as the synthetic estrogen EE2 (Fig. 2b) behave as total agonists as
154 they elicited maximal transactivation relative to E2. By contrast, partial transactivation curves
155 were observed with the mycoestrogen α -ZEA, the phytoestrogen genistein (Fig. 2a) and the
156 pharmaceuticals DES and hexestrol (Fig. 2b), as maximum transactivation was 60-70 %.

157 The industrial chemicals presented also different profiles in their potency and efficacy. The
158 alkylphenols 4-OP and 4-NP (Fig. 2c) were weakly estrogenic in this system, as 45 % of
159 transactivation was achieved at the highest tested concentration (10 μ M). Bisphenol A behaved
160 as a partial ER agonist. It significantly induced luciferase at a relatively low concentration (0.3
161 μ M) but this induction was limited as it reached a maximum response of 35 % relative to E2.

162 Interestingly, among the different xeno-estrogens tested, benzophenone derivatives most
163 efficiently induced luciferase in PELN-rtER cells (Fig. 2d), especially BP2 and THB that
164 behaved as total agonists for the rtER α , while BP1 induced partial activation of luciferase at 10
165 μ M and BP3 was found to be non active.

166 VTG induction by ER ligands in isolated trout hepatocytes

167 In order to compare our results with PELN-rtER with a well recognised *in vitro* fish system in
168 our laboratory conditions, some chemicals were tested for their ability to induced vitellogenin in
169 isolated rainbow trout hepatocytes (PRTH). As expected, the results summarised in Table 1
170 indicate that all tested compounds induced Vtg in a dose-response manner allowing EC₅₀
171 determination and were ranked: EE2<E2< α -ZEA<DES<Hexestrol<Genistein<BP2<4-
172 OP<THB<BP1.

173 Discussion

174 The stable fish reporter system (PELN-rtER) responded to a diversity of estrogenic compounds
175 with different transactivation potency and efficacy (Fig. 2). In order to determine how this cell
176 model compares with other established *in vitro* systems, the EC₅₀ and relative estrogenic
177 potencies (REP) were compared to values previously published using two other stable reporter
178 gene systems, namely the HELN-rtER (for HeLa-ERE-Luc-Neo transfected with the rtER)
179 (Pillon, 2005, Molina-Molina et al., 2008) and MELN (for MCF-7-ERE-Luc-Neo) cell lines
180 (Balaguer et al., 1999, Pillon et al., 2005) (Table 1). These two models consist of human cell
181 lines (HeLa and MCF-7 cells) that stably express the luciferase reporter gene under the control of
182 the rtER α and the human ER α (hER), respectively. In addition, the ability of chemicals to induce
183 VTG synthesis in primary cultures of rainbow trout hepatocytes (PRTH) has been evaluated and
184 is also reported in Table 1. Overall, the absolute sensitivity to E2 and a majority of the tested

185 compounds varied among the different assays, and were ranked: PRTH<PELN-rtER<HELN-
186 rtER<MELN. The relative estrogenic potency (REP) values allowed ranking of chemicals that
187 was fairly similar in all assays. Nevertheless, Pearson's correlation analyses showed that the fish
188 cell line was better correlated to HELN-rtER ($r^2=0.91$, $n=9$) and PRTH ($r^2=0.90$, $n=9$) than to
189 MELN ($r^2=0.80$, $n=9$), suggesting a good adequacy between the different fish-based assays.

190 The higher sensitivity of the human MELN assay can be partly attributed to the known lower
191 binding affinity of E2 for the rtER than for the hER (LeDréan et al., 1995, Matthews et al., 2000,
192 Molina-Molina et al., 2008), due to divergences in the amino acid sequences of the ligand
193 binding domain of these receptors (Pakdel et al., 2000). However, this loss of sensitivity is not
194 systematically found as certain xeno-estrogens, like polychlorobiphenyls (Matthews et al., 2000),
195 some alkylphenols (Olsen et al., 2005) or zearalenone and its derivatives (Le Guevel and Pakdel,
196 2001), have been shown to bind to and activate rtER at equal or lower concentrations than those
197 required to active hER.

198 In the present study, the most significant inter-assay difference concerns the estrogenic activity
199 benzophenone (BP) derivatives, which were almost equipotent in PELN-rtER and HELN-rtER
200 cells, and much less active in the human MELN cells. The good estrogenic potency of BPs
201 towards rtER has been already reported in mammalian cell or yeast-based assays (Kunz et al.,
202 2006, Molina-Molina et al., 2008). Here, we report that in a fish cell line, these compounds still
203 have a high estrogenic potency that is in the same order as that of natural ER ligands such as
204 estriol (Table 1). Our results strengthen the recent view that such emerging aquatic pollutants
205 present significant hazard to fish (Kunz et al., 2006) and present further evidence to support the
206 use of appropriate species-related assays to investigate hormonal activity.

