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Generalized concentration addition model predicts glucocorticoid activity bioassay responses to environmentally-detected receptor ligand mixtures

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Abstract

Many glucocorticoid receptor (GR) agonists have been detected in waste and surface waters domestically and around the world, but the way a mixture of these environmental compounds may elicit a total glucocorticoid activity response in water samples remains unknown. Therefore, we characterized 19 GR ligands using a CV1 cell line transcriptional activation assay applicable to water quality monitoring. Cells were treated with individual GR ligands, a fixed ratio mixture of full or partial agonists, or a non-equipotent mixture with full and partial agonists. Efficacy varied (48.09 to 102.5%) and potency ranged over several orders of magnitude (1.278×10^{-10} to 3.93×10^{-8} M). Concentration addition (CA) and response addition (RA) mixture models accurately predicted equipotent mixture responses of full agonists ($r^2 = 0.992$ and 0.987 , respectively). However, CA and RA models assume mixture compounds produce full agonist-like responses, and therefore they overestimated observed maximal efficacies for mixtures containing partial agonists. The generalized concentration addition (GCA) model mathematically permits $\leq 100\%$ maximal responses, and fell within the 95% confidence interval bands of mixture responses containing partial agonists. The GCA, but not CA and RA, model predictions of non-equipotent mixtures containing both full and partial agonists fell within the same statistical distribution as the observed values, reinforcing the practicality of the GCA model as the best overall model for predicting GR activation. Elucidating the mechanistic basis of GR activation by mixtures of previously detected environmental GR ligands will benefit the interpretation of environmental sample contents in future water quality monitoring studies.

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Keywords

glucocorticoid receptor; partial agonist; chemical mixtures; generalized concentration addition model; bioassay

Introduction

Environmental endocrine disrupting compounds have been of interest to scientists for the past several decades, with particular attention to compounds impacting estrogen and androgen signaling pathways. However, an increasing number of studies have reported detectable levels of glucocorticoid-active compounds in surface (Conley *et al.*, 2017; Jia *et al.*, 2016; Macikova *et al.*, 2014; van der Linden *et al.*, 2008) and waste waters (Chang *et al.*, 2007; Jia, et al., 2016; Macikova, et al., 2014; Schriks *et al.*, 2010; Suzuki *et al.*, 2015; van der Linden, et al., 2008), warranting further investigation into potential adverse effects to humans and wildlife alike.

Endogenous glucocorticoids, like cortisol and corticosterone, have been implicated in the numerous physiological processes, illustrating the risk of disruption to homeostatic endocrine signaling upon environmental exposure. For example, glucocorticoids are involved in growth regulation, reproduction, intermediary metabolism, immunosuppression and inflammatory endocrine signaling in humans (Hadley *et al.*, 2007; Lu *et al.*, 2006), as well a reproduction (Milla *et al.*, 2009; Witorsch, 2016) and social hierarchy establishment (Gilmour *et al.*, 2005) in fish species. Given their critical role in biological homeostasis, it is not surprising that glucocorticoids are among the most prescribed drugs, with therapeutic uses in allergies, asthma, inflammatory eye/ear/skin disorders and rheumatoid arthritis (Lu, et al., 2006). Over 4,000 kg of glucocorticoid pharmaceuticals were prescribed over the course of one year in the United Kingdom, surpassing prescription of (anti)estrogens, (anti)progestins, and (anti)androgens in the same time frame by 58.05%, 60.54%, and 58.32%, respectively (Runnalls *et al.*, 2010). In fact, an estimated 1.2% of the United States adult population have received an oral glucocorticoid (Overman *et al.*, 2013), which is greater than the most recent estimates for the United Kingdom (0.9%) (van Staa *et al.*, 2000) and Northeast Iceland (0.7%) (Gudbjornsson *et al.*, 2002).

Prolonged exposure to prescription glucocorticoids, or elevated endogenous levels, can initiate osteoporosis, hyperglycemia, suppression of the hypothalamus-pituitary-adrenal axis and the hypothalamus-pituitary-gonadal axis, and increase risk of infection (Curkovic *et al.*, 2013; Mazziotti *et al.*, 2006). Exposure to some glucocorticoids has been linked to low birth weight and hyperglycemia (Lindsay *et al.*, 1996; Nyirenda *et al.*, 1998; Reinisch *et al.*, 1978). Further, exposure to high concentrations of glucocorticoids increased circulating levels of vitellogenin protein (egg yolk precursor) in fish indicating disruption in normal reproductive endocrine signaling (Brodeur *et al.*, 2005; LaLone *et al.*, 2012); while exposure to low (environmentally-relevant) concentrations stimulated hyperglycemia (Kugathas *et al.*, 2011).

Despite the high volume of glucocorticoids prescribed domestically and around the world, not all waste water treatment methods effectively eliminate these compounds (Bain *et al.*,

2014; Jia *et al.*, 2015; Jia, et al., 2016). Glucocorticoid concentrations have been predicted to reach 30–854 ng/L in surface waters, based on established human metabolic and consumption rates (Kugathas *et al.*, 2012). Several groups have confirmed detection of GR agonists within this predicted concentration range using analytical chemistry methods (Chang, et al., 2007; Jia, et al., 2016; Macikova, et al., 2014; Schriks, et al., 2010; Weizel *et al.*, 2018).

