Environmental Science
Water Research & Technology

PAPER

View Article Online
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Cite this: *Environ. Sci.: Water Res. Technol.*, 2017, **3**, 450

Formation of bioactive transformation products during glucocorticoid chlorination†

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Received 29th January 2017,
Accepted 28th February 2017

DOI: 10.1039/c7ew00033b

rsc.li/es-water

Glucocorticoid (GC) release into the environment has led to widespread detection of glucocorticoid receptor (GR) activity in water resources that has been shown to persist throughout conventional and some advanced wastewater treatment processes. Here, we used high performance liquid chromatography, high resolution mass spectrometry and nuclear magnetic resonance spectroscopy to explore the reaction of natural (cortisone, cortisol) and synthetic (prednisone, prednisolone, dexamethasone) GCs with free chlorine (HOCl) to simulate their fate during chemical disinfection of water and wastewater. Generally, GCs react slowly ($t_{1/2}$ ~ 7–200 h) with HOCl when compared to other steroid classes, but they yield complex mixtures of transformation products, with at times the majority of product mass comprising structurally identifiable and likely bioactive steroids. For example, we frequently observed chlorination at the C-9 position (*e.g.*, 9-chloro-prednisone), a reaction known to increase GC activity 4-fold. We also identified reaction products in the adrenosterone family of androgens produced *via* cleavage of the C-17 side-chain on many GCs. Another common transformation pathway was the conversion of endogenous GCs to their more potent synthetic analogs *via* oxidation at the C-1/C-2 positions, with unsaturation reported to increase GR activity 4-fold (*e.g.*, cortisol to prednisolone). Despite identification of such products, *in vitro* assays generally suggest GR activity decreases with extent of parent decay during chlorination. Cortisol was the exception, with GR activity only decreasing 2-fold in product mixtures (based on measured EC_{50} values) despite a 95% reduction in parent concentration, a result attributable to formation of the more potent prednisolone during chlorination. Furthermore, our assay likely underestimates product bioactivity as it did not account for the activity of several identified GC byproducts that first require *in vivo* activation *via* C-11 reduction, nor did it consider androgen receptor (AR) activity associated with byproducts from the adrenosterone family. To avoid formation of product mixtures with conserved bioactivity, advanced chemical oxidation processes may represent a more promising approach; we show that GCs react much more rapidly with ozone ($t_{1/2}$ ~ 0.4–1.3 min) and produce no observable UV-active products. This suggests disruption of the GC conjugated π-electron and ring systems, thereby likely mitigating biological activity.

Water impact

For bioactive chemical classes, it is often assumed that environmental transformation eliminates associated ecosystem risks. Here, we show that reaction of glucocorticoids, a potent and ubiquitous steroid class, with free chlorine can yield mixtures of known and bioactive steroidal transformation products. This work calls attention to the likely formation of bioactive transformation products during engineered water and wastewater treatment, some of which may have adverse implications for ecosystem health.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ew00033b

Introduction

Recent decades of research have illustrated the potential ecological effects resulting from the production, use and discharge of myriad industrial, agricultural, medicinal and household chemicals to the environment.[1,2] Known as pollutants of emerging concern, they find their way into surface and drinking waters mainly from their persistence during wastewater treatment,[3] where experimental evidence suggests limitations of conventional approaches (*i.e.*, activated sludge) for their removal.[3] Of particular concern is the environmental fate of endocrine-active steroid hormones due to their widespread use in human and veterinary medicine, frequent detection in water resources, and proven adverse effects in aquatic organisms at trace levels.[2,4]

Glucocorticoids (GCs) are among the most widely prescribed pharmaceuticals in the United States, with over 100 million prescriptions dispensed in 2012 alone.[5] In addition to prescription-only GCs, there are also numerous over-the-counter (OTC) preparations available for human self-treatment, including some that are naturally produced by the body. For example, cortisone is an inactive, endogenous GC that is metabolically activated in the body by reduction to its 11-hydroxy form, cortisol (see Fig. 1). Once activated, cortisol has been assigned a relative anti-inflammatory potency of 1.[6] Cortisol (also known as hydrocortisone) is also available by prescription or OTC. Analogous to the endogenous GCs, prednisone is an inactive, synthetic GC that undergoes the same metabolic reduction to yield its active form, prednisolone. Prednisolone's 1,2-unstaturation in the A ring increases its anti-inflammatory potency 4-fold over cortisol.[6] Finally, dexamethasone, another synthetic GC, is 25-fold more potent than cortisol.[6] This increase in potency is brought about by 1,2-unsaturation, 9-fluorination, and 16-methylation.

Not surprisingly, GC release into the environment, primarily *via* mammalian excretion, has led to widespread and frequent detection of glucocorticoid receptor (GR) activity in water resources.[7–10] In such cases, concurrent chemical screening against known GCs can account for the majority of the GR bioactivity detected in some,[8] but not all,[9] instances. This raises concerns about harmful ecosystem impacts arising from the potency of certain GR agonists; for example, a recent study showed that environmentally relevant concentrations (10's of ng L^{-1}) of a synthetic GC and mineralocorticoid (fludrocortisone acetate) had adverse effects on adult zebrafish and their embryos, including decreased blood leukocyte numbers, significant alterations in gene expression and circadian rhythm, and increased heartrate and swimming activity.[11]

Studies have also demonstrated the persistence of GR activity during conventional wastewater treatment processes, as well as during some tertiary treatment techniques (*e.g.*, chlorination).[8,10] Persistence of GR activity during chlorination is notable, as others have demonstrated that chlorination can induce modest chemical transformations to other steroidal pollutants (*e.g.*, estrogens), potentially resulting in conserved or even enhanced bioactivity.[2,12] Such a scenario may also be plausible for GCs. This is exemplified by comparing cortisol to prednisolone (see Fig. 1), where a very minor structural modification (*i.e.*, dehydrogenation at C-1/C-2, an oxidation step) results in a 4-fold increase in bioactivity. Moreover, chemical transformations that only induce modest structural changes, while conserving the characteristic steroidal backbone, could also lead to products exhibiting activity across different biological endpoints.

In this study, we examine the potential for bioactive transformation products generated during engineered water and

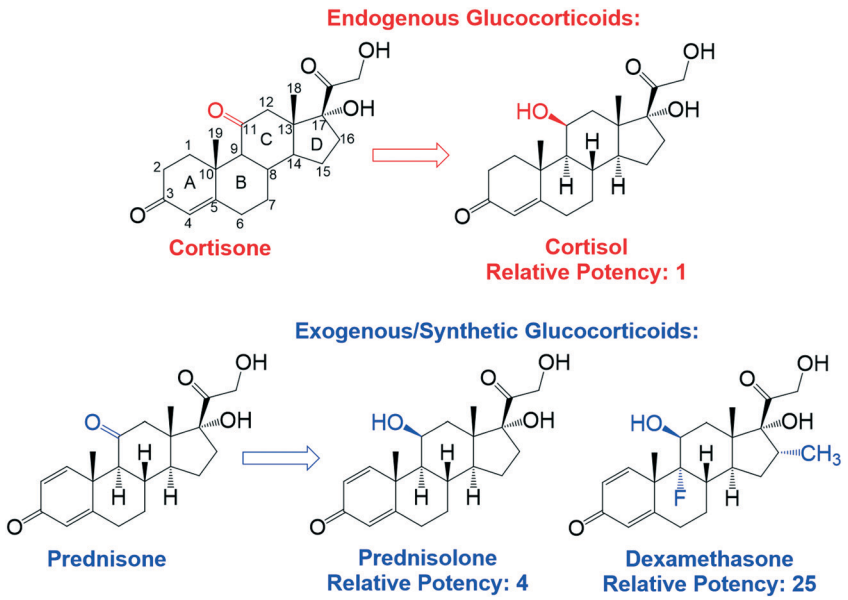

Fig. 1 Endogenous (top) and synthetic (bottom) GCs, as well as their reported relative anti-inflammatory potencies (see citations in the text). Letters in cortisone structure correspond to the conventional labeling of ring location, and numbers correspond to the conventional numbering of carbon position in steroids.

