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Endocrine disrupting compounds affecting corticosteroid signaling pathways in Czech and Swiss waters – potential impact on fish

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ABSTRACT

This study investigated the occurrence of corticosteroid signaling disruptors in wastewaters and rivers in the Czech Republic and in Switzerland. 36 target compounds were detected using HPLC-MS/MS, with up to 6.4 µg/L for azole antifungals that indirectly affect corticosteroid signaling. Glucocorticoid receptor (GR)-mediated activity was determined using the GR-CALUX bioassay with dexamethasone equivalent concentrations ranging from <LOD–2.6, 19–37, and 78–542 ng/L for river water, treated, and untreated wastewater, respectively. For most samples, the chemically predicted GR-mediated response was higher than that determined by the bioassay. Correspondingly, anti-glucocorticoid activity was observed in some fractions. The Fish Plasma Model (FPM), which predicts plasma concentrations, was applied to evaluate the potential of detected pharmaceuticals to cause receptor-mediated effects in fish. With one exception, medroxyprogesterone, the FPM applied to individual compounds predicted fish plasma concentrations to be below the level of human therapeutic plasma concentrations. To account for the activity of the sum of GR-active compounds, we introduce the “cortisol equivalents fish plasma concentration” approach, through which an increase in fish glucocorticoid plasma levels comparable to 0.9–83 ng/mL cortisol after exposure to the analyzed river waters was estimated. The results suggest that these chemicals may impact wild fish.

Introduction

The presence of endocrine disrupting compounds (EDCs) in the aquatic environment and their effects in aquatic organisms, especially the disruption of sex hormone signaling pathways, has been in the focus of ecotoxicologists for the past twenty years. Today, there is a growing concern that steroid hormones other than those directly related to reproduction could also pose a risk to aquatic organisms. These include natural and synthetic corticosteroids, i.e. glucocorticoids (GCs) and mineralocorticoids (MCs), and progestogens, which are frequently used as pharmaceuticals to treat a wide variety of conditions in human and veterinary medicine.

Corticosteroids are involved in the regulation of key physiological functions in vertebrates. GCs regulate energy metabolism, immune functions and stress response, while MCs are primarily involved in osmoregulation. The effects of these hormones are largely mediated by the glucocorticoid (GR) and the mineralocorticoid receptor (MR) that act as ligand-activated transcription factors and are well conserved among vertebrates.¹ In many vertebrates, including humans and fish, GR is specifically activated by the stress hormone cortisol, whereas MR is activated by aldosterone. MR can also be activated by cortisol, however, binding to MR is prevented by the 11 β -hydroxysteroid-dehydrogenase-2 (11 β -HSD2) that converts cortisol to cortisone in MR-expressing tissues.^{2,3} Corticosteroids are among the most frequently prescribed drugs, used in much greater amounts than estrogens or androgens.⁴ Synthetic glucocorticoids are used in the treatment of various inflammatory and immune diseases, including asthma, rheumatic disease, inflammatory bowel disease, allergies, and eye and skin diseases.^{2,5} MR-agonists are used in the treatment of adrenal insufficiency and MR-antagonists are applied to treat hypertension, excess urine protein excretion and heart failure.² Disturbed glucocorticoid action in humans has been associated with diseases including

osteoporosis, impaired development, obesity, type-2-diabetes and cardiovascular, inflammatory and autoimmune diseases.^{2,3}

The terms “glucocorticoid” and “mineralocorticoid” originate from mammalian studies. In teleost fish, the boundaries between glucocorticoid and mineralocorticoid action are less defined. Cortisol is an essential component of the stress response in fish, but also plays a significant role in osmoregulation via GR activation. MR appears to be involved in the brain dependent behavior, while its role in osmoregulation compared with GR-mediated signaling is minor.⁶ In various fish species, GCs were shown to modulate the expression of a number of genes involved in the innate immune system, the hypothalamic-pituitary-interrenal (HPI) axis, in glucose metabolism, and in cellular stress defense.⁷⁻⁹ The exposure to GCs can lead to growth reduction,⁷ immunosuppression,^{7,10} decreased regenerative capacity,¹¹ increased plasma glucose levels,¹² reduced fecundity,¹³ and fish masculinization.^{12,14,15} Many compounds can indirectly interfere with the corticosteroid signaling pathway by modulating gene expression and activity of enzymes involved in the production and metabolism of corticosteroids. For example, suppression of the steroidogenic acute regulatory gene (*StAR*) may affect steroidogenesis in general; 21-hydroxylase (CYP21) inhibition can lead to reduced cortisol synthesis and increased androgen synthesis; inhibition of 11 β -hydroxylase (CYP11B1) may block the last step of cortisol synthesis; disruption of 11 β -HSD1 and 11 β -HSD2, that convert cortisone to cortisol and *vice versa*, can cause hormonal imbalances.³ Additionally, other classes of steroid hormones, such as progestins, may act through GR/MR and/or modify the GR/MR gene expression, although their primary mode of action is through the progesterone receptor (PR).¹⁶⁻¹⁸ Finally, cross-talk with other nuclear receptors is possible, for example aryl-hydrocarbon receptor (AhR) activation might disrupt interrenal corticosteroidogenesis.^{19,20}