Contemporary water quality screening programs have incorporated effects-based *in vitro* chemical screening methods, or “bioassays,” which use biological endpoints to quantify the presence of biologically active compounds (Escher *et al.*, 2012). Despite several commercial options for glucocorticoid activity detection (Huang *et al.*, 2011; Jia, et al., 2015; Jia, et al., 2016; Suzuki, et al., 2015; Suzuki *et al.*, 2013; SwitchGearGenomics, 2010; van der Linden, et al., 2008), we recently used a novel non-commercial, yet highly sensitive, bioassay specific to glucocorticoid receptor-mediated transcriptional activation to detect glucocorticoid-like activity in impacted surface waters (Conley, et al., 2017). This economical detection method produces a considerable dynamic range (50–100 fold over control) and maintains its sensitivity over time due to transfection of the GR and GR-responsive promoter-luciferase reporter gene constructs upon each use.

The purpose of this study was two-fold. We aimed to further characterize the CV1-hGR screening bioassay, by assessing several glucocorticoid agonists (pharmaceuticals and endogenous corticosteroids) that had been previously detected in surface and waste waters. The concurrent presence of these compounds would imply potential simultaneous exposure events, possibly resulting in interactive responses *i.e.*, additivity, synergism or antagonism. Therefore, our second aim was to investigate how GR agonists behave in mixtures. Although, many groups have characterized estrogen (Bermudez *et al.*, 2010; Bermudez *et al.*, 2012; Conley *et al.*, 2016; Scholze *et al.*, 2014; Silva *et al.*, 2002), androgen (Blake *et al.*, 2010; Orton *et al.*, 2014), thyroid hormone (Ghisari *et al.*, 2009), and steroidogenesis-altering compound (Taxvig *et al.*, 2013) mixtures *in vitro*, to our knowledge none have completed similar studies with glucocorticoids.

There are several existing models used to predict responses of chemical mixtures; each with their own set of assumptions. The two most common approaches include the concentration addition model (CA; similar joint action), which assumes compounds share the same mechanism of action, (Bliss, 1939; Olmstead *et al.*, 2005), and the response addition model (RA; independent-joint action model), in which compounds elicit their effects through different mechanisms of action and do not interact with one another (Drescher *et al.*, 1995; Finney, 1942; Plackett *et al.*, 1948). Given that each of the selected environmentally-detected pharmaceuticals were designed specifically to bind the same nuclear receptor, we hypothesized that responses would be best predicted using the concentration addition mixtures model.

Although the CA model worked well to predict mixtures of full GR agonists, several of the screened glucocorticoids produced less than 100% maximal response in the CV1-hGR bioassay, violating a primary assumption of the CA model. The generalized concentration addition mixtures model (GCA), which mathematically permits partial agonists by

incorporating an additional variable for chemical-specific maximal efficacy (Howard *et al.*, 2009), has been applied previously to predict *in vitro* responses for chemical mixtures with partial agonists for multiple nuclear receptors including, the aryl hydrocarbon receptor (Howard *et al.*, 2010), peroxisome proliferator-activated receptors (Watt *et al.*, 2016), and steroidogenesis (Hadrup *et al.*, 2013). A comparison of predicted responses using both the CA and GCA model, and observed mixtures containing partial GR agonists are provided herein.

Methods

Test chemicals

The GR agonists dexamethasone (CAS: 50–02-2, purity: 98%), 21-hydroxyprogesterone (CAS: 64–85-7, purity: 97%), 6a-methylprednisolone (CAS: 83–43-2, purity: 98%), aldosterone (CAS: 52–39-1, purity: 95%), budesonide (CAS: 51333–22-3, purity: 99%), betamethasone (CAS: 378–44-9, purity: 98%), corticosterone (CAS: 50–22-6, purity: 98.5%), cortisone (CAS: 53–06-5, purity: 98%), dexamethasone 21-acetate (CAS: 1177–87-3, purity: 99%), flucinolone acetonide (CAS: 67–73-2, purity: 97.5%), flucinonide (CAS: 356–12-7, purity: 98%), fludrocortisone acetate (CAS: 514–36-3, purity: 98%), flutamethasone (CAS: 2135–17-3, purity: 99.6%), fluticasone propionate (CAS: 80474–14-2, purity: 98%), hydrocortisone (CAS: 50–23-7, purity: 98%), prednisolone (CAS: 50–24-8, purity: 99%), prednisone (CAS: 53–03-2, purity: 98%), triamcinolone acetonide (CAS: 76–25-5, purity: 99%), and one antagonist mifepristone (CAS: 84371–65-3, purity: 98%) were all purchased from Sigma-Aldrich. Test chemicals and mixtures were dissolved to desired concentrations and serially diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Final DMSO concentration in treatment wells < 0.1%.

Cell Culture and Transcriptional Activation Assay

CV1 cells (ATCC CCL70), were grown in 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) RPMI-1640 growth media using previously described culture techniques (Hartig *et al.*, 2007). Confluent cells were split at a ratio of 1:3 into 60 mm dishes and inoculated on day 7 (approximately 5×10^6 cells/dish) with adenoviral vectors containing the human GR and MMTV-Luc construct genes (CV1-hGR). The viral vector was diluted to yield a multiplicity of infection of 1 GR receptor to 50 reporter gene constructs. After 24 hr incubation with adenovirus, cells were rinsed, resuspended in media and seeded in 1–2 96-well plates.

