The Influence of Environmental Exposures on Glucocorticoid Receptor Signaling

By

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ABSTRACT

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Glucocorticoids (GCs) are hormones secreted in response to psychological stress. GCs have systemic effects on the endocrine, metabolic, cardiovascular, immune, reproductive, and central nervous systems. The physiological effects of GCs are mediated by the glucocorticoid receptor (GR), which is expressed in nearly every cell of the body. Environmental chemicals that disrupt GR signaling and/or cortisol homeostasis could adversely affect human health. A major challenge in identifying environmental chemicals that alter GR signaling in humans is a lack of adequate screening methods. The overall objective of this dissertation is to investigate the impact of environmental exposures on GR signaling and human GC levels. Chapter 1 discusses regulation of GC levels and presents evidence that environmental chemicals modulate GR signaling. Chapter 2 provides the field with a new bioassay to assess the endocrine disrupting effects of environmental chemicals on GR signaling. Results from this work demonstrate that this bioassay can be used to identify environmental chemicals that modulate GR activation and to screen serum samples for differences in total GC levels. Chapter 3 examines the persistent effect of early-life arsenic exposure on GC levels in a human population study. This is the first epidemiology study to investigate associations between early-life environmental exposures and GC levels in adulthood. Lastly, Chapter 4 evaluates prediction models of GR ligand mixtures. As a whole, this research aims to inform risk assessment of endocrine disrupting chemicals by developing methods to evaluate their impact on GR signaling and human health.

Dedication

I dedicate this dissertation to all the strong women in my life that taught me to embrace challenges with patience, perseverance, and pride.

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Chapter 1: Introduction

1.1 The Hypothalamic-Pituitary-Adrenal (HPA) Axis and Human Health

The HPA axis regulates the secretion of steroid hormones called glucocorticoids (GCs) that influence the metabolic, cardiovascular, immune, reproductive, and central nervous systems (Sapolsky et al. 2000). Cortisol is the endogenous GC found in humans. Corticotropin-releasing hormone (CRH) is secreted by the hypothalamus in response to stress, which stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland and induces the synthesis and release of cortisol from the adrenal cortex into the blood stream (Herman and Cullinan 1997). In blood, cortisol is mostly bound to corticosteroid-binding globulin (CBG) until it is released and enters a target cell where it binds the glucocorticoid receptor (GR) in the cytoplasm. Intracellular levels of cortisol are also regulated by 11β-HSD2, which converts cortisol to its inactive form called cortisone, and by 11β-HSD1 that catalyzes the reverse reaction (Tomlinson and Stewart 2001). Together, these factors regulate circulating cortisol levels.

HPA activity follows a circadian rhythm where the peak cortisol secretion occurs 30 minutes after waking and continues to decline throughout the day (Kirschbaum and Hellhammer 1989). The HPA axis is regulated by negative feedback loops that function to maintain homeostatic levels of circulating cortisol. Chronic activation of the HPA axis can cause "wear and tear" on these regulatory mechanisms and alter cortisol homeostasis (McEwen 1998). For example, there is evidence that chronic stress during early-life alters cortisol levels and GR signaling (Miller et al. 2009; Repetti et al. 2002). Moreover, aberrant HPA axis activity can also influence health and disease (DeMorrow 2018). Hypercortisolism, also known as Cushing's Syndrome, has been linked to adverse health effects including cardiovascular disease, osteoporosis, glaucoma, central obesity, hyperglycemia, and psychiatric disorders (Kadmiel and Cidlowski, 2013). In contrast, Addison's disease results in cortisol deficiency and is associated with impaired stress resistance, lymphoid tissue hypertrophy, weight loss, and hypoglycemia (Nieman and Chanco Turner 2006). Consequently, environmental exposures that act at any level of the HPA axis can have implications for human health. Odermatt et al. proposes that environmental chemicals can also modulate cortisol hormone action by disrupting:

1. Regulation of the HPA axis
2. Activity of enzymes with a role in steroidogenesis
3. Binding capacity of serum proteins
4. GC uptake into target cells
5. Intracellular metabolism of GCs by 11-HSD enzymes
6. Activation of GR
7. Function of GR-associated proteins
8. Binding to the promoter of a given target gene
9. GC export from the cell
10. Degradation and excretion of the steroid hormone

This dissertation aims to examine the influence of environmental chemicals on regulation of the HPA axis (1) and activation of GR (6).

1.2 Environmental Exposures and the HPA Axis

There is limited evidence that environmental chemicals disrupt HPA axis activity, which is typically assessed by measuring cortisol levels. Table 1.1 summarizes environmental exposures

associated with altered cortisol levels in epidemiological studies. The relationship between environmental exposures and cortisol varies by chemical. For example, arsenic (Sinha et al. 2014), cadmium (Bochud et al. 2018), dioxins (Kido et al. 2014; Manh et al. 2013), organophosphate pesticides (Cecchi et al. 2012), ozone (Miller et al. 2016), and particulate matter (Brook et al. 2010) were all associated with increased cortisol levels. Alternatively, blunted cortisol levels were observed in individuals exposed to bisphenol A (Giesbrecht et al. 2016), mercury (Gump et al. 2012; Schreier et al. 2015), and organochlorine pesticides (Araki et al. 2018). Despite inconsistencies in the direction of effect, it is clear that environmental exposures influence HPA axis activity.

Most studies to date have examined the relationship between lead exposure and HPA activity (Braun et al. 2014; Fortin et al. 2012, 2012; Gump et al. 2008; Souza-Talarico et al. 2017). Lead exposure has been associated with altered cortisol levels in both children and adults. However, results were inconsistent across studies, possibly due to differences in age, duration, and intensity of exposure. In children, the association between lead exposure and cortisol levels changed over a 6-12 month period demonstrating that this relationship is dynamic (Tamayo y Ortiz et al. 2016). No study has investigated the effect of early-life environmental exposures in adulthood. Since the diurnal cortisol rhythm develops in infancy and continues throughout early childhood, exposures that occur during this critical period may have long-term consequences on HPA axis activity (Gunnar and Quevedo 2007). Therefore, an objective of this dissertation is to examine the effect of early-life exposures on GC levels in adulthood.

1.3 Measuring Differences in Glucocorticoid Levels

GC concentrations are routinely measured using antibody-based methods, such as enzyme-linked immunosorbent assay (ELISA), or by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). While both of these techniques quantify the amount of a specific GC, they often require sample preparation, are expensive, and do not measure the biological effect of endogenous and exogenous GCs present in the biological matrix (Xu et al. 2014). Alternatively, receptor-based bioassays have also been used in epidemiologic studies to measure differences in plasma hormone bioactivity between individuals (Brouwers et al. 2011; Murk et al. 1997; Van Wouwe et al. 2004). These reporter assays link the expression of a luciferase gene to glucocorticoid response elements (GREs), thus providing a light-based readout that is proportional to the degree of glucocorticoid receptor activity (Figure 1.1). Several studies thus far have demonstrated that bioassays are able to measure differences in serum/plasma GC levels (Fejerman et al. 2016; Kajantie et al. 2004; Perogamvros et al. 2011; Raivio et al. 2002; Turner et al. 2010; Vermeer et al. 2003). For example, plasma GC levels were elevated after synthetic GC administration and differences in GR potency could be detected (Raivio et al. 2002). Most of these bioassays were created using mammalian cells that do not endogenously express GR (e.g. COS-1 and HEK293) and may not reflect the physiological receptor levels present in normal cells. One study did address this limitation by using transiently transfected cells that endogenously express GR (Perogamvros et al. 2011). This approach could be improved by using a stable cell line that increases reproducibility and power to detect smaller differences in GC levels between groups. Fejerman et al. identified an association between plasma GC levels and alcohol consumption using the stable AR/GR reporter cells, MDA-Kb2 (Fejerman et al. 2016). However, this cell line contains both the androgen and glucocorticoid reporter, which could affect specificity. Ideally, serum/plasma GC levels would be measured using a stable reporter

cell line that endogenously expresses GR, and no other nuclear receptor. Part of this dissertation work focuses on the development of a bioassay to detect GR modulation by environmental chemicals and GCs present in human serum/plasma.

1.4 Environmental Exposures and GR Signaling

HPA axis activity is controlled by negative feedback mechanisms mediated by GR (Myers et al. 2012). GR also mediates the physiological effects of GCs. After binding cortisol, GR undergoes a conformational change that allows it to dissociate from the heat-shock protein complex and translocate to the nucleus, where it regulates transcription of 10-20 percent of the human genome (Oakley and Cidlowski 2013). The ubiquitous expression of GR in almost all human tissues highlights the importance of this biological pathway (Pujols et al. 2002). Consequently, environmental chemicals that alter GR signaling can have drastic effects on HPA axis activity and human physiology.

Most studies thus far have focused on identifying environmental compounds that either induce (agonists) or inhibit (antagonists) GR transcription. The Tox21 program has tested over 8000 chemicals and identified 569 potential agonists and 472 antagonists of GR (US EPA 2017). Table 1.2 also provides a list of chemicals reported in the literature to modify GR transcription. Interestingly, two chemicals on the list (p,p-DDE and PCB-153) enriched GR signaling in the human cord blood transcriptome (Remy et al. 2016). However, few studies have examined the effect of GR modulators on human cortisol levels (Table 1). Furthermore, the mechanism of altered GR transcription for most of these compounds is unknown.

GR signaling can be modified through several different mechanisms. Chemicals can interfere with transcriptional activation by hindering cortisol binding, GR translocation, or GR binding to GREs in the promoter region of target genes. Chemically induced epigenetic modifications can also influence GR signaling. For example, cadmium enhanced methylation of the GR gene exon 1, which corresponded to lower GR expression levels (Castillo et al. 2012). The network of GR transcription is also determined by cellular and physiological context (Weikum et al. 2017). Therefore, chemicals that influence cell-specific cofactor interactions can also modify GR transcription. Arsenic was selected as the focus of this dissertation because it is the most comprehensively studied environmental compound for all of the above-mentioned mechanisms.

1.5 Arsenic Disruption of GR Signaling and HPA Axis Activity

Arsenic is a known human carcinogen that is also associated with adverse developmental, neurological, cardiovascular, metabolic and immunological effects (Naujokas et al. 2013). The mechanism by which arsenic causes these health effects is unknown. An analysis of 12 human pregnancy cohort studies found that GR signaling was associated with increased susceptibility to infectious disease (e.g. respiratory infection and diarrhea) from prenatal arsenic exposure (Rager et al. 2014). These results were confirmed using a chick embryo model where treatment with a GR inhibitor protected the embryos from developing arsenic-induced birth defects. Epidemiological studies have also observed increased GR methylation in placental tissues relative to prenatal arsenic exposure (Appleton et al. 2017; Cardenas et al. 2015). Together, these studies provide some evidence that arsenic-related disease may be mediated by GR signaling.

Multiple in vitro studies have demonstrated that arsenic disrupts GR transcriptional activity (Gosse et al. 2014; Bodwell et al. 2004; Kaltreider et al. 2001). Sodium arsenite treatments ranging from 0.3–3.3μM significantly reduced GC-induced expression of phosphoenolpyruvate carboxykinase, a GR target gene, in H4IIE rat hepatoma cells (Kaltreider et al. 2001). This same study demonstrated that arsenic does not affect GR nuclear localization or the ability of GCs to bind GR. Interestingly, a bi-phasic dose-response was observed between arsenic treatment and another GR target gene, tyrosine aminotransferase, in EDR3 hepatoma cells (Bodwell et al. 2004). Stimulatory effects on GR-mediated gene expression were observed for arsenic concentrations between 0.05-1μM. Conversely, treatment with higher arsenic concentrations produced inhibitory effects on GR-mediated gene expression. One possible mechanism by which arsenic decreases GR transcription is through impaired recruitment of co-activator proteins, such as CARM1, to GREs (Barr et al. 2009).

There is also strong evidence that arsenic disrupts HPA axis regulation in rodents. Adult male mice exposed to drinking water containing 50ppb of arsenic during the perinatal period show depressive-like behaviors, blunted stress responses to a predator-scent stressor, and a 2-fold increase in basal plasma GCs (Goggin et al. 2012; Martinez et al. 2008). Prenatally exposed mice also had reduced GR and glycosylated 11β-HSD1 protein expression in the hippocampus (Goggin et al. 2012). Expression of GR target genes important for learning and memory were reduced in the hippocampus of perinatally exposed mice (Martinez-Finley et al. 2011). Therefore, the reduction in hippocampal GR may link arsenic exposure to deficiencies in cognitive development. Similar decreases in GR and 11β-HSD1 protein were also observed at embryonic day 14 and 18 suggesting that prenatal arsenic exposure impairs negative feedback mechanisms of the HPA axis during development and that this effect is sustained into adulthood (Caldwell et al. 2015). While these studies indicate that early-life arsenic exposure alters HPA axis and GR signaling in mice, it is unclear whether similar responses occur in humans. This dissertation aims to conduct the first study to evaluate the effect of early-life arsenic exposure on GC levels in a human population.

1.6 Mixture Effects of Endocrine Disrupting Compounds

Studies on individual chemicals, such as arsenic, have demonstrated that environmental exposures can alter GR signaling and HPA axis activity. With over 80,000 chemicals on the U.S. market, the challenge lies in prioritizing which ones to evaluate for endocrine-related endpoints. Chemical screening programs like Tox21 have attempted to rank and prioritize chemicals based on their in vitro bioactivity profiles across hundreds of assay endpoints, including GR transcriptional activation and binding assays (Richard et al. 2016). However, testing individual chemicals is not representative of mixtures present in the human environment. For example, a survey conducted by the Environmental Working Group found that the average American adult uses 9 personal care products each day with 126 unique chemical ingredients (EWG 2004). Therefore, evaluating the effect of single chemicals may underestimate human health risk since it does not account for potential mixture effects (Kortenkamp and Faust 2018). Studies have also demonstrated that mixtures of endocrine disrupting compounds can elicit a response, even when each chemical is present at concentrations that individually produce unnoticeable effects (Orton et al. 2014; Silva et al. 2002). Researchers have attempted to model these additive effects using formulas based on knowledge of individual dose-response curves (Rider et al. 2018). Developing accurate prediction models not only improves estimation of additive effects but also helps inform

risk assessment of chemical mixtures. Most studies thus far have focused on modeling the mixture effects of chemicals on the estrogen, androgen, and thyroid receptors (Kortenkamp 2007). This dissertation work explores the application of a recently developed mixture model to predict the additive effect of chemicals on GR signaling.

1.7 Summary

The overall objective of this dissertation is to investigate the impact of environmental exposures on GR signaling and human GC levels. This work also provides the field with new methodologies to assess the endocrine disrupting effects of environmental chemicals on GR signaling. Chapter 2 describes the development of a bioassay to measure GR signaling and HPA axis activity. The effect of early-life arsenic exposure on human GC levels is discussed in Chapter 3. Results from this chapter will determine whether arsenic has persistent effects on HPA axis activity, thus providing a novel mechanism of arsenic-related disease. The final chapter of this dissertation uses the bioassay developed in Chapter 1 to evaluate prediction models of GR ligand mixtures. As a whole, this research aims to inform risk assessment of endocrine disrupting chemicals by developing methods to evaluate their impacts on GR signaling and human health.

Tables and Figures

Figure 1.1: Schematic of GR bioassay. Transcription of the luciferase reporter gene provides a light-based readout that is proportional to the degree of glucocorticoid receptor activity.

