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The quantification of pharmaceutical related biological activity in effluents from wastewater treatment plants in UK and Japan

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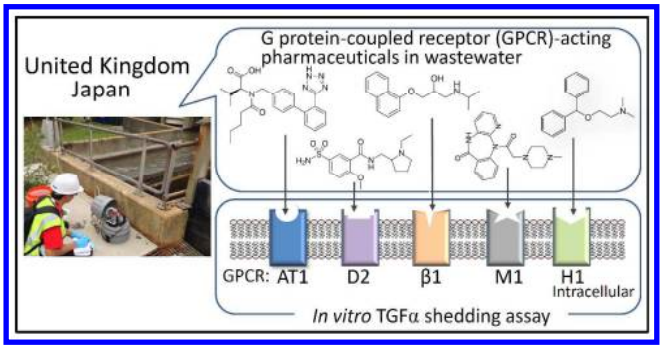
KEYWORDS

Antagonist, G protein-coupled receptor, biological activity of pharmaceutical, TGFα shedding assay, wastewater

ABSTRACT

Whilst pharmaceuticals are now routinely detected in aquatic environments, we know little of the biological activity their presence might provoke. It is estimated that nearly 40% of all marketed pharmaceuticals are G protein-coupled receptors (GPCRs)-acting pharmaceuticals. Here, we applied an *in vitro* assay, called the TGFα shedding assay, to measure the biological activities of GPCRs-acting pharmaceuticals present in effluents from municipal wastewater treatment plants in the United Kingdom (UK) and Japan from 2014 to 2016. The results indicated that compounds were present in the wastewater with antagonistic activities against angiotensin (AT1), dopamine (D2), adrenergic (β1), acetylcholine (M1) and histamine (H1) receptors in both countries. The most consistent and powerful antagonistic activity was against the H1, D2, and AT1 receptors at up to μg-antagonist-equivalent quantity/L. Chemical analysis of the same UK samples were also conducted in parallel. Comparing the results of the bioassay with the chemical analysis indicated; 1) the existence of other D2 or M1 receptor antagonist(s) besides sulpiride (D2 antagonist) or pirenzepine (M1 antagonist) in wastewater; and 2) there might be a mixture effect between agonist and antagonistic activities against β1 receptor. GPCR-acting pharmaceuticals should be paid more attention in the environmental monitoring and toxicity testing in future studies.

TOC Art of the present manuscript



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INTRODUCTION

Pharmaceuticals have been widely detected in effluents from wastewater treatment plants (WWTPs) and river water.[1–7] Because of their biological activity, concerns about their potential risks to aquatic organisms have been raised.[8–12] For protecting water ecosystems, effect-based *in vitro* assays have been increasingly used for water quality monitoring. For example, in the EU SOLUTIONS project, a suite of *in vitro* assays, which represent different cellular toxicity pathway including nuclear hormone receptors mediated effects (e.g. estrogen (ER), androgen (AR), progesterone (PR), glucocorticoid (GR), or thyroid (TR) receptor reporter gene assay), xenobiotic metabolism, mutagenicity, genotoxicity, oxidative stress, and cell viability, was applied.[13–16] These *in vitro* assays can provide useful information for the assessment of the mixture of hazardous chemicals present in the aquatic environment. However, until now, cellular toxicity pathway via G protein-coupled receptors (GPCRs) have not been considered in water quality monitoring. GPCR is the largest group of cell surface receptors, and participate in various physiological and pathophysiological processes. It is estimated that nearly 40% of all marketed pharmaceuticals act by binding to GPCRs.[17, 18]

In 2012, the *in vitro* transforming growth factor-α (TGFα) shedding assay, which is a high-throughput and sensitive assay to detect both agonism and antagonism of GPCRs, was developed.[19] So far, we have demonstrated that the TGFα shedding assay is useful to detect biological activity of GPCR-acting pharmaceuticals in wastewater.[20] Secondary effluent (SE) of WWTPs in Japan were extracted by the solid-phase extraction (SPE), and applied to the assay. As a result, antagonistic activities of several classes of GPCR-acting pharmaceuticals against angiotensin (AT1), dopamine (D2), adrenergic family members (β1), muscarinic acetylcholine (M1), and histamine (H1) receptors were detected for the first time.[20] However, so far, only our research group have applied the TGFα shedding assay to environmental waters; the situation in other countries remains unclear.

Contamination of wastewater with GPCR-acting pharmaceuticals is probably more serious in developed countries than in developing countries because 1) in general, the higher the country's gross domestic product, the higher the health expenditure including the cost of pharmaceuticals[21, 22]; 2) some classes of GPCR-acting pharmaceuticals (e.g., antagonists against AT1 or β1 receptors) are used to treat ageing-related and chronic disease such as hypertensive[23]; and 3) the percentage of elderly population in developed countries (e.g., Japan, Europe, and North America) are higher than those in developing countries (e.g., Africa and Latin America).[24] Pharmaceuticals which target other GPCRs are also expected to be

consumed more in developed countries than in developing countries. For example, antagonists against D2 receptor (e.g., antipsychotics) are used to treat schizophrenia[23], depressive disorders and dementia.[25] Antagonists against H1 receptor (e.g., antihistamines) are preliminary used to treat immunoglobulin E (IgE) immediate allergies.[23]

In this study, we aimed to investigate whether biological activities of GPCR-acting pharmaceuticals against AT1, D2, β1, M1, and H1 receptors could be detected by the TGFα shedding assay in wastewater in another developed country besides Japan. So far, our research group has investigated the occurrence of micropollutants in wastewater in the UK by chemical analysis[26, 27], and has established a system and facilities to conduct field surveys there. This is why we selected the UK as a research field in this study. To achieve the objective of this study, we conducted three experiments:

1) Detect and quantify agonistic and antagonistic activities against AT1, D2, β1, M1, and H1 receptors in effluent extracts from two UK activated sludge plants over the period 2014-16. As a reference, we also detect and quantify the activities in effluent from an activated sludge plant in Japan in 2015-16.

