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# Estrogenic activity of pharmaceuticals in the aquatic environment

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ABSTRACT

In the last years pharmaceuticals have aroused great interest as environmental pollutants for their toxic effects towards non target organisms. This study wants to draw attention to a further adverse effect of drugs, the endocrine interference. The most representative drugs of the widespread classes in environment were investigated. The YES-test and the E-screen assay were performed to detect the capability of these substances to bind the human estrogenic receptor α (hERα) in comparison with 17β-estradiol. Out of 14 tested pharmaceuticals, 9 were positive to YES-assay and 11 were positive to E-screen assay; in particular, Furosemide and the fibrates (Bezafibrate, Fenofibrate and Gemfibrozil) gave the maximal estrogenic response. Tamoxifen showed its dual activity as agonist and antagonist of hERα.



## 1. Introduction

In the last years, pharmaceuticals have become important as environmental pollutants for their extensive presence in the aquatic ecosystems (Kolpin et al., 2002; Zuccato et al., 2005). Being biologically active substances they may be of concern for their possible interactions with non-target organisms in the environment but also for humans because these compounds, after their pharmacological action, are excreted, unchanged or as metabolites, reach the environment in many ways and may go back up the aquatic trophic chain through surface waters, ground and drinking waters. Furthermore, pharmaceuticals may be stable in the environment or subjected to biotic and/or abiotic transformations, and among the abiotic transformations, the photodegradation is the most significant removal process, which sometimes leads to the formation of products much more toxic than parental compounds as reported by different studies (Boreen et al., 2003; Isidori et al., 2007).

Generally, pharmaceuticals do not have acute toxic effects on aquatic organisms because their low concentrations, in the order of ng–μg/L, but often, they may show subtle effects for their continuous introduction in the environment acting as pseudo-persistent pollutants (Calamari et al., 2003). Then, the ecotoxicological potential of drugs and their residues remains almost unknown (Daughton and Ternes, 1999; Fent et al., 2006) and in the last years, a particular effect detectable at low concentrations, the endocrine interference, has arisen the interest of researchers (Stuer-Lauridsen et al., 2000; Webb, 2001; Ingerslev et al., 2003; Fent et al., 2006).

Xenoestrogens have the capability to mimic the female steroid hormone, 17β-estradiol ($E_2$), which is primarily responsible for the development of the female reproductive system (Crisp et al., 1998). Different compounds act as endocrine disrupting chemicals (EDCs) and their effects in the aquatic environment are known since the end of the 1990s (Desbrow et al., 1998; Routledge et al., 1998). Starting from these studies, some researchers have worked to enhance the available information on the potential endocrine interference of drugs in the environment (Korner et al., 1999; Ingerslev et al., 2003; Klopman and Chakravarti, 2003; Snyder, 2003; Fent et al., 2006).

In the present work, we focused our attention on the endocrine interference of pharmaceuticals on human receptor ERα. The selected drugs belonged to the most frequently detected classes in the environment such as antibiotics, beta-blockers and anti-inflammatories (Gagne et al., 2005; Zuccato et al., 2005). The assays used to detect the endocrine interference were the YES-test and the E-screen assay: the first is a bioassay identifying compounds that can interact with the hERα, whose DNA sequence is stably integrated into the main chromosome of yeast cells, while the other is a test of cell proliferation with the human estrogen receptor-positive MCF-7 breast cancer cell line that is proved to be the most useful for the evaluation of the estrogenic activity in vitro in human model (Andersen et al., 1999).

## 2. Materials and methods

### 2.1. Test compounds

The substances analyzed in this study are listed in Table 1. Bezafibrate, Fenofibrate, Gemfibrozil, Ranitidine, Tamoxifen and Atenolol were kindly supplied by Prof. Previtera, Università Federico II, Napoli, Italy; Erythromycin, Furosemide, Ofloxacin have been provided by Sigma-Aldrich Chemicals (St. Lois, MO, USA); Ibuprofen, Naproxen, Paracetamol, Prednisone and Prednisolone by LabService Analytica srl (Anzola Emilia, BO, Italy).

For all the samples stock solutions in dimethylsulphoxide (DMSO) have been prepared and then diluted to the desired concentration in water. The final solvent concentration did not exceed 0.1% (v/v).

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**Table 1**
Pharmaceuticals analyzed.

| Therapeutic class | Pharmaceuticals |
|---|---|
| Antibiotics | Erythromycin |
| | Ofloxacin |
| Beta-blockers | Atenolol |
| Blood lipid lowering agents | Bezafibrate |
| | Fenofibrate |
| | Gemfibrozil |
| Anti-inflammatories | Ibuprofen (non-steroid) |
| | Naproxen (non-steroid) |
| | Paracetamol (non-steroid) |
| | Prednisone (steroid) |
| | Prednisolone (steroid) |
| Various other compounds | Furosemide (diuretic) |
| | Ranitidine (H2-receptor blocker) |
| | Tamoxifen (anti-estrogen) |

### 2.2. Yeast estrogen screen (YES)

*Saccharomyces cerevisiae*-RMY326 was used as strain test. These cells, besides containing hERα, also include expression plasmids carrying the reporter gene *lac-Z* (encoding the enzyme β-galactosidase), which is used to measure the receptors' activity. In this system, the hERα, activated by binding to estrogen or xenoestrogen, interacts with the estrogen-responsive-elements (ERE), situated within a promoter sequence before the *lac-Z* gene. This causes the expression of the reporter gene *lac-Z* and the produced enzyme (β-galactosidase) is secreted into the medium, where it metabolizes the chromogenic substrate, *orto*-nitrophenyl β-D-galactopyranoside (ONPG), which is normally colourless, into *orto*-nitrophenol (ONP), a yellow substance that can be measured at 420 nm.

