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Do progestins contribute to (anti-)androgenic activities in aquatic environments?☆

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ABSTRACT

Unknown compounds with (anti-)androgenic activities enter the aquatic environment via municipal wastewater treatment plants (WWTPs). Progestins are well-known environmental contaminants capable of interfering with androgen receptor (AR) signaling pathway. The aim of the present study was to determine if 15 selected progestins have potential to contribute to (anti-)androgenic activities in municipal wastewaters and the respective recipient surface waters. AR-specific Chemically Activated LUciferase gene eXpression bioassay in agonistic (AR-CALUX) and antagonistic (anti-AR-CALUX) modes and liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) methods were used to assess (anti-)androgenic activity and to detect the target compounds, respectively. The contribution of progestins to (anti-)androgenic activities was evaluated by means of a biologically and chemically derived toxicity equivalent approach. Androgenic (0.08–59 ng/L dihydrotestosterone equivalents – DHT EQs) and anti-androgenic (2.4–26 μg/L flutamide equivalents – FLU EQs) activities and progestins (0.19–75 ng/L) were detected in selected aquatic environments. Progestins displayed androgenic potencies (0.01–0.22 fold of dihydrotestosterone) and strong anti-androgenic potencies (9–62 fold of flutamide). Although they accounted to some extent for androgenic (0.3–29%) and anti-androgenic (4.6–27%) activities in influents, the progestins' contribution to (anti-)androgenic activities was negligible (≤2.1%) in effluents and surface waters. We also tested joint effect of equimolar mixtures of target compounds and the results indicate that compounds interact in an additive manner. Even if progestins possess relatively strong (anti-)androgenic activities, when considering their low concentrations (sub-ng/L to ng/L) it seems unlikely that they would be the drivers of (anti-)androgenic effects in Czech aquatic environments.

1. Introduction

Mixtures of many chemicals are continuously discharged by wastewater treatment plants (WWTPs) into aquatic environments. Some of these compounds may adversely affect the endocrine system of exposed organisms via androgen receptor (AR)-mediated signaling pathway (Gray et al., 2001; Kelce et al., 1998; Sohoni and Sumpter, 1998). To date, natural and synthetic estrogens have drawn great eco-toxicological interest due to their ability to induce intersex and feminization in freshwater fish (Leusch et al., 2017; Sumpter, 2005). Widespread feminization of male fish living downstream from WWTPs has been revealed to be caused not only by estrogens, however, but also by anti-androgenic compounds (Jobling et al., 2009). Moreover, androgenic contaminants of surface water can cause masculinization of resident female fish (Howell et al., 1980; Parks et al., 2001). (Anti-)androgenic activities have frequently been reported in aquatic environments worldwide (Bain et al., 2014; Boehler et al., 2017; Escher et al., 2014; Kinani et al., 2010; König et al., 2017; Urbatzka et al., 2007; van der Linden et al., 2008; Zhao et al., 2011). Compounds responsible for these activities often remain unidentified (Chen and Chou, 2016; Kinani et al., 2010; Leusch et al., 2014; Urbatzka et al., 2007).

Recently, progestins have come to be one of the groups of

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emerging pollutants drawing attention (Fent, 2015; Kumar et al., 2015). The group of substances termed progestins includes not only such natural hormones as progesterone but also synthetic substances designed to have biological activity similar to that of progesterone. Progestins are contained in contraceptives and other hormonal preparations (Sitruk-Ware, 2004). Both progesterone and synthetic progestins act primarily as progesterone receptor (PR) agonists (Africander et al., 2011), but they also cause off-target modulation of other steroid receptors (Bain et al., 2015; Besse and Garric, 2009; Stanczyk, 2003). Among other activities, progestins are known to act as both potent agonists and antagonists of human AR (Bain et al., 2015; Schindler et al., 2008). Androgenicity of some progestins has recently been observed also *in vivo* (Hua et al., 2015; Runnalls et al., 2013; Svensson et al., 2013, 2016; Zeilinger et al., 2009) and *in vitro* (Bain et al., 2015; Ellestad et al., 2014) within fish. Moreover, some of these progestins have been shown to inhibit synthesis of androgens *in vivo* (Fernandes et al., 2014) and possess anti-androgenic activity *in vitro* (Siegenthaler et al., 2017) in fish.

Surprisingly, no study to date has investigated whether environmental levels of progestins reach sufficient concentrations and have relative potencies strong enough to cause a substantial part of (anti-)androgenic activity observed in aquatic environments. The aim of the present study was to discover the extent to which progestins are responsible for (anti-)androgenic activities in Czech aquatic environments associated with municipal WWTPs. In parallel, we also assessed each sampling locality for the presence of a PR antagonist mifepristone and a selective PR modulator ulipristal acetate, because these compounds are suspected to be novel environmental contaminants (Golovko et al., 2018; Liu et al., 2010; Šauer et al., 2018). (Anti-)androgenic activities of mifepristone and ulipristal acetate and their contribution to overall sample activities were determined, as well.