2) Determine to what extent sulpiride (a D2 receptor antagonist) and pirenzepine (an M1 receptor antagonist) can explain the antagonistic activities at the D2 and M1 receptors, respectively

3) Determine to what extent propranolol, metoprolol and atenolol (antagonists for β1 receptor) can jointly explain the antagonistic activities at β1 receptor

Based on the activity of known agonist and corresponding antagonistic pharmaceuticals, activity detected in the effluent extracts were quantified as agonist or antagonist equivalent quantities (EQs), respectively. For antagonistic activity, valsartan (an antagonist for AT1 receptor), sulpiride, propranolol, pirenzepine, and diphenhydramine (an antagonist for H1 receptor) were used as reference pharmaceuticals for each GPCR, i.e., valsartan-EQ for AT1, sulpiride-EQ for D2, propranolol-EQ for β1, pirenzepine-EQ for M1, and diphenhydramine-EQ for H1 receptors, respectively.

In parallel to the TGFα shedding assay, concentrations of sulpiride, pirenzepine, and metoprolol, atenolol and propranolol (β-blockers) in effluents in UK were measured by chemical analysis. Thus, we determined to what extent these known pharmaceuticals could explain the antagonistic activities for D2, M1 and β1 receptors, respectively.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study are described in Supporting Information (SI) Methods S1.

Sampling and sample treatment for biological and chemical analyses

Sampling of WWTP effluents in UK was conducted as a part of field survey for the occurrence of pharmaceuticals and personal care products (PPCPs) in river basin and WWTPs in UK.[28, 29] Final effluent samples were collected from two municipal WWTPs in UK from 2014 to 2016 (SI Table S1, Samples ID1–4 and 5–8 from UK1 and UK2, respectively). Both WWTPs use activated sludge as secondary treatment, whilst UK2 uses sand filtration as a tertiary treatment. Effluent from final settling tanks after activated sludge process (secondary effluent, SE) from one municipal WWTP in Japan was also collected from 2015 to 2016 (Samples ID9–12 from JPN1). The characteristics of each WWTP are also summarized in SI Table S1.

For biological analysis, a total 3 L of each sample was collected in amber glass bottles, to which 1 g/L ascorbic acid was added as preservative. After collection, UK samples (ID1–8), and Japan samples (ID9–12) were transported to the laboratory in Centre for Ecology and Hydrology in UK or Kyoto University in Japan, respectively. All the samples were filtered and extracted within 24 h. The samples were stored at 4 °C before filtration.

Samples for the TGFα shedding assay were extracted by SPE as previously described (SI Methods S2).[30] These effluent extracts were serially diluted, and then applied to the TGFα shedding assay. The concentrations of effluent extracts during cell exposure were defined in terms of the relative enrichment factor (REF: the ratio of the enrichment factor (from the SPE step) to the dilution factor of the effluent extracts in the TGFα shedding assay). The Milli-Q water was also extracted by SPE in parallel as a blank control both in the laboratories in UK and Japan, which we confirmed to have no agonistic or antagonistic activity by the TGFα shedding assay.

Selection of GPCRs

We selected AT1, D2, β1, M1, and H1 receptors (Table 1), because strong antagonistic activities against these receptors were detected in effluent from WWTPs in Japan in our previous study.[20] We also selected a number of receptors in the same classes as these (D4, β3, M3, and H2), in order to compare the receptor specificity of the biological activity of the effluent extracts.

Agonists and antagonists used in this study

For each GPCR, known agonists and corresponding antagonists were used as positive controls for the bioassays, and as reference compounds for activity quantification (Table 1 and SI Methods S1). The activity of all the tested agonists and antagonists for AT1, D2, β1, M1 and H1 receptors had already been quantified by the TGFα shedding assay in our previous study.[20] In this study, agonist tests were repeated for each agonist.

In our previous study, olmesartan medoxomil (OM) was used as the standard antagonistic pharmaceutical against AT1 receptor to represent the antagonistic activity against AT1 receptor in effluent extracts.[20] However, in this study, valsartan was used as a standard instead of OM. Because OM is a pro-drug for olmesartan, its active form, OM is not appropriate as a standard.

Some antagonists were applied to multiple receptors belonging to the same class (D4, β3, M3, and H2) to confirm that the TGFα shedding assay could detect the specificity of receptor-antagonist binding affinities as previously described.[20]

Table 1. GPCRs and standard chemicals used in this study, and their EC_{50}, EC_{20}, IC_{50}, IC_{20}, and relative potency values