wastewater treatment to contribute to residual GR activity frequently reported in water resources. Specifically, we explored the reaction of natural (cortisone, cortisol) and synthetic (prednisone, prednisolone, dexamethasone) GCs (see Fig. 1) with free chlorine (HOCl) to simulate their fate during chemical disinfection of water and wastewater. We chose these GCs because of their widespread use and frequent detection in surface waters, as well as wastewater treatment plant (WWTP) influent and effluent (at concentrations on the order of 10–100 s ng L^{-1}).[7,8] In laboratory experiments, we examined the rate and extent of GC transformation through kinetic batch studies that allowed us to quantify rate constants for their oxidation *via* free chlorine. Using semi-preparative high performance liquid chromatography (HPLC), high resolution mass spectrometry (HRMS) and nuclear magnetic resonance spectroscopy (NMR), we then identified major transformation products. Finally, to determine whether bioactivity is conserved during GC chlorination, we used *in vitro* bioassays to evaluate the GR activity of select product mixtures. Outcomes of this work, including a comparison of GC fate during chemical oxidation with ozone, may help to guide future occurrence studies, while also promoting the design of efficient treatment systems for the removal of not only GCs but also residual GR and other receptor endpoint activity.

Materials and methods

Reagents

Chlorination experiments used sodium hypochlorite (NaOCl; Fisher Scientific; 5.65–6%), anhydrous potassium phosphate monobasic (KH_2PO_4; RPI; ACS grade), deionized water (Millipore, Q-Grad 2) and the following glucocorticoids: prednisone (Sigma; 98%), prednisolone (Sigma; 99%), cortisone (Steraloids, Inc.; ≥ 98%), cortisol (Sigma; 98%), and dexamethasone (Sigma; 98%). Adrenosterone and δ1-adrenosterone standards were purchased from Steraloids. Reagents used for free chlorine concentration analysis included anhydrous sodium phosphate dibasic (Na_2HPO_4; RPI; ACS grade), anhydrous potassium phosphate monobasic (KH_2PO_4; RPI; ACS grade), disodium ethylenediamine tetraacetic acid dihydrate (EDTA; Sigma; ACS grade), ferrous ammonium sulfate hexahydrate (FAS; J.T. Baker; ACS grade), and *N*,*N*-diethyl-*p*-phenylenediamine (DPD; Aldrich; 97%). Liquid–liquid extractions were performed with chloroform (Fisher Scientific; ACS grade). HPLC analysis used deionized water (Millipore, Q-Grad 2) and acetonitrile (Fisher Scientific; ACS HPLC grade) as the mobile phase.

Chlorination experiments

Aqueous stock solutions of HOCl were prepared by diluting concentrated NaOCl to ~5000 mg as Cl_2 L^{-1} in amber glass bottles sealed with Teflon-lined screw caps and were stored at 4 °C. Free chlorine concentrations of the HOCl stock solutions were measured *via* titration prior to use: 5 mL of each buffer reagent and the DPD indicator solution were added to 100 mL of diluted sample, with the solution turning pink in color in the presence of free chlorine. The free chlorine was then quantified by titration with FAS solution until the pink color was no longer visible.

Glucocorticoid chlorination experiments were conducted in amber glass bottles sealed with Teflon-lined screw caps. Reactors were loaded with 20 mL of 5 mM potassium phosphate buffer (pH 7) and an initial aqueous GC concentration of 5–100 μM (from freshly prepared saturated aqueous stock solutions; solubility ~0.1–0.28 mg mL^{-1} at 25 °C).[13] Reactions were then initiated by dosing reactors with an initial HOCl concentration of 0.1–100 mg Cl_2 L^{-1} (where GC : Cl_2 molar ratios ranged from 1 : 0.014 to 1 : 280 mol). Reactions were conducted at ambient temperature (~20 °C). Samples were withdrawn periodically over time to monitor HOCl concentration (*via* titration), and the concentration of the parent GC and any detectable transformation products (*via* HPLC analysis).

Notably, we avoided quenching reactions with sulfite to avoid altering product distributions. A limited number of experiments were conducted using excess (40 mM) sodium sulfite (Na_2SO_3) to quench residual free chlorine, and these generally showed parent GC and transformation product stability under these conditions. However, there were some instances where addition of sulfite altered product distributions (relative to those samples immediately analyzed without use of sulfite), suggesting reduction of some products generated during chlorination. Instead, for product analysis, samples were extracted into chloroform and concentrated to a residue by a stream of compressed air before further chemical analysis.

Ozonation experiments

Glucocorticoid ozonation experiments were conducted in amber glass bottles sealed with Teflon-lined screw caps. Reactors were loaded with 20 mL of 5 mM potassium phosphate buffer (pH 7), and an initial aqueous glucocorticoid concentration of 5 μM (from freshly prepared saturated aqueous stock solutions). Reactors also contained 350 μM (~26 mg L^{-1}) of aqueous *tert*-butanol, which was used as a model radical scavenger. The presence of *tert*-butanol suppresses the role of hydroxyl radical from ozone decomposition in GC transformation, allowing changes in GC concentration over time to be predominantly ascribed to direct oxidation *via* reaction with ozone (rather than indirect oxidation processes involving hydroxyl radical).[14,15]

Ozone stock solutions were prepared by bubbling an ozone/oxygen gas mixture generated by an LG-7 ozone generator into a flask of 5 mM phosphate buffer solution (pH 7) chilled with an ice bath. Reactions were then initiated by adding a volume of concentrated ozone stock solution (typically 25 mg L^{-1}) to the reactors for an initial diluted ozone concentration of 5–20 mg L^{-1}. Ozone concentrations were measured *via* direct UV absorbance measurements at 258 nm.[15] Reactions were conducted at ambient temperature (~20 °C) and samples were withdrawn periodically over time

for measurement of GC concentration and a preliminary assessment of transformation product occurrence. Because the primary goal of ozonation experiments was to provide a comparison of GC transformation rates relative to chlorination, 40 mM Na_2SO_3 (which exhibited no observable influence on parent GC stability) was used to quench reactions prior to HPLC analysis.

