Assessment of Endocrine Disruption Potential of Selected Pharmaceuticals Using an In-vitro Assay

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Abstract— Contamination of water bodies by pharmaceuticals has recently received much attention. Even at low concentration, pharmaceuticals can disrupt the natural hormonal responses (endocrine disruptors) leading to a multitude of toxicological effects in aquatic life. In the current study, the estrogenic effect of selected pharmaceuticals was evaluated using the E-Screen assay, which quantifies the cell proliferation of estrogen responsive MCF-7 breast cancer cells with respect to 17β-estradiol. In this assay, 17α ethinylestradiol and acetaminophen were found to yield a strong and weak estrogenic response, respectively, while diclofenac and carbamazepine caused antagonistic effect. Validity of the in-vitro assay was confirmed when these pharmaceuticals did not generate any response in the estrogen receptor negative breast cancer cell line, MDA-MB-231. Based on the level of residual pharmaceuticals reported in wastewater effluents in Mumbai, the results of this study indicate that estrogenic effects may be expected in wastewater effluents.

Keywords— Pharmaceuticals, endocrine disruptors, E-Screen assay.

I. Introduction

Recently, several studies have demonstrated endocrine disruption and chronic toxicity associated with pharmaceuticals that are abundantly present in water bodies. Many of the unit processes commonly used for treatment of wastewater cannot ensure complete removal of pharmaceutical residues [1,2,3,4,5] due to their complex chemical structures. Thus, pharmaceuticals enter the aquatic domain and continue to persist there. They are known to cause chronic toxic effects, such as, endocrine disruption, i.e., interference with the estrogenic, androgenic, thyroidal and neurological hormone systems (endocrine disruption) [6,7,8]. Such compounds are termed endocrine disrupting compounds (EDCs). EDC exposure has been linked with birth defects and deformities in human [7,8], higher risk of cancer, immunological and neuro-developmental disorders [9] and thyroid disorders [7]. Feminization of fish near wastewater treatment plant (WWTPs) discharge sites has also been reported [10].

Some commonly consumed pharmaceuticals are known to cause adverse effects on biosynthesis, metabolism and signaling of male and female sex hormones (androgens and estrogens) [11]. Interference to normal estrogen signaling is initiated via ligand binding to estrogen receptor (ER). Other than its natural ligand (17-β estradiol), exogenous compounds, such as, pharmaceuticals are also reported to have selective binding affinity for ERs [11]. EDCs can disrupt ER signaling by direct disruption of genomic and non-genomic ER action. These compounds can also cause ER disruption indirectly through modulation of metabolic enzymes that are crucial to steroidogenesis, such as aromatase [11]. While excellent biomonitoring and endocrine disruption effect data are available for certain chemicals, such as, phthalates and bisphenol-A, estrogenic effects of commonly utilized pharmaceuticals, which are abundantly present in wastewater effluents in India are scarce [1,12].

Characterization of estrogenicity of complex water samples, containing various EDCs at varying concentration, makes it difficult to predict estrogenicity. Biological tests may be employed to overcome this shortcomings. Biological tests have evolved from whole animal assays using rodents to new generation, high-throughput assays, such as, enzyme linked immunosorbent assays (ELISA) [13]. However, the new generation techniques are expensive and require highly skilled manpower. On the other hand, whole animal testing may not be sensitive enough to conclusively prove the disruption of a particular hormone. In-vitro assays, such as, E-Screen have been developed to overcome these shortcomings, [14,15,16]. In-vitro tests have the ability for rapidly distinguishing the biological activity of water samples in a cost effective manner.
Materials and Methods

A. Reagents

Foetal bovine serum (FBS) and charcoal-dextran-stripped foetal bovine serum (CD-FBS) were procured from HyClone (USA). Dulbecco’s modified eagle medium (DMEM), 1X Dulbecco’s phosphate buffer saline (D-PBS), sodium bicarbonate, 20X antibiotic-antimycotic solution, non-essential amino acids solution (NEAA), amphotericin B, streptomycin, penicillin G, phenol-red free DMEM media, 0.25% trypsin-EDTA, 17β-estradiol, fulvestrant, carbamazepine, 17α-ethinylestradiol, diclofenac, acetaminophen, cell freezing solution, MTT reagent, and solubilizing agent were purchased from Sigma-Aldrich. Breast cancer cell lines, MCF-7 and MDA-MB-231 were procured from the National Centre for Cell Science (NCCS, Pune, India).