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207 In addition, the different metabolic capacities of the cells may also have some influence on the
208 sensitivity of the assays to detect estrogenic activity (Olsen et al., 2005, Bursztyka et al., 2008).
209 HELN-rtER cells are derived from HeLa cells, which are poorly metabolically competent as
210 compared to PLHC-1 cells. The latter have retained significant metabolic capacities including
211 phase I and II biotransformation and efflux transporter proteins (Zaja et al., 2007), which may
212 contribute to reduce the intracellular availability of chemicals for the receptors. In trout
213 hepatocytes, which maintain substantial metabolic capacities in culture, the different tested
214 chemicals were generally active at significantly higher concentrations than in cell lines, although
215 the use of different endpoints (i.e. luciferase activation versus VTG detection) likely influenced
216 the sensitivity of the response. Nevertheless, the observation that EC_{50} values in PELN-rtER
217 were generally closer to PRTH than to HELN-rtER, could reflect, at least in part, different
218 metabolic capacities in the assays and again a good agreement between the two fish cell models.
219 In summary, the establishment of a reporter cell line that stably expresses rtER-mediated
220 luciferase within a fish hepatoma-derived cell context is reported for the first time. It is proposed
221 that such tool is useful to identify species-specific responses, as shown with benzophenone
222 derivatives. However, the lower sensitivity of the response to a majority of chemicals than in
223 similar human-derived reporter cell lines could lead one to conclude that these cells have
224 limitations for a chemical screening purpose since false negative may occur when assessing
225 weak estrogenic compounds. Nevertheless, correlation with the well-recognised vitellogenin
226 assay in PRTH cells supports the use of PELN-rtER cells to investigate estrogenicity in fish
227 hepatic cell context. Furthermore, the metabolic capacities and in particular the high expression
228 of functional aryl hydrocarbon receptor (AhR) in PLHC-1 cells make the PELN-rtER cell line a

229 relevant mechanistic tool to study ER/AhR interaction on receptor transactivation by
230 environmental chemicals in a fish cellular context (Aït-Aïssa et al., in preparation).

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Figure legends

Figure 1. (a) Influence of temperature on rtER transactivation (expressed as percentage of maximal luciferase induction) by 17 β -estradiol (E2) in PELN-rtER cells after 24 h of exposure. (b) Kinetics of luciferase induction (expressed as relative luminescence units or RLU) by 17 β -estradiol in PELN-rtER cells at 25°C. Values are means \pm SD of triplicates.

Figure 2. Typical dose response curves of luciferase induction (expressed as percentage of luciferase induction by E2 1 μ M) in PELN-rtER by (A) natural (xeno)estrogens: 17 β -estradiol (E2), estrone (E1), estriol (E3), α -zearalanol (α -ZEA), genistein (Gen), (B) pharmaceutical compounds: diethylstilbestrol (DES), hexestrol (Hex), 17 α -ethynylestradiol (EE2), (C) alkylphenols: 4-nonylphenol (4-NP), 4-*t*-octylphenol (4-OP), bisphenol A (BPA) and (D) benzophenone derivatives: 2,4-dihydroxybenzophenone (benzophenone 1 or BP1), 2,2',4,4'-tetrahydroxybenzophenone (benzophenone 2 or BP2), 2-hydroxy-4-methoxybenzophenone (benzophenone 3 or BP3), 2,4,4'-trihydroxybenzophenone (THB). Cells were exposed for 48 hours at 25°C; values are means \pm SD of triplicates.

Table 1: Effective concentrations (EC₅₀) and relative estrogenic potencies (REP) of various ER ligands in fish (PELN-rtER, PRTH) and human (HELN-rtER, MELN) cell-based *in vitro* assays.

a: Except for benzophenone derivatives, HELN-rtER and MELN data were taken from Pillon (2005) and Pillon et al. (2005), respectively. Effect of benzophenone derivatives in MELN, HELN-rtER and PRTH were from Molina-Molina et al. (2008); *b:* PRTH data were obtained as described in the Materials and Method section.

n.d.: not determined ; n.a.: not active ; -: not applicable; *n:* number of independent experiments; SEM: standard error of the mean.