Analytical methods

High performance liquid chromatography. Samples were analyzed by HPLC for reaction progress and post-extraction for extraction efficiencies with an Agilent 1200 series HPLC-Diode Array Detector (DAD) system. The method used either an Agilent Zorbax Eclipse XDB-C_{18} (4.6 × 150 mm, 3.5 μm) or Agilent PREP-C_{18} Scalar (4.6 × 150 mm, 5 μm) column with acetonitrile/water gradient elution (25–80% acetonitrile over 11 min; 1 mL min^{-1}) and scanning 200–400 nm wavelength detection. HPLC separations were performed using a Beckman System Gold instrument with a model 166P variable wavelength detector (VWD) connected to a 128P solvent module, with acetonitrile/water gradient elution (2 mL min^{-1}), 254 nm wavelength detection, and an Apollo C_{18} semi-preparative (10 × 250 mm, 5 μm) column.

Nuclear magnetic resonance spectroscopy. ^1H NMR, Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectra were recorded using a Bruker AVANCE-600 spectrometer. Chemical shift values were referenced to the residual solvent signal for $CDCl_3$ (δ_H/δ_C, 7.26/77.2). All NMR data were processed using either MestReNova 10.0 or Bruker TopSpin 3.5 software.

High resolution electrospray ionization time-of-flight mass spectrometry (HRESITOFMS). HRESITOFMS data were obtained using a Waters Premier Q-TOF instrument. We used the same chromatographic method as described above (for HPLC-DAD) with the addition of 0.1% formic acid. Analyses were conducted using a reference standard of Leu-enkephalin and positive electrospray ionization over a mass range of 120–1000 Da under the following instrument parameters: 20 μL injection volume; 2.8 kV capillary, 35.0 V sampling cone, 4.0 V extraction cone and 2.0 V ion guide voltages; 110 °C source temperature and 400 °C desolvation temperature.

Glucocorticoid receptor assays

GR activity was determined with the GeneBlazer GR HEK293 T cell assay system (Life technologies, Madison, WI) as previously described.[16] In brief, division-arrested cells were plated at a density of 50 000 cells per well into a 96-well plate in assay medium. Transformation products were diluted in DMSO (Fisher Scientific) and a dilution series of four concentrations was added to cells at an optimal DMSO concentration of 0.5% (prednisolone: 100–3.6 nM, cortisol: 180–6.6 nM; dexamethasone: 20–0.08 nM). Dexamethasone (nine concentrations between 500–0.08 nM) was run as a reference compound. For cortisol and prednisolone transformations, a standard curve of parent cortisol (540–2.2 nM) and prednisolone (100–3.6 nM) was added. Cell-free wells were run as controls. Cells were incubated overnight for 16 hours at 37 °C in 5% CO_2. The following day, the 6× substrate solution was prepared according to the manufacturer's instructions and 20 μL was added to each well. The plate was incubated for 2 hours in the dark at room temperature while covered in adhesive film. Fluorescent intensity of blue (460 nm) and green (530 nm) emissions was measured by a Wallac Victor2 multilabel plate reader (PerkinElmer, Waltham, MA). Background fluorescence was determined by subtracting cell free controls. The ratio between the blue and green fluorescence was determined, and a DMSO background control was subtracted. Percent GR receptor activation was calculated dividing the compound of interest by the maximum dexamethasone activation. EC_{50} values, defined as the half-maximal effective concentration, were calculated *via* the probit method.

Results and discussion

Timescales and rate coefficients for glucocorticoid chlorination

To quantify rate coefficients for glucocorticoid (GC) chlorination, initial experiments were conducted with 100 mg Cl_2 L^{-1} and 10 μM of GC, where excess chlorine allowed GC decay to be approximated as pseudo-first order (see Fig. S1 and associated discussion in the ESI†). Indeed, measured HOCl concentrations revealed minimal variation across the reaction progress in these systems. Accordingly, based on measured pseudo-first order rate coefficients (k_{obs} values), we observed the following reactivity trend among the GCs considered, where values in parentheses indicate half-lives [$t_{1/2}$ values = $\ln(2)/k_{obs}$] estimated from exponential decay model fits (see Fig. S2†) and standard deviations from 3 replicates: cortisol (7 ± 1 h) > cortisone (20 ± 1 h) ≫ prednisolone (65 ± 3 h) >

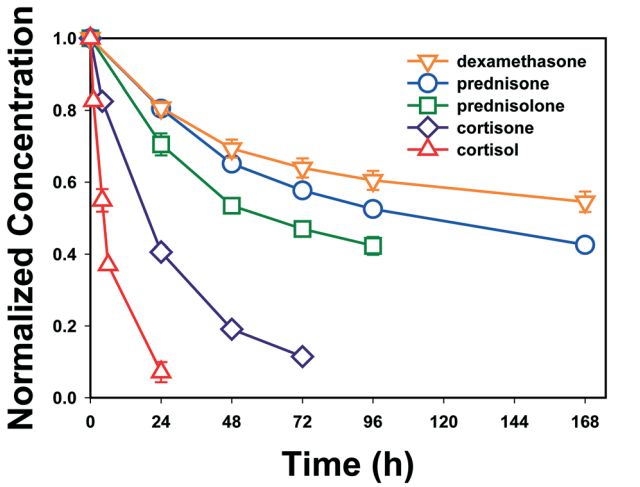

Fig. 2 Concentration of GCs over time during reaction with free chlorine. Reaction conditions: initial GC concentration of 10 μM, initial chlorine concentration of 100 mg Cl_2 L^{-1}, and pH 7. Error bars represent one standard deviation from n = 3 replicates.

Table 1 Second order rate coefficients for chlorination of GCs at pH 7. To calculate k_2 values, we assumed HOCl to be the major reactive species and [HOCl] = 0.75 × TOT_{OCl} at pH 7 based upon the reported acid dissociation coefficient for hypochlorous acid.[25] Uncertainties represent one standard deviation from n = 3 replicates

Compound	k_2 value (M^{-1} s^{-1})
Cortisol	$2.9 \pm 0.47 \times 10^{-2}$
Cortisone	$9.2 \pm 0.28 \times 10^{-3}$
Prednisolone	$2.8 \pm 0.12 \times 10^{-3}$
Prednisone	$1.8 \pm 0.04 \times 10^{-3}$
Dexamethasone	$9.2 \pm 0.43 \times 10^{-4}$

prednisone (100 ± 3 h) ≫ dexamethasone (200 ± 20 h) (Fig. 2). Further, with a constant chlorine concentration we also estimated second-order rate coefficients for the oxidation of each GC by HOCl {k_2 in (M^{-1} s^{-1}) = k_{obs}/[HOCl]} and these k_2 values are summarized in Table 1. We note that these estimated k_2 values assume HOCl is the major reactive species in our experimental systems, although other free chlorine species (*e.g.*; Cl_2, Cl_2O or OCl^-) may also contribute to the observed reactivity and merit additional exploration.

Because rate constants reported in Table 1 were measured at chlorine concentrations far exceeding those typically used in water and wastewater treatment,[17–21] we also conducted select studies at more environmentally relevant reagent concentrations. For example, 50 μg L^{-1} cortisol reacted with 5 mg Cl_2 L^{-1} with a half-life of ~120 hours (±15 h standard deviation from 3 replicates), a much longer timescale due to the lower reagent concentrations (as expected for a second-order kinetic process). Under these conditions, as will also be the case during water treatment, HOCl remains in excess, thereby allowing us once again to invoke an exponential decay model. Notably, the second-order rate coefficient obtained *via* these data at environmentally relevant concentrations ($3.0 \pm 0.45 \times 10^{-2}$ M^{-1} s^{-1}; Fig. S3†) agrees well with the value reported in Table 1.