Receptor class	Receptor name	Agonist used [abbr.]	$EC_{50(agonist)}$ (M)	$EC_{20(agonist)}$ [a] (M)	Antagonist used [abbr.]	$IC_{50(antagonist)}$ (M)	$IC_{20(antagonist)}$ [a] (M)
Angiotensin II	AT1	Angiotensin II [ANG II]	3.4×10^{-10}	8.2×10^{-11}	Valsartan [VAL]	2.9×10^{-9}	7.2×10^{-10}
Dopamine	D2	Dopamine [DA]	6.7×10^{-9}	1.8×10^{-9}	Sulpiride [SUL]	1.9×10^{-7}	4.4×10^{-8}
	D4		1.6×10^{-8} [b]			6.8×10^{-6}	
Adrenoceptor	β1	Isoproterenol [ISO]	3.2×10^{-8}	8.1×10^{-9}	Propranolol [PRO]	8.1×10^{-9} (RP = 1.0 [c])	2.1×10^{-9}
					Metoprolol [MET]	6.4×10^{-8} (RP = 1.3×10^{-1} [c])	
					Atenolol [ATE]	4.2×10^{-7} (RP = 2.0×10^{-2} [c])	
	β3		2.9×10^{-6} [b]		PRO	2.5×10^{-6}	
Acetylcholine	M1	Acetylcholine [ACh]	4.4×10^{-8}	1.2×10^{-8}	Pirenzepine [PIR]	2.6×10^{-8}	6.5×10^{-9}
	M3		5.4×10^{-9} [b]			2.0×10^{-6}	
Histamine	H1	Histamine [HIS]	1.2×10^{-8}	3.2×10^{-9}	Diphenhydramine [DIP]	2.5×10^{-7}	5.5×10^{-8}
	H2		8.1×10^{-8} [b]			$> 10^{-5}$ [d]	

a: $EC_{20(agonist)}$ and $IC_{20(antagonist)}$ of reference compounds only for AT1, D2, β1, M1, and H1 receptors are shown here, which were used to calculate agonist equivalent quantities (EQs) or antagonist EQs of wastewater extracts.

b: Data was cited from our previous study.[20]

c: Relative potency (RP) = $IC_{50(propranolol)}$ / $IC_{50(propranolol,\ metoprolol\ or\ atenolol)}$.

d: Inhibition of AP-TGFα release was not observed at the test concentration.

In vitro TGFα shedding assay

The principle of the TGFα shedding assay for agonistic activity is agonist-induced accumulation of alkaline phosphatase-tagged TGFα (AP-TGFα), a reporter enzyme, in the media harvested from cultured cells (i.e., conditioned medium (CM)). The TGFα shedding assay was conducted as previously described[19, 20] with slight modifications (SI Methods S3). Briefly, GPCR-expressing plasmid was transiently transfected into a cultured cell line (HEK 293 cells). By selecting the GPCR expression plasmid in cells, we can measure agonistic and antagonistic activities against each GPCR. Transfected cells were reseeded in a 96-well plate,

and then exposed to a reference compound or effluent extract 1 h. Accumulation of AP-TGFα in the CM (AP-TGFα release (%)) was calculated, and then normalized to the maximum activity of the reference agonist (SI Methods S4 and Figure S1A and B). Dose–response data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Then, agonistic effects of the effluent extracts were determined as an agonist equivalent quantities (EQ) (SI Methods S5 and Figure S2A and B). When the AP-TGFα release from a given effluent extract reached >20% of the maximum AP-TGFα release induced by the corresponding agonist (e.g., ANG II for AT1 receptor), it was defined as 'detected'.

For antagonistic activity, cells were pretreated with the test antagonist or effluent extract 5 min before stimulation with a known agonist corresponding to the tested GPCR. Concentrations of corresponding agonists (angiotensin II for AT1, dopamine for D2, isoproterenol for β1, acetylcholine for M1, and histamine for H1 receptors) are equal to the concentrations that induce more than 80% activation of each receptor (i.e., EC_{80}). If antagonistic pharmaceuticals are present in the effluent extracts, agonist-induced AP-TGFα release decrease. Accumulation of AP-TGFα in the CM (AP-TGFα release (%)) was calculated, and then normalized to the maximum activity of the reference agonist (SI Methods S4 and Figure S1C and D). The antagonistic effects of the effluent extracts were determined as an antagonist EQ (SI Methods S5 and Figure S2C and D). When agonist-induced AP-TGFα release was inhibited by a given effluent extract by >20%, it was defined as 'detected'.

All assays were performed in triplicate for all GPCRs. In the case of GPCRs for which agonist and/or antagonistic activity was detected in wastewater extracts, assays were performed at least twice, and total 6–9 data sets were obtained.

Before being analyzed for agonistic and antagonistic activity, the dilution range of effluent extracts in which GPCR-acting pharmaceuticals in effluent extracts show the specific interaction with a GPCR was determined in mock transfection condition test (SI Methods S6). The cytotoxicity of each effluent extract was analyzed by the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Japan).[31] Based on the results, we conducted the TGFα shedding assay on effluent extracts with a maximum REF value of 63.2 (ID1–3, 6, 7, and 9–12) or 20 (ID4, 5, and 8) (SI Figure S3). We confirmed that the Milli-Q water extract showed neither activity under mock transfection conditions nor cytotoxicity at all dilutions (data not shown).

Data presentation for *in vitro* assay

EC_{20}, IC_{20}, agonist EQ, and antagonist EQ of wastewater extracts were calculated using the linear concentration-effect curves approach as previously described[14, 32–34] with slight modification (SI Methods S5 and Figure S2). Briefly, the linear part of the concentration–effect curves was used to determine the EC_{20} value of each effluent extract ($EC_{20(extract)}$: the REF that gave a 20% activation) and IC_{20} value of each effluent extract ($IC_{20(extract)}$: the REF that gave a 20% reduction of agonist-induced AP-TGFα release) (SI Figure S2). The EC_{20} value of the corresponding agonist ($EC_{20(agonist)}$) and the IC_{20} value of the corresponding antagonist ($IC_{20(antagonist)}$) were determined from the dose–response curves of corresponding agonists and antagonists (SI Figure S4). The agonist EQ (ng-agonist-EQ/L) for each GPCR was then determined as $EC_{20(agonist)}/EC_{20(extract)}$. Similarly, antagonist EQ (ng-antagonist-EQ/L) was determined as $IC_{20(antagonist)}/IC_{20(extract)}$. For each GPCR, the limit of detection (LOD) for agonist EQ and antagonist EQ were determined based on the $EC_{20(agonist)}$ and $IC_{20(antagonist)}$, respectively (SI Methods S5).