Even though the potential adverse effects are significant, little is known about the presence and effects of corticosteroid signaling disruptors in the environment. A limited number of studies have detected gluco- and mineralocorticoids in wastewater treatment plant (WWTP) influents and effluents, as well as in river waters.²¹⁻²⁵ Recently, it was documented that exposure to municipal wastewater effluents perturbs the functioning of the cortisol stress axis and evokes cellular stress response in fish *in vivo*.²⁶ The goal of our study was to estimate the potential of unintended long-term effects in fish related to the exposure to a complex mixture of environmentally present gluco- and mineralocorticoids, as well as non-steroidal compounds that could affect the corticosteroid signaling indirectly. Modes of action of target compounds with respect to the corticosteroid signaling pathway are listed in Table 1. First, an analytical method was developed in-house that allows a simultaneous quantification of a set of target compounds in wastewater and river water samples.²⁷ Further, GR-dependent activity of environmental samples and standard compounds was measured using the *in vitro* GR-CALUX assay in order to reveal compounds that bind to the GR and thereby directly interfere with GR signaling. Finally, the fish plasma model (FPM) was used to extrapolate the potential of unintended long-term effects of pharmaceuticals in fish *via* a comparison of human therapeutic plasma concentrations (HPC_T) with estimated fish steady-state concentrations (FPC_{SS}).²⁸ We furthermore extended the FPM in order to estimate the potential risk of the mixture of compounds acting through the GR by introducing the so-called “cortisol equivalents fish plasma concentration” (^{CEQ}FPC).

Experimental

Chemicals and solvents

Standard compounds were obtained from Sigma-Aldrich (Table 2) and used without further purification to prepare standards in ethanol or dimethylsulfoxide (DMSO), stored at -20°C. Organic solvents were of HPLC gradient grade purity (Acros or Scharlau).

Sampling

Samples of wastewater (WW) and river waters were collected in October and November 2011 in the Czech Republic and in Switzerland (Figure 1). Samples included untreated hospital WW, influents and effluents of WWTPs treating both municipal WW and hospital WW (composite samples), river samples upstream and downstream of WWTPs and affected by agriculture (grab samples). For more details see Supporting Information (SI).

Sample preparation and chemical analysis

See Ammann et al.²⁷ for details. Briefly, samples were divided into 4 replicates; 2 replicates, together with one blank (1L purified water), were spiked with 5 deuterated internal standards for chemical analysis, the other 2 were used in the bioassay. Samples of 0.5 L (untreated WW) and 1 L (treated WW, river water) were filtered through glass fibre filters (Whatman GF/F), pH adjusted to 6.50–6.85, and mixed-mode solid phase extraction (SPE) was performed. SPE cartridges were eluted with different solvent mixtures to obtain four fractions (F1–F4) with decreasing polarities: F1 contained slightly acidic compounds, F2 slightly basic compounds, F3 medium polar compounds and F4 lipophilic compounds. The total extract was obtained by pooling F1–F4. The samples were then analyzed by LC-MS/MS for chemical data.

GR-CALUX bioassay combined with viability determination

The GR-CALUX bioassay was obtained from BioDetection Systems (BDS, Amsterdam, NL) and performed as described elsewhere^{24,29} and complemented with a non-destructive assessment of cell viability using AlamarBlue and CFDA-AM dyes (Invitrogen, USA).³⁰ Cells at 10,000 cells/well were seeded into 96-well plates with DMEM/F-12 medium without phenol red, supplemented with DCC stripped fetal bovine serum (Invitrogen, USA). After 24h incubation (37°C, 5% CO₂), the medium was replaced by medium containing standard compounds (0.1% EtOH) or environmental samples (0.5% DMSO) in a concentration range providing dose-response curves. The exposure was done in triplicate, each plate contained a solvent control (0.1% EtOH or 0.5% DMSO) and the reference compound dexamethasone (Dex) in the concentration range of 3×10^{-11} to 10^{-7} M. After 24h exposure, the medium was removed and a solution of AlamarBlue and CFDA-AM in PBS added. After 30 min of incubation in the dark at room temperature, fluorescence was measured on the plate reader Infinite M200, Tecan, Switzerland (AlamarBlue exc/em: 530/595 nm, CFDA-AM exc/em: 493/541 nm) to determine cell viability.³⁰ The solution was then removed from the plate, cells were lysed with lysis buffer (Promega, USA) and luminescence was quantified on the luminometer (Berthold, Germany) after addition of luciferase substrate prepared according to standard protocols (BDS, Amsterdam, NL). The experiments were repeated at least three times for pure chemicals and twice for each environmental sample replicate and fraction. Anti-glucocorticoid activity of samples was determined in competition with Dex. IC₂₀ values were calculated from dose-response curves compared to the signal of the Dex competitor concentration of 6×10^{-10} M, which was considered as 100% response. Blank values were obtained by testing the extract of 1L Milli-Q water without IS, handled like a sample.

Data analysis

Standard chemicals were analyzed with the GR-CALUX assay in order to obtain relative potencies (REPs) related to the reference compound dexamethasone (Dex). In a similar way, GR-mediated activity was determined in environmental samples. Dexamethasone equivalents predicted from chemical analysis (^{CHEM}DEQ) and measured in the GR-CALUX (^{BIO}DEQ) were determined (see SI).