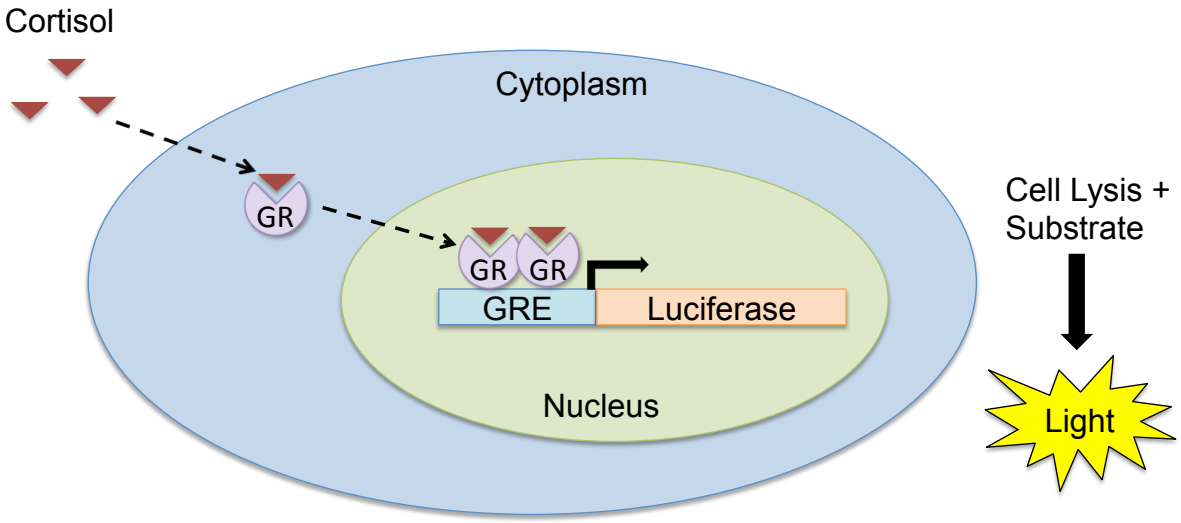

Table 1.1: Effect of environmental exposures on human cortisol levels.

Chemical	Population	Mean Age (Years)	Effect	Reference
Arsenic	Women (N=267)	38	Increased serum cortisol levels	Sinha et al. 2014
Bisphenol A	Pregnant women (N=174)	31.5	Decreased salivary cortisol at waking & flatter daytime pattern	Giesbrecht et al. 2016
Cadmium	Adults (N=1000)	47.3	Increased urinary cortisol metabolite	Bochud et al. 2018
Dioxins	Lactating women (N=109)	26.7	Increased salivary and serum cortisol levels	Kido et al. 2014
	Lactating women (N=25)	23.2	Increased salivary cortisol levels	Manh et al. 2013
Lead	Older Adults (N=126)	65.9	Increased salivary cortisol levels	Souza-Talarico et al. 2017
	12-month-old infants (N=255)	-	Decreased salivary cortisol levels	Tamayo y Ortiz et al. 2016
	18–24-month-old infants (N=150)	-	Increased salivary cortisol levels	Tamayo y Ortiz et al. 2016
	Pregnant women (N=936)	27.8	Decreased cortisol awakening response & flatter diurnal slope	Braun et al. 2016
	Occupationally exposed males (N=70)	46.4	Decreased serum cortisol levels	Fortin et al. 2012
	Children (N=169)	2.6	Increased salivary cortisol response to stressor	Gump et al. 2008
Mercury	Pregnant women (N=732)	27.4	Blunted morning cortisol response with high stress	Schreier et al. 2015
	Children (N=100)	10	Blunted diurnal cortisol levels	Gump et al. 2012
Organochlorine pesticides (Mirex & trans-nonachlor)	Pregnant women (N=514)	30.4	Decreased cortisol levels in cord blood	Araki et al. 2018
Organophosphate pesticides	Pregnant women (N=97)	24	Increased serum cortisol levels in 1st trimester	Cecchi et al. 2012
Ozone	Health young adults (N=24)	25.6	Increased serum cortisol levels	Miller et al. 2016
Particulate matter (<2.5μM)	College students (N=55)	20.2	Increased serum cortisol levels	Li et al. 2017

Table 1.2: Chemicals that modify GR transcription

Chemical	Effect	Reference
Bisphenols		
Bisphenol A	Agonist	Sargis et al. 2010
	Antagonist	Kojima et al. 2019; Roelofs et al. 2015; Vrzal et al. 2015
Bisphenol AF	Antagonist	Kojima et al. 2019
Bisphenol AP	Antagonist	Kojima et al. 2019
Bisphenol B	Antagonist	Kojima et al. 2019
Bisphenol F	Agonist	Kolšek et al. 2015
	Antagonist	Roelofs et al. 2015
BHEPS	Agonist	Kolšek et al. 2015
Brominated Flame Retardants		
BDE-47	Agonist	Wilson et al. 2016
BDE-85	Antagonist	Kojima et al. 2009
BDE-99	Antagonist	Kojima et al. 2009
BDE-100	Antagonist	Kojima et al. 2009
TBBPA	Antagonist	Roelofs et al. 2015
4-MeO-BDE-90	Antagonist	Kojima et al. 2009
4'-HO-BDE-49	Antagonist	Kojima et al. 2009
4'-HO-BDE-17	Antagonist	Kojima et al. 2009
Heavy Metals		
Barium chloride	Antagonist	Zhang et al. 2018
Cadmium chloride	Antagonist	Simons et al. 1990
Cobalt chloride	Antagonist	Zhang et al. 2018
Copper chloride	Antagonist	Zhang et al. 2018
Lead Nitrate	Antagonist	Zhang et al. 2018
Lithium chloride	Antagonist	Zhang et al. 2018
Tin chloride	Antagonist	Zhang et al. 2018
Zinc chloride	Antagonist	Zhang et al. 2018
Sodium arsenite	Antagonist	Kaltreider et al. 2001; Simons et al. 1990
	Biphasic	Bodwell et al. 2006
Organochlorine Pesticides		
Endrin	Agonist	Sargis et al. 2010
Methoxychlor	Antagonist	Zhang et al. 2016
o,p'-DDT	Antagonist	Zhang et al. 2016
p,p'-DDE	Antagonist	Wilson et al. 2016; Zhang et al. 2016
p,p'-DDT	Antagonist	Zhang et al. 2016
Organophosphate Flame Retardants		
TPHP	Antagonist	Kojima et al. 2016
HO-m-TPHP	Antagonist	Kojima et al. 2016
HO-p-TPHP	Antagonist	Kojima et al. 2016
Organotins		
Dibutyltin	Antagonist	Gumy et al. 2008
Tributyltin	Agonist	Gumy et al. 2008
Triphenyltin	Agonist	Gumy et al. 2008

Chemical	Effect	Reference
Parabens		
Butylparaben	Agonist	Klopčič et al. 2015; Kolšek et al. 2015
Ethylparaben	Agonist	Kolšek et al. 2015
Methylparaben	Agonist	Kolšek et al. 2015
Propylparaben	Agonist	Klopčič et al. 2015; Kolšek et al. 2015
Perfluorinated compounds (PFCs)		
PFOS	Agonist	Wilson et al. 2016
PFDA	Agonist	Wilson et al. 2016
Phthalates		
Benzylbutyl phthalate	Agonist	Sargis et al. 2010
Diethylhexyl phthalate	Agonist	Klopčič et al. 2015
Polychlorinated biphenyls (PCBs)		
PCB101	Agonist	Antunes-Fernandes et al. 2011
PCB118	Agonist	Antunes-Fernandes et al. 2011
PCB153	Agonist	Antunes-Fernandes et al. 2011
PCB19	Antagonist	Antunes-Fernandes et al. 2011
PCB28	Antagonist	Antunes-Fernandes et al. 2011
PCB47	Antagonist	Antunes-Fernandes et al. 2011
PCB51	Antagonist	Antunes-Fernandes et al. 2011
PCB52	Antagonist	Antunes-Fernandes et al. 2011
PCB53	Antagonist	Antunes-Fernandes et al. 2011
PCB95	Antagonist	Antunes-Fernandes et al. 2011
PCB100	Antagonist	Antunes-Fernandes et al. 2011
3'OH-PCB180	Antagonist	Antunes-Fernandes et al. 2011
3'OH-PCB182	Antagonist	Antunes-Fernandes et al. 2011
4'OH-PCB172	Antagonist	Antunes-Fernandes et al. 2011
5-OH-PCB183	Antagonist	Antunes-Fernandes et al. 2011
30 OH-PCBs	Antagonist	Takeuchi et al. 2011
3-MeSO2-CB101	Antagonist	Johansson et al. 2005
3-MeSO2-CB149	Antagonist	Johansson et al. 2005
Pyrethroids		
Bifenthrin	Antagonist	Zhang et al. 2016
λ-cyhalothrin	Antagonist	Zhang et al. 2016
Cypermethrin	Antagonist	Zhang et al. 2016
Resmethrin	Antagonist	Zhang et al. 2016
3-PBA	Antagonist	Zhang et al. 2016
Tetramethrin	Agonist	Klopčič et al. 2015
Other pesticides		
Atrazine	Antagonist	Zhang et al. 2016
Ethiofencarb	Antagonist	Zhang et al. 2016
M2 (Vinclozolin Metabolite)	Antagonist	Molina-Molina et al. 2006
Paraquat	Antagonist	Vrzal et al. 2015
Parathion	Antagonist	Vrzal et al. 2015
Tolylfluanid	Agonist	Sargis et al. 2010
	Antagonist	Zhang et al. 2016

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Chapter 2: Development of a Cell-Based Bioassay to Screen Environmental Chemicals and Human Serum for Glucocorticogenic Activity

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Abstract
Glucocorticoids (GCs) are hormones secreted in response to psychological stress. GCs have systemic effects on the endocrine, metabolic, cardiovascular, immune, reproductive, and central nervous systems. The physiological effects of GCs are mediated by the glucocorticoid receptor (GR), which is expressed in nearly every cell of the body. Environmental chemicals that disrupt GR signaling and/or cortisol homeostasis could adversely affect human health. A major challenge in identifying environmental chemicals that alter GR signaling in humans is a lack of adequate screening methods. To address this, we constructed a novel cell-based bioassay that specifically measures GR activity and screened a library of 176 structurally diverse environmental chemicals at concentrations up to 10μM both in the absence and presence of cortisol (100nM). Although we did not identify any GR agonists or antagonists in the chemical screening library, three chemicals amplified cortisol induced GR activity. In addition to screening chemicals, we also optimized this bioassay to measure GC levels in human serum using samples collected from 12 healthy individuals at four time points over a year. Serum GC estimates from the bioassay were highly correlated with a cortisol enzyme-linked immunosorbent assay. Notably, the between-person variability for these subjects was much greater than the within-person variability. Technical variability accounted for <2% of total variability in serum GC levels. These results demonstrate that our novel GR bioassay can be used to identify environmental chemicals that modulate GR activity and to screen serum samples for differences in total GC levels.

2.1 Introduction

Glucocorticoids are steroid hormones that affect the cardiovascular, metabolic immune, reproductive, and central nervous systems (Sapolsky et al. 2000). GC secretion is regulated by the hypothalamus-pituitary-adrenal (HPA) axis in a circadian and stress-associated manner (Biddie et al. 2012). Cortisol is the predominant GC produced by humans and its excess production is associated with multiple chronic diseases, such as atherosclerosis, diabetes, and depression (McEwen 1998). The physiological effects of GCs are mediated by the glucocorticoid receptor (GR), which is expressed in nearly every cell of the body (Pujols et al. 2002). The ubiquitous expression of GR in almost all human tissues highlights the importance of this biological pathway for human health. Upon ligand-binding, GR undergoes a conformational change that allows it to dissociate from the heat-shock protein complex and translocate to the nucleus where it activates transcription of genes containing glucocorticoid response elements (GREs) (Evans 1988). GR influences transcription of 10-20 percent of the human genome (Oakley and Cidlowski 2013). Consequently, altered GR signaling can have drastic effects on gene transcription profiles and cellular function.

Various xenobiotic compounds modify human GR signaling (Odermatt and Gumy 2008; Gulliver 2017). For example, synthetic GCs are often prescribed to treat many inflammatory and autoimmune diseases (Coutinho and Chapman 2011). Synthetic GCs have also been detected in waste and surface water samples collected globally, suggesting broad environmental exposure to these compounds (Schriks et al. 2010; Kolkman et al. 2013; Macikova et al. 2014; Suzuki et al. 2015; Jia et al. 2016). In addition to synthetic GCs, in silico and in vitro methods have also demonstrated that environmental compounds can bind and affect GR transcriptional activity. Some examples include organochlorine and pyrethroid pesticides, parabens, phthalates, bisphenols, and organotins (Gumy et al. 2008; Kolšek et al. 2014; Zhang et al. 2016; Zhang et al. 2017; Kojima et al. 2019). Furthermore, the Tox21 program tested over 8000 chemicals and identified 569 potential agonists and 472 antagonists of GR (US EPA 2017 Nov 1). Environmental exposures can also influence human GR signaling in target cells by modifying circulating cortisol levels. There is some evidence that environmental exposures, such as lead and organophosphate pesticides, can alter basal cortisol levels in humans (Cecchi et al. 2012; Fortin Marie C. et al. 2012; Braun et al. 2014; Tamayo y Ortiz et al. 2016). However, the number of epidemiological studies examining this relationship remains limited.

Cell-based bioassays are one approach to identify environmental chemicals that impact human GR signaling. This method relies on cells that contain a reporter gene driven by a GRE, which produces a measurable response proportional to the degree of GR activation. A luciferase reporter model is frequently used since the assay is rapid, simple, relatively inexpensive, sensitive, and has a broad linear range (Smale 2010). Not only has this technology helped to identify chemicals that interfere with receptor-mediated effects, but studies have also demonstrated that GR bioassays can quantify total GC levels in human serum and plasma (Raivio et al. 2002; Vermeer et al. 2003; Kajantie et al. 2004; Turner et al. 2010; Perogamvros et al. 2011; Fejerman et al. 2016). While these studies highlight that cell-based bioassays can be used to evaluate the effect of environmental chemicals on GC levels, they are not without limitations. For instance, most bioassays were generated with mammalian cell lines that lack endogenous GR expression (e.g. COS-7, U2OS, HEK293, and CV-1) (Sedlák et al. 2011;

Campana et al. 2015). Cellular context is important since the absence of cell-specific co-regulators could impact GR transcriptional activity (Weikum et al. 2017). Furthermore, the high level of homology between the glucocorticoid, mineralocorticoid, androgen, and progesterone receptors makes it particularly difficult to design specific bioassays (Kino 2017). For example, the MDA-Kb2 cell line is a commercially available bioassay derived from human breast cancer cells that responds to both androgens and GCs (Wilson et al. 2002). These bioassays could be improved by selecting a cell line devoid of homologous nuclear receptors and that endogenously expresses GR to preserve cellular context and attain specificity.

This paper describes the development of a novel bioassay that measures GR activity. We stably transfected MDA-MB-231, a triple negative breast cancer cell line, with a luciferase reporter gene driven by three tandem GREs. These cells endogenously express high levels of GR and lack the androgen and progesterone receptors (Horwitz et al. 1978). We first characterized specificity of the bioassay by testing the response of 6 GCs, a GR antagonist, and 4 non-GC steroid hormones. We then used the bioassay to screen a library of 176 structurally diverse environmental chemicals for altered GR activity. Lastly, we optimized the bioassay to measure GC levels in human serum samples. These results were also compared to estimates from a cortisol enzyme-linked immunosorbent assay (ELISA) and the MDA-Kb2 bioassay. Our work establishes the first specific, breast cancer-derived GR bioassay that can be used to identify environmental exposures that alter GR activity and serum GC levels.

2.2 Methods

2.2.1 Chemicals

Dexamethasone, hydrocortisone (cortisol), betamethasone, prednisolone, triamcinolone, corticosterone, mifepristone, aldosterone, estradiol, dihydrotestosterone and hydroxyflutamide were all purchased from Sigma-Aldrich. Compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The chemical library was provided by Dr. Bruce Hammock (University of California, Davis) and contained 176 compounds at 10mM in DMSO. Chemicals included in the library plates are listed in Supplemental Material Table 2.1. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from VWR.

2.2.2 Cell Culture

The human triple negative breast cancer cell line, MDA-MB-231, was obtained from the Cell Culture Facility at the University of California, Berkeley and authenticated using short tandem repeat profiling. MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) at 37°C in an incubator with 5% CO_2.

MDA-Kb2 (ATCC CRL-2713), a human triple negative breast cancer cell line, was obtained from the American Tissue Culture Collection (ATCC). MDA-Kb2 cells were cultured in Leibovitz's L-15 (L-15) (Gibco) supplemented with 10% FBS at 37 °C in an incubator without CO_2. One week prior to luciferase experiments, cells were maintained in phenol red-free L-15 supplemented with 10% charcoal-dextran FBS.