Cell passage numbers 197–265 were used to measure GR transcriptional activation upon exposure to individual chemicals and chemical mixtures as described previously (Conley, et al., 2017). CV1-hGR cells were seeded using 100 μ L cell suspension per well and treated with 100 μ L of additional 5% (DCC-FBS) RPMI-1640 media containing 2x solution of DMSO vehicle control, dexamethasone, or treatment chemical/mixture. After 24 hr exposure, overt toxicity was assessed and any wells with > 25% cells with cytopathological changes were excluded from further analysis. Viable cells were rinsed with PBS, lysed (luciferase cell culture lysis 5x reagent, Promega), mixed for 10 min and frozen at -80°C for 1 hr prior to luminescence readings. Wells containing cell lysate were thawed to room

temperature. Luciferase reaction buffer and firefly luciferase substrate, *Renilla* D-luciferin, were auto-injected immediately before luciferase readings every 0.2 sec for 5 sec at 3900 nm using a Fluostar luminometer (BMG LABTECH Inc., Cary, NC USA).

Chemical Exposures

GR ligands were selected based on reported presence in surface and/or waste waters. One known glucocorticoid antagonist, mifepristone, and 18 known agonists were screened in the CV1-hGR transcriptional assay to obtain potency and relative efficacy compared to the glucocorticoid reference compound dexamethasone. Each ligand was tested in a concentration-response manner concurrent with a dexamethasone standard curve. Ligand concentration treatments were replicated 4 times on each 96-well plate (4 wells per concentration), each chemical dose-response was replicated 3 times ($n = 3$ plates). Ligand concentrations for concentration-response experiments spanned several orders of magnitude increasing by half-log concentrations. The relative potency factor (RPF; compared to dexamethasone reference) where,

$$Relative\ Potency\ Test\ Ligand = \frac{EC_{50\ Dexamethasone}}{EC_{50\ Test\ Ligand}}$$

slope, EC_{50} , and maximum efficacy were determined for each agonist ligand.

Chemical Mixtures and Mixtures Modeling

Cell responses from exposure to equipotent and non-equipotent mixtures of glucocorticoid ligands were compared using mathematical models and experimental observations. Equipotent glucocorticoid mixtures responses were predicted using a concentration addition mixtures model (Olmstead, et al., 2005):

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^n \frac{C_i}{EC_{50,i}} \right)^{p'}}}$$

where R is the response to the mixture, C_i is the concentration of chemical i in the mixture, $EC_{50,i}$ is the concentration of chemical i that causes a 50% response, and p' is the average power associated with the chemicals with similar mechanisms of action. Additionally, responses of equipotent mixtures were modeled using the response addition mixtures model:

$$R = 1 - \prod_{i=1}^N (1 - R_i)$$

where R is the mixture response and R_i is the response of individual chemical i .

Responses for equipotent mixtures were also experimentally observed. Individual chemical concentrations in equipotent ligand mixtures were determined using

$$[GR\ ligand] = RPF * \frac{\% \text{ Response}_{Dex}}{n}$$

where n is the number of compounds in the ligand mixture. Subsequent half-log serial dilutions of the highest mixture concentration maintained equipotency while reducing overall mixture concentration *e.g.* 300nM, 100nM, 30nM, 10nM DexEqs. Concentrations for each ligand in each well can be found in Table S1. Equipotent mixtures were repeated 4 times (n = 4 96-well plates)

Equipotent mixtures containing partial agonists were significantly different from predicted responses of the concentration and/or addition mixtures models. Concentrations for each ligand in each well can be found in Tables S2 (dexamethasone and 21-hydroxyprogesterone) and S3 (dexamethasone and corticosterone). Therefore, responses of non-equipotent two-chemical mixtures containing either one full and one partial agonists or two full agonists were modeled using the generalized concentration addition model (Howard, et al., 2009):

$$R = f_{AB}([A], [B]) = \frac{\alpha_A \frac{[A]}{K_A} + \alpha_B \frac{[B]}{K_B}}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B}}$$

where R is the response of a mixture of two agonists at concentration [A] and [B], and α and K represent maximum efficacy and EC₅₀ values, respectively.

Cell responses to non-equipotent GR ligand mixtures containing either two full, or one full and one partial agonist were also experimentally observed. Cells were exposed using an 8 by 8 factorial matrix mixture exposure design originally described by *Bermudez et al* (Bermudez, et al., 2010; Bermudez, et al., 2012). Briefly, each well of cells was exposed to a variable combination of eight different concentrations (dexamethasone: 10pM-10nM; prednisolone 300pM- 300nM; 21-hydroxyprogesterone 3nM- 3μM; corticosterone 1nM- 1μM) of two chemicals totaling 64 mixtures treatment wells and 32 wells for a dexamethasone standard curve, including DMSO control. Matrix designed exposure experiments were repeated eight times (n = 8 96-well plates).

Calculations and Statistical Analysis

Data analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, LaJolla California, USA). Relative light unit (RLU) values for test chemical, test mixtures and dexamethasone were normalized to mean RLU of concurrent DMSO vehicle control-treated cells, and exposure concentrations were log₁₀-transformed. A non-linear four-parameter dose-response curve was fit to each data plot. Test ligand Y-values were normalized to the calculated top of the curve for concurrent dexamethasone concentration-response curve to determine final percent response values for test ligand treatments. A non-linear four-parameter dose-response curve was generated using the mean relative percent

response values for replicated test ligand concentration-response experiments (96-well plates) to obtain overall EC₅₀ and maximum response values (Table 1).