Fish plasma model (FPM)

The FPM compares the human therapeutic plasma concentration (HPC_T) with an estimated fish steady-state concentration (FPC_{SS}) of a given drug, assuming that drug targets are conserved across species.²⁸ The lowest effective human drug peak plasma concentrations (C_{max}) reported in literature were used as HPC_T .

The partitioning of a drug between the aqueous phase and the arterial blood in fish was calculated based on work by Fitzsimmons et al.³¹ (Eq. (1)). Additionally, we used a pH corrected partitioning coefficient ($\log D_{OW}$) to account for the pH-dependent ionization processes, which limit the reliability of $\log K_{OW}$ (Eq. (2), e.g. reviewed in Rand-Weaver et al.).³² The FPC_{SS} for a drug was estimated using a bioconcentration model assuming equilibrium partitioning, by multiplying the drug's blood:water distribution coefficient ($K_{Blood:Water}$) by the measured concentrations (c) (Eq. (3)), and effect ratios were calculated (ER; Eq. (4)). An $ER \leq 1$ indicates that the expected FPC_{SS} is equal to or greater than the C_{max} and thus receptor-mediated responses in fish and potential endocrine disrupting effects. C_{max} , $\log K_{OW}$ values estimated with EPISUITE,³³ and $\log D_{OW}$ values estimated with ACD/Labs³⁴ used in the FPM are provided in SI (Table S1).

$$\log K_{Blood:Water} = 0.73 * \log K_{OW} - 0.88 \quad (1)$$

$$\log K_{Blood:Water} = 0.73 * \log D_{OW} - 0.88 \quad (2)$$

$$FPC_{SS} = c * K_{Blood:Water} \quad (3)$$

$$ER = \frac{C_{max}}{FPC_{SS}} \quad (4)$$

In addition, we introduce the so-called “cortisol equivalents fish plasma concentration” (^{CEQ}FPC , Eq. (5)). The ^{CEQ}FPC links the FPC_{SS} of GR-agonists with their REP (this study), divided by the REP of cortisol. The resulting ^{CEQ}FPC provides an estimation of the potential increase of GR-agonists concentration in plasma relative to cortisol in fish after exposure to a complex mixture of GR-active compounds.

$$^{CEQ}FPC = \frac{\sum_{i=1}^n (FPC_{SSi} * REP_i)}{REP_{cortisol}} \quad (5)$$

Results and discussion

Endocrine disrupters assessed in this study were selected based on the mode of action with respect to the corticosteroid signaling pathway (Table 1). Only compounds approved for use in both countries were included in the list. In Switzerland, drug sale figures are treated as proprietary, but data on dispensation of human prescribed drugs could be obtained from the Czech Republic (SI, Table S2), with the MR-antagonist spironolactone, used to treat hypertension, excess urine protein excretion and heart failure,² ranking highest in amounts dispensed (831 kg in 2011). Prednisone had the highest dispensation among the glucocorticoids (110 kg), followed by methylprednisolone (39 kg) and hydrocortisone (36 kg). This compares well to the situation in the UK, where hydrocortisone (1811 kg) and prednisolone (1488 kg) use were highest among GCs in 2006. In the same study, veterinary use of GCs was estimated to constitute approximately 7% of total GCs use.³⁵ Data on veterinary use were unavailable in both the Czech Republic and Switzerland.

Occurrence of corticosteroids in wastewater and river water samples

With the targeted trace analysis method described in Ammann et al.,²⁷ some compounds with equal transitions coeluted and hence were reported as the sum: betamethasone and

dexamethasone, beta- and dexamethasone 21-acetate, cortisol and cortisone, and prednisolone and prednisone.

Concentrations of all compounds quantified in different environmental samples are given in Table 2. The combined concentration of 17 assessed GCs in river water samples was in the range of 23–57 ng/L, with beta-/dexamethasone, prednisolone/prednisone and cortisol/cortisone being the most abundant GCs. A modelling approach applied to the streams of the River Thames catchment in the UK predicted mean concentrations of up to 30 ng/L as a best case scenario or 850 ng/L as a worst case scenario for the total of the 28 GCs accounted for.³⁵ Concentrations measured in our study rather pertain to the best case scenario. Further, fludrocortisone acetate, which was found to be active in the GR-CALUX (see below), and compounds that could indirectly affect GR signaling through interference with steroidogenesis, such as clotrimazole, fluconazole, genistein and glycyrrhetic acid, were detected in river waters in concentrations >10 ng/L. The presence of GCs in river water collected upstream of WWTPs indicates other sources of GCs. A slight increase in the total GC concentration was observed downstream of WWTPs compared to upstream sites. The results also indicate that WWTP effluents were diluted after a certain distance and only slightly affected river water quality. Lesser dilution might arise during dry months at low river water flows, and resulting lesser dilution of the WWTP effluents. Further, concentration of pharmaceuticals in waters shows temporal changes due to varying consumer use as well as degradation depending on light and temperature.³⁶

The highest concentrations of chemicals were detected in extracts of untreated hospital wastewaters and in the WWTP influent. At 2 µg/L, dexamethasone/betamethasone were the most abundant glucocorticoids in the Swiss hospital WW. Further, prednisolone/prednisone, cortisol/cortisone and methylprednisolone were among the most concentrated GCs in hospital WW in both the Czech Republic and Switzerland. The fungicide fluconazole was detected in

both Swiss and Czech untreated hospital WW (up to 6 µg/L), confirming earlier results from the Baden hospital WW.³⁷