2.2.3 Stable Transfection

The pGRE-Luc2P plasmid was provided by Dr. Zdenek Dvorak (Palacky University) and contained a luciferase reporter gene driven by three tandem GREs (Novotna et al. 2012). MDA-MB-231 cells were seeded at a density of 2.5×10^4 in a 60 mm culture dish and transfected the following day with 5ug of pGRE-Luc2P using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were placed on selection media containing 0.5mg/ml of hygromycin B (Calbiochem) 24 hours post-transfection. Selection media was changed every 2-3 days for two weeks. Surviving cells were cloned by limited dilution in 96-well plates and maintained on selection media for two more weeks. Clones were transferred to 24 well plates and kept under selection for an additional three weeks prior to assessing dexamethasone-induced luciferase activity. The stable clone with the largest fold-change was renamed 231GRE.

2.2.4 Screening Chemicals for GR Activity

One week prior to luciferase experiments, 231GRE cells were maintained in phenol red-free DMEM (Hyclone) supplemented with 10% charcoal-dextran FBS (Atlanta Biologicals) to reduce interference from hormones present in media. 231GRE cells were seeded at a density of 2.5×10^4 cells/well in white 96-well plates (Thermo Scientific Nunc). The next day, cells were incubated with chemical treatments for 18 hours at 37°C. Chemical treatments included: vehicle (DMSO, 0.1%), dexamethasone (10pM-10μM), cortisol (100pM-100μM), betamethasone (10pM-10μM), prednisolone (10pM-10μM), triamcinolone (10pM-10μM), corticosterone (100pM-200μM), aldosterone (10pM-100μM), estradiol (10pM-10μM), dihydrotestosterone (10pM-10μM), or progesterone (10pM-10μM). Cells were also treated with RU486 (10pM-10μM), a competitive antagonist, in the presence of 100nM dexamethasone. Following the incubation period, cells were rinsed with PBS and lysed (1x cell lysis buffer, Promega). Luciferase activity was measured using a Berthold Centro XS3 LB 960 microplate luminometer with automatic injection of Luciferase Assay Reagent (Promega). All chemical treatments were conducted in triplicate and repeated as three independent experiments.

The chemical library screen was performed in part with the High-Throughput Screening Facility at UC Berkeley, which provided the Agilent V11 Bravo Automated Liquid Handler and the Thermo Scientific Multidrop Combi Reagent Dispenser. The 10mM chemical library plate was diluted in DMSO to concentrations of 1mM, 100μM, 10μM, and 1μM with the Agilent V11 Bravo liquid handler. For the agonist screen, 2μL of the chemical libraries were transferred to 998μL of phenol-red free DMEM using a liquid handler. This same instrument was used to transfer 100μL of diluted compounds to the assay plates, bringing the final volume in each well to 200μL. All agonist assay plates included media only, negative control (0.1% v/v DMSO only), and positive control (100nM cortisol) wells. Cortisol was selected as a positive control since it is the endogenous GR ligand.

The library compounds were also screened in the presence of 100nM cortisol to test for antagonism. This concentration was selected based on the half-maximal response concentration (EC_{50}) of cortisol. For the antagonist screen, chemical libraries were diluted 1:250 in phenol-red free DMEM and 50μL of diluted compounds were transferred to assay plates. An additional 50μL of cortisol-containing media was dispensed to all wells at a final volume and concentration of 200μL and 100nM, respectively. Each antagonist assay plate included media only, DMSO only, 100nM cortisol, and 100nM RU486 control wells.

231GRE cells were dispensed into 96-well plates with a Multidrop Combi Reagent Dispenser (Thermo Scientific) and treated the following day. There were three replicate plates for each treatment. The final concentration of chemicals for both assays ranged from 1nM-10μM. All assay plates were covered with a rayon film (VWR) and incubated at 37°C for 18 hours prior to measuring luciferase activity with a BioTek Cytation 5 microplate reader.

Luminescence measured in negative control wells was averaged and subtracted from all values on the plate. Background corrected relative light units (RLUs) were then normalized by dividing by luminescence measured in the positive control well. Cytotoxic concentrations of compounds, assessed by MTT and/or >20% reduction in basal luciferase activity, were not included in the statistical analysis.

2.2.5 Cell Viability Assay

The MTT assay was used to evaluate cytotoxicity of all tested compounds. 231GRE cells were plated in clear bottom 96-well plates and allowed to attach overnight. Cells were treated and incubated at 37°C for 18 hours. Afterwards, the media was replaced with 100μL media containing 0.5 mg/mL MTT and incubated for three hours at 37°C before removing the media and adding 100μl of DMSO to each well. Absorbance was measured at 570nm with a BioTek Cytation 5 microplate reader.

2.2.6 Measuring Serum GC Levels

Serum samples were collected from healthy individuals four times approximately 2-4 months apart over a one-year period. A sample size of N=12 healthy subjects were included in the study (exclusion criteria for volunteer subjects were chronic illness or pregnancy at the time of blood draws). A single blood sample was obtained from fasted participants between the hours of 8-10AM. Serum was collected with glass BD Vacutainer® tubes and stored at -80°C until analysis. The Internal Review Board within the University of California Berkeley's Human Research Protection Program approved data collection for this study and informed consent was obtained from all participants.

231GRE cells were seeded at 2.7×10^4 cells/well in white 96-well plates and incubated at 37°C for 24 hours. Media was then removed and 100 μL of hormone-depleted media containing diluted human serum was added to wells in quadruplicate. Plates were incubated at 37°C overnight prior to measuring luciferase activity. Cortisol standards were included on each plate at the following concentrations: 0 (0.1% v/v DMSO only), 3.13, 6.25, 12.5, 25, 50nM. Readings for quadruplicate samples were averaged and converted to cortisol concentrations based on standard curves fit with a quadratic function. These values were multiplied by the dilution factor to obtain cortisol equivalent values.

A similar protocol was used to screen human serum with MDA-Kb2 cells for comparison with 231GRE results. However, the androgen receptor is also capable of activating the luciferase reporter present in these cells. To address this limitation, treatments were performed in the presence of 1μM hydroxyflutamide, an androgen receptor inhibitor. Serum from female and male subjects was diluted 10 and 40-fold, respectively, to limit androgen receptor activation.

Serum cortisol was measured by competitive enzyme-linked immunoassay (ELISA) according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI) and compared to concentrations obtained by the two GR cell-based assays.

2.2.7 Statistics

Dose–response curves were fit with a four-parameter Hill function using the R drc package to obtain half-maximal activity concentration (AC_{50}) and maximum response values (Ritz et al. 2015). All positive hits in the chemical screen had a statistically significant AC_{50}<10μM. In the agonist screen, positive hits were defined as chemicals that induced a statistically significant increase in the maximum response parameter (p-value <0.05). Antagonists were defined as chemicals that inhibited activation of the reporter by cortisol. Chemicals that enhanced cortisol-induced reporter activity were also considered hits.

Comparisons between human serum treatments and media controls were made using one-way ANOVA with Dunnet's post-hoc test. Differences with *p*-value <0.05 were considered statistically significant. A coefficient of variation (CV=100×standard deviation/mean) was computed for each serum sample to assess technical variability. Concordance correlation coefficients (and 95% confidence intervals) were calculated to compare cortisol equivalent values obtained by the bioassays to concentrations measured by ELISA. This estimate evaluates how far the observed data deviates from the line of perfect concordance. Values closer to 1 indicated very good agreement between the bioassay and ELISA results.

A random effects model was used to identify sources of variability in measured serum GC levels. The inter-individual (σ_h^2), time-specific (σ_i^2), intra-individual (σ_{hi}^2), and within sample (σ_e^2) variability components were defined by the following equation:

$$Y_{hij} = \log(X_{hij}) = \mu_y + \alpha_h + \beta_i + \gamma_{hi} + \varepsilon_{hij}$$

for h=1,…,12 individuals; i= 1, 2, 3, 4 time points; and j= 1, 2, 3, 4 replicate samples, where X_{hij}= the serum GC levels for the h^{th} individual at the i^{th} time point for the j^{th} replicate and Y_{hij} represents the natural log transformation of X_{hij}. In this model, μ_y represents the true logged mean of serum GC levels and α_h represents the random effect of the h^{th} individual. β_i represents the time-specific random effect and γ_{hi} is the random effect of the h^{th} individual at the i^{th} timepoint. Lastly, ε_{hij} is the random-error effect of the j^{th} replicate sample from the h^{th} individual at the i^{th} timepoint. It is assumed that α_h, β_i, γ_{hi}, and ε_{hij} are mutually independent and normally distributed with means of zero. The respective variance components of α_h, β_i, γ_{hi}, and ε_{hij} were σ_h^2 (variability between individuals), σ_i^2 (variability over time), σ_{hi}^2 (variability within individuals), and σ_e^2 (within sample + residual variability).

2.3 Results

2.3.1 Characterization of 231GRE Cell Line

MDA-MB-231 cells were stably transfected with the pGRE-Luc2P reporter plasmid. Five monoclonal cell lines were generated and evaluated for dexamethasone-induced luciferase activity. Clone #7 exhibited the highest reporter activity in response to dexamethasone (1μM) with a fold change of 438 (Supplemental Material Figure 1). Luciferase activity was also detected in this clone for up to 24 passages, demonstrating stable integration of the reporter

plasmid (Supplemental Material Figures 2). Therefore, clone #7 was renamed 231GRE based on its wide dynamic range and stability.

Specificity of the 231GRE cell line was then evaluated by testing multiple GCs and other non-GC steroid hormones (Figure 2.1). Dose-response curves were generated for six GCs: dexamethasone (DEX), cortisol, betamethasone (BMZ), prednisolone (PRED), triamcinolone (TAC), and corticosterone. All GCs induced luciferase activity and produced EC_{50} values comparable to previously published bioassays (Table 2.1). Dexamethasone-induced reporter activity was also suppressed by RU486, a GR antagonist. Aldosterone (ALDO), a mineralocorticoid hormone, elicited a response since it is a partial GR agonist. As expected, estradiol (E2), progesterone (PROG), and dihydrotestosterone (DHT) had no effect on reporter activity. Collectively, these results demonstrate specificity of the 231GRE bioassay for GR ligands.

2.3.2 High-throughput Screen of Environmental Chemicals

A library of 176 structurally diverse environmental chemicals was tested for GR activity. 231GRE cells were treated with 10-fold serial dilutions of the chemical library at concentrations ranging from 1nM-10μM. Cortisol (100nM) was the positive control and consistently induced GR activation. However, none of the environmental compounds produced a statistically significant increase in reporter activity (Supplemental Table 2.2). 231GRE cells were also treated with library compounds in the presence of 100nM cortisol to test for antagonism. Except for the positive control (RU486), none of the screened compounds inhibited cortisol-induced GR activity (Supplemental Table 2.3). Interestingly, three compounds (isopropyl-N-phenylcarbamate, 3,4,4'-trichlorocarbanilide, and 2-(4-chlorophenyl)-benzothiazole) potentiated the effect of 100nM cortisol (Figure 2.2, Supplemental Table 2.3). Although the chemical screen did not identify any GR agonists or antagonists, this approach indicated chemicals that enhanced cortisol-induced GR activity.

2.3.3 Measuring Serum GC Levels

The 231GRE bioassay was also optimized to measure serum GC levels. Cells were treated with human serum obtained from a healthy individual that was diluted 10, 20, 30, 40 and 50-fold in cell culture media. All tested dilutions induced a statistically significant increase in reporter activity above the media only control (Figure 2.3). Relative luciferase units were then converted to cortisol equivalent values and directly compared to the concentration measured by ELISA (Table 2.2). The cortisol concentration was best estimated by serum diluted 10 and 20-fold. Variability in cortisol equivalent values was also lowest in the 1:10 serum dilution (CV=3.7%). Therefore, serum was diluted 10-fold for all other experiments.

Serum GC levels were then measured in samples collected from twelve healthy individuals at four different time points over a one-year period with the 231GRE bioassay. Cortisol equivalent values were also compared to estimates from the MDA-Kb2 bioassay and concentrations determined by ELISA (Figure 2.4). The concordance correlation coefficients (r_c) between the 231GRE and ELISA concentrations were all statistically significant and ranged from 0.69 to 0.94. However, concordance between MDA-Kb2 and ELISA concentrations was much lower (r_c range: 0.06-0.20) and yielded 95% confidence intervals that contained zero, indicating a lack of

statistical significance. These comparisons demonstrate that cortisol concentrations were more closely approximated by the 231GRE bioassay than by MDA-Kb2.

The 231GRE bioassay was also used to identify sources of variability in serum GC levels (Figure 2.5). Variability between replicates was minimal with intra-assay CVs ranging from 0.6-11.8% (median: 4.7%). A random effects model estimated that the inter-individual, time-specific, intra-individual, and within-sample variability were 64.5%, <0.01%, 33.6%, and 1.9%, respectively. These results demonstrate that morning serum GC levels varied more between individuals than within the same individual over a one-year period and that technical variability of the 231GRE bioassay was remarkably low.

2.4 Discussion

This paper describes the development of a specific and stable breast cancer-derived GR bioassay called 231GRE. We demonstrated that 231GRE cells were highly specific for GR ligands and did not respond to compounds from other steroid classes. Furthermore, all GR ligands had EC50 values comparable with those reported for other stable reporter cell lines. We also conducted a high-throughput screen of 176 environmental chemicals with 231GRE cells to identify compounds that altered GR activity. None of the tested compounds were agonists or antagonists of GR. However, we identified three chemicals that potentiated cortisol-induced GR activity. We also optimized the 231GRE bioassay to measure GC levels in human serum. ELISA cortisol concentrations were in high concordance with 231GRE estimates. Additionally, we observed greater variability in serum GC levels between individuals than within the same individual over a one-year period. Collectively, these results highlight applications of the 231GRE cell line and how it could be used to identify environmental compounds that alter GR activity and/or serum GC levels.

To our knowledge, this is the first specific GR bioassay generated in a breast cancer cell line. The only other available stable breast cancer-derived GR bioassay is the MDA-Kb2 cell line, which contains a reporter driven by the mouse mammary tumor virus (MMTV) promoter that responds to both androgens and GCs (Wilson et al. 2002). To overcome this limitation, we performed the stable transfection with a luciferase reporter gene driven by three tandem GREs, which provides greater specificity than the MMTV promoter. Additionally, MDA-MB-231 cells were selected for the bioassay since it endogenously expresses highly levels of GR and lacks homologous nuclear receptors, such as the progesterone and androgen receptors (Horwitz et al. 1978). MDA-MD-231 cells are frequently utilized in breast cancer research and were recently used to show how GR activation promotes breast cancer metastasis and reduced survival in mice (Obradović et al. 2019). Therefore, our GR bioassay provides a valuable tool to evaluate the contribution of environmental exposures in the development and progression of breast cancer.

The high-throughput screen did not identify any potential agonists or antagonists of GR in 231GRE cells. While it is possible that concentrations used in this study were not high enough to observe changes in GR activity, human exposure to these compounds is unlikely to exceed 10μM. Rappaport et al. found that blood pollutant levels ranged from 10^{-7}μM to 10μM (median: 2.4×10^{-4} μM), suggesting that concentrations tested in the present study reflect relevant human exposure levels. It is also intriguing that chemicals previously shown to modulate GR activity had no effect when tested in 231GRE cells. For example, pyrethroids (bifenthrin, λ-cyhalothrin,

cypermethrin, resmethrin) and organochlorine pesticides (o,p′-DDT, p,p′-DDT, methoxychlor) antagonized GR transcriptional activation in a Chinese hamster ovarian cell line that contained a MMTV-luciferase reporter (Zhang et al. 2016). This same study showed that chemicals had differential effects on downstream GR target genes, even within the same cell line. Therefore, the effect of chemicals on GR transcription may vary by gene promoter. It is also possible that the 231GRE bioassay lacks certain cofactors that influence the effect of chemicals on GR transcriptional activity. However, additional studies are needed to evaluate the influence of environmental chemicals on GR signaling across multiple cell types.