Generally, the timescales we report for GC chlorination are slow relative to other steroid classes. We conducted additional chlorination experiments with 17β-trenbolone (a synthetic androgen) and estrone (an endogenous estrogen), and rapid degradation ($t_{1/2}$ ~ 1 h) was observed for both species at low initial chlorine concentrations (initially 2.5 mg Cl_2 L^{-1}; Fig. S4†). These trends in reactivity among steroid classes are consistent with literature data.[22,23] Based on published second order rate coefficients, estrogens react much more rapidly with free chlorine (reported k_2 values from 1.1–1.3 × 10^2 M^{-1} s^{-1}, several orders of magnitude greater than for GCs)[22] due to their phenolic moiety that acts as a good nucleophile toward the electrophilic HOCl species. Likewise, others have ascribed the reactivity of 17β-trenbolone (reported k_2 value = 2.5×10^1 M^{-1} s^{-1})[23] to its extended conjugated π-bond system (*i.e.*, trienone moiety), which renders the compound susceptible to various acid or base catalyzed oxidation reactions. Incidentally, testosterone (an endogenous androgen) and progesterone (an endogenous progestin) have been reported to exhibit little-to-no observable reactivity toward free chlorine (conditions: 0.25 mg L^{-1} testosterone, 4 mg Cl_2 L^{-1} for 7 days and 0.32 mg L^{-1} progesterone, 14 mg Cl_2 L^{-1} for 30 minutes).[22,24] Notably, these two steroids share some common functional groups with these GCs, such as a cyclic enone or secondary alcohol, both of which are known to have little-to-no reactivity toward free chlorine at water treatment levels.[25]

Collectively, our results suggest that GCs will be, at most, only partially degraded in water and wastewater treatment systems during chemical disinfection. Typical chlorine doses for municipal wastewater disinfection range from 5–20 mg Cl_2 L^{-1} over 30–120 minutes of contact time, with higher doses (up to 60 mg Cl_2 L^{-1}) and longer contact times required for low quality wastewater (*e.g.*, primary or trickling filter effluents).[17,19,26] For municipal drinking water disinfection, typical chlorine doses range from 1–10 mg Cl_2 L^{-1} with a range of distribution system contact times from chlorination to first consumer of 1–20 hours and up to 150 hours in some instances.[20,21] Moreover, chlorine residuals at the first consumer have an average concentration of ~1 mg Cl_2 L^{-1} and up to 2 mg Cl_2 L^{-1} in some municipalities.[20] Accordingly, if we assume representative values of chlorine residual (1 mg L^{-1}) and reaction time (85 h, including treatment and distribution), second-order rate coefficients in Table 1 estimate transformation of GCs up to ~10% for the most reactive species (assuming a constant and excess chlorine residual).

Transformation product identification

Although the extent of GC transformation is limited, we contend it is nevertheless important to identify reaction products because of the high use of GCs (and thus high probable environmental concentration) and the potential for risks associated with structurally analogous, bioactive transformation products and their mixtures. Initial HPLC assessment of reaction mixtures generally showed formation of multiple, more hydrophobic transformation products (Fig. S5†). To assess how product distributions and yields evolved as a function of chlorine exposure, we conducted chlorination reactions across a range of HOCl to GC molar ratios ([HOCl]:[GC] from ~14:1–140:1). As shown in Fig. 3, generally we observed little difference in the yields and identity of major products as a function of parent transformation across the molar ratios considered, with product formation increasing with chlorine exposure as expected. In some instances, secondary, presumably higher order chlorination products were observed under very high exposure conditions (*i.e.*, high [HOCl]:[GC] ratios). Given the likelihood of only partial transformation during treatment, however, we focused our attention on the identity of the initial (primary) transformation products or those that were observed and persistent across all exposure conditions.

An overview of the products identified for each GC are discussed below, and summarized in Table S1 of the ESI.† Where possible, we report product yields on a percent basis, calculated as mole product/mole parent consumed, for compounds for which we were able to acquire an analytical

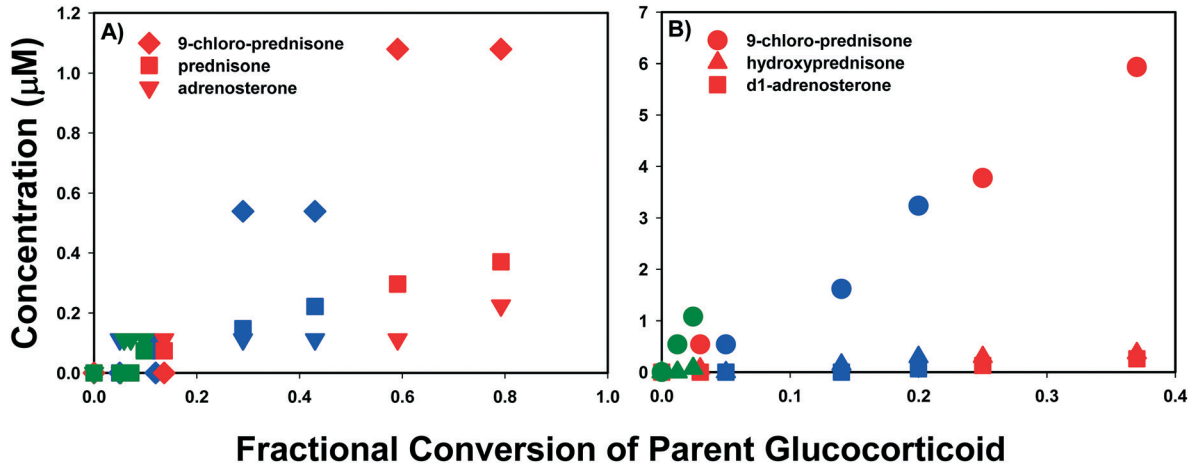

Fig. 3 Product formation (in μM) as a function of parent GC transformation during chlorination of (A) cortisone and (B) prednisone. Data are shown for different chlorine exposures, where product formation at a low molar ratio ([HOCl] : [GC] ~ 14 : 1) is shown in green, a medium molar ratio ([HOCl] : [GC] ~ 70 : 1) is shown in blue and a high molar ratio ([HOCl] : [GC] ~ 140 : 1) is shown in red. Concentrations were calculated from calibration curves of known standards (although in the case of hydroxyprednisone, it was assumed that its molar absorptivity was the same as that reported for prednisone).

standard. In the subsequent discussion, patterns in product formation emerged, and it became evident that our previously reported reactivity trend can be rationalized by the oxidative transformations most routinely observed. These are: (i) oxidation at C-1/C-2 in the A ring (cross-conjugated enone formation); (ii) oxidation at C-11 in the B ring (alcohol to ketone transformation); and (iii) C-17 side-chain cleavage in the D ring (ketone formation).