The yeast cells were grown overnight at 26 °C by shaking in minimal medium (Yeast Nitrogen Base in sterile water plus) and enriched with a solution of amino acids and glucose (2% w/v). After 24 h, an aliquot of the culture was diluted in fresh minimal medium to reach again, in further 16–18 h, a cell concentration of 2 $10^7$ cells/mL (exponential phase) in the presence of the samples to be tested. Five concentrations for each compound and 17β-estradiol (from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-4}$ mM), as positive control, were assayed. Then, yeast cells were collected by serial centrifugations at 4000 rpm for 5 min and re-suspended in Z-buffer (30 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 5 mM KCl, 0.5 mM $MgSO_4$) plus a 0.025% β-mercaptoethanol, $CH_2Cl_2$, SDS 0.1%.

The β-galactosidase activity was determined by the addition of 700 μL of ONPG (4 mg/mL in Z-buffer) and post-incubation at 26 °C. The chromogenic reaction was stopped when the negative control began turning yellow (in about 8–10 min) by the addition of 500 μL of $Na_2CO_3$ 1 M. Then, the cell debris was removed by centrifugation at 14000 rpm for 2 min, and the absorbance at 420 nm of the sample was measured (Isidori et al., 2006a).

The β-gal units (Miller units) were determined by the following formula:

$$OD_{420} \times 1000 / t \times V \times OD_{600}$$

$t$ = elapsed time (in minutes) of incubation; $V$ = volume of culture used in mL; $OD_{600} = A_{600}$ of culture.

The EC50 values, defined as the concentration of compound giving 50% of the maximal response induced by 17β-estradiol, were calculated from the dose–response assays using standard nonlinear regression methods (Toxcalc™, 1996).

The Relative Inductive Efficiency (RIE) was determined as the ratio of the maximal β-galactosidase activity induction with test compound to 17β-estradiol × 100.

### 2.3. Cell line and cell culture conditions

Estrogen receptor-positive human breast cancer MCF-7 cells were obtained from the Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Bari, Italy. For routine maintenance, cells were grown in Dulbecco's modification of Eagle's medium (DMEM) with 15 mg/L phenol red (Gibco, Italy), at 37 °C in an atmosphere of 5% $CO_2$/95% air under saturating humidity. The culture medium was supplemented with 5% fetal bovine serum (FBS), 2% L-Glutamine, 2% HEPES and 1% of a penicillin/streptomycin solution.

### 2.4. The E-screen assay

The proliferation test (E-screen assay) was carried out according to the method of Soto et al. (1995) modified by Minervini et al. (2005). Sub confluent MCF-7 cells grown in 25-$cm^2$ flasks (Sarstedt, Verona, Italy) in DMEM were trypsinized and re-suspended in steroid-free experimental medium, phenol red-free DMEM supplemented with 5% dextran-coated charcoal treated FBS (DCC-FBS), 2% L-Glutamine, 2% HEPES and 1% of penicillin/streptomycin solution. Cells were seeded into 96-well plates (Sarstedt) at a density of 30000 cells/well and let to attach for 24 h.

Then, the medium was aspirated and replaced by experimental medium, containing concentrations of test compounds. Each of the five concentrations was tested in six replicates.

A negative control (hormones free) and the positive control, 17β-Estradiol at the concentration of $10^{-6}$ mM, were included on each plate. After five days of incubation, the cell proliferation was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] test, based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The formazan is spectrophotometrically quantified (590 nm).

The basic endpoint of the E-screen assay is the cell number in comparison with the negative control.

The proliferative effect (PE) is the ratio of the highest cell number achieved with $E_2$ or the test compound and the cell number of the negative control:

PE = absorbance $E_2$ or compound/absorbance negative control.

The estrogenic activity of a test compound is evaluated by determination of the relative proliferative effect (RPE). The RPE compares

**Table 2**
Estrogenic activity of $E_2$ and tested compounds in the YES-assay.

| Compound | Range of Concentration concentration (mg/L) | $EC_{50}$ (mg/L) | RIE% max |
|---|---|---|---|
| $E_2$ (standard) | $2.72 \cdot 10^{-6}$–$2.72 \cdot 10^{-2}$ | $2.72 \cdot 10^{-6}$ ($1.6 \cdot 10^{-6}$–$7.9 \cdot 10^{-5}$) | 100 |
| Atenolol | 6.25–100 | 40.80 (25.66–131) | 51.7 |
| Bezafibrate | 6.25–100 | 44.65 (30.29–96.53) | 52 |
| Erythromycin | 15–240 | 21.34 (17.38–24.99) | 85 |
| Fenofibrate | 6.25–100 | 9.24 (7.31–11.03) | 83 |
| Furosemide | 0.06–0.99 | 0.99 (0.66–1.52) | 62 |
| Gemfibrozil | 10–100 | n.d. | 7 |
| Ibuprofen | 6.25–100 | n.d. | 31 |
| Naproxen | 6.25–100 | n.d. | 6 |
| Ofloxacin | 7.5–120 | n.d. | 13 |
| Paracetamol | 5–80 | 6.54 (5.81–7.22) | 84 |
| Prednisone | 8.75–140 | 76.12 (56.96–119.90) | 50 |
| Prednisolone | 10–160 | 27.50 (22.56–36.11) | 59 |
| Ranitidine | 5.65–91.06 | n.d. | 32 |
| Tamoxifen | 0.125–2 | 0.34 (0.27–0.47) | 55.5 |

In brackets confidence limits (95% probability).
*RIE% = Relative Inductive Efficiency; n.d. = not determinable.