Calculation of relative potency value of propranolol, metoprolol and atenolol, and predicted propranolol-EQs values

For β1 receptor, we measured and compared biological activities of three β-blockers, metoprolol, atenolol, and propranolol, by the TGFα shedding assay. Propranolol showed the highest activity among these three β-blockers (see Results and Discussion). Therefore, propranolol was used as a reference pharmaceutical to calculate antagonist EQ of effluent extracts for β1 receptor in the TGFα shedding assay (i.e., propranolol-EQ). Relative potency (RP) values of propranolol, metoprolol and atenolol were determined as $IC_{50(propranolol)}$ / $IC_{50(propranolol,\ metoprolol\ or\ atenolol)}$. Predicted propranolol-EQs of effluent extracts were calculated based on the concentration addition model from the molar concentrations (mol/L) of propranolol, metoprolol, and atenolol by chemical analysis, and their RP values (SI Methods S7).

Recovery of antagonistic activities during solid-phase extraction

Before applying the TGFα shedding assay to wastewater extracts, recovery rates of activity of reference GPCR-acting pharmaceuticals for AT1, D2, β1, M1, and H1 receptors during the SPE procedure for the TGFα shedding assay were investigated. We tested the recovery of activities of valsartan, sulpiride, propranolol, pirenzepine and diphenhydramine by spike testing (SI Methods S8).

Chemical analysis of pharmaceuticals

Six UK samples (ID1–4, 7, and 8) were collected for chemical analysis in parallel with the samples for the TGFα shedding assay, and extracted by the SPE procedure. These sampling were conducted as a part of field survey[28, 29], where the concentrations of 53 PPCPs in river basin and WWTPs in UK were measured by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS/MS) and quantified using the recovery of corresponding or representative surrogate internal standard as previously described.[35] Concentration data of sulpiride, pirenzepine, propranolol, metoprolol and atenolol are shown in our previous study.[28] We used these concentration data in this present study. Thus, the sulpiride-EQ, pirenzepine-EQs, and propranolol-EQ measured by the TGFα shedding assay were compared with concentrations of sulpiride, pirenzepine, and three β-blockers to determine to what extent these known pharmaceuticals could explain the antagonistic activities for D2, M1 and β1 receptors, respectively.

Statistical analysis

The significance of the difference of antagonistic EQs measured by the TGFα shedding assay between UK1 and JPN1 WWTPs, and UK2 and JPN1 WWTPs were assessed by *t*-test, respectively, using GraphPad Prism 5 software.

RESULTS AND DISCUSSION

Activity of known agonists and antagonists

The concentration–response curves of reference agonist are shown in SI Figure S4 (Agonist). The $EC_{50(agonist)}$ and $EC_{20(agonist)}$ values were calculated from these curves (Table 1), and used to calculate the agonist EQs of the effluent extracts. Similarly, the concentration–response curves of reference antagonist are shown in SI Figure S4 (Antagonist). The $IC_{50(antagonist)}$ and $IC_{20(antagonist)}$ values were calculated from these curves (Table 1), and used to calculate the antagonist EQs of the effluent extracts.

As for the β1 receptor, antagonistic activities of three β-blockers, propranolol, metoprolol, and atenolol, were analyzed by the TGFα shedding assay (SI Figure S4, Antagonist, β1). The most potent was found to be propranolol (Table 1, IC_{50} value: 8.1×10^{-9} M) followed by metoprolol (IC_{50} value: 6.4×10^{-8} M) and atenolol (IC_{50} value: 4.2×10^{-7} M). This trend is consistent with previous studies showing the binding affinity of β-blockers to β1 receptor.[36, 37] Therefore, propranolol was used as a standard antagonistic pharmaceutical in this study. Relative potency values of propranolol, metoprolol, and atenolol to propranolol are calculated

to be 1.0, 1.3×10^{-1}, and 2.0×10^{-2}, respectively (Table 1).

Some antagonists were applied to multiple receptors belonging to the same class (SI Figure S4, D4, β3, M3, and H2). For example, diphenhydramine was applied to H1 and H2 receptors. The results show that the TGFα shedding assay could detect the specificity of receptor-antagonist binding affinities as previously described.[20]

Recovery rates of antagonistic activity by the SPE cartridge

Recovery rates of antagonistic activity against each GPCR are shown in SI Figure S5. The recovery of all the tested pharmaceuticals in the Milli-Q water was higher than 70% (SI Figure S5A and B, Milli-Q). Recoveries of antagonistic activity of valsartan, propranolol, and diphenhydramine in SE were 77, 70, and 72%, respectively (SI Figure S5A, SE). These results indicate that recoveries of antagonistic activities against AT1, β1, and H1 receptors during the SPE procedure used for the TGFα shedding assay are acceptable.[38] Therefore, in this study, antagonist EQs for these receptors measured by the TGFα shedding assay were not corrected for their activity recoveries. Propranolol-EQs measured by the assay were directly compared with the predicted propranolol-EQs based on the concentrations of propranolol, metoprolol, and atenolol measured by chemical analysis (see below).