Our results demonstrate that environmental chemicals can potentiate the effect of cortisol on GR activity. For example, we observed that 3,4,4'-trichlorocarbanilide (triclocarban) increased the amount of reporter activity induced by 100nM cortisol. Another study conducted with MDA-Kb2 cells also found that 2μM triclocarban enhanced cortisol-induced GR activity (Kolšek et al. 2015). Triclocarban is an antimicrobial agent that was recently banned by the US Food and Drug Administration in 2016 from consumer antiseptic washes due to its endocrine disrupting effects. However, this compound is still allowed in household plastics, industrial cleaning and hospital supplies, and other personal care products not covered by the regulation (Halden et al. 2017). Based on our findings, future regulation of triclocarban and other industrial chemicals should consider the additive effect of these compounds on endogenous hormone signaling. Enhanced cortisol-induced GR activity was also observed with the carbamate herbicide isopropyl-N-phenylcarbamate (Propham) and the benzothiazole derivative 2-(4-chlorophenyl)-benzothiazole. Christodoulou et al. 2018 identified benzothiazole derivatives that influenced GR activity through allosteric binding. Moving forward, molecular docking is a promising approach to evaluate whether these compounds bind GR and modulate receptor-ligand interactions. Additionally, larger screens should be conducted to identify other environmental chemicals that enhance cortisol-induced GR signaling.

Our work provides the field with a tool to measure total GC levels present in human serum. This technique is a rapid, sensitive, and cost-effective method to quantify cortisol levels. Serum cortisol concentrations are routinely measured using antibody-based methods, such as ELISA or by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). While both of these techniques quantify the amount of a specific compound present in a biospecimen, they often require sample preparation, are expensive, and do not measure the biological effect of exogenous and endogenous compounds present in serum (Xu et al. 2014). Alternatively, several studies thus far have used cell-based bioassays to detect differences in serum GC levels (Raivio et al. 2002; Vermeer et al. 2003; Kajantie et al. 2004; Turner et al. 2010; Perogamvros et al. 2011; Fejerman et al. 2016). For example, elevated serum GC levels were detected after synthetic GC administration (Raivio et al. 2002). Therefore, cell-based bioassays such as ours can be used to quantify the overall net effect of both endogenous and exogenous molecules present in human serum. This method can also be coupled with other analytical approaches (e.g. LC-MS/MS) to identify environmental GR agonists and antagonists present in human serum (Smith et al. 2015; Smith et al. 2019).

Comparisons with a cortisol ELISA demonstrated that plasma concentrations were more closely approximated by 231GRE than MDA-Kb2. It should be noted that although concordance between MDA-Kb2 and ELISA was relatively low (r_c=0.14), these two assays were still highly

correlated (r=0.6). This means that both GR bioassays can be used to infer relative differences in plasma GC levels, but absolute differences are more accurately estimated by 231GRE. Our results also suggest that 231GRE may be more sensitive to GR activation than MDA-Kb2. However, the reason for this is unclear. There is some evidence that the androgen receptor (AR) and GR influence each other's transcriptional activity (Chen et al. 1997). Therefore, androgens present in serum and/or hydroxyflutamide might interfere with GR activation in MDA-Kb2, since these cells also express AR.

Characterizing sources of variation in biomarkers is important when designing epidemiological studies. Therefore, we measured sources of variability in serum GC levels using samples collected from healthy adults. Intra-assay CVs were below 15% for all tested serum samples indicating low technical variability of the 231GRE assay. Reproducibility of the bioassay was further supported by the fact that within-sample variance only accounted for <2% of variability in total serum GC levels. Interestingly, serum GC levels varied less within the same individual (intra-individual) than between individuals (inter-individual). This result corresponds with previous research that reported a high level of individual stability in morning cortisol concentrations (Huizenga et al. 1998). Evidence from twin studies suggests that genetic factors may play a role in regulating morning cortisol levels (Maxwell et al. 1969; Meikle et al. 1988). Consequently, the high amount of inter-individual variability in serum GC activity may reflect genetic differences in cortisol secretion and regulation between individuals. Collectively, these results suggest that our bioassay is reproducible and can be used to measure morning serum GC activity in epidemiological studies.

In summary, we developed a novel method that can be used to identify environmental chemicals that modulate GR transcriptional activity and to screen human serum samples for differences in total GC levels. Given the significance of the GR pathway in human health and in development of disease, greater emphasis should be placed on identifying environmental chemicals that perturb GR signaling and cortisol homeostasis.

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Tables and Figures

Table 2.1: Comparison of EC_{50} (M) values for GR ligands between 231GRE and other published stable GR bioassays

Chemical	231GRE	AZ-GR (Novotna et al. 2012)	GR-BLA (TOX21)	GR-CALUX (Bovee et al. 2011)
Dexamethasone (DEX)	9.5E-9	9.5E-9	2.8E-9	2.2E-9
Cortisol	1.4E-7	6.6E-8	3.8E-8	3.7E-8
Betamethasone (BMZ)	1.3E-8	1.5E-8	4.9E-9	1.1E-8
Prednisolone (PRED)	6.0E-8	4.5E-8	2.3E-8	1.2E-8
Triamcinolone (TAC)	5.9E-8	5.4E-8	3.0E-8	1.9E-8
Corticosterone	8.2E-7	2.4E-7	8.0E-8	8.0E-8
Aldosterone (ALDO)	6.4E-6	1.1E-6	-	5.0E-7

Table 2.2: Comparison of ELISA concentration to cortisol equivalent (CortEq) values for human serum dilutions

Dilution	CortEq (nM)	SD	CV (%)
1:10	93	3.5	3.7
1:20	144	9.6	6.6
1:30	196	11.4	5.8
1:40	225	14.7	6.5
1:50	260	19.6	7.5
ELISA	118		

Figure 2.1: Dose-response curves of glucocorticoid receptor ligands and other steroid hormones. Plots represent mean data from three independent experiments (N=3) that were fit with a 4-parameter hill function.

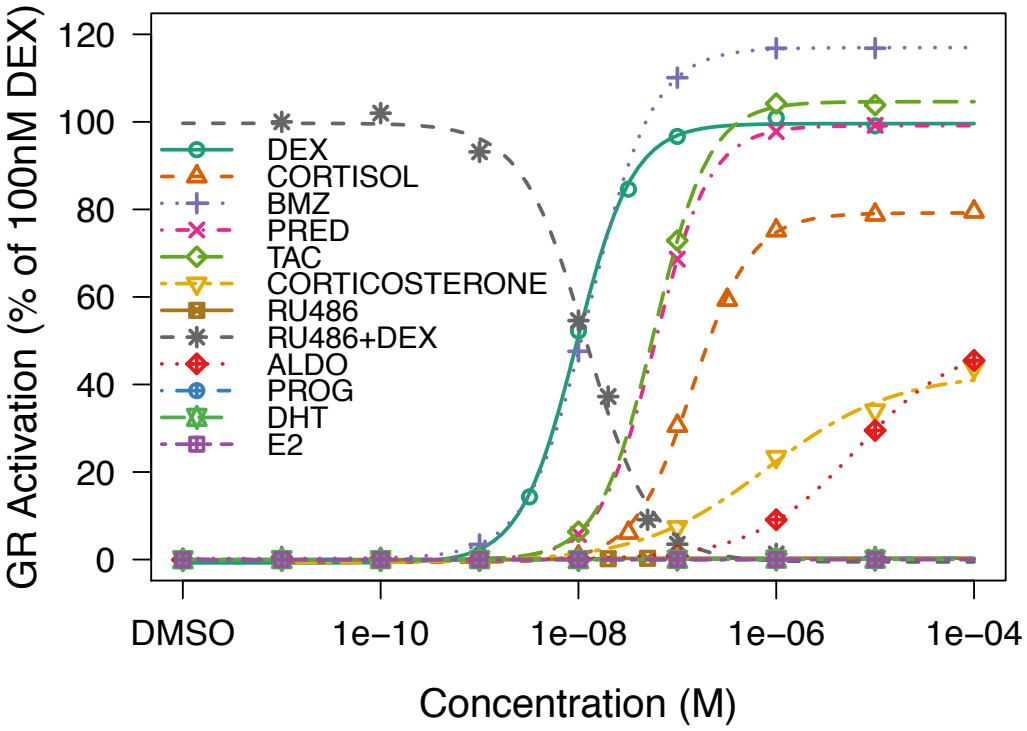


Figure 2.2: Dose-response curves for chemicals that enhanced cortisol-induced GC activity. Data from triplicate wells for each concentration were used to plot 4-parameter Hill functions. The dashed line represents baseline activity of cortisol alone (100%).

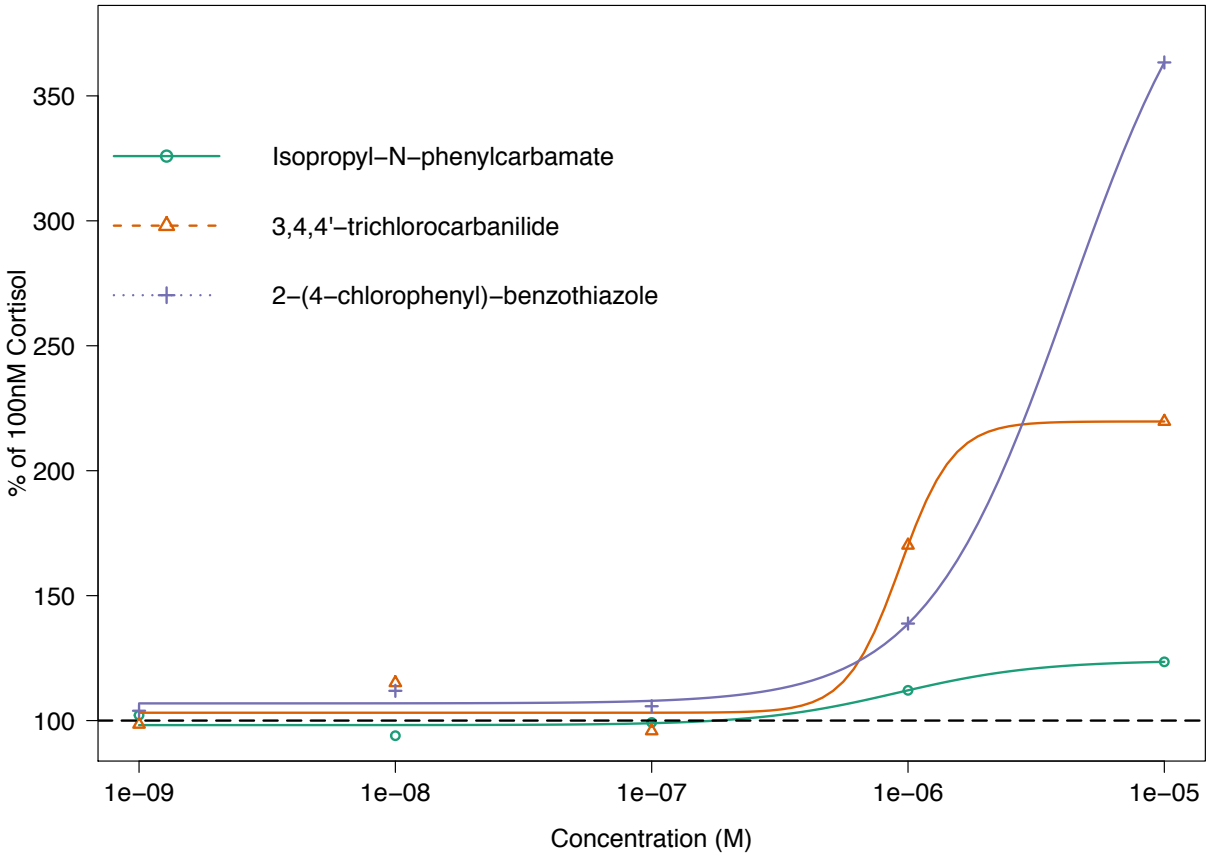


Figure 2.3: Response of 231GRE cells treated with diluted human serum. The bars represent the average relative luciferase units measured for each dilution factor. Error bars represent the standard error of the mean (SEM) of quadruplicate wells. * Indicates a statistically significant difference between serum dilution and media only control (P-value <0.05).

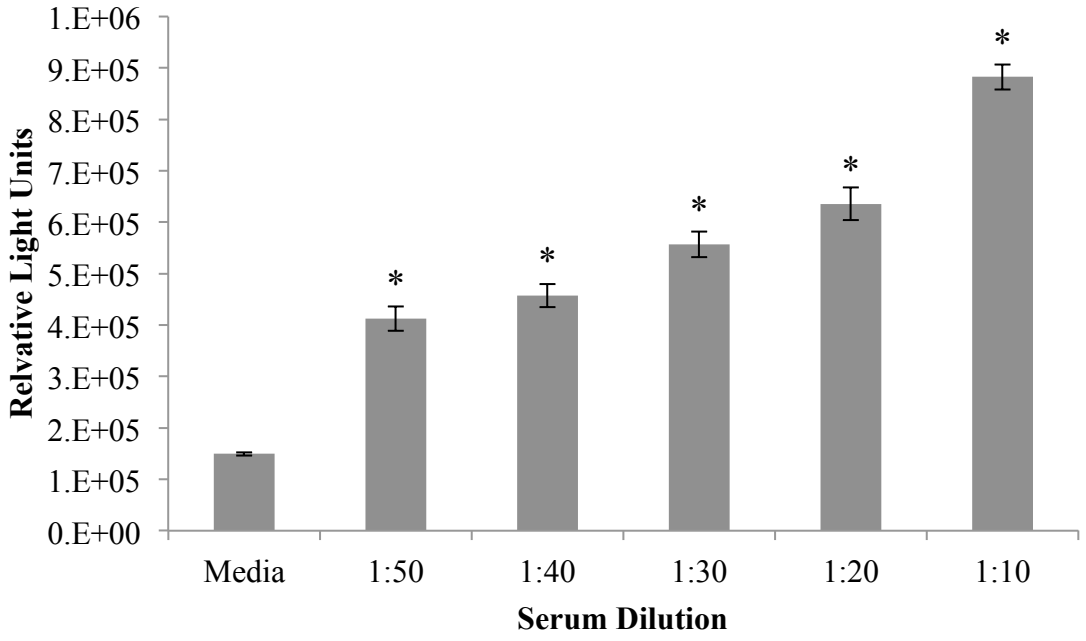


Figure 2.4: Correlation between serum GC activity and cortisol ELISA concentrations for each time point. Cortisol equivalent values from the bioassays were plotted on the x-axis and ELISA concentrations on the y-axis. All concentrations are on the logarithmic scale. Fit lines for 231GRE (red) and MDA-Kb2 (blue) were also plotted and compared to the black identity line (y=x). Concordance correlation coefficients for each bioassay are included in the plot legends.