When comparing the Swiss hospital WW and the municipal WWTP influent, a decrease in concentrations of almost all analytes was observed, possibly due to the dilution of untreated hospital WW effluent by municipal WW, as well as due to sorption and transformation of the compounds on their way to the WWTP. However, concentrations of corticosterone, ketoconazole, clotrimazole, daidzein and genistein were increased in the WWTP influent, indicating a higher consumption of these compounds in households than in hospitals. An alternative explanation could be that the WWTP influent was collected during 20 hours, while the hospital WW was collected during 3 hours only. Thus, the influent corresponds only in part to the hospital effluent. In the Swiss WWTP influent, the prednisolone/prednisone concentration was the highest of all GCs, followed by cortisol/cortisone and beta-/dexamethasone (Table 2). In comparison, cortisol and cortisone were dominant GCs in Chinese WWTP influents (up to 120 ng/L and 86 ng/L, respectively), while all other GCs together contributed less than 10 ng/L.³⁸

Both Czech and Swiss WWTP effluents (CZ2ef, CH2ef) contained a comparable concentration of total GCs (96 and 54 ng/L, respectively). Both WWTPs have comparable mechanical and biological treatment. The overall elimination of GCs, which was computable only for WWTP Turgi, was 92% (Table 2). Although our study was not aimed at determining WWTP elimination efficiencies, our results point to rather high elimination of GCs, which is in agreement with other studies (Table S3).^{21,38,39} Additionally, a high removal was observed for mineralocorticoids. To our knowledge, this is the first study to report removal efficiencies of fludrocortisone acetate, spironolactone and aldosterone, which were 66, 94, and 90%, respectively. The elimination of azole antifungals ketoconazole and miconazole was rather high, whereas clotrimazole and fluconazole showed low removal. While fluconazole is known

to be poorly removed by conventional activated sludge treatment, clotrimazole, ketoconazole and miconazole are typically removed by more than 80% (Table S4).⁴⁰⁻⁴³ Ketoconazole is partly biotransformed, whereas clotrimazole and miconazole are more likely to sorb onto sewage sludge.⁴² Thus, actual over-saturation of the sewage sludge could be a reason for the low elimination of clotrimazole observed in WWTP Turgi.

GR-mediated activity of pure chemicals

A number of chemical compounds, detected in at least two samples with concentrations > 10 ng/L, were tested for their ability to interact with the glucocorticoid receptor (see SI Figures S1, S2, for concentration-response curves). Relative potencies (REPs) in the GR-CALUX assay were calculated for GR-agonists (Table 3). For some compounds, REP values were reported previously²⁴ and were comparable with our results in the GR-CALUX assay. Four compounds – fluticasone propionate, clobetasol propionate, budesonide, and flumethasone – showed a substantially higher GR-agonistic activity than Dex. Moreover, fluticasone propionate and clobetasol propionate produced GR-mediated luminescence >100% induction of Dex, whereas, 21-hydroxyprogesterone did not reach more than 37% induction of maximal Dex response (Figure S2). Therefore, the REP for 21-hydroxyprogesterone was calculated based on EC20. Some compounds suspected to interfere with GCs signaling (e.g., clotrimazole and fluconazole) and also inactive forms of GC hormones (cortisone, prednisone) were found to be inactive in the GR-CALUX, as expected (Table 3). None of the assessed chemicals showed anti-glucocorticoid activity (data not shown). EC50 values were compared to those obtained in the transactivation experiment with trout GR1 and GR2.¹⁰ Dex, betamethasone, and prednisolone elicited the transactivation activity of mammalian and trout GR2 in the same order of magnitude. Budesonide was 10-times more potent in GR-CALUX compared to trout GR2, whereas cortisol was 12-times less potent in GR-CALUX compared to trout GR2 (Table S5).

GR-mediated activity of river water and wastewater extracts

Glucocorticoid-like activity was found only in the slightly acidic and hydrophilic fraction 1 (F1) of wastewater and river water extracts. No GR-agonistic activity was determined in fractions F2 to F4 in any of the samples and including blank. The highest activity observed in the bioassay was in untreated hospital WW, up to 542 and 119 ng/L ^{BIO}DEQs in average in samples CH1 and CZ1, respectively (Table 2). The activity of the Swiss WWTP influent (CH2in) containing the hospital as well as the municipal wastewater reached up to 78 ng/L ^{BIO}DEQs, whereas the activity of the corresponding treated effluent (CH2ef) decreased to 37 ng/L. Even lower GR-mediated activity was observed in the Czech WWTP effluent (19 ng/L ^{BIO}DEQs in average), despite the fact that higher GC concentrations were detected. Relatively low GR-activity was determined in river water samples (0.8–2.6 ng/L Dex; Figure 2). The total reconstituted extract of samples was similarly or less active in the biotest than F1 of the corresponding sample; the lower activity can partly be explained by the loss of compounds after pooling and drying under N₂. However, presence of GR-antagonists in the total extract may play a role as well. Glucocorticoid-like activity in the same order of magnitude was reported previously in the total extracts of hospital WW, treated effluents and surface waters in the Netherlands.^{24,29} Dexamethasone equivalents in the range of <0.4–2.7 ng/L were determined using GR-CALUX in the river Rhine over one year, with maximum loads observed in winter and minimum loads in summer.³⁶