Full agonism was determined for each test chemical using overlapping 95% confidence intervals for max efficacy compared to the reference compound, dexamethasone. EC₅₀, slope, and maximum response values were used for mixtures response modeling, and best fit model was determined using r^2 for equipotent mixtures (Rider *et al.*, 2005). The Mann-Whitney (Wilcoxon rank-sum) statistical test was used to determine the best fit mixtures model for two chemical matrix exposure experiments, as described elsewhere (Howard, *et al.*, 2010; Watt, *et al.*, 2016).

Results

CV1-hGR Assay and Individual Ligand Screening

To apply this CV1-hGR transcriptional activation assay in agonist/antagonist screening and effects-based water quality monitoring, we evaluated assay sensitivity and responsiveness using dexamethasone as the reference compound and an approach similar to the one used by Suzuki *et al.* (Suzuki, *et al.*, 2015). The average maximum fold induction, or luciferase expression at 3.0×10^{-8} M dexamethasone divided by expression of vehicle control (DMSO), was 59-fold. This value is approximately equal to (Suzuki, *et al.*, 2015), or higher than (Suzuki, *et al.*, 2013; van der Linden, *et al.*, 2008) maximum fold values produced by similar experiments using the CALUX assay, indicating high assay responsiveness. The z-factor, an overall measurement of assay quality, was 0.730. Therefore, the CV1-hGR bioassay falls into the “excellent category” in regards to the initial chemical screen (Zhang *et al.*, 1999). The average EC₅₀ for dexamethasone ($n = 54$) was 2.32×10^{-10} M (95% CI = 2.21×10^{-10} to 2.4×10^{-10} M), indicating comparable sensitivity to existing assays (Bain, *et al.*, 2014; Macikova, *et al.*, 2014; Schriks *et al.*, 2013; Schriks, *et al.*, 2010; Suzuki, *et al.*, 2013; van der Linden, *et al.*, 2008).

Of the 19 GR ligands screened, one was a known human GR antagonist, mifepristone (RU486). When cells were co-treated with mifepristone and an optimized (80% of maximal) reference compound concentration of 1 nM dexamethasone, mifepristone IC₅₀ = 1.431×10^{-9} M (Figure 1). The remaining 18 ligands were GR agonists, causing increases in response as exposure concentrations increased (Figure 2). The mean curve slope for all agonist curves was 1.22 and ranged from 0.5349 (prednisone) to 2.171 (corticosterone) (Table 1). Agonists separated into one of two categories; full or partial agonists. Compounds that produced a maximum response statistically similar to the 100% max response of the reference compound, dexamethasone, were considered full agonists (Figure 2A). While compounds with an upper 95% confidence interval around their maximal response that was less than the lower 95% confidence interval around the maximal response of dexamethasone were considered partial agonists (Figure 2B). Hydrocortisone, corticosterone, aldosterone and 21-hydroxyprogesterone were classified as partial agonists. The highest tested concentration for 21-hydroxyprogesterone (100μM) was cytotoxic (> 25% cytopathological changes in each treatment well) and not used in further analysis, while prednisone and cortisone did not produce a maximal response value due to their limits of solubility in our bioassay.

The tested compounds spanned several orders of magnitude in potency, with EC₅₀ values ranging from 1.28e-10 to 3.93e-8 M. Observed EC₅₀ values are reported in Table 1, and relative compound potency was as follows: flucinolone acetonide > flumethasone > fluticasone propionate > triamcinolone acetonide > budesonide > flucanide > betamethasone > dexamethasone > dexamethasone-21-acetate > fludrocortisone acetate > 6α-methylprednisolone > prednisolone > hydrocortisone > corticosterone > aldosterone > 21-hydroxyprogesterone > prednisone > cortisone. Notably, all the partial agonists were also all less potent than the full agonists (Figure 2).

Full Agonist Mixture

Mixtures of various GR agonists have been detected in waters around the world, therefore we modeled the response of an equipotent mixture of the 12 full GR agonists (individual compounds depicted in Figure 2A) using both the concentration (CA) and response addition mixtures models (RA). The same equipotent GR full agonist mixture was evaluated experimentally using the bioassay (Figure 3). The EC₅₀ values for the CA, RA and observed concentration response curves were 2.24×10^{-10} , 2.28×10^{-10} , 2.55×10^{-10} , while the average Hill Slopes were 1.21, 1.55, 1.50, respectively.

We would expect a higher r^2 value for the CA since all the mixture agonists were synthesized to bind and activate the GR as their primary mechanism of action; an assumption when using the CA to evaluate chemical mixtures. However, the concentration-response curves from both models fit within the 95% prediction interval bands of the observed data indicating both accurately predict the observed values. In fact, the r^2 values for the CA and RA were very similar, 0.992 and 0.987, respectively.