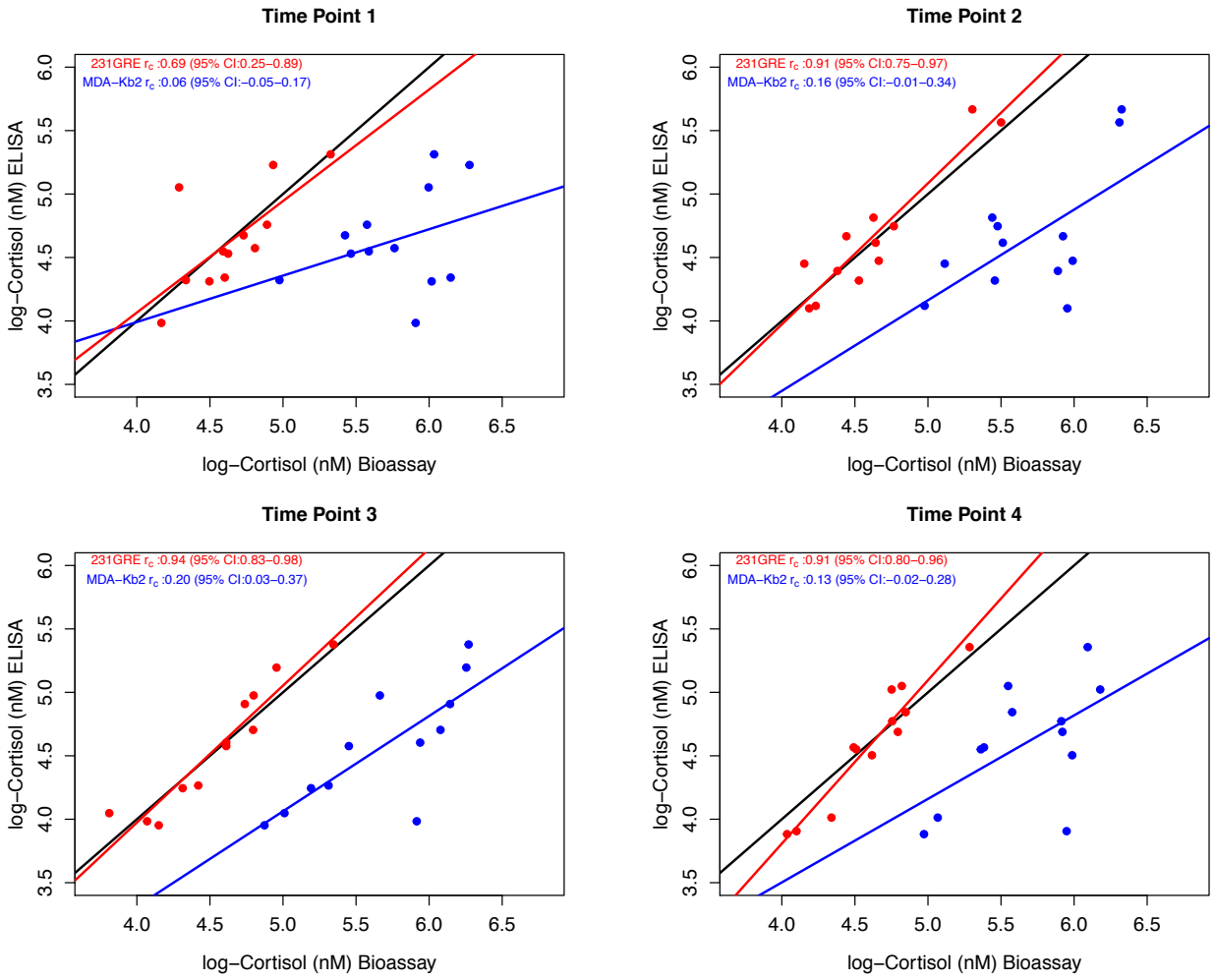
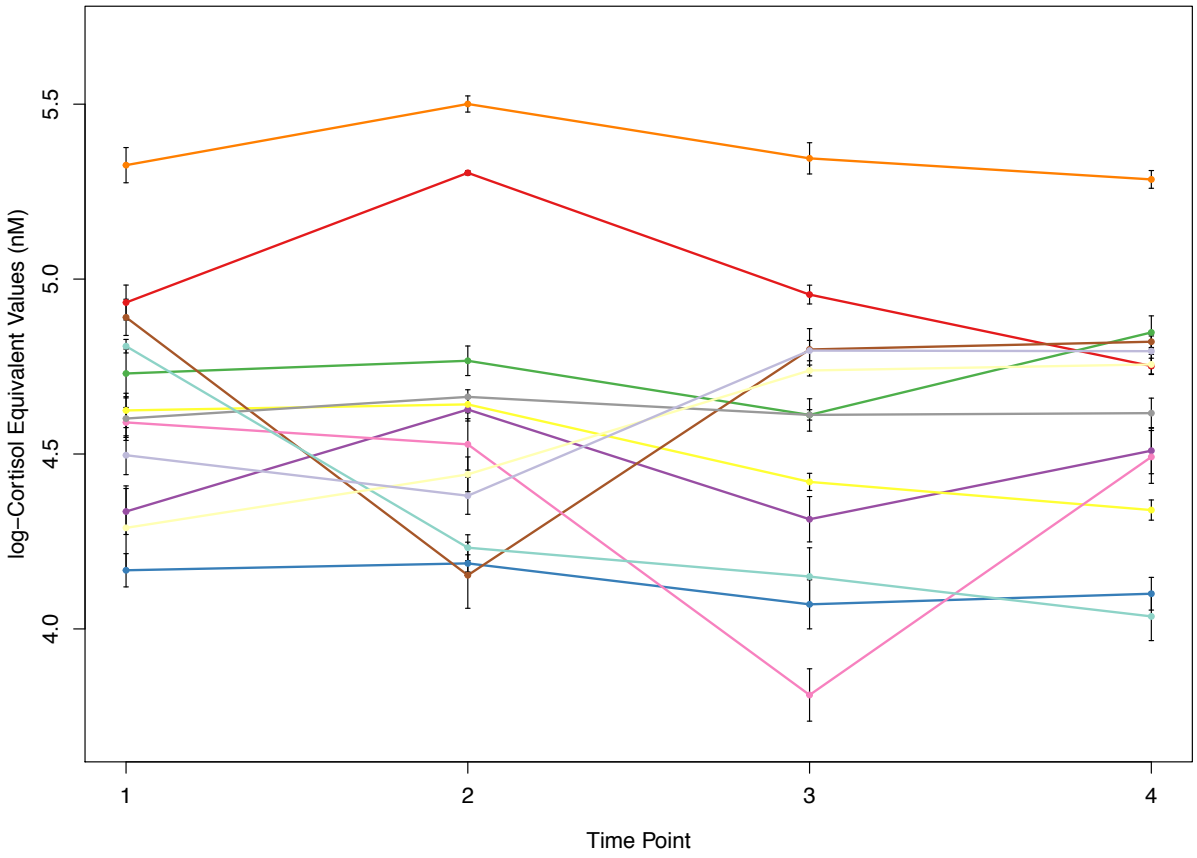


Figure 2.5: Variability in serum GC activity. Cortisol equivalent values were plotted by time point. Each line represents a different individual. Error bars represent the standard deviation of quadruplicate wells.



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Supplementary Materials

Supplementary Table 2.1: List of chemicals in library plates

Chemical	Plate	Well
Atrazine	1	A2
2-chloro-4-ethylamino-6-amino-s-triazine	1	A3
Carbaryl	1	A4
DNBP	1	A5
Paraquat dichloride	1	A6
Diphenylacetonitrile	1	A7
Eptam	1	A8
Pirimiphos - ethyl	1	A9
3,5,6-trichloro-2-pyridinol	1	A10
Tributyl (2,4-dichlorobenzyl)phosphonium chloride	1	A11
2,4,5-T	1	A12
Simazine	1	B2
Ammelide	1	B3
Propoxur	1	B4
4.6-Dinitro-o-cresol	1	B5
Diethyl phthalate	1	B6
Maleic acid hydrazide	1	B7
CDEC	1	B8
Diazinon	1	B9
o,o-diethylthiophosphate	1	B10
Tributyl phosphorotrithioite	1	B11
p,p-DDT	1	B12
Simetryn	1	C2
Ammeline	1	C3
Aldicarb	1	C4
Triclopyr	1	C5
Bromacil	1	C6
Nicotine	1	C7
Ferbam	1	C8
Malathion	1	C9
Methidathion	1	C10
Phosdrin	1	C11
o,p-DDD	1	C12
Cyanuric acid	1	D2
Cyanazine	1	D3
Aldoxycarb	1	D4
Fluroxypyr	1	D5
Rotenone	1	D6
Ziram	1	D7
Maneb	1	D8
Chlorpyrifos	1	D9
6-chloromethyl-4-hydroxy-2-isopropyl pyrimidine	1	D10
Carbophenothion	1	D11
p,p-DDD	1	D12
Propazine	1	E2
Terbutryn	1	E3
Isopropyl-N-[m-chlorophenyl]carbamate	1	E4
Clopyralid	1	E5
Captan	1	E6

Chemical	Plate	Well
Nabam	1	E7
Zineb	1	E8
Chlorpyrifos oxon	1	E9
2-methoxy-3,5,6-trichloropyridine	1	E10
Dichlorvos (DDVP)	1	E11
p,p-DDE	1	E12
Ametryn	1	F2
Prometon	1	F3
Isopropyl-N-phenylcarbamate	1	F4
Picloram	1	F5
Folpet	1	F6
Metam sodium	1	F7
Tetramethylthiuram disulfide	1	F8
2-diethylamino-6-methylpyrimidin-4-ol	1	F9
Parathion	1	F10
o,o-dimethyl phosphochloridothioate	1	F11
o,p-DDE	1	F12
Prometryn	1	G2
2-chloro-4,6-diamino-s-triazine	1	G3
Oryzalin	1	G4
Mecoprop	1	G5
Cacodylic acid, Na salt	1	G6
Molinate	1	G7
s-propyl butylethylthiocarbamate	1	G8
Methamidophos	1	G9
Des-N-isopropyl isophenphos oxygen analog	1	G10
Dichlorprop	1	G11
2,4-dichlorophenoxybutyric acid (2,4-DB)	1	G12
2-chloro-4-isopropyl-6-amino-s-triazine	1	H2
Dazomet	1	H3
2-methylheptyl-4,6-dinitrophenyl Crotonate	1	H4
Glyphosate	1	H5
Chloranocryl	1	H6
Thiobencarb	1	H7
Pirimiphos - methyl	1	H8
Diethyl phosphate	1	H9
Des-N-isopropyl isophenphos	1	H10
2,4-Dichlorophenoxyacetic acid (2,4-D)	1	H11
Dalapon	1	H12
Heptachlor	2	A2
2,4,6-trichlorophenol	2	A3
2,4,5-Trichlorophenoxyacetic acid, isopropyl ester	2	A4
Thiodan	2	A5
Asana - Chemservice	2	A6
cis-cypermethrin	2	A7
1-Naphthaleneacetic acid	2	A8
3-Indolebutyric acid	2	A9
Irgasan	2	A10
Triton X-100	2	A11
Amgard CJ	2	A12
Heptachlor epoxide	2	B2
Chloranil	2	B3

Chemical	Plate	Well
Silvex	2	B4
4,4'-Dichloro-a-(trichloromethyl)benzhydrol	2	B5
zeta-cypermethrin	2	B6
Oxyfluorfen	2	B7
1-Naphthaleneacetic acid, methyl ester	2	B8
Gibberellic acid	2	B9
Finasteride	2	B10
SDS	2	B11
Phosphoric acid triphenyl ester	2	B12
Aldrin	2	C2
Dichlone	2	C3
Benzene hexachloride	2	C4
Methoxychlor	2	C5
Deltamethrin	2	C6
Diuron	2	C7
Chloracetic acid	2	C8
N-m-Tolylphthalamic acid	2	C9
Clomipramine	2	C10
Phenanthrene	2	C11
Carbamazepine	2	C12
Dieldrin	2	D2
o-Chlorophenoxyaceric acid	2	D3
Lindane	2	D4
Baythroid	2	D5
Pyrethrum	2	D6
Diflubenzuron	2	D7
2,2-dichloropropionic acid	2	D8
o-Dichlorobenzene	2	D9
Anthracene	2	D10
Tween - 20	2	D11
Fluoxetine HCl	2	D12
2,2'-methylenebis(4-chlorophenol)	2	E2
p-Chlorophenoxyaceric acid	2	E3
Chlorodane	2	E4
a-Cypermethrin	2	E5
Pyrethrum	2	E6
Monuron	2	E7
Trichloroacetic acid	2	E8
p-Dichlorobenzene	2	E9
Bis 2-Ethylhexyl phthalate (BEHP)	2	E10
n-dodecyl phosphoric acid	2	E11
1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane	2	E12
Pentachlorophenol	2	F2
2-methyl-4-chlorophenoxyacetic acid (MCPA)	2	F3
Endrin	2	F4
d-(cis/trans)phenothrin	2	F5
Cypermethin (mix of isomers)	2	F6
Fenuron -	2	F7
2-Naphthoxyacetic acid	2	F8
Naphthalene	2	F9
Butylated hydroxyanisole (BHA)	2	F10
Clofibric acid	2	F11

Chemical	Plate	Well
N-cyclohexyl-2-benzothiazyl sulfenamide	2	F12
2,3,4,6-Tetrachlorophenol	2	G2
2,4-Dichlorophenoxyacetic acid, butyl ester	2	G3
Toxaphene	2	G4
Resmethrin	2	G5
trans-Cypermethrin	2	G6
3,4,4'-trichlorocarbanilide	2	G7
Phenoxyacetic acid	2	G8
1-Nitronaphthalene	2	G9
Butylated hydroxytoluene (BHT)	2	G10
2,2',4,4'-tetrabromodiphenyl ether (PBDE-47)	2	G11
2-(4-chlorophenyl)-benzothiazole	2	G12
2,4,5-trichlorophenol	2	H2
2,4-Dichlorophenoxyacetic acid, isopropyl ester	2	H3
Tedion	2	H4
Bifenthrin	2	H5
Sanmarton	2	H6
1-Naphthaleneacetamide	2	H7
2-Phenoxypropionic acid	2	H8
Siduron	2	H9
Bisphenol A	2	H10
Pyrovatex CP	2	H11
2hydroxybenzothiazole	2	H12

Supplemental Table 2.2: Hill function maximum and AC_{50} values for agonist screen

Chemical	Max (%)	P-value	AC50 (M)	P-value
Isopropyl-N-[m-chlorophenyl]carbamate	1.67	0.42	3.96E-06	0.75
MCPA	1.46	0.96	2.85E-09	0.99
2,4-Dichlorophenoxyacetic acid, butyl ester	1.46	0.96	2.85E-09	0.99
Anthracene	1.27	0.85	1.17E-06	0.98
BEHP	1.27	0.85	1.17E-06	0.98
Aldrin	1.26	0.97	1.62E-10	0.87
Diethyl phthalate	1.20	0.96	8.07E-08	0.99
N-cyclohexyl-2-benzothiazyl sulfenamide	1.13	0.88	1.09E-07	0.98
Tween - 20	0.95	0.92	3.72E-07	0.98
PBDE-47	0.94	0.90	4.22E-09	0.98
Diflubenzuron	0.83	0.72	3.96E-06	0.92
Carbaryl	0.79	0.01	1.59E-06	0.72
Propoxur	0.79	0.01	1.59E-06	0.72
Resmethrin	0.73	0.95	8.75E-07	0.99
Bifenthrin	0.73	0.95	8.75E-07	0.99
Isopropyl-N-phenylcarbamate	0.73	0.03	1.64E-06	0.70
1-Naphthaleneacetic acid	0.60	0.88	2.01E-07	0.99
Phosphoric acid triphenyl ester	0.59	0.91	4.22E-09	0.96
2,4,5-Trichlorophenoxyacetic acid, isopropyl ester	0.56	0.89	2.91E-07	0.99
2hydroxybenzothiazole	0.54	0.96	5.33E-06	0.99
Captan	0.52	0.99	1.31E-08	0.99
Chloranocryl	0.49	0.00	1.49E-06	0.83
Ziram	0.49	0.96	2.62E-09	0.99
trans-Cypermethrin	0.46	0.98	5.07E-08	0.99
cis-cypermethrin	0.45	0.88	2.95E-06	0.98
Pyrovatex CP	0.41	0.96	8.17E-08	0.99
2,4,5-trichlorophenol	0.39	0.75	1.38E-06	0.95
2,4-Dichlorophenoxyacetic acid, isopropyl ester	0.39	0.39	4.09E-06	0.90
Dichlone	0.38	0.94	9.20E-09	0.97
Chloranil	0.36	0.89	3.07E-07	0.97
n-dodecyl phosphoric acid	0.36	0.92	8.72E-06	0.90
Methoxychlor	0.33	0.98	3.33E-07	0.99
Finasteride	0.29	0.91	4.11E-07	0.97
Malathion	0.28	0.97	1.19E-08	1.00
Heptachlor	0.28	0.91	1.83E-07	0.99
Simetryn	0.28	0.89	9.72E-09	0.97
Ferbam	0.26	0.95	1.36E-09	0.98
Methamidophos	0.24	0.96	3.71E-09	0.99
Silvex	0.24	0.88	1.26E-08	0.98
Tedion	0.23	0.24	1.46E-09	0.88