For the D2 receptor, when 5.0×10^{4} of sulpiride were spiked into effluent, recovery was only 42%, however, it was improved to 89% when the spiked concentration was reduced to be 5.0×10^{2} ng/L (SI Figure S5B, sulpiride). Similarly, for M1 receptor, the recovery of activity was only 45% when 2.0×10^{4} of pirenzepine were spiked into effluent, however it was improved to be 82% when the spiked concentration was reduced to be 2.0×10^{2} ng/L (SI Figure S5B, pirenzepine). These results indicate that for D2 and M1 receptors, at a few hundred ng-antagonist-EQ/L, recovery of antagonistic activities during the SPE procedure is acceptable[38], and sulpiride-EQs or pirenzepine-EQs measured by the assay are directly comparable to the concentrations of sulpiride or pirenzepine by chemical analysis, respectively.

Agonistic and antagonistic activities found in the effluent extracts

For all the effluent samples, the concentration–response curves of agonistic activity, and the concentration–inhibition curves of antagonistic activity were obtained from the results of the TGFα shedding assay (SI Figures S6, S7, and S8 for effluent extracts from UK1, UK2, and JPN1 WWTPs, respectively). The linear form of the concentration–effect curves was used to determine EC_{20} and IC_{20} values for each wastewater extract (SI Figures S9 and S10 for UK1, Figures S11 and S12 for UK2, and Figures S13 and S14 for JPN1). The Milli-Q water extract

showed no response with all the tested GPCRs (data not shown), which demonstrates that all the agonistic and antagonistic activity was wastewater-specific.

Agonistic activities were detected only with the D2, β1, and M1 receptors in the effluent extract from UK2 WWTP in September 2014 (SI Figure S11, ID6, H) and August 2015 (ID8, Q–S). In other samples, agonistic activities were lower than LOD with tested GPCRs (SI Figures S9, S11, and S13).

In the antagonistic test, effluent extracts from UK1 WWTP (ID1–4) showed the inhibition of agonist-induced AP-TGFα release with all tested GPCRs frequently through the sampling campaign (SI Figure S10). Effluent extracts from UK2 WWTP (ID5–8) also frequently showed antagonistic activities against AT1, D2, β1, and H1 receptors, but only one occasion for M1 (SI Figure S12). Effluent extracts from JPN1 WWTP (ID9–12) also showed antagonistic activities against all GPCRs (SI Figure S14). Notably, antagonistic activities against AT1 and H1 receptors were strong in all the samples: IC_{20} values were lower than those for other receptors (SI Figure S14A, F, K, and P for AT1 receptor, and E, J, O, and T for H1 receptor).

We confirmed the receptor specificity of antagonistic activity detected in effluent extracts (SI Figure S15). For example, sample ID1 showed antagonistic activities against D2, β1, M1, and H1 receptors but no antagonistic activity was observed against receptors in the same class, which shared the same endogenous agonists (D4, β3, M3, and H2). The results show that antagonistic activities against AT1, D2, β1, M1 and H1 in Japan as well as UK samples were receptor specific. These results indicate that activities were attributable to highly selective GPCR-acting pharmaceuticals, but not to nonreceptor-mediated pathway, such as adsorption of the agonist by large organic molecules, as previously described.[20]

Agonist and Antagonist equivalents of effluent extracts

From the linear concentration–effect curves of agonistic activity of the effluent extract from UK2 WWTP in September 2014 and August 2015 (SI Figure S11, ID6 and 8), agonist EQ values were calculated: 19 ng-DA-EQ/L for D2 receptor, 43 and 1.3×10^2 ng-ISO-EQ/L for β1 receptor, and 1.2×10^2 ng-ACh-EQ/L for M1 receptor, respectively (SI Table S2).

From the linear concentration–effect curves of antagonistic activity (SI Figures S10, S12, and S14), antagonist EQ values were calculated for the effluent extracts (Figure 1, and SI Table S3). For AT1 receptor, valsartan-EQs in effluents from the JPN1 WWTP (Figure 1, AT1, 3.5×10^2–4.0×10^2 ng-VAL-EQ/L) were significantly higher than those in UK1 (36–1.1×10^2 ng-VAL-EQ/L) ($p < 0.001$, *t*-test) and UK2 WWTPs (22–1.9×10^2 ng-VAL-EQ/L)

(p = 0.0004, t-test). Similarly, for H1 receptor, diphenhydramine-EQs in the effluent from the JPN1 WWTP (4.1×10^3–5.8×10^3 ng-DIP-EQ/L) were significantly higher than those in the UK1 (1.7×10^3–2.0×10^3 ng-DIP-EQ/L) (p = 0.0003, t-test) and the UK2 WWTPs (8.1×10^2–1.5×10^3 ng-DIP-EQ/L) (p = 0.0001, t-test). For the D2 receptor, sulpiride-EQs were at similar levels among UK1 (4.8×10^2–1.5×10^3 ng-SUL-EQ/L), UK2 (4.9×10^2–1.2×10^3 ng-SUL-EQ/L), and JPN1 WWTPs (3.7×10^2–1.2×10^3 ng-SUL-EQ/L). Similarly, for β1 receptor, the propranolol-EQs were at similar levels among UK1 (41–62 ng-PRO-EQ/L), UK2 (40–47 ng-PRO-EQ/L), and JPN1 WWTPs (38–56 ng-PRO-EQ/L). For the M1 receptor, antagonistic activities were detected for all samples in JPN1 (89–2.5×10^2 ng-PIR-EQ/L) and for three samples in UK1 (53–80 ng-PIR-EQ/L), but detected in only one sample in UK2 (49 ng-PIR/L). For both the UK and Japan samples, the antagonist EQs for the H1 receptor had the highest activity among the five GPCRs tested in this study, followed by D2 and AT1, and then finally β1 and M1 receptors.