Mixtures Containing Partial Agonists

Responses from equipotent fixed ratio mixtures containing one full agonist, dexamethasone, and one partial agonist, 21-hydroxyprogesterone (Figure 4A) or corticosterone (Figure 4B), were predicted using both CA and RA using individual chemical data (Table 1), as well as evaluated experimentally using the CV1-hGR bioassay. Neither the CA nor RA model worked well to predict the observed results of an equipotent mixture when it contained a GR partial agonist. R^2 values for the 21-hydroxyprogesterone and dexamethasone mixture were 0.315 (CA) and 0.424 (RA). For an equipotent mixture of corticosterone and dexamethasone the r^2 values were 0.414 (CA) and 0.432 (RA). Predicted mixture responses over-estimated the observed responses and fell outside the 95% confidence interval bands of the experimental data, especially at the highest chemical mixture concentrations where cellular response plateaued below 100% (Figure 4). In fact, the maximal response of both mixtures with partial agonists were more comparable to maximal responses of respective partial agonist alone (Figure 4).

Since equipotent partial agonist mixtures generated responses less than the maximum responses predicted by CA and RA models, we conducted multiple two-chemical mixtures experiments with variable mixture concentration ratios to evaluate how each compound may be contributing to an overall response. The CA (highest r^2 for full agonist mixtures), as well as the generalized concentration addition model (Howard, et al., 2009) which

mathematically permits <100% maximum efficacy, were used to predict assay responses. Observed (*in vitro* exposure) and model predicted responses to 21-hydroxyprogesterone, corticosterone or prednisolone and dexamethasone are shown in Figures 4, 5, and 6, respectively. Model-predicted and observed responses (gradient solid plane) were concomitantly graphed with $\pm$ SEM of observed responses (mesh planes) on three-dimensional plots for visual comparison.

Even at high concentrations of the full agonist dexamethasone, mixtures with partial agonists did not reach 100% max efficacy compared to the reference compound alone (Figure 5A, Figure 6A). In general, the CA model overestimated responses in the presence of high concentrations of partial agonists, evident by +SEM mesh plane below gradient plane (Figure 5B, Figure 6B). However, the generalized concentration addition model plane generally fell within the $\pm$ SEM mesh planes (Figure 5C, Figure 6C).

A statistical comparison of response value distributions (Mann-Whiney) between model predictions and observed responses were used to determine a superior model for predicting mixtures of known GR ligands. With a rejection limit of $p > 0.05$, only the GCA model values were from the same statistical distribution suggesting that the GCA was the best fit model for partial agonists (Table 2). Responses to non-equipotent mixtures of full agonist prednisolone and dexamethasone also were evaluated *in vitro* (Figure 7A), and using the CA (Figure 7B) and GCA (Figure 7C) models, to determine if the GCA model also could be effectively applied to mixtures of full agonists (no partial agonists). Both the CA and GCA models resulted in p-values below the rejection limit suggesting GCA could indeed be used for two chemical mixtures containing only full agonists.

Discussion

Herein we further characterized a recently developed *in vitro* glucocorticoid receptor activation assay applicable to effects-based water quality screening, by initially determining the potency and efficacy of 19 GR ligands previously detected in surface and waste waters. Further, sufficient evidence is provided for the selection of the GCA model, compared to other additive response chemical mixture models, for predicting responses of GR agonist mixtures containing a partial GR agonist.

We used dexamethasone to produce assay reference standard curves since the pharmaceutical has been well-established as the reference compound of choice for glucocorticoid receptor activation assays due to its high potency. Suzuki *et al.* describes the measured potency (EC_{50}) of dexamethasone in GR assays as a measure of assay responsiveness which can be compared to other assays quantifying the same biological endpoint. We report an EC_{50} value of $2.24E^{-10}$ M (Table 1) for GR activation by dexamethasone in the CV1-hGR bioassay. This is a comparable value to those reported for GR CALUX assays, ($1.6E^{-9}$ (Schriks, et al., 2013); $5.3E^{-10}$ (Macikova, et al., 2014); $7.3E^{-10}$ (van der Linden, et al., 2008); $8.1E^{-10}$ (Suzuki, et al., 2013); $8.4E^{-10}$ (Schriks, et al., 2010); and $2.5E^{-9}$ M (Bain, et al., 2014)). In general, the reported $EC_{50(DEX)}$ values for GR activation using GR-GeneBLAzer and GR-switchgear indicate that the CV1-hGR is a more sensitive bioassay method. For example, $EC_{50(DEX)}$ values for GR-GeneBLAzer were $2.5E$

⁻⁹ (Jia, et al., 2015); and $1.8\text{E } 10^{-9}$ M (Huang, et al., 2011), while GR-switchgear $\text{EC}_{50(\text{DEX})} = 2.6\text{E}^{-9}$ M (Jia, et al., 2015).

Moreover, quantifying activation of the specified biological endpoint using the CV1-hGR bioassay comes with the added benefit of reduced cost compared to commercially available counterparts (BioDetectionSystems, 2018; SwitchGearGenomics, 2010), as well as a high assay Z-score (0.730). This dimensionless parameter indicates a degree of confidence in reporting positive hits. (Zhang, et al., 1999). A value > 0.5 supports the continued use of this assay in medium to high-throughput screening for GR activation in future water quality screens.

We assessed many known GR ligands that had been previously detected in waste and/or surface waters around the world. Evaluated compounds included endogenous glucocorticoids, mineralocorticoids, and progestins, as well as synthetically-derived glucocorticoids, each with pharmaceutical applications (Table 1). Notably, all environmental compounds ascertained in our literature search were either endogenous hormones or engineered (GR-specific pharmaceuticals) to bind our desired receptor target. Although two groups recently reported environmental compounds with non-pharmaceutical/non-therapeutic origins, including imidazole fungicides and organophosphate flame retardants, that exhibit GR antagonistic qualities at relatively high concentrations ($\mu\text{g/L}$) (Duret *et al.*, 2006; Zhang *et al.*, 2017).