B. Cell culture conditions

A monolayer of both MCF-7 and MDA-MB-231 cell lines were routinely grown using DMEM supplemented with 10% FBS, 1% antibiotic antifungal solution (final concentration: 100 units/mL penicillin, 0.1 μg/mL streptomycin, 2.5 ng/mL amphotericin B) and 10 nM 17β-estradiol at 37°C out of a humidified atmosphere of 5% CO₂. This is referred as the maintenance medium. The cells were sub-cultured after attaining 70-80% confluency (every 3 days) by trypsinization using 0.05% trypsin-EDTA solution. The cells were subsequently re-seeded at a 1:20 split ratio.

For the experiments, cells were grown in DMEM without phenol red; supplemented with NEAA, penicillin, streptomycin and amphotericin B as specified above and with 5% charcoal-dextran stripped FBS. Henceforth, this is referred as the experimental medium.

C. Preparation of stocks and standards

The positive control used was E2. Stock solution of each pharmaceutical was prepared by dissolving it in 100% ethanol. Standards of varying concentration were prepared by dilution with estrogen free culture medium containing 0.1% ethanol. The concentration of solvent that does not adversely affect the assay response was predetermined by performing experiments with solvent controls. In studies with the test pharmaceuticals, the solvent concentration was kept below the limit identified.

To ensure that a positive agonist response reflects an endocrine receptor (ER)-mediated activity, the test pharmaceutical was re-tested in the presence of fulvestrant (ER antagonist). Triplicate measurements were performed for each test substance.

<table>
<thead>
<tr>
<th>Table 1: Selected Concentration Range of Pharmaceuticals</th>
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<tbody>
<tr>
<td>Pharmaceuticals</td>
</tr>
<tr>
<td>17β-estradiol</td>
</tr>
<tr>
<td>17α-ethinyl estradiol</td>
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<tr>
<td>Acetaminophen</td>
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<tr>
<td>Diclofenac</td>
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<tr>
<td>Carbamazepine</td>
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<tr>
<td>Fulvestrant</td>
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</table>
**D. Cell proliferation assay (E-Screen)**

Before performing the E-Screen assay, MCF-7 cells were developed in the maintenance medium for 3 days and later preconditioned in the experimental medium for 3 days. For initiating the E-Screen assay, the cells were seeded into a 96 well plate at a concentration of 4000 cells/well (200 µL/well). A control containing only the medium was maintained for determining the background absorption value.

After a day, the seeding medium was aspirated out, the wells were washed twice with D-PBS (1 X) and refilled with 200 µL of the experimental medium supplemented with the test pharmaceutical at the desired concentration (Table 1). For every pharmaceutical, trials were led with the test chemical alone and with the test substance in combination with 10^{-11} M E2. For pharmaceuticals that demonstrated a proliferative impact, 10^{-7} M fulvestrant was added to affirm an estrogenic effect. Controls containing 0% and 0.1% ethanol, 10^{-11} M E2, and 10^{-7} M fulvestrant were also included. Every 48 h, the supernatant was removed and 200 µL of the experimental medium supplemented with the test pharmaceutical was replenished. Cell proliferation was determined after 6 days (late exponential stage). The bioassay was ended by removing the media from the wells and washing each well. The proliferation indices were measured after termination of the study.

The concentration of fulvestrant was selected based on screening studies where proliferation of MCF-7 cells grown in the presence of 10^{-11} M E2 was tested using fulvestrant concentration over the range 10^{-8} to 10^{-5} M. Potential cytotoxic effect of fulvestrant that could have been misinterpreted as anti-estrogenic effect on MCF-7 cells was further tested using the ER negative MDA-MB-231 cells. These cells were also maintained under similar conditions as the MCF-7 cells. Cell proliferation in presence of each pharmaceutical was tested using the MTT assay.