Agonistic activity was detected only in the UK2 WWTP in September 2014 (ID6) and August 2015 (ID8). In contrast, antagonistic activity was detected in many effluent extracts from WWTPs in both the UK and Japan against all GPCRs tested in this study. These greater detection frequencies of antagonistic activity than agonistic activity coincide well with the results in our previous study focusing on Japan.[20] This might be expected since most of the currently marketed GPCR-acting pharmaceuticals are antagonists[20] based on the information on the DrugBank online database. Mixture effects between the agonist and antagonistic activity also might play a part (see below next section).

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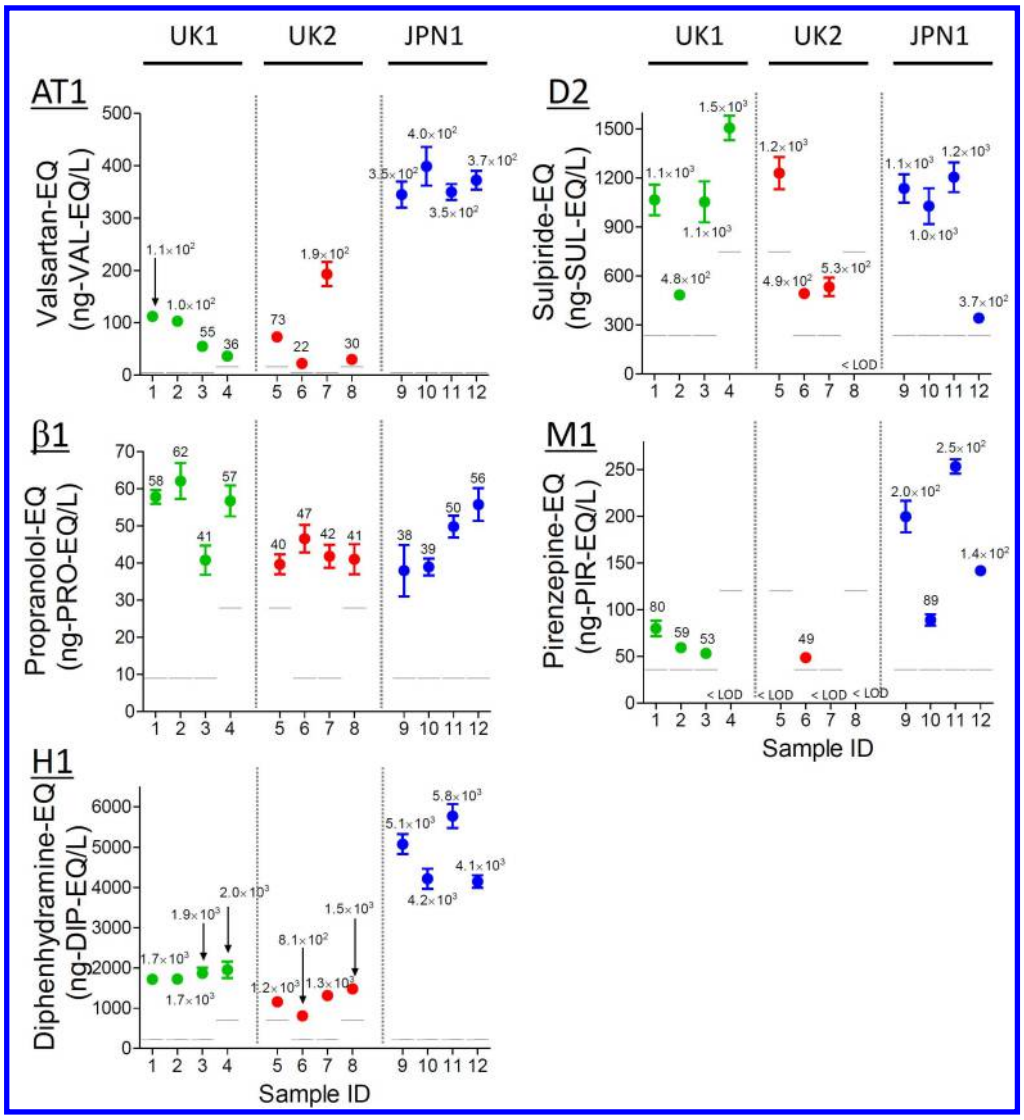

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356 **Figure 1. Summary of antagonistic activities of wastewater extracts.**

357 Plots represent mean ± SEM, n = 6. Lines are limit of detection (LOD) of activities. VAL: valsartan;
358 SUL: sulpiride; PRO: propranolol; PIR: pirenzepine; DIP: diphenhydramine.

359

360 **Comparison between antagonist equivalents derived from the bioassay and measured**
361 **concentrations of corresponding pharmaceuticals**

362 Concentrations of sulpiride, pirenzepine, propranolol, metoprolol and atenolol in UK samples

(ID1–4, 7, and 8) were measured by UPLC/MS/MS in parallel with the TGFα shedding assay. Concentration values are used from our previous study[28] (SI Table S4). Sulpiride-EQs in samples ID1–4, 7, and 8 measured by the TGFα shedding assay (4.8×10^2–1.5×10^3 ng-SUL-EQ/L) were at least 5 times higher than concentrations of actual sulpiride measured in these samples (15–1.2×10^2 ng/L) (Figure 2, D2). In addition, at thousands ng-SUL-EQ/L level, some parts of sulpiride-EQ might be loss during SPE process (SI Figure S5). Similarly, pirenzepine-EQs in samples ID1–3 measured by the assay (53–80 ng-PIR-EQ/L) were at least 10 times higher than concentrations of pirenzepine measured by chemical analysis in these samples (0.5, 6.1 and 3.8 ng/L) (Figure 2, M1). These results indicate that, at least two WWPTs in the UK investigated in this study, besides sulpiride or pirenzepine, other D2 or M1 antagonistic pharmaceuticals occur in wastewater (see below "Pharmaceuticals potentially responsible for the observed AT1, H1, D2, M1 and β1 receptors activity" section).