Prednisone. Analysis of product mixtures *via* HRESITOFMS and NMR (select reactions) revealed that chlorination of prednisone resulted in the formation of four primary transformation products. For example, at an initial [HOCl] : [prednisone] ratio of 140 : 1, ~65% of the initial parent mass remained after 48 h of reaction. The corresponding product mixture included 9-chloro-prednisone (~80% yield) and a hydroxyprednisone derivative, the latter of which we failed to isolate for further characterization. Two other products were known androgens, d1-adrenosterone (~5% yield) and a chlorinated d1-adrenosterone derivative (only at greater HOCl exposure), both of which were generated *via* C-17 side-chain cleavage (a base-catalyzed mechanism for such a process has previously been reported during the synthesis of 17-ketosteroids).[26,27] The structure of 9-chloro-prednisone was determined by detailed analysis of MS and NMR data, with the molecular formula of the product established by HRESITOFMS analysis and the chlorination position assigned

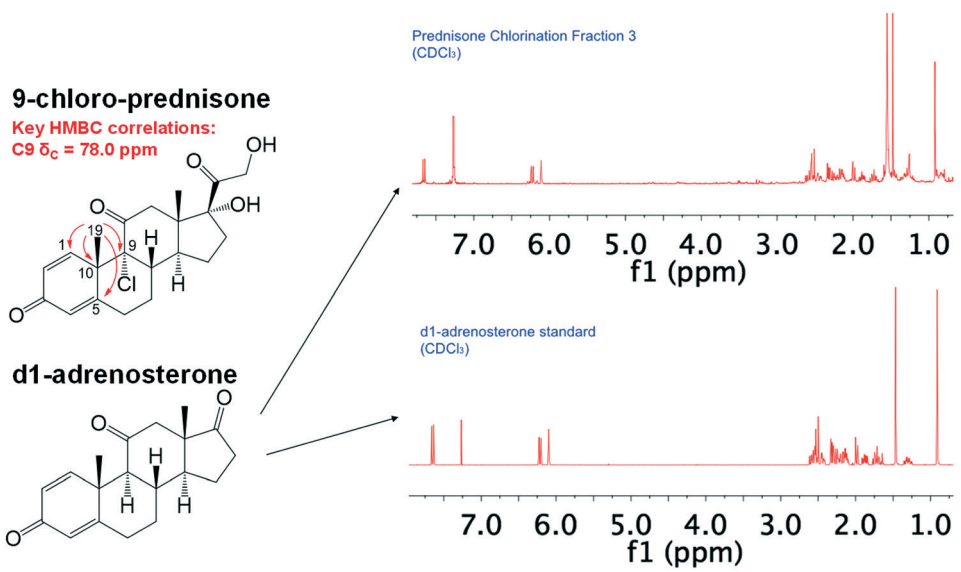

Fig. 4 Key HMBC correlations of 9-chloro-prednisone and ^{1}H NMR spectra of prednisone chlorination fraction 3 (top) and d1-adrenosterone standard (bottom). Signal at 1.6 ppm in prednisone chlorination fraction 3 corresponds to residual trace of water in $CDCl_3$.

on the basis of HMBC data (Fig. 4, see Table S2† for complete NMR assignments). The HMBC spectrum showed expected correlations from H-19 to C-1, C-5, and C-10, with an additional correlation to a carbon at 78.0 ppm (C-9), which is typical of a halogenated carbon. In addition, formation of d1-adrenosterone was also confirmed by comparison of the ^{1}H NMR spectrum of the isolated product sample to that of a known standard (see Fig. 4).

We note that the lack of a commercial or isolated standard for the hydroxylated GC transformation product precludes us from reporting its definitive yield. However, if we assume that hydroxylation does little to effect the molar absorptivity of prednisone, as is the case when comparing 11-desoxycortisol (ε = 17 323 at 242 nm) with cortisol (ε = 16 129 at 242 nm),[13] then the yield of hydroxyprednisone can be estimated as ~10%.

The observation of these NMR-confirmed products is noteworthy, as both 9-chloro-prednisone and d1-adrenosterone are previously reported to display bioactivities.[28–30] Chlorination at the C-9 position of prednisone has been reported to enhance its GC activity 4-fold.[31] In addition, d1-adrenosterone is known to have weak anabolic and androgenic activity (20% and 5% as potent as testosterone, respectively),[29] implying that interconversion of GCs to androgens can occur readily upon chlorination treatment and could therefore impart residual androgen receptor (AR) activity in treated effluents or finished water supplies.

Prednisolone. The chlorination of prednisolone exhibited similar trends in product formation and yields. At a [HOCl]:[prednisolone] ratio of 140:1, ~60% of the initial parent mass remained after 48 h of reaction and six transformation products were detected. Two products were identified as prednisone (~30% yield), *via* C-11 oxidation, and the known androgen 11β-hydroxyboldione, which was formed from C-17 side-chain cleavage of the parent prednisolone. Assuming that 11β-hydroxyboldione has approximately the same molar absorptivity as d1-adrenosterone, as is the case when comparing prednisolone (ε = 15 000 at 242 nm) with prednisone (ε = 15 500 at 238 nm),[13] then the yield of 11β-hydroxyboldione is estimated at ~5%. At greater HOCl exposure (or higher [HOCl]:[prednisolone] ratios), three more products were identified: chlorinated prednisone and prednisolone derivatives and the known androgen d1-adrenosterone (~5% yield), which was also formed *via* C-17 side-chain cleavage of the reaction product prednisone. d1-Adrenosterone then reacted further to yield a chlorinated d1-adrenosterone derivative. While the precise location of chlorination was not rigorously determined for the chloroprednisone derivative, we speculate that it is also occurring at C-9 by analogy to the results described above for 9-chloro-prednisone (*e.g.*, both have the same retention time and λ_{max} 240 nm). This chlorinated prednisone is produced at a yield of ~20%.

Likewise, the chloroprednisolone product was not isolated and therefore the location of chlorination was not determined. We estimate its yield between 1–10%. The lower end of this estimate assumes that chlorination does not affect its molar absorptivity relative to prednisolone (*i.e.*, a prednisolone standard was used to calculate this yield). However, as found through our isolation of 9-chloro-prednisone, chlorination can have a significant impact on molar absorptivity. Thus, the upper end of this range accounts for such a scenario (*i.e.*, the isolated 9-chloro-prednisone was used as a standard to estimate yield).

After accounting for these products, roughly a third of the parent GC appears to be transformed to as-yet unidentifiable products. We did observe a relatively more polar product with a maximum absorbance wavelength of less than 220 nm, but it did not readily ionize during repeated attempts to characterize it further *via* MS (see Fig. S5†). We speculate that the unaccounted mass lies in one or more products resulting from localized A-ring reactivity that eliminates the enone chromophore, thereby also limiting its ionization potential. Given their greater polarity and presumed structural changes, it is likely that these unidentified products exhibit little or no residual GC activity (*e.g.*, both the 3-keto group and C-4/C-5 double bond are required for GR binding).[32]

Prednisone is a product that will retain biological activity, after *in vivo* 11-keto reduction back to prednisolone. In addition, as with d1-adrenosterone, 11β-hydroxyboldione is a known, weak androgen, reported to be <5% as potent as testosterone.[29] Moreover, 11β-hydroxyboldione has been shown to be an inhibitor of estrone-induced growth of the uterus (*i.e.*, antiuterotrophic activity), with potency ~40% of that reported for progesterone.[30] Finally, as previously discussed, chlorination at the C-9 position (as in 9-chloro-prednisone) is known to result in a 4-fold increase in GC activity.[31] Based upon this reported biological activity, we speculate that the chlorinated prednisolone derivative is at least as active as parent prednisolone.