Chemical	Max (%)	P-value	AC50 (M)	P-value
2-Naphthoxyacetic acid	0.16	0.68	5.51E-06	0.79
4.6-Dinitro-o-cresol	0.16	0.92	8.50E-09	0.98
Heptachlor epoxide	0.15	0.93	2.56E-08	0.99
Cyanuric acid	0.13	0.95	2.53E-10	0.95
Propazine	0.13	0.95	2.53E-10	0.95
d-(cis/trans)phenothrin	0.13	0.94	8.25E-09	0.99
Ametryn	0.13	0.96	4.88E-09	0.98
Diphenylacetonitrile	0.11	0.08	9.49E-07	0.40
2,4-D	0.11	0.03	1.02E-08	0.37
2,4,5-T	0.11	0.03	1.02E-08	0.37
Atrazine	0.11	0.99	2.67E-08	1.00
Simazine	0.11	0.99	2.67E-08	1.00
Chlorpyrifos oxon	0.10	0.06	4.59E-08	0.82
2-diethylamino-6-methylpyrimidin-4-ol	0.10	0.06	4.59E-08	0.82
Des-N-isopropyl isophenphos	0.10	0.08	6.83E-08	0.72
Tributyl (2,4-dichlorobenzyl)phosphonium chloride	0.10	0.08	6.83E-08	0.72
Tributyl phosphorotrithioite	0.10	0.08	6.83E-08	0.72
Rotenone	0.10	0.99	4.08E-09	1.00
Carbophenothion	0.10	0.02	3.76E-07	0.96
Paraquat dichloride	0.08	0.04	1.26E-07	0.74
Metam sodium	0.08	0.16	4.71E-07	0.85
DNBP	0.08	0.07	1.67E-08	0.90
o-Chlorophenoxyaceric acid	0.08	0.89	4.62E-10	0.96
Diazinon	0.07	0.98	3.30E-07	1.00
o,o-diethylthiophosphate	0.07	0.17	9.51E-08	0.38
Methidathion	0.07	0.17	9.51E-08	0.38
p,p-DDT	0.06	0.13	1.48E-10	0.90
Bromacil	0.06	0.18	1.21E-07	0.58
BHT	0.06	0.25	1.18E-08	0.73
Phenoxyacetic acid	0.05	0.98	2.92E-09	0.99
2-methoxy-3,5,6-trichloropyridine	0.05	0.32	2.50E-08	0.88
Parathion	0.05	0.32	2.50E-08	0.88
Endrin	0.05	0.38	1.04E-07	0.55
DDVP	0.05	0.29	7.69E-08	0.62
Fluoxetine HCl	0.04	0.48	9.80E-09	0.23
Pentachlorophenol	0.03	0.60	3.69E-10	0.81
Oxyfluorfen	0.02	0.71	9.33E-10	0.87
Pyrethrum2	0.01	0.87	3.38E-07	0.98
Clomipramine	0.00	0.95	1.20E-07	0.48
Gibberellic acid	0.00	0.95	3.93E-10	0.73
Molinate	-0.01	0.84	1.24E-07	0.77
Maleic acid hydrazide	-0.03	0.51	1.43E-07	0.83

Chemical	Max (%)	P-value	AC50 (M)	P-value
Nabam	-0.03	0.57	2.55E-07	0.84
Cyanazine	-0.04	0.17	6.51E-09	0.04
Terbutryn	-0.04	0.17	6.51E-09	0.04
Prometon	-0.04	0.17	6.51E-09	0.04
2-chloro-4,6-diamino-s-triazine	-0.04	0.17	6.51E-09	0.04

Chemicals not listed were either: cytotoxic, model did not converge, or AC50 >10μM

Supplemental Table 2.3: Hill function parameters for antagonist screen

Chemical	Min	P-value	Max	P-value	AC50	P-value
2-(4-chlorophenyl)-benzothiazole	106.88	8.76E-09	432.84	1.22E-13	4.26E-06	0.001
3,4,4'-trichlorocarbanilide	103.14	3.00E-09	219.70	3.29E-10	9.30E-07	0.007
Isopropyl-N-phenylcarbamate	98.19	1.23E-13	124.07	2.08E-11	9.09E-07	0.028
Chloranocryl	98.61	6.15E-12	113.79	1.39E-12	1.15E-07	0.122
Simetryn	97.80	2.59E-15	104.70	1.28E-15	1.28E-07	0.138
Isopropyl-N-[m-chlorophenyl]carbamate	99.77	9.84E-14	169.15	1.32E-11	1.43E-06	0.155
Diflubenzuron	98.48	4.32E-10	144.36	2.55E-09	7.05E-07	0.268
2-Phenoxypropionic acid	-76.71	9.89E-06	109.71	2.06E-12	3.70E-10	0.303
3-Indolebutyric acid	-76.71	9.89E-06	109.71	2.06E-12	3.70E-10	0.303
Anthracene	99.93	2.06E-11	133.93	7.03E-10	1.41E-06	0.334
Fluoxetine HCl	101.49	9.57E-12	119.87	4.27E-10	5.84E-07	0.334
Thiobencarb	102.53	8.07E-11	112.71	3.02E-11	1.13E-07	0.381
2-methylheptyl-4,6-dinitrophenyl Crotonate	101.34	6.64E-12	109.01	3.03E-12	9.97E-08	0.392
2,4,5-trichlorophenol	100.31	1.05E-11	107.64	4.13E-11	3.90E-08	0.405
Irgasan	99.03	1.29E-08	110.74	6.03E-14	3.56E-06	0.470
Cyanazine	99.25	7.56E-15	112.68	1.62E-14	2.89E-07	0.481
1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane	104.03	6.43E-10	115.19	1.90E-09	3.30E-08	0.553
MCPA	71.50	1.45E-02	99.79	1.34E-08	2.82E-06	0.570
o,p-DDE	93.36	1.23E-06	111.57	1.12E-15	1.77E-06	0.624
Benzene hexachloride	92.65	5.98E-11	97.42	8.99E-14	1.36E-08	0.656
o,o-dimethyl phosphochloridothioate	93.11	2.71E-10	103.43	4.50E-15	3.31E-06	0.658
BHA	93.28	2.20E-10	108.72	1.89E-10	4.35E-08	0.659
Pyrethrum1	78.09	2.66E-09	102.09	8.09E-14	2.52E-06	0.672
DDVP	92.09	2.05E-10	105.89	2.89E-15	3.10E-06	0.672
2,4-DB	90.46	1.86E-07	104.43	2.27E-15	4.10E-06	0.676
Dieldrin	29.24	3.29E-02	99.30	5.95E-07	3.62E-10	0.684
Chlorpyrifos oxon	91.03	2.66E-09	104.39	8.99E-16	3.48E-06	0.718
n-dodecyl phosphoric acid	99.54	5.87E-11	125.77	2.29E-05	2.89E-06	0.719
Clofibric acid	99.54	5.87E-11	125.77	2.29E-05	2.89E-06	0.719
Asana - Chemservice	91.23	8.18E-10	108.20	2.85E-14	3.44E-06	0.726
Carbaryl	118.79	3.50E-08	85.60	2.05E-01	1.26E-10	0.727
Siduron	25.17	2.76E-02	199.98	4.77E-10	1.93E-07	0.728
Heptachlor	94.93	2.86E-08	106.02	5.52E-11	6.83E-07	0.730
Silvex	93.28	3.58E-11	100.93	9.19E-12	1.56E-07	0.737
Phenanthrene	98.42	7.39E-13	102.81	3.29E-13	4.98E-08	0.738
Dalapon	89.13	8.58E-08	110.31	5.46E-14	3.97E-06	0.747
Methamidophos	96.41	1.50E-11	101.54	5.98E-11	2.43E-07	0.754
Diuron	91.72	1.03E-07	108.06	5.95E-06	2.73E-07	0.762
Diazinon	93.00	5.49E-02	106.96	2.04E-14	7.87E-06	0.763
o-Dichlorobenzene	84.52	1.36E-03	104.73	6.50E-06	2.75E-10	0.774
Des-N-isopropyl isophenphos oxygen analog	84.65	2.99E-05	102.80	1.66E-13	3.04E-06	0.775

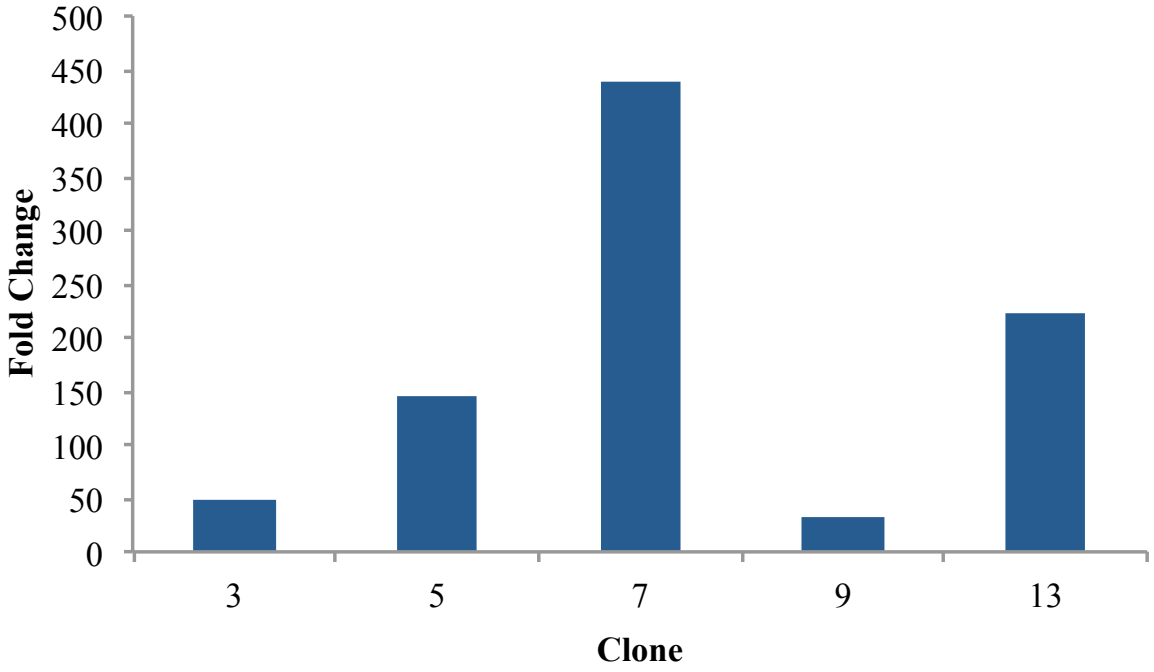
Chemical	Min	P-value	Max	P-value	AC50	P-value
Trichloroacetic acid	92.92	1.70E-11	104.76	4.18E-11	3.37E-08	0.796
Fenuron -	96.25	2.18E-11	104.99	7.63E-11	3.21E-08	0.797
Parathion	97.85	3.26E-06	103.02	9.27E-16	5.00E-06	0.816
BEHP	66.49	1.15E-01	102.50	1.86E-08	4.47E-06	0.818
N-cyclohexyl-2-benzothiazyl sulfenamide	40.34	1.82E-03	185.93	9.97E-10	1.63E-07	0.820
Chloracetic acid	93.39	5.47E-14	100.03	2.64E-15	3.17E-08	0.821
Terbutryn	89.51	1.02E-06	110.31	3.78E-08	1.23E-08	0.828
Malathion	87.82	3.63E-06	104.71	1.05E-13	2.15E-06	0.831
2,3,4,6-Tetrachlorophenol	99.87	1.40E-10	105.54	6.85E-10	3.39E-08	0.831
Maneb	75.51	1.83E-01	103.57	1.95E-13	3.35E-06	0.832
Zineb	75.51	1.83E-01	103.57	1.95E-13	3.35E-06	0.832
2-Naphthoxyacetic acid	57.88	9.61E-05	144.37	1.37E-08	5.00E-08	0.846
BHT	96.17	8.24E-11	111.63	1.47E-10	2.79E-08	0.853
Bisphenol A	96.17	8.24E-11	111.63	1.47E-10	2.79E-08	0.853
Chlorpyrifos	81.62	4.87E-02	103.62	3.66E-16	3.55E-06	0.855
Phosphoric acid triphenyl ester	74.33	1.00E-04	104.15	1.66E-11	3.92E-06	0.859
2-diethylamino-6-methylpyrimidin-4-ol	32.92	7.27E-03	164.76	4.30E-09	1.78E-07	0.868
Bromacil	49.54	2.83E-04	145.76	9.70E-09	1.34E-07	0.869
o,o-diethylthiophosphate	64.97	3.26E-05	133.56	2.92E-08	2.04E-07	0.870
Tedion	94.35	4.62E-06	102.77	3.43E-13	4.23E-06	0.876
Resmethrin	92.63	2.55E-05	101.87	1.02E-09	1.31E-06	0.878
Thiodan	74.49	6.48E-01	105.60	1.37E-13	6.77E-06	0.880
Pentachlorophenol	92.19	8.98E-11	103.88	2.20E-10	3.36E-08	0.886
a-Cypermethrin	-2.03	8.42E-01	194.11	6.90E-10	5.70E-08	0.886
Deltamethrin	42.20	1.20E-03	143.32	1.46E-08	1.97E-07	0.905
Aldrin	89.12	2.12E-03	97.07	2.62E-13	2.98E-09	0.908
PBDE-47	103.40	1.09E-11	124.35	4.27E-01	8.73E-06	0.909
Amgard CJ	95.85	1.19E-06	144.10	1.88E-08	3.51E-08	0.910
Triclopyr	90.81	1.95E-02	99.14	4.63E-16	5.51E-06	0.915
Carbophenothion	69.10	6.01E-08	104.91	3.85E-13	3.18E-06	0.919
Methoxychlor	74.82	8.15E-01	96.95	8.84E-15	8.32E-06	0.923
Ziram	89.90	5.82E-05	102.09	5.87E-12	4.21E-06	0.929
Ammelide	79.28	4.07E-06	119.44	7.58E-08	1.66E-07	0.932
Phenoxyacetic acid	77.12	7.31E-06	128.31	4.73E-08	3.40E-08	0.935
Clopyralid	-1.14	9.12E-01	201.09	5.10E-10	8.79E-09	0.935
2,4-Dichlorophenoxyacetic acid, butyl ester	95.33	8.19E-08	103.31	2.65E-07	6.03E-09	0.940
d-(cis/trans)phenothrin	75.49	4.50E-05	98.49	1.11E-12	3.05E-06	0.941
2hydroxybenzothiazole	85.16	4.28E-01	115.95	5.41E-08	1.97E-09	0.946
Ametryn	70.59	2.24E-05	131.30	4.90E-08	2.98E-08	0.947
2,4,5-Trichlorophenoxyacetic acid, isopropyl ester	99.60	3.10E-03	108.23	1.37E-09	5.01E-07	0.952
Heptachlor epoxide	82.99	7.39E-01	99.29	1.14E-15	7.08E-06	0.954
Naphthalene	57.05	9.45E-01	149.69	8.58E-01	4.39E-10	0.961