Ligand specificity and affinity for protein receptors often increases as newer generations of pharmaceuticals are developed with fewer adverse side effects to target species (D'Haens, 2016; Hochhaus, 2004), and sometimes to the detriment of non-target aquatic species exposed to agricultural or waste effluents (Ellestad *et al.*, 2014). Although designated GR pharmaceutical classes distinguish compounds based on similar potency, classification systems vary based on route of administration and formulation (FERENCE *et al.*, 2009; Kerscher *et al.*, 2006). Therefore, it is difficult to determine whether any standardized classification system coincides with our observed potency (EC_{50}) of the individual chemicals for GR activation. However, functional groups present on the steroid D-ring were highly variable among the selected ligands. In general, increases in molecular weight and structural complexity of these functional groups coincided with observed increases in chemical potency for individual compounds. For example, D-ring functional group molecular weight and ligand potency for fluticasone propionate (167.14 g/mol) > dexamethasone (92.26 g/mol) > cortisol (76.22 g/mol). He *et al.* attributed both the lack of D-ring functional groups and increased flexibility associated with a double-bonded set of carbon atoms in the steroid A-ring with cortisol's decreased affinity for the GR compared to other more potent ligands (He *et al.*, 2014).

Not only did the selected GR ligands vary in potency, but they also varied in efficacy. Hydrocortisone, corticosterone, aldosterone and 21-hydroxyprogesterone were partial agonists because they did not produce 100% max response of dexamethasone. Even in mixture experiments that involved a full agonist, mixture responses remained low when despite dexamethasone concentrations that were high enough to invoke a maximal response (30 nM dexamethasone alone). Other studies with human nuclear receptors have

characterized similar phenomena with mixtures containing partial agonists (Hadrup, et al., 2013; Howard, et al., 2010; Watt, et al., 2016), and have defined the occurrence of reduced maximal response as “competitive antagonism” (Watt, et al., 2016). These differences in activity have been attributed to the structural conformation of the characteristic α helix 12, which correlates to the peak ligand-induced transcriptional activation (maximal efficacy) (Bledsoe *et al.*, 2002; Kauppi *et al.*, 2003).

While defining an appropriate mixtures model for GR receptor activation is useful, characterizing mixture responses (especially below the lowest observable effects concentrations for individual chemicals) is fundamental to employing effects-based tools for their intended environmental screening applications. Several *in vivo* studies have illustrated how mixtures of chemicals, even at very low concentrations, can invoke an additive response for an adverse outcome when in a mixture with several other compounds with the same mechanism of action (the “something from nothing” hypothesis) (Brian *et al.*, 2005; Conley *et al.*, 2018; Deneer *et al.*, 1988; Silva, et al., 2002). Further *in vivo* and *in vitro* responses to mixtures of estrogen and androgen receptor ligands are well-characterized (Bermudez, et al., 2010; Blake, et al., 2010; Brian, et al., 2005; Conley, et al., 2016; Howdeshell *et al.*, 2008; Rider *et al.*, 2008; Rider *et al.*, 2010; Silva, et al., 2002), but to our knowledge this is the first characterization of its kind using the human glucocorticoid receptor.

Quintessential to effects-based assays, like the CV1-hGR, is their ability to positively identify summed concentrations of biologically active compounds at concentrations below which the compounds could be detected individually using alternative methods. Our previous study applying this bioassay illustrates the importance of developing such GR detections methods. Despite identifying GR activity using the CV1-hGR bioassay in multiple samples from across the country (Conley, et al., 2017), the compounds included in the targeted chemical analyses were not sufficient to account for the observed activity (Bradley *et al.*, 2017). This lack of association between target analytes and GR activity could be due to the absence of the relevant compounds from the analyte list, suggesting the analyte list should be broadened, or unrecognized chemical-receptor interactions might exist.

Taken together, the potential for activation of adverse outcome pathways by mixtures of individual compounds at low concentrations and that environmental glucocorticoid-active compounds may exist below other existing method detection limits, illustrates the importance of continually improving our methods, as well as our understanding of this bioanalytical screening tool. Finally, the economy and utility of the CV1-hGR bioassay, as well as the inability for some existing waste water treatment practices to fully remove glucocorticoids (Jia, et al., 2016), reinforces its role as one part of a screening assay suite in future water quality screening programs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mifepristone concentration-response curve.

CV1-hGR cells were co-treated with 1 nM dexamethasone and a range of concentrations of a known antagonist, mifepristone, to obtain IC_{50} value (1.431×10^{-9} M) in the bioassay.

Data reported as mean % response relative to dexamethasone standard curve and error bars represent $\pm$ SEM ($n = 3$).

Figure 2. Glucocorticoid receptor full and partial agonists.

CV1-hGR cells were treated with individual glucocorticoid receptor full (A) and partial (B) agonists over a range of concentrations to determine EC₅₀, potency, and max efficacy for each compound in the bioassay (Table 1). Data reported as mean % response relative to concurrent dexamethasone standard curve $\pm$ SEM (n = 3). The 95% confidence interval bands were used to determine type of agonist (full or partial), bands were omitted from full

agonist graph for clarity. (A complete concentration-response curve was unattainable for cortisone due to compound solubility. For this reason, the confidence interval around the curve was not predicted and therefore was not included in graph B.)