**MTT assay:**

Cell proliferation of estrogen responsive and estrogen non-responsive cell-lines (MCF-7 and MDA-MB-231, individually) were measured by the MTT assay [20, 21]. The medium from each well was removed precisely after incubation for 6 days. Each well was washed 2-3 times with DMEM without FBS and 100 µL fresh experimental medium and 10 µL MTT (5 mg/mL) was added to each well. The plate was incubated for 6-7 h. Subsequently, 100 µL of solubilizing reagent was added to each well, blended and left for 45 s. Presence of viable cells was visualized by the development of purple color due to formation of formazan crystals. Cell proliferation was quantified by measuring absorbance at 570 nm using an Elisa plate reader (Cole Parmer, Germany). Absorbance values lower than the controls indicate a reduction in the rate of cell proliferation and vice versa. Concentration of the test pharmaceutical causing 50% cell proliferative effect (EC_{50}) and that causing 50% inhibition of viability (IC_{50}) were determined graphically. %Response (Y) indicating cell proliferation in presence of the test chemical after blank correction and normalization of the absorbance values with respect to the negative control was determined (Eqn. 1).

\[
\% \text{Response (Y)} = \left[ \frac{(A_T - A_B)}{(A_C - A_B)} \right] \times 100 \quad \text{.........Eqn. 1}
\]

where, \(A_T\), \(A_B\) and \(A_C\) are the absorbance in presence of the test chemical (along with cells, media and solvent), blank (only media, no cells) and the negative control (cells, media and solvent), respectively.

A dose response profile (Y versus C) was prepared and the Hill equation (Eqn. 2, \(Y_{max}\) and \(Y_{min}\) correspond to the maximum and minimum response, respectively) was fitted by non-linear regression to obtain the EC_{50} (concentration yielding 50% response) and the Hill coefficient (\(\theta\)).

\[
Y = Y_{min} + \frac{(Y_{max} - Y_{min})}{1 + \left(\frac{C}{EC_{50}}\right)^\theta} \quad \text{.........Eqn. 2}
\]

This approach was used for determining EC_{50} of both the test chemical and the positive control. For test compounds that caused an antagonistic effect and reduced the estrogenic response on MCF-7 cells compared to that of E2, IC_{50} was determined instead of EC_{50}.

**Quantitative evaluation based on E-Screen assay:**

Proliferative behavior of a population is best estimated using the doubling time (\(t_d\)). Determining \(t_d\) requires measuring cell yields at several time points over the exponential proliferation phase, however, in the experiment design used, MCF-7 cell yields were measured only at 6 days, and hence, other measures of proliferative behavior were used. Estrogenic activity of pharmaceuticals was assessed by determining the relative proliferative effect (RPE) and the relative proliferative potency (RPP). The proliferative effect (PE) of a sample (Eqn. 3) is defined as the ratio of maximum absorbance obtained for the test chemical (\(A_{T,max}\)) to that obtained for the negative control (\(A_{C,max}\)). RPE compares maximum proliferation induced by the test chemical to that induced by estradiol (E2, positive control) and is determined based on PE of the test chemical (PE_{T}) and PE of E2 (PE_{E2}, Eqn. 4). Thus, high and low agonistic activity would be characterized by RPE values close to 100 and much lower than 100, respectively [16].
\[ PE = \frac{A_{T,\text{max}}}{A_{C,\text{max}}} \]  \hspace{1cm} \text{Eqn. 3}

\[ RPE = \left( \frac{P_{E2}}{P_{E2-1}} \right) \times 100 \]  \hspace{1cm} \text{Eqn. 4}

RPP also referred as estradiol equivalents (EEQ) was determined based on EC\textsubscript{50} of E2 (EC\textsubscript{50,E2}) and EC\textsubscript{50} of the test chemical (EC\textsubscript{50,T}) as shown in Eqn. 5.

\[ RPP = \frac{EC_{50,E2}}{EC_{50,T}} \]  \hspace{1cm} \text{Eqn. 5}

In case of antagonistic effect, EC\textsubscript{50} was replaced with IC\textsubscript{50} in Eqn 5.

### III Results and Discussion

The selection of pharmaceuticals for the study was done based on their prevalence in treatment plant effluents, frequency of prescription and published data regarding their bioavailability and excretion patterns. Four pharmaceuticals, belonging to various drug classes were selected for the study. EE2 was chosen as it is a synthetic estrogen and its endocrine disruption and persistence in water bodies is well documented [21]. Acetaminophen and diclofenac are reported to be two of the most frequency prescribed as well as sold over the counter drugs. Acetaminophen is reported to be frequently present in wastewater treatment plant effluents in India [1]. Carbamazepine is not frequently prescribed as it is an anti-epileptic. However, it is reported to be highly recalcitrant, non-biodegradable and persistent [22]. Additionally, it is also reported to be present in Indian wastewater effluents [1]. All the pharmaceuticals chosen have distinctly different biological action and are typically used to treat different illnesses. None of them were designed or branded to cause direct estrogen dependent responses, except for 17-α ethinylestradiol (EE2), which is used as a synthetic hormone and contraceptive.