There was a very good match of the ^{BIO}DEQ values between technical replicates (Figure 2) which proves that the procedure applied to wastewater and river water samples is robust. Glucocorticoid activity predicted from the chemical analysis (^{CHEM}DEQs) was similar to or higher than the GR-activity observed in the GR-CALUX (^{BIO}DEQs) for most samples (Figure 2, Table 2). This suggests the potential presence of GR-antagonists in the environmental samples. In contrast, a higher than chemically predicted GR-CALUX response was measured

for most wastewater and surface water samples in the Netherlands,^{24,29} but there only a limited number of GCs were monitored chemically. The only sample in our study with a higher measured GR-CALUX response compared to the predicted activity was the Swiss WWTP effluent (CH2ef). The GR-mediated activity of the treated effluent decreased to approximately 50% of the corresponding influent activity (F1 of each sample), whereas, the overall removal of GCs based on chemical analysis was 92%. This finding may be explained by (i) the presence of active compounds in the effluent that were not monitored, e.g. some GC metabolites created during treatment, (ii) compounds with high GR-activating potency but incomplete removal that maintain the activity in the effluent (e.g. flumethasone), and/or (iii) compounds with very high potency in GR-CALUX, such as clobetasol propionate or fluticasone propionate that contribute to the activity in the bioassay but could not be reliably quantified because their concentration was close to the LOD of the analytical method. The mixture of beta-/dexamethasone contributed roughly 30% on average in all samples to the predicted GR-CALUX response. The contribution of other compounds was lower and dependent on the type of sample (Table 3). For example, budesonide and flumethasone were responsible for 23 and 17%, respectively, of the predicted glucocorticoid activity in river water samples. Both compounds were detected in low concentrations in surface waters, but due to their relatively high REPs they contributed substantially to the predicted dexamethasone equivalents.

The anti-glucocorticoid activity of samples was much less pronounced than the agonistic activity. The inhibition of GR-mediated activity in competition with Dex was observed in hospital wastewaters (CH1, CZ1) and in Czech and Swiss river water samples (CH2us, CH2ds, CH3, CZ2us, CZ2ds, CZ3); the respective anti-GR effects were very similar (Figure S3). This indicates that antagonists were present in the samples. Since none of the analyzed

standards exerted anti-glucocorticoid activity in GR-CALUX, the identity of the antagonists remains unknown.

Potential adverse effects in fish

Recently, Kugathas et al.¹² assessed the effects of beclomethasone dipropionate, a potent synthetic glucocorticoid, on adult fathead minnows. They reported that very low $\mu\text{g/L}$ concentrations reduced plasma glucose concentrations, lymphocyte count, and plasma cortisol levels, and increased the GR gene expression; all of these GCs-related effects were concentration-dependent.¹² In contrast, LaLone et al.¹³ reported rather minor effects on fathead minnows after exposure to 500 $\mu\text{g/L}$ Dex. In our study, the total GCs concentration expressed as Dex equivalents was in the lower ng/L range in rivers. It therefore would be important to assess the concentrations, or the induction equivalents, of these chemicals in laboratory and wild fish tissue.

The fish plasma model (FPM)²⁸ was applied in this study in order to indicate risks for fish connected with the exposure to pharmaceuticals analyzed in river water and wastewater samples. Estimated fish steady-state concentrations (FPC_{ss}) of individual drugs were compared to the lowest human peak plasma concentration after drug administration (C_{max}) found in the literature⁴⁴⁻⁵¹ and effect ratios (ERs) were calculated (Table S1). An $\text{ER} \leq 1$, which indicates that a drug response in fish might occur,²⁸ was found for medroxyprogesterone in the Swiss river water sample downstream of the WWTP (CH2ds) and in untreated Swiss wastewater samples CH1 and CH2in. The ER of medroxyprogesterone in river water samples CH2us and CH3 was very close to 1. Medroxyprogesterone has a low C_{max} value for humans (0.1 ng/mL)⁴⁴ and it remains to be tested whether it is similarly active in fish in order to predict risks for fish. So far, its immunosuppressive effects were demonstrated *in vivo* in carp.⁵²

While the FPM predicts plasma levels for individual compounds, we also aimed to predict an overall impact by the mixtures. Thus, we introduced the so-called “cortisol equivalents fish plasma concentration” (^{CEQ}FPC) in this study. ^{CEQ}FPC provides an estimation of the bio-concentration of GR-agonists in fish plasma, which is recalculated to cortisol equivalents. We calculated that GR-agonists bio-concentrated in fish plasma during exposure to river water analyzed in our study could cause an increase in the levels of GR-active compounds in plasma comparable to 0.9–19 ng/mL and 1.1–83 ng/mL cortisol equivalents based on $\log K_{OW}$ and $\log D_{OW}$, respectively (Table 2, last two rows). None of the GR-active compounds is dissociable, therefore, the differences between ^{CEQ}FPC calculated using $\log K_{OW}$ versus $\log D_{OW}$ are caused by the algorithm estimating the values in the different softwares. The natural level of plasma cortisol in wild fish under non-stressed conditions is typically below 10 ng/mL, but some species show higher values (e.g. *Oncorhynchus mykiss* 19–34 ng/mL or *Lepomis macrochirus* 25–125 ng/mL).⁵³ Baseline plasma cortisol levels depend on many variables, including life stage and age, season and time of day, environment (e.g. salinity, temperature), and social status.⁵⁴ If GR-agonists would increase natural plasma cortisol level equivalents by 19 or even 83 ng/mL ^{CEQ}FPC , as calculated for the Swiss sample collected downstream WWTP (CH2ds), it could result in internal cortisol levels being much higher than is natural for many wild fish species. Such an increase has the potential to cause chronic stress in fish, resulting in a variety of adverse effects. This is a clear indication that the risks connected to the exposure to GR-active compounds cannot be neglected despite the low concentrations of individual compounds detected in river waters.