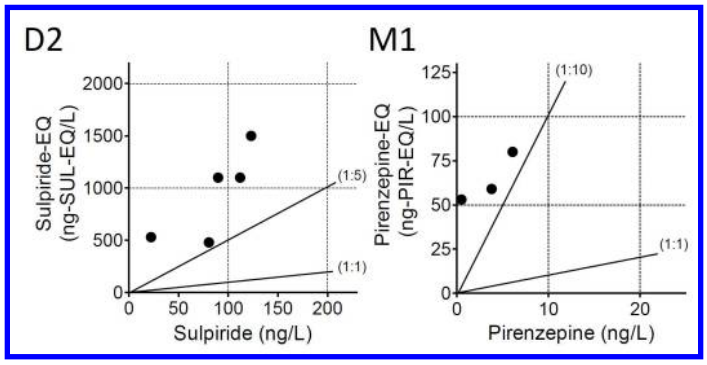

Figure 2. Comparison between sulpiride-EQs and concentration of sulpiride (antagonist for D2 receptor), and pirenzepine-EQs and concentration of pirenzepine (antagonist for M1 receptor).

Antagonistic activities against M1 receptor in samples ID4, 5, 7, and 8 were below LOD. Therefore, pirenzepine-EQs for samples ID1–3 are compared with concentration of pirenzepine. Lines are the ratios of the concentrations to the EQs. For example, sulpiride-EQs deviate upward from 1:5 line (D2), which indicates sulpiride-EQs are more than 5-time higher than concentration of sulpiride. SUL: sulpiride; PIR: pirenzepine.

Predicted propranolol-EQs for samples ID1–4, 7, and 8 based on the measured concentrations of propranolol, atenolol and metoprolol by chemical analysis were compared with the measured propranolol-EQs from the TGFα shedding assay (Figure 3). The

contribution of propranolol to predicted propranolol-EQs was dominant (gray bars), which indicate that, between the different putative β-blockers, propranolol was the most important in causing antagonistic activity against β1 receptor in wastewater in the UK. For samples ID1, 2, 4, and 8, measured propranolol-EQs were lower than the predicted propranolol-EQs. This might be due to the competition between agonist and antagonistic activity in these effluent extracts. In the case of endocrine disrupting chemicals, it has been demonstrated that estrogenic and antiestrogenic compounds compete for the estrogen receptor (ER) in wastewater, and, as a result, the observed estrogenic activity is less than the predicted activity.[30, 39] Similarly, agonist and antagonist compounds operating at the β1 receptor might compete with each other leading to the observed propranolol-EQ being less than predicted.

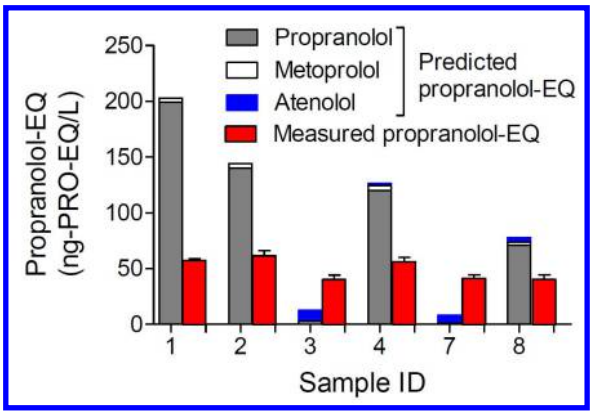

Figure 3. Comparison between predicted propranolol-EQs and measured propranolol-EQs.

Predicted propranolol-EQs of samples ID1–4, 7, and 8 were calculated based on the concentrations of propranolol, metoprolol, and atenolol in these samples (SI Table S4), and their relative potency (RP) values. Propranolol is not considered to calculate predicted propranolol-EQs for samples ID3 and 7, because the concentration data is not available for these samples. RP values of propranolol, metoprolol, and atenolol to propranolol are 1.0, 1.3×10^{-1}, and 2.0×10^{-2}, respectively. Measured propranolol-EQ values are from SI Table S3.

Comparison of biological activities of GPCR-acting pharmaceuticals in effluent extracts among WWTPs

The antagonistic activities against for all GPCRs were found at similar levels between UK1 and UK2 WWTPs (Figure 1). For D2 and β1 receptors, the antagonistic activities in JPN1 were also found at similar levels with UK1 and UK2 WWTPs (Figure 1, D2 and β1). On the

other hand, activities against AT1 and H1 receptors in JPN1 were significantly higher than those in UK1 and UK2 (Figure 1, AT1 and H1). The characteristic of individual WWTPs covered in this study, such as the type of influents (i.e., municipal wastewater), the population equivalent served, and the treatment efficiency, were comparable (SI Table S1). Therefore, the differences observed in the TGFα shedding assay might come from the different usage patterns of pharmaceuticals between the UK and Japan. For example, pharmaceuticals which target the AT1 receptor, antihypertensive, might be consumed more in Japan than UK because of the higher proportion of the population of elderly people (age ≥ 60) in Japan (33%) compared to that of the UK (24%).[24]

The higher activity against the H1 receptor found in JPN1 compared to those in UK1 and UK2 might be due to the sampling in the UK in different seasons (in summer and winter) from that in Japan (in spring). In spring, about 27% of Japanese people suffer from hay-fever, particularly with cedar pollinosis, and take H1 antagonists to treat its symptoms.[40, 41] If we took wastewater in the UK in spring, antagonistic activity against the H1 receptor in UK WWTPs might be as high as that in Japan.