Chemical	Min	P-value	Max	P-value	AC50	P-value
Cyanuric acid	-8.36	9.98E-01	214.17	9.37E-01	6.10E-10	0.963
Propazine	-8.36	9.98E-01	214.17	9.37E-01	6.10E-10	0.963
Aldicarb	61.45	5.89E-05	133.98	2.93E-08	7.89E-09	0.965
p,p-DDD	57.63	8.93E-01	110.07	3.19E-14	3.45E-06	0.966
SDS	88.53	2.84E-06	116.34	1.92E-07	8.31E-07	0.969
4,4'-Dichloro-a-(trichloromethyl)benzhydrol	75.90	8.75E-01	97.76	2.65E-13	6.81E-06	0.970
Phosdrin	53.16	9.33E-01	147.76	8.33E-01	1.47E-07	0.974
p-Dichlorobenzene	84.94	3.16E-06	113.72	1.76E-07	9.95E-09	0.975
o-Chlorophenoxyaceric acid	96.32	9.20E-06	80.39	9.51E-01	1.19E-07	0.977
CDEC	73.17	8.17E-01	125.10	7.20E-01	2.16E-07	0.979
Simazine	73.06	1.74E-05	123.03	1.03E-07	4.80E-09	0.979
Methidathion	29.82	9.69E-01	173.90	8.27E-01	1.41E-08	0.981
2-methoxy-3,5,6-trichloropyridine	26.53	9.73E-01	168.29	8.41E-01	4.11E-07	0.982
Diethyl phosphate	18.52	9.82E-01	201.29	8.11E-01	3.99E-09	0.982
s-propyl butylethylthiocarbamate	95.85	2.27E-07	107.60	7.05E-08	2.71E-10	0.982
Pirimiphos - methyl	95.85	2.27E-07	107.60	7.05E-08	2.71E-10	0.982
Monuron	69.15	2.57E-05	132.25	4.31E-08	9.02E-07	0.983
6-chloromethyl-4-hydroxy-2-isopropyl pyrimidine	8.38	9.94E-01	203.99	8.64E-01	1.95E-08	0.983
2,4,5-T	48.03	9.57E-01	181.61	8.43E-01	1.36E-08	0.985
Diphenylacetonitrile	57.95	9.39E-01	158.32	8.44E-01	2.40E-08	0.985
Sanmarton	65.24	9.34E-01	145.70	8.63E-01	2.80E-08	0.985
cis-cypermethrin	65.24	9.34E-01	145.70	8.63E-01	2.80E-08	0.985
Oxyfluorfen	65.24	9.34E-01	145.70	8.63E-01	2.80E-08	0.985
p,p-DDE	56.44	9.52E-01	155.67	8.78E-01	6.50E-08	0.985
4.6-Dinitro-o-cresol	85.16	7.44E-01	114.42	6.65E-01	4.67E-10	0.986
Pirimiphos - ethyl	40.95	9.62E-01	173.56	8.50E-01	1.92E-08	0.986
Mecoprop	65.20	9.10E-01	133.84	8.17E-01	1.18E-09	0.986
3,5,6-trichloro-2-pyridinol	24.30	9.85E-01	190.10	8.89E-01	3.66E-08	0.986
2-chloro-4-ethylamino-6-amino-s-triazine	65.04	9.09E-01	145.93	8.11E-01	3.34E-08	0.986
p-Chlorophenoxyaceric acid	40.63	9.97E-01	99.99	1.24E-06	1.27E-07	0.987
Eptam	70.80	9.28E-01	142.22	8.65E-01	4.39E-08	0.987
Ferbam	66.49	9.12E-01	136.49	8.30E-01	3.46E-08	0.987
Tetramethylthiuram disulfide	63.28	9.23E-01	141.77	8.38E-01	2.50E-08	0.987
Tributyl (2,4-dichlorobenzyl)phosphonium chloride	59.91	9.40E-01	154.65	8.54E-01	2.70E-08	0.987
Tributyl phosphorotrithioite	59.91	9.40E-01	154.65	8.54E-01	2.70E-08	0.987
Ammeline	71.31	8.90E-01	130.33	8.12E-01	1.96E-08	0.989
Nabam	91.00	4.19E-01	103.94	3.81E-01	1.87E-08	0.989
Prometon	64.22	9.13E-01	137.49	8.17E-01	2.56E-09	0.991
Metam sodium	16.55	9.95E-01	182.19	9.49E-01	1.11E-07	0.993
Molinate	16.55	9.95E-01	182.19	9.49E-01	1.11E-07	0.993
Cacodylic acid, Na salt	29.88	9.83E-01	169.35	9.09E-01	2.50E-09	0.994

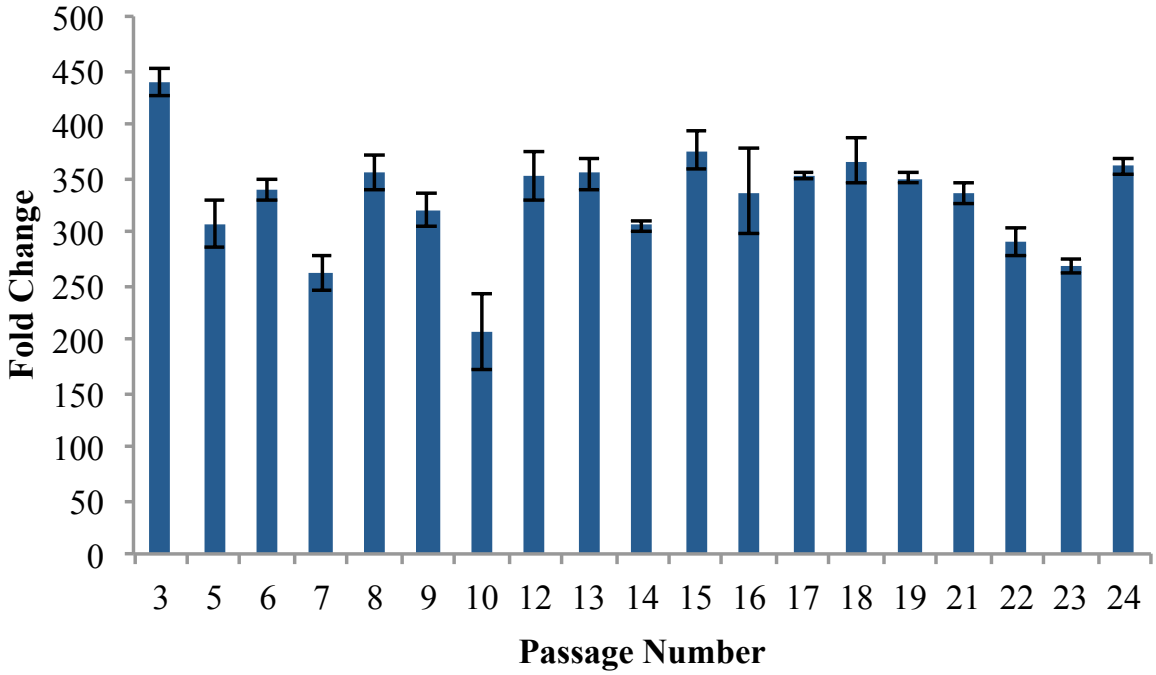
Chemical	Min	P-value	Max	P-value	AC50	P-value
Aldoxycarb	65.54	9.30E-01	135.08	8.68E-01	1.31E-08	0.994
Captan	1.67	1.00E+00	199.45	9.46E-01	3.56E-08	0.995
Nicotine	90.94	7.31E-01	108.72	7.02E-01	6.22E-08	0.995
Paraquat dichloride	104.58	5.03E-01	107.25	5.40E-01	1.39E-08	0.996
Dichlorprop	43.78	9.71E-01	152.83	9.10E-01	2.46E-08	0.997
Cypermethin (mix of isomers)	74.39	1.64E-07	98.97	4.86E-12	2.94E-06	0.998
Rotenone	88.28	9.25E-01	103.03	9.36E-01	2.82E-09	0.998

Chemicals not listed were either: cytotoxic, model did not converge, or AC50 >10μM

Supplementary Figure 2.1: Fold-change in reporter activity induced by 1μM dexamethasone for different stably transfected clones



Supplementary Figure 2.2: Fold-change in 231GRE reporter activity over 24 passages



Chapter 3: Long-Term Effect of Early-life Arsenic Exposure on Plasma Glucocorticoid Levels

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Abstract
Over 100,000 individuals were exposed to arsenic-contaminated drinking water in Antofagasta, Chile from 1958-1970. To date, very high rates of cancer, lung disease, cardiovascular disease, hypertension and other outcomes have been documented in people born during this high exposure period. The mechanism by which arsenic causes these health effects is unknown. The objective of this study was to investigate the long-term effect of early-life arsenic exposure on morning plasma glucocorticoid (GC) levels. Data on lifetime arsenic exposure and plasma GC levels were collected from a convenience sample recruited from Antofagasta in 2013 and 2017. Participants included 121 individuals born in Antofagasta during the high exposure period and 129 subjects born elsewhere who are now ages 36-59. Information on self-reported weight, height, education, smoking, and other factors were also obtained. Plasma GC levels did not differ by in utero arsenic exposure. Cumulative arsenic exposure was associated with decreased GC levels. In females, a 20% reduction in GC levels was observed with cumulative arsenic exposure. The relationship between cumulative arsenic exposure and adjusted cortisol equivalent values was modified by obesity status. GC levels decreased in a dose-dependent manner among non-obese individuals (p-trend=0.01). This is the first study to investigate the effect of early-life arsenic on GC levels in humans. Our work provides preliminary evidence that arsenic may act as an endocrine disruptor by altering GC levels. Future studies should assess whether this mechanism links other early-life exposures to disease in adulthood.

3.1 Introduction

Millions of people worldwide are exposed to arsenic-contaminated drinking water, including 50 million in Bangladesh, 30 million in India, 15 million in China, and tens of millions more in Europe and South and Central America (Ravenscroft et al., 2009). In the US, approximately 5% of all public water systems (2,302 of 43,443) have arsenic concentrations greater than 10 μg/L, the current US standard (US EPA, 2000). Arsenic also occurs in foods like apple juice, rice and rice products (US FDA, 2011, 2013). Overall, many individuals in the US and worldwide are exposed to elevated levels of arsenic through contaminated drinking water and food.

An exposure of this magnitude poses a threat to global public health since chronic arsenic ingestion is an established cause of lung, bladder, and skin cancer, and has also been associated with skin lesions, cardiovascular disease, hypertension, diabetes, and other conditions (National Research Council, 2013). Early-life arsenic exposure is also associated with adverse developmental and reproductive outcomes (Farzan et al., 2013). For example, high arsenic exposure has been associated with increased risk of infant mortality (Milton et al., 2005; von Ehrenstein et al., 2006), spontaneous abortion (Milton et al., 2005), and stillbirth (Rahman et al., 2010). Furthermore, early-life arsenic exposure has been linked to increased disease risk in adulthood. Ecological studies conducted in Northern Chile found that young adults exposed to high levels of arsenic in contaminated drinking water in utero and during early childhood had higher mortality rates from bronchiectasis (Smith et al., 2006), acute myocardial infarction (Yuan et al., 2007), and cancer (Yuan et al., 2010; Smith et al., 2012) than those who were lesser exposed or exposed only as adults. Follow-up studies in this population also observed impaired lung-function (Dauphiné et al., 2011; Steinmaus et al., 2016) and higher risk of lung and bladder cancer (Steinmaus et al., 2014) among individuals with early-life arsenic exposure. These studies indicate that early-life exposure may enhance susceptibility to arsenic-related disease.

The biological processes underlying the persistent effects of early-life arsenic exposure are largely unknown. Some of the proposed mechanisms include altered epigenetic reprogramming, immune modulation, and oxidative stress (Bailey et al., 2016; Vahter, 2008). However, there is also increasing evidence that early-life arsenic exposure may disrupt the endocrine system (Sun et al., 2016). Prenatal exposure to 50 ppb of arsenic increased plasma glucocorticoid (GC) levels 2-fold in adult male mice (Martinez et al., 2008). GCs are steroid hormones that have widespread effects on the metabolic, cardiovascular, immune, reproductive, and central nervous systems (Sapolsky et al., 2000). Secretion of GCs is regulated by the hypothalamic-pituitary-adrenal (HPA) axis in a circadian and stress-related manner. Mice prenatally exposed to arsenic also had impaired HPA axis regulation and exhibited depressive-like behaviors later in life (Martinez et al., 2008; Goggin et al., 2012). Therefore, disrupted GC levels may partially explain associations between early-life arsenic exposure and adverse health effects observed in adulthood.

No study thus far has looked at the effect of early-life arsenic exposure on circulating GC levels in humans. In fact, only one epidemiologic study has ever examined the effect of arsenic exposure on plasma cortisol levels, the endogenous GC present in humans. Women from West Bengal, India currently exposed to drinking water containing11-50 μg/L of arsenic had almost 2-fold higher plasma cortisol levels than women with less that 10 μg/L of exposure (Sinha et al., 2014). While this study suggests that arsenic may alter cortisol levels in humans, additional

epidemiological studies are needed to further evaluate the influence of arsenic exposure on GC homeostasis.

For several reasons, Northern Chile is one of the best places in the world to study the human health effects of arsenic. This area includes the Atacama Desert, one of the driest places on earth. Because it is so dry, there are few water sources and essentially each city has its own single water supply. Records on arsenic concentrations in all major water sources in the area are available for past decades. Except for the installation of arsenic treatment plants in some areas, these concentrations have been remarkably stable over time (Ferreccio et al., 2000). Until recently few people consumed bottled water or used water filters. Because there are relatively few water sources and because there are good historical records on each, accurate estimates can be made of people's lifetime arsenic exposure, from birth through adulthood, simply by knowing the cities in which they have lived. This type of information on lifetime exposure is unique in environmental epidemiology. Another unique feature is that the largest city in the area, Antofagasta, had a well-documented distinct period of past high exposure. This began in 1958 when a growing population led to supplementation of the city's water supply with rivers with arsenic concentrations near 800 μg/L, and ended in 1970 when a treatment plant was installed. Overall, this resulted in a 13-year period during which over 100,000 people were exposed to arsenic concentrations of about 860 μg/L. Since there was no other water source in the city, essentially everyone was exposed. This scenario, with its distinct start and stop, large population, and good exposure records is unprecedented in environmental epidemiology. This exposure situation has provided a rare opportunity to investigate the long-term consequences of early-life arsenic exposure. To date, very high rates of cancer, lung disease, cardiovascular disease, hypertension and other outcomes have been documented in people born during this high exposure period (Hall et al., 2017; Smith et al., 2012; Steinmaus et al., 2014). Evidence from this population suggests that early-life arsenic exposure induces persistent alterations that promote disease in adulthood.

Here, we present the first study to investigate the effect of early-life arsenic exposure on plasma GC levels in humans. We used an exposure situation in Northern Chile to examine the impacts of early-life arsenic exposure on GC production in adults. Plasma samples were collected from 250 adults currently living in Antofagasta and included individuals born in Antofagasta during the high exposure period in addition to subjects who were born elsewhere in Chile with lower levels of exposure. A rapid and low-cost cell-based bioassay was used to measure differences in plasma GC levels between individuals with high versus low early-life arsenic exposure. This exposure situation provides a rare opportunity to investigate the long-term consequence of early-life arsenic exposure on basal GC levels in adulthood.

3.2 Methods

3.2.1 Participants

Participants were a convenience sample of employees from the Antofagasta Hospital and University of Antofagasta. Recruitment occurred during two time periods: November 2013-January 2014 and May-July 2017. A total of 250 participants were included in the study. All participants were residents of Antofagasta at the time of recruitment and were of the age where they would have been *in utero* during the 1958-1970 high exposure period in Antofagasta. The study consisted of 129 participants who were born in Antofagasta and 121 comparison

participants who lived in Antofagasta at the time of recruitment but were born elsewhere and moved there after the high exposure period. Exclusion criteria included antibiotic use in the past 3 months, use of enemas or laxatives more than once per month or use of steroids or immunosuppressants. Institutional review boards within the University of California, Berkeley and the School of Medicine at the Pontificia Universidad Católica de Chile approved data and sample collection for this study.

3.2.2 Data Collection

Using a standard questionnaire, participants were asked to provide all residences lived in ≥6 months, water source at each residence (e.g. bottled water, tap), all jobs held ≥6 months, and workplace exposures like silica and asbestos. Questions regarding tobacco covered age smoking began, periods quit, cigarettes per day, and secondhand smoke. Subjects were asked about height and typical weight, all medical conditions and medications, highest education or grade achieved, and typical daily water intake currently and 20 years ago. A single fasted blood sample was collected in EDTA tubes from each willing participant between the hours of 6:00AM and 12:00PM. Research personnel recorded blood collection times for all participants. All samples were processed on site and frozen at -80˚C for 2-8 weeks before being transported on dry ice to the University of California, Berkeley where they were stored until analysis.

3.2.3 Arsenic Exposure

Arsenic water measurements were obtained for >90% of all subject residences from government agencies, water companies, and other sources (Ferreccio et al., 2000). For each subject, city of residence was linked to a water arsenic measurement for the years they lived there so that an arsenic concentration could be assigned to each year of each subject's life. High in utero exposure was defined as having been born in Antofagasta during the high exposure period. In addition, the study included participants that previously lived in other cities in Northern Chile that also had arsenic water concentrations ≥10 ug/L in the past. Therefore, lifetime cumulative arsenic exposure was calculated for each participant by summing the yearly concentrations. Cumulative arsenic exposure categories were defined by quartiles. Sensitivity analyses were also conducted to evaluate whether changing exposure cut-offs had any impact on results.