Further, exposure to compounds that indirectly affect corticosteroid signaling and steroidogenesis was shown to induce adverse effects in fish. For example, effects on testicular physiology occurred in zebrafish following exposure to clotrimazole,⁵⁵ whereas ketoconazole altered steroidogenesis and reproductive success⁵⁶ and decreased fecundity⁵⁷ in fish. In this

study, a relatively low removal (Table 2) and low effect ratio (Table S1) was calculated for clotrimazole detected in Swiss waters, which may pose a risk for aquatic organisms. The concentration of genistein, that at mg/L range exposure caused malformations, reduced blood circulation and up-regulation of steroidogenesis in fish embryos,⁵⁸ was low in the river water samples and no risks connected to genistein exposure were revealed by the FPM. Detection limits for the natural compounds quercetin and resveratrol were relatively high compared to the other analytes and hence ER values could not be calculated for river water. So far, these compounds were shown to have rather positive effects on fish, such as improved immune functions,⁵⁹ prolongation of lifespan, enhancement of cognitive and locomotive activity, and reduction of neurodegeneration.⁶⁰

The assumptions behind the FPM and ^{CEQ}FPC approach are that biological targets are conserved across species and react to the same extent, i.e. have similar receptor affinities. However, GCs may have different affinity to fish GR2 versus GR1.^{10,61} Another limitation is the use of the GR-CALUX, based on a human derived cell line, in the estimation of ^{CEQ}FPC for fish because this requires extrapolation from human cells to fish. The latter disadvantage could be addressed in the future by using a fish-derived cell line as done for GR receptor transactivation by Becker et al.⁶¹ However, it was shown that the affinity to mammalian GR (GR-CALUX) and trout GR2 was similar for most studied GCs (Table S5).¹⁰ Additionally, the results depend on the differences in logK_{OW}/logD_{OW} values estimations.

Predicting internal concentrations of “hormone equivalents“, such as cortisol equivalents (^{CEQ}FPC) in this study, and comparing this to endogenous levels provides a benchmark for deciding whether any effects can be expected. Considering the difficulty encountered with studies investigating the effects of mixtures of endocrine disrupting compounds on fish populations, such an approach could facilitate the environmental risk assessment of pharmaceuticals acting through the same receptor-mediated mode of action. Additionally,

given the fact that different species display different sensitivity towards chemicals, the risks of azole antifungals should be further studied. In conclusion, our study demonstrates risks for disruption of the GR/MR pathway and supports the notion that the studied chemicals may impact fish in the environment.

SUPPORTING INFORMATION AVAILABLE

Detailed methodology of both sampling and data analysis; parameters used in FPM calculations and calculated effect ratios (Table S1); dispensation of prescribed GCs in the Czech Republic (Table S2); removal of GCs in WWTPs (Table S3); comparison of mammalian GR and trout GR2 and GR1 transactivation by GCs (Table S5); concentration-response curves of test chemicals (Figures S1, S2). This information is available free of charge via the Internet at <http://pubs.acs.org/>.

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Table 1. Mode of action of compounds affecting corticosteroid signaling pathway

	Mode of action (with respect to corticosteroid signaling)	Reference
Glucocorticoids		
Cortisol (Hydrocortisone)	endogenous hormone, GR-, MR-agonist	²
Cortisone	inactive form of hydrocortisone	²
Betamethasone	synthetic GR-agonist	⁶²
Dexamethasone (Dex)	synthetic GR-agonist	²
Betamethasone 21-acetate	pro-drug of betamethasone, synthetic GR-agonist	⁶²
Dexamethasone 21-acetate	pro-drug of Dex, synthetic GR-, MR- agonist	⁶²
Budesonide	synthetic GR-agonist	⁶²
Clobetasol propionate	synthetic GR-agonist	⁶²
Corticosterone	endogenous hormone (e.g. in rodents), GR-, MR-agonist	²
Flumethasone	synthetic GR-agonist	⁶²
Fluorometholone	synthetic GR-agonist	⁶²
Fluticasone propionate	synthetic GR-, MR-agonist	⁶²
6 α -Methylprednisolone	pro-drug of prednisolone, synthetic GR-agonist	⁶²
Prednisolone	synthetic GR-agonist	²
Prednisone	pro-drug of prednisolone	⁶²
Triamcinolone acetonide	synthetic GR-agonist	⁶²
Mifepristone (RU-486)	GR-antagonist, alters GR and MR gene expression	^{2,17,18}
Mineralocorticoids and other steroids		
Aldosterone	endogenous hormone, MR-agonist	²
21-Hydroxyprogesterone	MR-agonist; weak GR-agonist	⁶³ ; see Table 3
Fludrocortisone acetate	MR-, GR-agonist	^{2,63}
Eplerenone	MR-antagonist	²
Spironolactone	MR-antagonist	²
Medroxyprogesterone	GR-agonist, MR-antagonist	⁶⁴