Pharmaceuticals potentially responsible for the observed AT1, H1, D2, M1 and β1 receptors activity

So far, one AT1 receptor antagonist (valsartan), and three H1 antagonists (diphenhydramine, fexofenadine, and loratadine) have been detected in wastewater in the UK by chemical analysis.[42–45] In Japan, two AT1 receptor antagonists (losartan and candesartan)[46] and one H1 receptor antagonist (diphenhydramine)[46, 47] have been detected. Other AT1 receptor antagonists (e.g., olmesartan, irbesartan, telmisartan, and eprosartan)[48–51], and H1 receptor antagonists (e.g., cinnarizine, cetirizine, cyproheptadine, and loratadine)[52] have been detected in wastewater in other countries. Whilst in this study, the concentrations of these pharmaceuticals were not measured by chemical analysis, it is possible they were contributing to the antagonistic activities detected against the AT1 and H1 receptors.

Although two H2 antagonists, ranitidine and cimetidine, have been detected in wastewater in the UK by chemical analysis at hundred to thousand ng/L range in previous studies[42, 43, 45], H2 antagonistic activity was lower than LOD (3.0×10^2 ng-famotidine-EQ/L) in all samples tested for H2 activity here (SI Figure S15, H2). This gap might be due to differences in usage of pharmaceuticals in local catchment areas, differences in treatment efficiency of WWTP, low recovery of H2 antagonist during the SPE processing, and/or weak activity of H2 antagonists (SI Discussion S1).

Concentrations of sulpiride and pirenzepine could explain only small parts of sulpiride-EQs and pirenzepine-EQs detected in effluent extracts in the UK, respectively (Figure 2). Looking at the pharmaceutical consumption data in the UK in 2014 that is available from the National Health Service (NHS) online database[53], we can find many D2 receptor antagonists besides sulpiride such as quetiapine, amisulpiride, domperidone, chlorpromazine, promazine, metoclopramide, promethazine, and olanzapine. Similarly, for the M1 receptor, other than pirenzepine, we can find many antagonists such as quetiapine and olanzapine (also known as D2 antagonists), solifenacin, flavoxate, trospium, oxybutynin, disopyramide, and tolterodine. These antagonistic pharmaceuticals might also contribute to the sulpiride-EQs and pirenzepine-EQs as well. Of these D2 and M1 antagonists, quetiapine, amisulpride, and olanzapine have been detected by chemical analysis in wastewater in other countries.[51, 54, 55] However, other D2 and M1 antagonists have been overlooked and so far are not being measured by the chemical analysis. Attention should be paid to these pharmaceuticals for environmental monitoring in future studies.

Agonistic activity was detected only in the UK sample which was collected at UK2 WWTP in September 2014 (ID6) and August 2015 (ID8). Based on the pharmaceutical consumption data available from the NHS in the UK[53], levodopa and pilocarpine, which are agonistic pharmaceuticals against D2 and M1 receptors, respectively, are sold in the UK. These agonistic pharmaceuticals might contribute to the agonistic activity detected in the UK wastewater extracts.

Future research needs in environmental monitoring and toxicity testing

In this study, biological activity of GPCR-acting pharmaceuticals which act on AT1, D2, β1, M1, and H1 receptors were detected in wastewater in the UK by the TGFα shedding assay for the first time. Such activity is clearly not unique to wastewater in Japan. Further efforts to identify GPCR-acting pharmaceuticals responsible for the observed AT1, H1, D2, M1 and β1 receptors activity in wastewater will be needed in future studies. Looking at the pharmaceutical consumption data (e.g., NHS online database in the UK) is a useful means of identifying new targets.

In addition to the chemical concentration, knowledge of the activity (i.e., potency) of the individual chemicals is also required to be able to understand the adverse effects on aquatic organisms of GPCR-acting pharmaceuticals. Thus far, one AT1 antagonist (valsartan), six H1 antagonists (diphenhydramine, cyproheptadine, azelastine, ketotifen, oxatomide, and pyrilamine), one D2 antagonist (sulpiride), three β1 antagonists (propranolol, metoprolol, and

atenolol), and one M1 antagonist (pirenzepine) have been analyzed for the potency using by the TGFα shedding assay in this study or in our previous studies.[19, 20] However, other GPCR-acting pharmaceuticals have not. This should be a subject of future study.

Investigations of the mixture effect of GPCR-acting pharmaceuticals are also necessary to understand its adverse effects on aquatic organisms. The results of this study indicate that there might be a mixture effect between agonist and antagonistic activities against the β1 receptor. Similarly, the mixture effect could occur in other GPCRs in complex environmental samples.

SUPPORTING INFORMATION

Sampling information, summary of agonistic and antagonistic activities of effluent extracts, concentrations of antagonistic pharmaceuticals in effluents measured by chemical analysis, dose–response curves of known agonists and antagonistic pharmaceuticals, the results of mock transfection conditions experiments, dose–response curves of effluents from WWTPs in the UK and Japan, receptor specificity of effluents, methods for other experiments, and discussion about the absence of H2 receptor antagonistic activity in UK samples. This material is available free of charge at http://pubs.acs.org/.

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