Chemicals	PELN-rtER (fish PLHC-1 cells, rainbow trout receptor)				HELN-rtER ^a (human HeLa cells, rainbow trout receptor)		MELN ^a (human MCF-7 cells, endogenous receptor)		PRTH ^b (isolated rainbow trout hepatocytes, endogenous receptor)	
	EC50 (nM)	SEM	<i>n</i>	REP	EC50 (nM)	REP	EC50 (nM)	REP	EC50 (nM)	REP
17β-estradiol (E2)	5.5	1.5	9	1	0.25	1	0.018	1	22	1
Estrone (E1)	130	25	3	0.04	12	0.02	0.69	0.03	n.d.	-
Estriol (E3)	176	58	3	0.03	7.8	0.03	0.10	0.18	n.d.	-
17α-ethynylestradiol (EE2)	3.6	0.4	3	1.54	0.18	1.39	0.01	2.56	12	1.94
Hexestrol (Hex)	11	5	5	0.50	0.2	1.25	n.d.	-	206	0.11
Diethylstilbestrol (DES)	23	10	5	0.24	0.8	0.31	0.18	0.10	130	0.18
α-zearalanol (α-ZEA)	26	14	5	0.22	0.12	2.08	0.14	0.13	41	0.56
α- zearalenol (α-ZEE)	47	-	2	0.12	0.25	1.0	n.d.	-	n.d.	-
β- zearalenol (β-ZEA)	504	-	2	0.011	2.5	0.1	n.d.	-	n.d.	-
Genistein (Gen)	498	-	1	0.010	220	1.1E-03	27	6.7E-04	1702	0.013
Bisphenol A (BPA)	352	83	3	0.016	400	6.3E-04	96	1.9E-04	n.d.	-
4-octylphenol (4-OP)	1938	-	2	2.9E-03	300	8.3E-04	54	3.3E-04	36271	6.3E-04
4-nonylphenol (4-NP)	23028	-	1	2.4E-04	600	4.2E-04	339	5.3E-05	n.d.	-
Benzophenone 1 (BP1)	3507	2515	3	1.6E-03	3477	7.2E-05	9192	1.9E-06	100000	2.3E-04
Benzophenone 2 (BP2)	384	142	4	0.014	161	1.6E-03	3284	5.5E-06	30000	7.6E-04
Benzophenone 3 (BP3)	n.a.	-	3	-	18426	1.4E-05	20315	8.8E-07	n.d.	-
Trihydroxybenzophenone (THB)	620	460	3	0.009	578	4.3E-04	4012	4.5E-06	60000	3.8E-04