2. Material and methods

2.1. Chemicals and material

All progestins, mifepristone and ulipristal acetate prescribed in the Czech Republic (Golovko et al., 2018) were chosen as target compounds. In addition, medroxyprogesterone was included because it has recently been found in Czech aquatic environments (Macikova et al., 2014a; Šauer et al., 2018). All tested compounds were of high purity as follows: altrenogest (≥99%), chlormadinone acetate (99.7%), cyproterone acetate (≥98%), dienogest (99.9%), drospirenone (99.9%), dydrogesterone (99.5%), etonogestrel (≥98%), flutamide (≥99%), gestodene (≥98%), levonorgestrel (≥99%), medroxyprogesterone (98.5%), medroxyprogesterone acetate (≥97%), megestrol acetate (≥99%), mifepristone (≥98%), nomegestrol acetate (≥98%), norethisterone (≥98%), progesterone (99.9%), and ulipristal acetate (≥98%). All were purchased from Sigma-Aldrich (Czech Republic). Classification of the studied compounds and their physicochemical properties are described in more detail in the Supplementary Material (Table S1). As internal standards, Altrenogest 19,19,20,21,21-d5, Chlormadinone-d6 Acetate, Cyproterone Acetate-13C2,d3, Medroxy Progesterone-d3, 6-epi-Medroxy Progesterone-d3, 17-Acetate, Megestrol Acetate-d3, Mifepristone-d3, Progesterone-d9, and Ulipristal Acetate-d3 were purchased from Toronto Research Chemicals (Canada). Individual stock solutions of native and internal standards were prepared for chemical analysis at 1 mg/mL concentration in methanol and stored at −20 °C. A spiking mixture of internal standards was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg/mL for each compound. AR-CALUX cells, illuminate mix, lysis mix, and dihydrotestosterone standards prepared in dimethyl sulfoxide (≥99.5% purity) were purchased from BioDetection Systems (the Netherlands). Ultrapure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

2.2. Collection of samples, sample preparation, and solid-phase extraction

Samples were collected from wastewaters (influents and effluents) of four WWTPs located in the Czech Republic and from the receiving surface waters (upstream and downstream). The WWTPs receive domestic and industrial wastewaters and at two sites, WWTPs at Prachatice and České Budějovice, hospital wastewaters. While all the studied WWTPs are based on mechanical–biological treatment with activated sludge secondary treatment, they differ slightly in their biological treatment (Table S2). Grab or time proportional (15-minute interval) 24 h composite samples (3–4 L) were collected (Table S2). Grab surface water sampling was performed up- and downstream from the respective WWTPs at a distance of 50 m from WWTP outlets at the same side of the point of discharge. Grab samples were taken using a 2 L bottle fastened to a stick and then poured into 1 L amber glass bottles. Surface water samples were collected at the same time as were samples of effluents. The collected samples were transported to the laboratory and stored at 4 °C in darkness until extraction, which was carried out within 24 h.

In order to preconcentrate the target compounds, a recently developed protocol for solid-phase extraction (SPE) and sample evaporation was used (Golovko et al., 2018), albeit with a slight change wherein some samples were acidified prior to extraction to find out the influence of acidification on the extraction efficiency of SPE (see section 2.3.). Briefly, an SPE-DEX 4790 automated solid-phase extractor (Horizon Technology, Salem, NH, USA) was employed to extract 1 L water samples. Atlantic C18 SPE disks (Horizon Technology) were used as a sorbent and preconditioned with acetonitrile for liquid chromatography mass spectrometry (Sigma-Aldrich, Czech Republic) and demineralized water. The samples were filtered through Atlantic Fast Flow glass fiber filters of pore sizes 5 and 1 μm (Horizon Technology). After a sample had been passed through the Atlantic C18 SPE disks, the entire extraction system was rinsed with demineralized water. The Atlantic C18 SPE disks were air dried for 15 min. The retained target compounds were then eluted with total volume of 10 mL acetonitrile. The SPE extracts thus obtained were evaporated by gentle nitrogen stream until dryness at 37 °C using a Termovap TV10 + sample concentrator (ECOM, Czech Republic). The extracts were redissolved either in 2 × 50 μL of acetonitrile for chemical or in 2 × 20 μL of dimethyl sulfoxide for biological analyses.

2.3. pH test

Because sample pH is an important factor influencing extraction efficiency (Kuster et al., 2009; Vulliet et al., 2008), we tested the effect of sample acidification prior to SPE. An advantage of sample acidification prior to SPE is that it inhibits biological activity of microorganisms potentially present in samples and thereby may help in preventing biotransformation and bioconcentration of target compounds. Sample acidification also may influence the dissociation of ionizable compounds, however, and thus cause problems with retention of analytes on SPE sorbents. C18 SPE sorbents such as Atlantic disks best retain neutral forms of polar compounds, but some analytes may be affected due to sample acidification depending upon their dissociation constants (Table S3). Thus, we assessed whether sample pH adjustment had an effect on retention of progestins, mifepristone and ulipristal acetate on Atlantic C18 SPE disks. The effect was evaluated