Cortisone. At an initial [HOCl]:[cortisone] ratio of 140:1, chlorination of cortisone resulted in ~40% of the initial parent mass remaining after 24 h of reaction and the formation of five identifiable transformation products. One product was prednisone (~15% yield), generated *via* C-1/C-2 unsaturation. Two chlorinated prednisone derivatives were also observed, one of which is presumably 9-chloro-prednisone (*i.e.*, it had the same retention time and λ_{max} 240 nm as described above, corresponding to ~15% yield), and another that was only observed at very high [HOCl]:[GC] molar ratios (*i.e.*, 140:1). Another product detected was adrenosterone (~5% yield), a known androgen produced *via* C-17 side-chain cleavage of the parent cortisone. As with prednisone and prednisolone, formation of d1-adrenosterone (~5% yield) was again observed after long chlorine exposures (or alternatively at very high [HOCl]:[GC] molar ratios), produced *via* side-chain cleavage of the reaction product prednisone. Some of the remaining product mass was accounted for by two additional products (observed M + H values at *m*/*z* 411 and 359), which were observed by MS but not isolated. One, likely a hydroxylated cortisone derivative, also has an incorporated single chlorine atom due to the diagnostic chlorine isotopic pattern in the MS data, and absorbs at wavelengths less than 220 nm. The other product

has the same mass as prednisone (M + H 359), but with a shorter retention time, and thus likely slightly more polar.

The oxidation of cortisone to form prednisone will result in a 4-fold increase in GC activity, after metabolic reduction to the active 11-hydroxy form. Additional enhancement of bioactivity will result from the formation of 9-chloro-prednisone (an 8-fold increase in GC activity over parent cortisone). Thus, cortisone oxidation to yield a synthetic analogue (prednisone) and a chlorinated derivative would be expected to preserve some degree of bioactivity through the chemical disinfection process.

Formation of adrenosterone also has potential ecosystem implications. Not only is it a weak endogenous androgen in mammals and fish, where it is ~25% as potent as testosterone,[33] it is also enzymatically converted *in vivo* by the action of 17β-hydroxysteroid dehydrogenase (17βHSD) to form 11-keto-testosterone, a potent androgen (equivalent in potency to testosterone) and the main endogenous androgen in fish.[34–36] The transformation of adrenosterone to 11-keto-testosterone has also been shown to occur in a common green alga (*Scenedesmus quadricauda*).[37] Moreover, 11-keto-testosterone can be further enzymatically reduced by the action of 5α-reductase (SRD5A) to the androgen 11-keto-dihydrotestosterone, which is equivalent in potency to dihydrotestosterone, the most potent natural androgen.[35] Lastly, as with 11β-hydroxyboldione, adrenosterone has been shown to have antiuterotrophic activity, with potency roughly half of that observed for progesterone.[30]

Cortisol. At the [HOCl]:[cortisol] ratio of 140:1, ~60% of the initial parent mass remained after 3 h of reaction and four transformation products were identified. One of these products was prednisolone (~20% yield; structure confirmed by NMR), presumably generated *via* the same C-1/C-2 unsaturation process observed in the conversion of cortisone to prednisone. Another product identified was cortisone (~10% yield), formed *via* C-11 oxidation. Two chlorinated prednisolone derivatives were also identified by MS, only one of which was generated across the full range of chlorine exposures and [HOCl]:[cortisol] molar ratios investigated (estimated between 10–70% yield, using the same assumptions previously applied to the chloroprednisolone product).

The conversion of cortisol to prednisolone results in a 4-fold increase in bioactivity and shows that GC interconversion from an endogenous steroid to an exogenous analog can occur readily during chlorination. In fact, a recent study monitoring GC levels in WWTP effluent and treated effluent showed a slight decrease in cortisol and increase in prednisolone levels after chlorination, which could, in part, be explained by the C-1/C-2 oxidation process evident from our results.[8] In addition, cortisone is a product that will retain biological activity, after *in vivo* 11-keto reduction back to cortisol. Also, based upon the reported biological activity of 9-chloro-prednisone,[31] we speculate that the chlorinated prednisolone derivatives are at least as active as prednisolone, with a possible 4-fold increase in GR activity over parent cortisol.

Dexamethasone. Chlorination of dexamethasone resulted in the formation of two known compounds, 11-keto-dexamethasone and 17-oxo-dexamethasone. Although oxidized, 11-keto-dexamethasone retains its GR binding affinity (as potent as dexamethasone), in contrast to the other C-11 keto GCs.[38] In addition, unlike the other adrenosterone class of weak androgens discussed previously, 17-oxo-dexamethasone does not exhibit any androgenic or antiuterotrophic activity.[29,30] Both 11-keto and 17-oxo-dexamethasone were only found to be formed under the very high [HOCl]:[GC] molar ratios (140:1), therefore, they are not likely to be major byproducts of chlorination (*i.e.*, we expect dexamethasone to persist through chlorination).

Summary of reaction pathways observed for GC chlorination

Overall trends in product formation suggest several common transformation pathways shared across endogenous and exogenous GCs. These include GC interconversion (hereafter "GI"), GC to androgen transformation (hereafter "G to A") and formation of chlorinated steroidal byproducts (hereafter "+Cl"). These pathways are summarized in Fig. 5 for the chlorination of cortisol, cortisone, prednisolone, and prednisone.

From the perspective of conserved or enhanced bioactivity through environmental transformation, among the most notable products and pathways are: (i) 9-chloro-prednisone, from the direct chlorination of prednisone and from C-1/C-2 unsaturation, followed by chlorination of cortisone (with an anticipated 4 and 8-fold increase in GC activity, respectively); (ii) prednisone, from either C-1/C-2 unsaturation of cortisone (with an anticipated 4-fold increase in GC activity) or C-11 oxidation of prednisolone (conservation of GC activity); and (iii) prednisolone, from C-1/C-2 unsaturation of cortisol (with an anticipated 4-fold increase in GC and GR activity). Further, notable examples of transformation products with receptor endpoints distinct from their parent compound include: (i) adrenosterone, from C-17 side-chain cleavage of cortisone; (ii) d1-adrenosterone, from C-17 side-chain cleavage of prednisone; and (iii) 11β-hydroxyboldione, from C-17 side-chain cleavage of prednisolone.