Progesterone	GR-agonist, MR-antagonist, 11 β -HSD2 inhibitor, alters GR, MR, CYP11B gene expression	16-18,64,65
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Non Steroids

Clotrimazole, fluconazole, ketoconazole, miconazole	imidazole antifungals, inhibitors of ergosterol biosynthesis; disrupt <i>StAR</i> gene expression; CYP11B1 inhibitor	3,66,67
Daidzein, genistein	CYP21 inhibitor (natural phytoestrogen)	3
Glycyrrhetic acid	11 β -HSD1/2 inhibitor	3
Metirapone	CYP11B1 inhibitor	3
β -Naphthoflavone	AhR-agonist, reduces <i>StAR</i> expression, upregulates CYP11B1	3,20
Pravastatin	cholesterol synthesis inhibitor	68
Quercetin	disturbs the MR and heat shock proteins complex	69
Resveratrol	AhR-antagonist	70

Table 2. Concentration of compounds and dexamethasone equivalents (chemically predicted, ^{CHEM}DEQs; bioassay measured, ^{BIO}DEQs) detected in wastewater and river water samples from the Czech Republic and Switzerland (ng/L; average from two sample replicates), removal efficiency of WWTP Turgi (%), and cortisol equivalents fish plasma concentration (^{CEQ}FPC; ng/mL) calculated for the samples

CAS number		Concentration (ng/L)											
		wastewater			removal (%)	river water			wastewater		river water		
CH1	CH2in	CH2ef	CH2us	CHds		CH3	CZ1	CZ2ef	CZ2us	CZ2ds	CZ3		
<i>Glucocorticoids</i>													
Cortisol (Hydrocortisone)	50-23-7	378	160	26	83.8	7	8	10	939	29	5	6	7
Cortisone	53-06-5												
Betamethasone	378-44-9	1720	106	15	85.8	13	10	8	31	14	8	15	12
Dexamethasone	50-02-2												
Betamethasone 21-acetate	987-24-6	4	<2	4	*	4	13	<1	36	8	<1	2	<1
Dexamethasone 21-acetate	1177-87-3												
Budesonide	51333-22-3	4	1	<1	-	4	4	1	7	5	<1	2	2
Clobetasol propionate	25122-46-7	7	7	<1	>84.6	<1	1	<1	3	1	<1	<1	<1
Corticosterone	50-22-6	14	21	5	76.2	4	6	5	3	2	1	1	2
Flumethasone	2135-17-3	5	6	3	50.0	1	2	2	6	5	1	2	1
Fluorometholone	426-13-1	2	3	<1	>60	1	<1	1	1	<1	<1	<1	<1
Fluticasone propionate	80474-14-2	5	4	<1	>71.4	<1	<1	<1	2	<1	<1	<1	<1
6α-Methylprednisolone	83-43-2	36	8	1	93.8	3	3	4	393	6	4	4	3
Prednisolone	50-24-8	1221	336	<5	>98.5	13	12	10	241	24	5	3	5
Prednisone	53-03-2												
Triamcinolone acetonide	76-25-5	14	6	1	83.3	<1	<1	<1	2	5	<1	<1	1
Mifepristone (GR-antagonist)	84371-65-3	17	<2	<1	-	<1	<1	<1	<2	<1	1	<1	2
Sum of GCs		3423	656	54	91.8	49	57	39	1660	96	23	32	31
<i>Mineralocorticoids and other steroids</i>													
Aldosterone	52-39-1	22	19	2	89.5	<1	2	2	7	4	1	1	2
21-Hydroxyprogesterone	64-85-7	11	5	<2	>55.6	1	1	3	7	2	<1	<1	<1
Fludrocortisone acetate	514-36-3	82	36	12	66.2	7	5	14	15	8	3	5	8
Eplerenone	107724-20-9	11	6	4	27.3	2	3	3	18	8	<1	<1	3
Spironolactone	52-01-7	130	36	2	94.4	1	4	3	231	25	<1	1	1
Medroxyprogesterone	520-85-4	42	6	<1	>81.8	1	5	2	2	2	<1	<1	<1
Progesterone	57-83-0	15	4	<1	>75	5	10	4	91	<1	<1	<1	<1