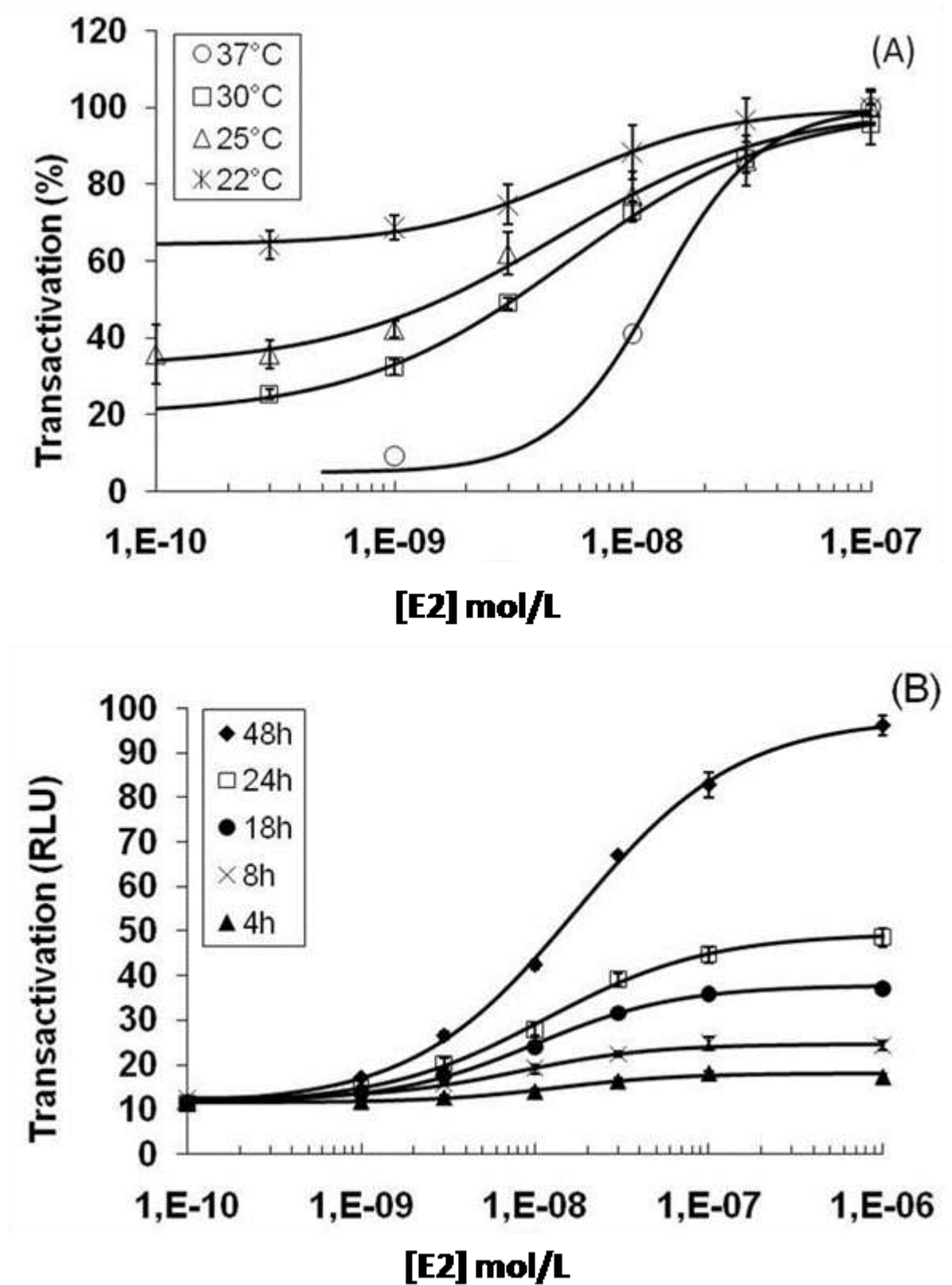


Figure 1

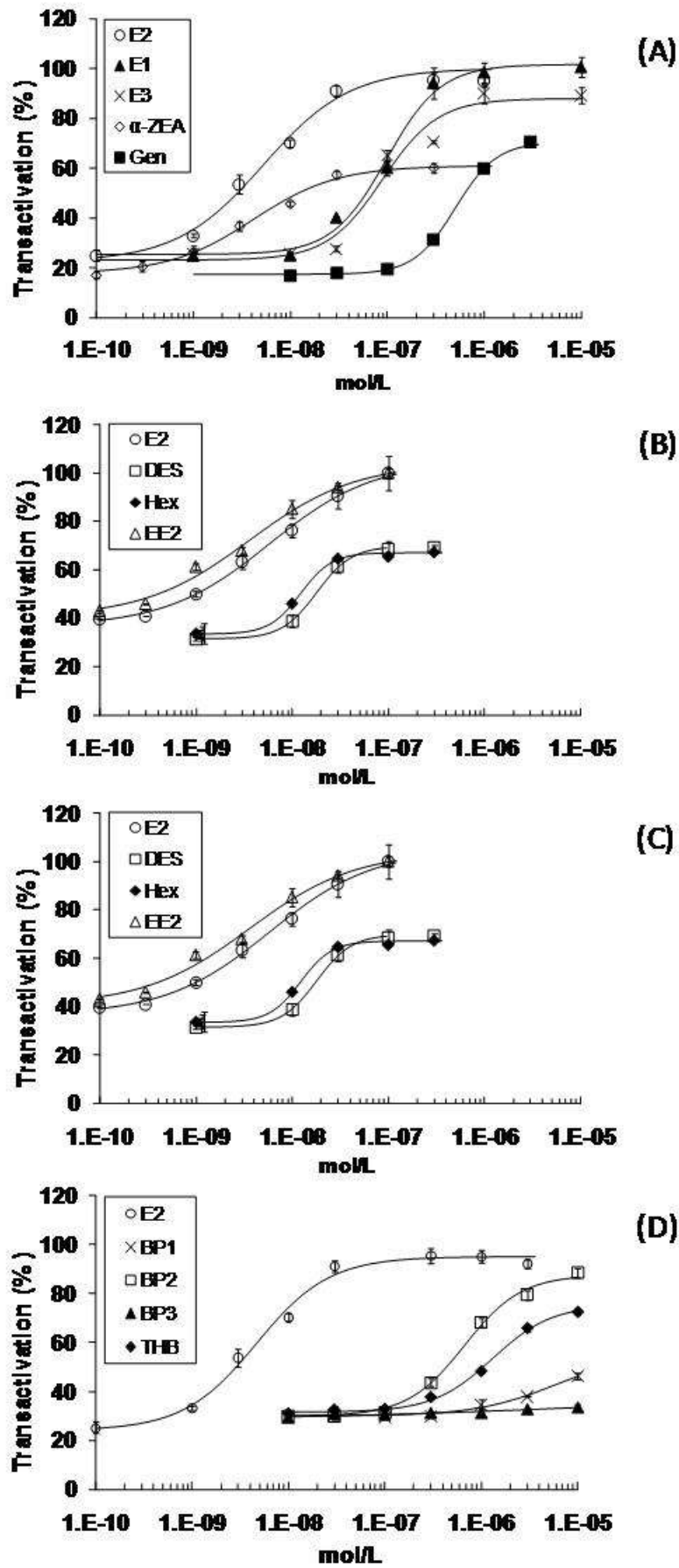


Figure 2