Given the consistency of transformation pathways across different GCs and the ubiquity of GCs in therapeutic use, we can reasonably extend the above transformation pathways to predict products of other, potent GCs not included in this study including 6α-methylprednisolone (potency: 5), betamethasone (potency: 25) and triamcinolone acetonide (potency: 30)[6] (see Fig. S6 in the ESI†). For example, we predict 6α-methylprednisolone to behave analogously to prednisolone, yielding two main products, 6α-methylprednisone and 11β-hydroxy-6α-methylboldione, both of which are known, bioactive steroids.[39,40] 6α-Methylprednisone is predicted to form *via* C-11 oxidation of parent 6α-methylprednisolone (analogous to formation of prednisone from prednisolone) and is a product that will retain biological activity, after *in vivo* 11-keto reduction back to 6α-methylprednisolone.[39] In addition, 11β-hydroxy-6α-methylboldione is known to possess anti-lipaemic activity and is suspected to form *via* C-17 side-chain cleavage of parent 6α-methylprednisolone (analogous

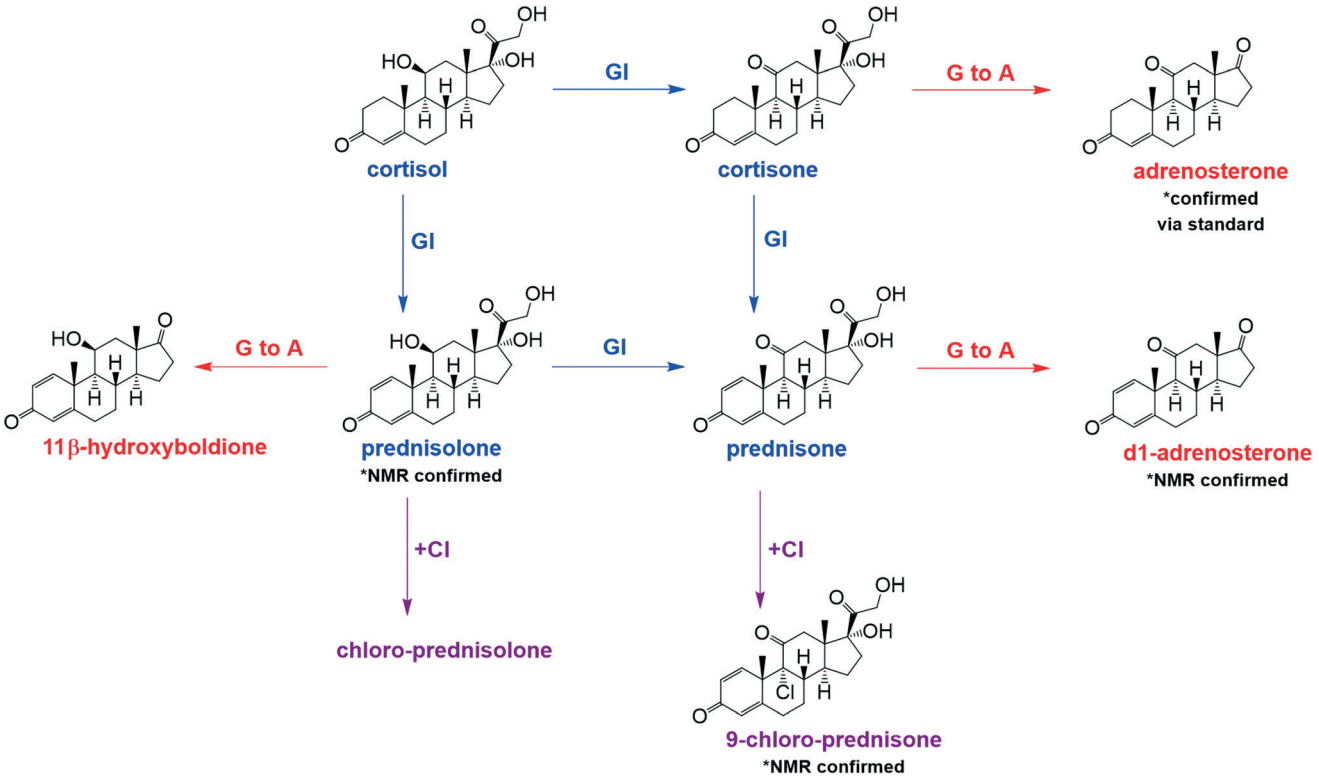

Fig. 5 Generalizable trends in product formation during GC chlorination under low HOCl exposure conditions (*i.e.*, initial chlorine concentration of 50 mg Cl_2 L^{-1}). Common pathways include: GC interconversion (GI) shown in blue, GC to androgen transformation (G to A) shown in red, and GC chlorination (+Cl) shown in purple. Product prednisolone, 9-chloro-prednisone, and d1-adrenosterone were confirmed *via* NMR. Adrenosterone was confirmed *via* HRMS and by matching retention time to a standard.

to formation of 11β-hydroxyboldione from prednisolone).[40] Although, to the best of our knowledge, the androgenic activity of 11β-hydroxy-6α-methylboldione has not been reported, methylation at C-6 is known to increase the androgenic activity of testosterone,[2] therefore, we would suspect 11β-hydroxy-6α-methylboldione to have a higher androgenic activity compared to that of 11β-hydroxyboldione.

In contrast, evidence suggests that betamethasone and triamcinolone acetonide will react similarly to dexamethasone, given their structural similarities (*e.g.*, cross-conjugated enone in ring A, fluorine atom at position C-9, and methyl or protected alcohol at position C-16). Thus, it is highly likely that these potent GCs will be relatively resistant to chlorination and persist through the disinfection process, as we observed experimentally for dexamethasone. Indeed, a recent study showed WWTP effluent and treated effluent levels of betamethasone, triamcinolone acetonide, and dexamethasone to be relatively stable throughout the chlorination process.[8]

Glucocorticoid receptor bioassays

Despite reported product bioactivity noted in the discussion above, *in vitro* assays generally suggest GR activity loss correlating with the extent of parent decay during chlorination (Fig. 6). This is exemplified by assay results for dexamethasone (3-fold reduction in potency as EC_{50} value with 80% reduction in parent concentration) and prednisolone (4-fold reduction in potency with 70% reduction in parent concentration). Cortisol was the exception, however, with assays of this reaction mixture suggesting only a 2-fold reduction in potency (increase in EC_{50}) despite a 95% reduction in parent concentration. This result is not surprising, as formation

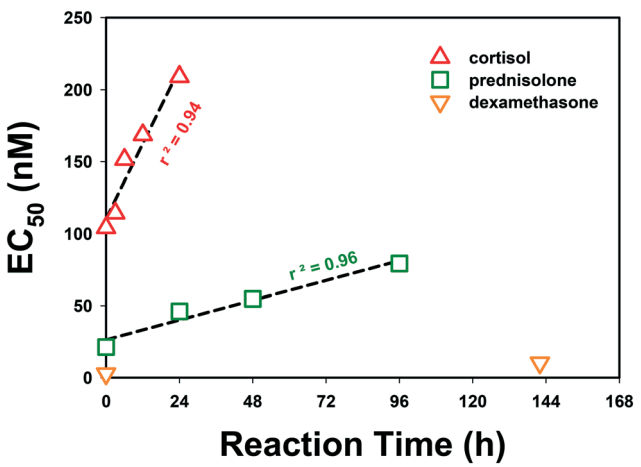

Fig. 6 Product mixture EC_{50} values from GC receptor bioassays as a function of reaction time for cortisol, prednisolone, and dexamethasone. Product mixtures were generated at pH 7 using high molar ratio ([HOCl] : [GC] ~ 140 : 1) conditions for GC chlorination.

of the more potent prednisolone and chloro-prednisolone products contribute significantly to residual GR activity.