Figure 3. Fixed ratio mixture of twelve glucocorticoid receptor full agonists.

Response produced by CV1-hGR cells upon exposure to a fixed ratio equipotent mixture of twelve full GR agonists was modeled (concentration and response addition mixtures models) and experimentally tested ($n = 4$, individual ligand concentrations can be found in Table S1). Model predictions and observed responses were compared using best fit curve. Data for observed concentration-response curve reported as mean % response relative to concurrent dexamethasone standard curve $\pm$ SEM ($n = 4$).

Figure 4. Partial agonist and fixed ratio mixtures with full and partial agonist concentration-response curves.

Responses produced by CV1-hGR cells upon exposure to a partial agonist A) 21-hydroxyprogesterone or B) corticosterone and fixed ratio equipotent mixtures of a full agonist, dexamethasone, and each partial agonist were determined. Mixtures were modeled (concentration and response addition mixtures models) and experimentally tested. Data for observed mixture concentration-response curve reported as mean % response relative to

concurrent dexamethasone standard curve $\pm$ SEM (n = 4; individual ligand concentrations can be found in Table S2 and S3).

Figure 5. Dexamethasone and 21-hydroxyprogesterone matrix mixture experimental observation and model prediction comparisons.

Responses of CV1-hGR cells to chemical mixtures with variable ratios of dexamethasone (full agonist) and 21-hydroxyprogesterone (partial agonist) were A) measured experimentally (observed; $n = 8$) and predicted using B) the concentration addition and C) generalized concentration addition models. A) *In vitro* responses and B, C) model predictions are plotted in solid gradient planes concurrently with the $\pm$ SEM of observed responses (mesh planes), for visual comparison of observed and predicted results.

Figure 6. Dexamethasone and corticosterone matrix mixture experimental observation and model prediction comparisons.

Responses of CV1-hGR cells to chemical mixtures with variable ratios of dexamethasone (full agonist) and corticosterone (partial agonist) were A) measured experimentally (observed; $n = 8$) and predicted using B) the concentration addition and C) generalized concentration addition models. A) *In vitro* responses and B, C) model predictions are plotted in solid gradient planes concurrently with the $\pm$ SEM of observed responses (mesh planes), for visual comparison of observed and predicted results.

Figure 7. Dexamethasone and prednisolone matrix mixture experimental observation and model prediction comparisons.

Responses of CV1-hGR cells to chemical mixtures with variable ratios of dexamethasone (full agonist) and prednisolone (full agonist) were A) measured experimentally (observed; $n = 8$) and predicted using B) the concentration addition and C) generalized concentration addition models. A) *In vitro* responses and B, C) model predictions are plotted in solid gradient planes concurrently with the $\pm$ SEM of observed responses (mesh planes), for visual comparison of observed and predicted results.

Table 1:
Referenced and Observed Values for Glucocorticoid Receptor Ligands Screened in the CV1-hGR Bioassay

Detected Compound	CAS #	Previously Reported Values			Experimental Values from CV1-hGR Bioassay				
		Environmental Concentration (ng/L) [†]	EC ₅₀ (nM)	Relative Potency (DexEqs)	EC ₅₀ (nM)	Relative Potency	Hill Slope	Max Efficacy (% Dex)	95% CI Max Efficacy
Flucinolone acetate	67-73-2	3.69 (Jia, et al., 2016)	0.24 (Jia, et al., 2016)	7.398 (Jia, et al., 2016)	0.032	6.933	1.34	98.03	92.61 to 103.7
Triamcinolone acetate	76-25-5	14.0 (Jia, et al., 2016), 5 (Macikova, et al., 2014)	0.79 (Jia, et al., 2016), 0.37 (Schriks, et al., 2010)	2.265 (Jia, et al., 2016), 2.3 (Schriks, et al., 2010), 1.12 (Macikova, et al., 2014)	0.089	2.513	1.35	99.21	96.93 to 101.5
Fluticasone propionate	80474-14-2	1.43 (Jia, et al., 2016), <1 (Macikova, et al., 2014)	0.025 (Jia, et al., 2016)	70.88 (Jia, et al., 2016), 57 (Macikova, et al., 2014)	0.088	2.560	0.97	102.5	97.4 to 108.1
Flucuronide	356-12-7	0.27 (Jia, et al., 2016)	1.89 (Jia, et al., 2016)	0.948 (Jia, et al., 2016)	0.13	1.753	1.51	94.74	91.11 to 98.46
Budesonide	51333-22-3	0.36 (Jia, et al., 2016), 5 (Macikova, et al., 2014)	0.26 (Jia, et al., 2016)	6.895 (Jia, et al., 2016), 6.1 (Macikova, et al., 2014)	0.11	2.023	0.90	97.24	94.06 to 100.6
Flumethasone	2135-17-3	5 (Macikova, et al., 2014)	0.36 (Jia, et al., 2016)	5.032 (Jia, et al., 2016), 4.0 (Macikova, et al., 2014)	0.079	2.849	1.11	98.43	96.18 to 100.7
Dexamethasone 21-acetate	1177-87-3	8 [*] (Macikova, et al., 2014)	NA	1.36 (Macikova, et al., 2014)	0.24	0.920	1.09	96.88	92.75 to 101.3
Dexamethasone	50-02-2	0.16 (Jia, et al., 2016), 15 [*] (Macikova, et al., 2014)	1.79 (Jia, et al., 2016), 0.84 (Schriks, et al., 2010), 9.7E-10 (Suzuki, et al., 2015)	1	0.024	1	1.25	99.85	96.45 to 103.4
Betamethasone	378-44-9	0.66 (Jia, et al., 2016), 15 [*] (Macikova, et al., 2014)	2.83 (Jia, et al., 2016), 1.02 (Schriks, et al., 2010), 1.3E-09 (Suzuki, et al., 2015)	0.634 (Jia, et al., 2016), 0.8 (Schriks, et al., 2010), 0.59 (Macikova, et al., 2014)	0.19	1.158	1.39	92.26	90.49 to 94.07
Fludrocortisone acetate	514-36-3	14 (Macikova, et al., 2014)	9.67 (Jia, et al., 2016)	0.185 (Jia, et al., 2016)	0.49	0.454	1.16	93.27	89.15 to 97.75
6α-methylprednisolone	83-43-2	1.53 (Jia, et al., 2016), 6 (Macikova, et al., 2014)	6.79 (Jia, et al., 2016), 2.25 (Schriks, et al., 2010), 2.4E-09 (Suzuki, et al., 2015)	0.264 (Jia, et al., 2016), 0.4 (Schriks, et al., 2010), 0.54 (Macikova, et al., 2014)	0.64	0.348	1.35	95.67	92.34 to 99.23