Estradiol (E2) was found to stimulate the response of MCF-7 cells in a dose dependent manner over the concentration range 10\textsuperscript{-14} M to 10\textsuperscript{-7} M (Fig. 1). As expected, it caused negligible dose dependent response in ER negative MDA-MB-231 cells. The estrogenic potency of estradiol is well established since it is typically used as positive control. The maximum response of E2 (533%) was observed to occur at a concentration of 10 nM. The EC\textsubscript{50} and Hill coefficient based on this profile were 0.032±0.005 nM and 0.57±0.05, respectively. The error in parameter estimates are depicted as standard error (SE). Hill model provided an adequate fit to the data. Similarly, the Hill coefficient and EC\textsubscript{50} / IC\textsubscript{50} of all the test compounds were quantified by fitting the Hill equation to the dose response data (Table 2).

It was observed that EE2 also exhibited a proliferative effect similar to that of E2 (Fig. 2a), whereas, the antipyretic drug, acetaminophen showed a very weak proliferative effect on MCF-7 cells (Fig. 2b). The response elicited by EE2 was expected as it is a synthetically prepared hormone analog of E2. This pharmaceutical molecule is designed such that it can selectively bind to ER just as E2 does. In fact, there are some studies that suggest an increased estrogenic activity of EE2 relative to E2 owing to its increased hydrophilicity. A much higher concentration of acetaminophen was required to elicit a proliferative response compared to that of EE2. Thus, the EC\textsubscript{50} value of acetaminophen was higher compared to that of EE2, i.e., 0.098±0.014 mM and 0.393±0.006 nM, respectively. Further, it was found that EE2 showed a maximum response that was only 1.3 fold lower while acetaminophen showed maximum response 2 fold lower compared to E2. Thus, EE2 was inferred to have estrogenic activity comparable to E2 while acetaminophen was inferred to have only a weak estrogenic activity. This weak estrogenic effect of acetaminophen may be attributed to the presence of phenolic group. Many authors report that presence of hydroxylated aromatic groups or phenolic groups can increase selectivity to bind to ER [21, 23]. Both the pharmaceuticals had no effect on proliferation of MDA-MB-231 cells, confirming their estrogenic activity.

In the presence of the competitive inhibitor (10\textsuperscript{-8} M fulvestrant), the proliferative effect of EE2 on MCF-7 cells was found to be decreased. This confirmed that the proliferative response shown by EE2 (Fig. 2a) is indeed due to its ability to bind to ER and cause endocrine disruption. Although acetaminophen was found to be much less estrogenic compared to estradiol, the acetaminophen concentrations that could stimulate MCF-7 cell proliferation are reported to be pharmacologically relevant [4]. Additionally, as was observed in case of EE2,
fulvestrant blocked its proliferative effect as well, confirming that acetaminophen also elicits weak estrogenic effects. However, recent studies also indicate that acetaminophen exhibits weak estrogenicity through pathways triggered without ER binding [24]. Hence, E-Screen assay can be a suitable tool for screening estrogenic or anti-estrogenic compounds that have multiple modes of action. Traditional assays such as rat uterine microsomal ER binding assays can only be useful for compounds that illicit an endocrine disruptive effect by ER binding.

Interestingly, anti-proliferative effects were observed when cells were subjected to the NSAID, diclofenac and the anti-epileptic drug, carbamazepine in the concentration range over 10⁻⁸ mM to 1 mM and 1 μM to 196 μM, respectively (Fig. 3a-b). The IC₅₀ values of these pharmaceuticals are shown in Table 2. It was seen that both diclofenac and carbamazepine exhibited a marked decrease in cell proliferation with respect to the negative control.