We caution that these GR bioassay results do not account for the entirety of known, bioactive GC transformation products that we observed. For example, cortisone chlorination yields primarily prednisone and 9-chloro-prednisone, neither of which are directly GR active because C-11 is in its oxidized (ketone) form (this also applies to cortisone).[41] However, it is known that ~95% of an administered dose of prednisone is reduced to its GR active, 11β-hydroxy form (prednisolone) by the action of 11β-hydroxysteroid dehydrogenase.[41] Thus, although prednisone and 9-chloro-prednisone will not function as GR agonists, they are expected, after metabolism, to be 4 and 8 times more biologically active than cortisone, respectively. Further, our evaluation of bioactivity only considered one receptor endpoint (*i.e.*, GR), when similar analyses with androgen receptor (AR) assays are also necessary given the known androgens produced during GC chlorination.

Ozonation for chemical oxidation

As an alternative treatment approach for managing persistent GR activity, we explored the ability of chemical oxidation with ozone to increase the rate of GC transformation and limit formation of known, bioactive transformation products. As shown in Fig. 7, with conditions representative of water/wastewater treatment, GCs reacted much more rapidly with ozone ($t_{1/2}$ ~ 0.4–1.3 min) relative to free chlorine. We reiterate that *tert*-butanol was used as a model radical scavenger in these systems, allowing changes in GC concentration over time to be primarily ascribed to direct oxidation *via* reaction with ozone. Moreover, no UV-active products were detected *via* LC-DAD analysis. This was the case regardless of whether reactions were either quenched with sulfite or allowed to proceed to completion. Thus, for ozonation, we report the following reactivity trend (with half-lives estimated from exponential decay model fits): cortisone (0.4 min at 5 mg L^{-1} O_3) ~ prednisone (0.5 min at 5 mg L^{-1} O_3) ~ cortisol (0.7 min at 5 mg L^{-1} O_3) > prednisolone (0.5 min at 15 mg L^{-1} O_3) > dexamethasone (1.3 min at 20 mg L^{-1} O_3).

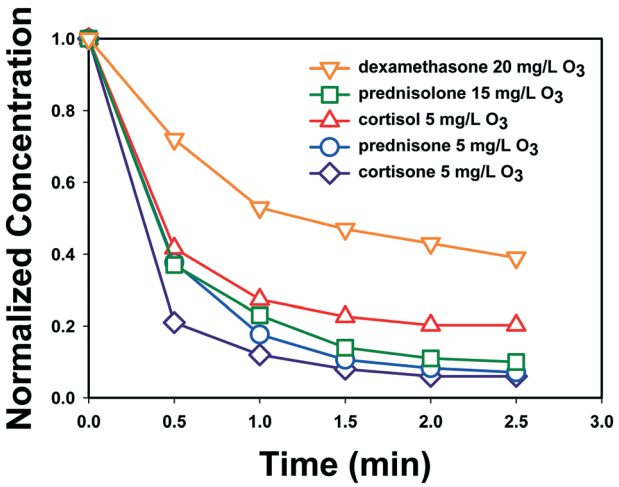

Fig. 7 Normalized GC concentration over time during reaction with ozone. Reaction conditions: 5 μM aqueous GC (~2 mg L^{-1}), 5–20 mg L^{-1} of O_3 (as indicated), 350 μM aqueous *tert*-butanol as a model radical scavenger, and pH 7.

Most likely, ozone reacts to disrupt the GC conjugated π-electron and ring systems, presumably altering the structures sufficiently to mitigate or eliminate biological activity. Indeed, previous research suggests that α/β-unsaturated cyclic ketones undergo an oxidative ring-opening reaction with ozone, through an ozonide intermediate.[42] Likewise, ozonation of progesterone, an endogenous progestin that shares structural commonalities with GCs (*e.g.*, cyclic enone in the A-ring), produced oxidative A-ring-opening over similar timescales ($t_{1/2}$ ~ 1 min) to that observed herein for GCs.[43] Typical ozone doses for municipal wastewater disinfection are 5–15 mg L^{-1} and 15–30 minutes of contact time, with higher doses (up to 40 mg L^{-1} O_3) and longer contact times required for low quality wastewater (*e.g.*, raw wastewater and primary effluents).[19,44] Thus, our results are within environmentally relevant range of doses of ozone and contact times and indicate ozonation to be a more promising approach to chemical oxidation of GCs that also limits the formation of bioactive transformation products compared to the use of chlorination.

Conclusions

Overall, GCs are relatively slowly degraded by HOCl. It is reasonable to expect, therefore, at best partial degradation during chemical disinfection, with mixtures of parents and products discharged to water distribution systems and in WWTP effluent. Chlorination generally results in interconversion of GCs (*e.g.*, cortisol to prednisolone), production of androgens, and formation of chlorinated steroid derivatives. These transformations appear common for GCs as a class, suggesting they can reasonably be extended to other GCs not explicitly investigated herein to predict their likely extent of reaction and products during chemical disinfection with free chlorine.

Although many identifiable GC transformation products represent known steroids with established bioactivity, *in vitro* assays conducted herein generally indicated that GR activity decreases with the extent of parent decay. However, our assays likely under-predict the bioactivity of transformation product mixtures because they do not account for activity arising from 11-keto GC products (which require metabolic activation through enzymatic reduction), nor did we assess the androgenicity of products formed *via* C-17 side-chain cleavage in the D ring. Ultimately, therefore, we conclude that transformation products arising from GC chlorination are likely to contribute to some, but not all, of the unaccounted for GR activity often reported for surface water resources.

More broadly, this work provides yet another example where a common environmental transformation process (chemical disinfection, particularly by free chlorine) does not entirely eliminate ecosystem risks associated with emerging pollutant classes. Conserved, enhanced, or broader spectrum receptor bioactivity through environmental transformation

processes challenges our current regulatory and risk assessment approaches, while also complicating prioritization of analytical targets for environmental monitoring. Notably, as we showed herein, management of steroidal bioactivity during engineered treatment may require the more widespread adoption of advanced technologies, like ozonation, with demonstrated ability to limit the formation of bioactive transformation products. Collectively, outcomes of this work may help to guide future occurrence studies for persistent and bioactive GC transformation products, while also guiding the design of more efficient treatment systems for removal of not only GCs but also all associated GR activity.

Acknowledgements

The authors would like to acknowledge support provided by the UI OVPRED and NSF (CHE-1609791 and CBET-1335711) to NCP, JBG and DMC. Additional funding was also provided through the UCR USDA/AES resource allocation program and the USGS/NIWR (2015SC101G) to AK and DS. We would like to thank Dr. Santhana Velupillai of the University of Iowa Central NMR Facility and Vic Parcell of the University of Iowa High Resolution Mass Spectrometry Facility for their support in product analysis. We would also like to thank Dr. Eric Patterson of Stony Brook University for his helpful discussions. The authors would also like to acknowledge two anonymous reviewers whose recommendations improved the overall quality of this work.

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