<i>Non Steroids</i>													
Clotrimazole	23593-75-1	17	27	23	13.2	31	47	38	168	18	3	6	7
Fluconazole	86386-73-4	4640	236	200	15.1	4	10	18	6426	182	9	27	15
Ketoconazole	65277-42-1	4	142	15	89.4	<1	<1	<1	77	6	<1	1	<1
Miconazole	22916-47-8	2	15	<1	>93.1	<1	<1	<1	<1	<1	<1	<1	<1
Daidzein	486-66-8	671	1538	<3	>99.8	<1	<1	1	1096	3	1	2	8
Genistein	446-72-0	456	2049	28	98.7	6	5	8	623	6	30	36	1
Glycyrrhetic acid	471-53-4	2829	85	13	85.3	23	<1	18	198	126	6	<6	7
Metirapone	54-36-4	<10	<10	<5	-	<3	<3	<3	<10	8	<3	<3	<3
β-Naphthoflavone	6051-87-2	1	<1	<1	-	1	<1	<1	<1	<1	2	<1	1
Pravastatin	81093-37-0	69	39	<5	>87.0	4	8	3	21	10	3	6	7
Quercetin	117-39-5	<200	<200	<100	-	<60	<60	<60	<200	<100	<60	<60	<60
Resveratrol	501-36-0	<300	<300	<150	-	<90	<90	<90	434	246	<90	<90	<90
<i>Dexamethasone equivalents, cortisol equivalents fish plasma concentration</i>													
CHEMDEQs fraction 1 (ng/L)		1067	264	8	97.1	20	14	8	269	33	42	24	23
BIODEQs total extract (ng/L)		162	47	30	35.7	ND	ND	ND	89	13	ND	0.6	0.9
BIODEQs fraction 1 (ng/L)		542	78	37	53.2	2.1	2.6	1.2	119	19	ND	0.9	0.8
CEQFPC (ng/mL) ^a		356	202	3.9		6.3	19	2.8	116	21	0.9	3.5	3.0
CEQFPC (ng/mL) ^b		1530	1144	5.6		22	83	6.6	536	87	1.1	9.7	8.5

* increased concentration after treatment; ND – compounds not detected; for sample abbreviations see Figure 1

^a calculation based on logKow values estimated with EPISUITE³³

^b calculation based on logDow values (pH=7.4) estimated with ACD/Labs³⁴

Table 3. Relative potency (REP) of compounds measured in the GR-CALUX bioassay and their contribution (%) to the GR-CALUX response predicted based on measured chemical concentrations

Compound	REP	95% confidence interval	Untreated wastewaters ¹	Treated WWTP effluents ²	River water samples ³
Average contribution (%) ± SD					
Cortisol (Hydrocortisone)	0.036 (0.07*)	0.031 - 0.041	2 ± 2	2 ± 1	1 ± 1
Cortisone	NA (<0.0008*)	NA			
Betamethasone	0.59 (0.8*)	0.51 - 0.69			
Dexamethasone	1	0.93 - 1.08	30 ± 34	34 ± 28	30 ± 16
Betamethasone 21-acetate	NA	NA			
Dexamethasone 21-acetate	1.36	0.97 - 1.96	2 ± 2	13 ± 5	6 ± 9
Budesonide	6.1	5.2 - 7.1	4 ± 4	0 ± 0	23 ± 20
Clobetasol propionate	38	32 - 46	19 ± 14	13 ± 22	0 ± 0
Corticosterone	0.033	0.029 - 0.038	0 ± 0	0 ± 0	0 ± 0
Flumethasone	4.0	3.5 - 4.6	3 ± 3	17 ± 14	17 ± 17
Fluorometholone	0.98 (1.4*)	0.84 - 1.15	1 ± 1	0 ± 0	0 ± 0
Fluticasone propionate	57	43 - 76	13 ± 14	0 ± 0	6 ± 21
6α-Methylprednisolone	0.54 (0.4*)	0.44 - 0.68	17 ± 24	9 ± 4	5 ± 5
Prednisolone	0.13 (0.2*)	0.11 - 0.16	6 ± 5	5 ± 5	4 ± 4
Prednisone	ND (<0.002*)	ND			
Triamcinolone acetonide	1.12 (2.3*)	0.95 - 1.31	1 ± 0	2 ± 4	1 ± 2
Aldosterone	0.0037 (0.008*)	0.0030 - 0.0048	0 ± 0	0 ± 0	0 ± 0
Fludrocortisone acetate	0.33	0.28 - 0.40	3 ± 2	6 ± 3	7 ± 5
21-Hydroxyprogesterone	0.00079	0.00070 - 0.00089	0 ± 0	0 ± 0	0 ± 0
GR-mediated activity not detected:					
Eplerenone	Genistein				
Spironolactone	Glycyrrhetic acid				
Clotrimazole	Pravastatin				
Fluconazole	Quercetin				
Daidzein	Resveratrol				

NA - not assessed for GR activity

ND - no GR activity detected

* REP reported previously²⁴

¹ average contribution calculated from the following samples: CH1, CH2in, CZ1 (2 replicates of each sample)

² average contribution calculated from the following samples: CH2ef (1 sample replicate), CZ2ef (2 replicates)

³ average contribution calculated from all Swiss and Czech river water samples (2 replicates of each sample)

CH1	untreated hospital WW	CZ1
CH2in	untreated WWTP influent	-
CH2ef	treated WWTP effluent	CZ2ef
CH2us	river water upstream WWTP	CZ2us
CH2ds	river water downstream WWTP	CZ2ds
CH3	river water (agricultural influence)	CZ3

Figure 1. Map of sampling sites with an overview of collected samples

Figure 2. Predicted (^{CHEM}DEQ) and measured GR-CALUX response (^{BIO}DEQ); A: in untreated and treated wastewater samples (ng Dex/L), and B: in river water samples (ng Dex/L) in fraction 1 (F1) and in the total extract.