		Previously Reported Values			Experimental Values from CV1-hGR Bioassay				
Detected Compound	CAS #	Environmental Concentration (ng/L) [†]	EC ₅₀ (nM)	Relative Potency (DexEq)	EC50 (nM)	Relative Potency	Hill Slope	Max Efficacy (% Dex)	95% CI Max Efficacy
Prednisolone	50–24–8	0.34 (Jia, et al., 2016), 24 [*] (Macikova, et al., 2014)	17.7 (Jia, et al., 2016), 3.68 (Schriks, et al., 2010), 5.7E-09(Suzuki, et al., 2015)	0.101 (Jia, et al., 2016), 0.2 (Schriks, et al., 2010), 0.13 (Macikova, et al., 2014)	3.90	0.057	1.12	98.4	94.93 to 102
Hydrocortisone (cortisol)	50–23–7	1.57 (Jia, et al., 2016), 29 [*] (Macikova, et al., 2014)	11.4(Schriks, et al., 2010), 1.4E-08 (Suzuki, et al., 2015)	0.07 (Schriks, et al., 2010), 0.036 (Macikova, et al., 2014)	3.90	0.057	1.44	90.13	86.37 to 94.3
Corticosterone	50–22–6	6 (Macikova, et al., 2014)	NA	0.033 (Macikova, et al., 2014)	10.83	0.021	2.17	80.54	77.6 to 83.55
Aldosterone	52–39-1	4 (Macikova, et al., 2014)	112.2 (Schriks, et al., 2010)	0.008 (Schriks, et al., 2010), 0.0037 (Macikova, et al., 2014)	61.25	0.004	1.09	77.36	73.98 to 81.16
21-hydroxyprogesterone	64–85–7	3 (Macikova, et al., 2014)	NA	0.00079 (Macikova, et al., 2014)	80.97	0.003	1.33	48.09	42.98 to 53.5
Prednisone	53–03–2	24 [*] (Macikova, et al., 2014)	>500 (Jia, et al., 2016), >500 (Schriks, et al., 2010)	<0.004 (Jia, et al., 2016), <0.002 (Schriks, et al., 2010)	NA	1.06E-05	0.53	N/A	N/A
Cortisone	53–06–5	0.51 (Jia, et al., 2016), 29 [*] (Macikova, et al., 2014)	>500 (Jia, et al., 2016), >1000 (Schriks, et al., 2010)	<0.004 (Jia, et al., 2016), <0.0008 (Schriks, et al., 2010)	NA	1.39E-10	0.79	N/A	N/A
Mifepristone	84371–65-3	1 (Macikova, et al., 2014)	-	(IC50: Dex)	0.04987 (IC50: 1nM Dex)	Antagonist	–1.01	N/A	N/A

CAS numbers, referenced values (environmental concentrations and potency), and measured values from CV1-hGR bioassay are provided for each tested glucocorticoid receptor ligand.

[†] Previously detected in waste and surface waters; environmental concentrations reported in respective references.

^{*} Compound was co-eluted from environmental sample with another GR ligand and was therefore reported as a summed concentration of the both compounds.

Table 2
Statistical Summary for Variable Model Predictions.

The distribution of values for the Concentration Addition Mixtures Model (CA) and the Generalized Concentration Addition Mixtures Model (GCA) were compared to the distribution of observed values. A significant difference ($p < 0.05$) indicates the modeled and observed data sets are from different distributions and that the model did not accurately predict observed values.

Observed Data	Model	Mann-Whitney p-value	Significant Difference ($p < 0.05$)
21-hydroxyprogesterone	CA	<0.0001	yes
21-hydroxyprogesterone	GCA	0.9111	no
corticosterone	CA	0.0004	yes
corticosterone	GCA	0.2488	no
prednisolone	CA	0.5479	no
prednisolone	GCA	0.7342	no