Diclofenac showed up to 27 fold decrease and carbamazepine showed up to 30 fold decrease. Later, the assay was repeated by addition of E2 at its EC₅₀ concentration to verify if the estrogenic effect of E2 would be lowered by the test chemicals thereby confirming their anti-estrogenic effect. In the presence of 10 nM E2, an inhibitory effect of diclofenac on cell proliferation was observed (up to 146 fold decrease with respect to estradiol). Similarly, carbamazepine also showed a comparable inhibitory effect on cell proliferation (up to 160 fold decrease with respect to estradiol). From these results, diclofenac and carbamazepine are both demonstrated as potent anti-estrogens. These two pharmaceutical molecules also have multiple benzene rings in their structure, although they are not hydroxylated. Carbamazepine has an amide bond and diclofenac has a carboxylic acid functionality. This may promote binding to ER. However, since there are no phenolic groups, the anti-estrogenic effects of selected pharmaceuticals were identified by E-Screen assay and subsequently, the PE, RPE and RPP values were obtained. EE2 had efficacy equivalent to E2, giving a maximum RPE of 73±7% (Table 2). The concentration of E2 and EE2 yielding maximum response was determined as 10⁻⁸ nM and 10⁻⁷ nM, respectively. Thus, the agonist, EE2, depicted potency comparable to E2 (RPP=0.82) indicating its potent estrogenic nature (Table 2). The partial agonist, acetaminophen, showed low proliferative effect and low potency with RPE and RPP of 20±2% and 0.32 x10⁻⁶, respectively (Table 2).

Irrespective of the concentration range used, none of the pharmaceuticals exhibited any effect (proliferative or non-proliferative) on the ER negative MDA-231 cells. This conclusively confirmed that the effects shown by the pharmaceuticals are solely estrogen dependent responses.

The estrogenicity and anti-estrogenicity of selected pharmaceuticals were identified by E-Screen assay and subsequently, the PE, RPE and RPP values were obtained. EE2 had efficacy equivalent to E2, giving a maximum RPE of 73±7% (Table 2). The concentration of E2 and EE2 yielding maximum response was determined as 10⁻⁸ nM and 10⁻⁷ nM, respectively. Thus, the agonist, EE2, depicted potency comparable to E2 (RPP=0.82) indicating its potent estrogenic nature (Table 2). The partial agonist, acetaminophen, showed low proliferative effect and low potency with RPE and RPP of 20±2% and 0.32 x10⁻⁶, respectively (Table 2).

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<table>
<thead>
<tr>
<th>Pharmaceuticals</th>
<th>PE</th>
<th>RPE (%)</th>
<th>EC₅₀</th>
<th>RPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>8.7±0.7</td>
<td>100</td>
<td>0.032±0.005 nM</td>
<td>1</td>
</tr>
<tr>
<td>EE2</td>
<td>6.6±0.4</td>
<td>73±7</td>
<td>0.039±0.006 nM</td>
<td>0.82</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>2.5±0.1</td>
<td>20±2</td>
<td>0.098±0.014 mM</td>
<td>0.32 x10⁻⁶</td>
</tr>
<tr>
<td>Pharmaceuticals</td>
<td>PE</td>
<td>RPE (%)</td>
<td>IC₅₀</td>
<td>RPP</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>1.0±0.1</td>
<td>0.57±0.06</td>
<td>0.016±0.074 mM</td>
<td>1.9 x10⁻⁶</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>1.1±0.1</td>
<td>0.77±0.08</td>
<td>47.4±16.7 μM</td>
<td>6.8 x10⁻⁷</td>
</tr>
</tbody>
</table>

Uncertainty in PE and RPE are propagated SD based on triplicate experiments; and that for EC₅₀ is SE based on nonlinear regression.
complex mixtures consisting of parent compounds and their by-products, whose potential endocrine disrupting nature is so far unidentified [12, 25]. The current study is a first step towards understanding the effects of the various pharmaceuticals on ER dependent endocrine disruption effects.

IV Conclusion

Since pharmaceuticals are routinely being discharged into the aquatic compartment, it is important to know their effects on organisms in the ecosystem. This work illustrates that pharmaceuticals may act both as agonists and antagonists and quantitatively demonstrates their estrogenic effects with respect to estradiol using the E-Screen assay. Of the various pharmaceuticals selected for the study, it was observed that EE2 acted as an estrogen mimic and could elicit estrogenic effects as potent as its natural counterpart, E2. In contrast, although acetaminophen showed an enhanced proliferative effect, it was only weakly estrogenic. Diclofenac and carbamazepine were found to be anti-estrogenic in nature and could even suppress the estrogenic effect of estradiol when added simultaneously.

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