3.2.4 Plasma GC Measurement

The 231GRE cell-based bioassay that we developed and that is described elsewhere was used to measure plasma GC levels (manuscript in submission). Briefly, the MDA-MB-231 cell line was stably transfected with a luciferase reporter gene plasmid driven by three copies of a simple glucocorticoid-response element. 231GRE cells wereå cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) at 37°C in an incubator with 5% CO_2. Cells were switched to phenol red-free DMEM (Hyclone) containing charcoal-dextran FBS (Atlanta Biological) one week prior to luciferase experiments to remove interference from hormones present in media. To quantify plasma GC levels, 231GRE cells were seeded at 2.7×10^4 cells/well in white 96-well plates and incubated at 37°C and allowed to attach overnight. The next day, media in quadruplicate wells was replaced with 100μL of diluted human plasma samples (1:20 dilution in hormone-depleted media). A common reference plasma sample was included on every plate. Cortisol standards were also included on each plate at the following concentrations: 0 (0.1% v/v DMSO only), 1.56, 3.13, 6.25, 12.5, 25nM. Cells were incubated with plasma treatments for 24 hours at 37°C prior to rinsing with

PBS and lysing with 1x cell lysis buffer (Promega). Luciferase activity was measured using a Berthold Centro XS3 LB 960 microplate luminometer with automatic injection of Luciferase Assay Reagent (Promega). Luciferase production was measured as relative light units (RLUs), which are proportional to the degree of glucocorticoid receptor activity. RLUs were adjusted for batch and plate effects based on the reference plasma sample since each collection period was run separately. Standard curves were used to convert adjusted RLUs to cortisol concentrations. Quadruplicate samples were averaged and multiplied by the dilution factor (20) to obtain cortisol equivalent values. Intra-assay coefficient of variation (CV) values ranged from 0.7 to 26.3% (median: 6.4%).

3.3.5 Statistical Analysis

Socioeconomic variables were compared between subjects with and without high in utero arsenic exposures using Fisher's exact test. Wilcoxon tests and correlation coefficients were used to evaluate differences in unadjusted cortisol equivalent values by sociodemographic variables. Males and females were also analyzed separately since cortisol equivalent values differ by sex. Cortisol equivalent values were right-skewed and therefore log-transformed for regression analyses. Multivariate regression was used to compare differences in mean log-transformed cortisol equivalent values across early-life arsenic exposure levels in analyses adjusted for sex, age (<50 vs. ≥50 years old), education (≤ high school vs. >high school), obesity (body mass index ≥30), time of sample collection (before vs. after 9AM), and study collection period (2013 vs. 2017).

3.3 Results

A total of 250 subjects were recruited for the study and included 121 participants (48.4%) with high in utero arsenic exposure. Sex, age, collection time, obesity, smoking, education, and collection period was not associated with in utero exposure (Table 3.1). Individuals were most exposed to arsenic between birth and the age of 10 years (Figure 3.1). Subjects with high cumulative exposure were more likely to be older and have less education (Table 3.1). Cumulative exposure was also higher in the second study collection period since it included older participants.

Table 3.2 lists mean cortisol equivalent values stratified by sociodemographic variables. For all subjects combined, lower cortisol equivalent values were associated with later collection time (further from 6:00 AM) ($p<0.001$), higher BMI ($p=0.01$), and the second study collection period ($p=0.03$). Median cortisol equivalent values were higher for male than female participants (175.5 vs. 143.9, $p<0.001$). In the sex-stratified analysis, cortisol equivalent values were inversely associated with collection time for both females and males. Cortisol equivalent values were also lower among female participants with higher BMI and less education. Age and current smoking status were not associated with cortisol equivalent values in all subjects or when stratified by sex.

The relationship between early-life arsenic exposure and log-transformed cortisol equivalent values was examined with linear regression (Table 3.3). There was no statistically significant difference in cortisol equivalent values between individuals with high versus low in utero arsenic exposure, even after adjusting for sex, age, obesity, education, collection time, and study collection period. Similar null results were observed in analyses stratified by sex. Interestingly, all quartiles of cumulative arsenic exposure were associated with a statistically significant

decrease in cortisol equivalent values. After adjusting for potential confounders, there was an 11.8% decrease in cortisol equivalent values among participants in the highest exposure quartile (p=0.05). Cumulative arsenic exposure was also associated with a decreasing trend in cortisol equivalent values that was borderline statistically significance (p-trend=0.07). The inverse relationship between cumulative arsenic exposure and cortisol equivalent values was stronger in females than in males. For example, females in the second and highest quartile of exposure had a 20.2% (p=0.02) and 21.1% (p=0.02) reduction in plasma cortisol equivalent levels, respectively. Decreases in plasma cortisol levels were also observed in male participants with cumulative arsenic exposure, but differences were much smaller and not statistically significant. The relationship between cumulative arsenic exposure and adjusted cortisol equivalent values was also modified by obesity status (p-interaction=0.04). In non-obese individuals, cortisol equivalent values decreased in a dose-dependent manner (p-trend=0.01). Similar results were not seen in analyses confined to obese participants.

3.4 Discussion

This study provides the first evidence that early-life arsenic exposure has long-term consequences on plasma GC levels in humans. Morning GC levels were measured in adults currently living in Antofagasta using a rapid and low-cost cell-based bioassay. Cumulative arsenic exposure was associated with decreased GC levels. This association was stronger among female and non-obese participants. In females, a 20.1% reduction in plasma GC levels was seen with cumulative arsenic expsosure above 1287.9 μg/L-years. Our results demonstrate that cumulative arsenic exposure during early-life is associated with decreased morning GC levels in adulthood.

Our results for sociodemographic factors and GC levels were consistent with the literature. For example, GC levels decreased with sample collection time since peak cortisol secretion occurs 30 minutes after waking and continue to decline throughout the day (Kirschbaum and Hellhammer, 1989). Additionally, plasma GC levels were lower in females possibly due to the fact that they produce less cortisol than men (Vierhapper et al., 1998; Zumoff et al., 1974). Obesity was also associated with decreased morning GC levels in females suggesting a sex-dependent effect. This is most likely because obesity influences cortisol metabolism differently in males and females. Obesity enhances cortisol metabolism by 5α‐reductase cortisol in both males and females (Andrew et al., 1998). However, reactivation of cortisol from cortisone is also enhanced in obese males (Andrew et al., 1998).

The decrease in morning GC levels observed among female participants suggests that early-life arsenic exposure may have caused long-term or persistent changes in HPA axis regulation that lead to a blunting of diurnal cortisol variation (lower in the morning higher in the evening) in adulthood. According to the "attenuation hypothesis," chronic over-activation of the HPA axis by long-term and severe exposure to an external stressor eventually down regulates cortisol secretion (Gunnar and Vazquez, 2001; Susman, 2006; Trickett et al., 2010). Decreased cortisol secretion is an adaptive response to prolonged cortisol exposure, which has deleterious effects on brain structures (e.g. hippocampus and frontal cortex) as well as cardiovascular and immunological functions (Trickett et al., 2010). Lower morning GC levels have been previously linked to both early-life exposures to environmental and social stressors (e.g smoking, lead, childhood adversity) and higher levels of cardiovascular risk factors including blood pressure

(Stroud et al., 2014; Braun et al., 2014; Power et al., 2012; Kuras et al., 2017; Rosmond and Björntorp, 2000). Consequently, early-life arsenic exposure may increase the risk of cardiovascular disease via altered cortisol secretion and HPA axis regulation. It is unclear why associations were observed in females and not men. One possibility is that estrogens influence the association between early-life arsenic exposure and GC levels. Estrogen administration has been shown to decrease morning cortisol levels (Edwards and Mills, 2008). No study has examined the effect of arsenic on estradiol levels, but in utero arsenic exposure increased estrogen receptor expression and signaling in the lungs of female mice (Shen et al., 2007). The relationship between arsenic, estrogen, and GC levels should be further investigated in this population.

These results are inconsistent with the only other study that assessed the relationship between arsenic exposure and cortisol. Sinha et al. investigated the effect of low-level arsenic exposure (11-50 μg/L) on serum cortisol in females from West Bengal and observed a 2-fold increase in serum cortisol levels (Sinha et al., 2014). Thus, differences between the two studies might be related to differences in exposure levels (860 vs. 50 μg/L). Another possible reason for these contradicting results is that women in West Bengal were currently exposed as adults, whereas individuals in Antofagsta were only exposed during early-life. Age at exposure may influence the effects of arsenic on cortisol. For example, in utero exposure was not associated with plasma GC levels in this study. Cumulative arsenic concentrations capture both intensity and duration of exposure, whereas in utero exposure only reflects concentration levels at birth. Since basal cortisol patterns develop from the age of 3 until the end of puberty (Netherton et al., 2004; Panagiotakopoulos and Neigh, 2014; Watamura et al., 2004), individuals cumulatively exposed to arsenic during this critical period may have lasting effects on HPA axis programming. For example, women that experienced childhood abuse had decreased basal cortisol levels in adulthood, suggesting that early-life exposures can have persistent effects on the HPA axis (Heim et al., 2001). Additional studies are needed to better characterize the dose-response relationship and to identify critical windows of susceptibility.

Altered GC levels were observed 40-50 years after the exposure period ended, suggesting that arsenic has a persistent effect on HPA axis function. The HPA axis is controlled by negative feedback mechanisms mediated by the glucocorticoid receptor (GR) (Myers et al., 2012). Consequently, epigenetic regulation of the GR gene is a potential mechanism linking early-life arsenic exposure to long-term changes in GC levels. Studies have shown that epigenetic marks set by early-life exposures at the GR gene locus remain stable throughout life (McGowan et al., 2009; Radtke et al., 2011). The most commonly studied method of epigenetic regulation is DNA methylation. Increased GR methylation has previously been linked to altered cortisol reactivity in adulthood (Edelman et al., 2012). Epidemiological studies have also shown that prenatal arsenic exposure was associated with increased GR gene methylation in placental tissues (Appleton et al., 2017; Cardenas et al., 2015). Therefore, early-life arsenic exposure may alter GC levels by increasing GR methylation, but further studies are needed to confirm this mechanism.

A dose-dependent decrease in GC levels was also observed with increasing arsenic exposure only among non-obese participants. These results may reflect opposing effects of arsenic and obesity on 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) the enzyme that converts

cortisone to cortisol. Adult mice prenatally exposed to arsenic have decreased 11β-HSD1 gene and protein expression (Goggin et al., 2012). Therefore, arsenic may decrease plasma GC levels by blocking reactivation of cortisol by 11β-HSD1. This effect is most likely masked in obese individuals since obesity is associated with increased 11β-HSD1 expression in adipose tissue (Paulsen et al., 2007). Future studies should investigate whether arsenic influences cortisol metabolite levels and the implications for human health.

Total GC levels were measured using a cell-based bioassay and could reflect changes in other factors present in plasma. For example, arsenic itself has been shown to impair glucocorticoid receptor signaling (Gosse et al., 2014; Bodwell et al., 2004; Kaltreider et al., 2001). However, arsenic is rapidly excreted following ingestion, has a short half-life in plasma, and all study participants lived in Antofagasta during the collection period and were not currently exposed to arsenic concentrations in drinking water ≥ 10 ug/L. Therefore, it is unlikely that arsenic present in plasma explains observed reductions in GC levels. In a study of 12 healthy human subjects, serum GC estimates from the cell-based bioassay were highly correlated with a cortisol enzyme-linked immunosorbent assay (manuscript in submission). Therefore, GC levels measured in this study most likely reflect plasma cortisol concentrations but should be confirmed by other analytical techniques such as mass spectrometry.

A limitation of this study was the relatively small sample size, despite including participants from both the 2013 and 2017 study collection periods. Similar patterns were seen when study collection periods were analyzed separately (data not shown). However, combining the two collection periods may have also increased variability and consequently reduced the ability to detect differences. Many samples are needed to detect changes in cortisol levels given the high amount of inter-individual variability (Almeida et al., 2009). Despite the small sample size, statistically significant associations were still observed. Associations between early-life arsenic exposure and plasma GC levels should be investigated in a larger sample with adequate statistical power.

Confounding is also possible but adjustments for other factors including current smoking status and chronic medical conditions (hypertension, diabetes, or cancer) had little impact on results. Misclassification of arsenic exposure may have occurred in our study from inaccurate recall of residential history or non-water sources of arsenic. However, inaccurate recall of residential history is unlikely since individuals usually know where they live. Furthermore, similar results were seen with arsenic exposure metrics that incorporated information on drinking water source. Arsenic exposure may also occur through food or air, but most food in this population is imported from areas with low arsenic water concentrations since climate in the study area is so dry. Intra-individual variability in plasma GC levels could also lead to misclassification. Plasma samples were collected and analyzed from all subjects using the same protocols. Therefore, the resulting bias would most likely be non-differential and towards the null. GC levels were also only measured in a single morning plasma sample. Future studies should collect samples that capture the diurnal variation of GC levels to better evaluate the effect of early-life arsenic exposure on HPA axis regulation.

There could also be possible confounding of cumulative exposure by age. However, age was not strongly associated with GC levels, both in all subjects and when stratified by sex. Furthermore,

effect sizes changed very little with adjustment for age. For example, the association between the highest cumulate arsenic exposure group and GC levels in female participants was β= -0.24 and β= -0.23 with and without adjustment for age, respectively. Effects were still present when adjusting for age as a continuous variable. Therefore, effects observed with cumulative arsenic exposure are unlikely due to age.

Studies examining the relationship between environmental exposures and basal cortisol levels in humans remain limited. This work provides preliminary evidence that arsenic may have endocrine disrupting effects in humans. The adverse effect of arsenic on endocrine-related endpoints should be considered in regulatory standards. Future studies should assess whether GC levels mediate the association between early-life arsenic exposure and disease later in life.

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Tables and Figures

Table 3.1: Associations between arsenic exposure and sociodemographic variables

			Arsenic *In Utero*			Arsenic Cumulative[1]	
Variable	Category	No	Yes	OR (95% CI)	Low	High	OR (95% CI)
Sex	Female	56	56	Ref	73	65	Ref
	Male	73	65	1.23 (0.66, 1.91)	52	60	1.29 (0.76, 2.20)
Age	≤50 years	66	48	Ref	84	30	Ref
	>50 years	63	73	1.59 (0.94, 2.72)	41	95	6.43 (3.60, 11.76)
Obesity	BMI <30	83	79	Ref	87	75	Ref
	BMI ≥30	46	42	1.16 (0.67, 2.03)	38	50	1.52 (0.88, 2.67)
Smoking	Non-smoker	88	86	Ref	89	85	Ref
	Current	41	35	0.87 (0.49, 1.55)	36	40	1.16 (0.65, 2.07)
Education	High School or Below	60	71	Ref	52	79	Ref
	Beyond High School	68	49	0.67 (0.39, 1.14)	72	45	0.42 (0.24, 0.71)
Collection Time	Before 9am	67	59	Ref	60	66	Ref
	After 9am	62	62	1.14 (0.67, 1.92)	65	59	0.83 (0.49, 1.40)
Collection Period	2013	59	42	Ref	63	38	Ref
	2017	70	79	1.58 (0.92, 2.73)	62	87	2.32 (1.34, 4.04)

Abbreviations: BMI, body mass index; Ref, reference group; SD, standard deviation
1. Cut-off points for low vs. high cumulative exposure was 3584 μg/L (median)

Table 3.2: Cortisol equivalent values (means and medians) by sociodemographic variables

		All subjects						Females						Males					
Variable	**Group**	**N**	**Median**	**Mean**	**SD**	**R**	**p**	**N**	**Median**	**Mean**	**SD**	**R**	**p**	**N**	**Median**	**Mean**	**SD**	**R**	**p**
Sex	Female	112	143.9	152.9	54.5			112	143.6	152.2	54.3								
	Male	138	175.5	184.2	59.7		**<0.001**							138	175.5	184.2	59.7		
Age	≤50 years	114	170.2	175.9	61.6			45	145.9	158.6	63.2			69	173.8	187.1	58.3		
	>50 years	136	156.7	164.8	57.2	-0.09	0.17	67	143.3	147.9	47.4	-0.07	0.47	69	178.4	181.2	61.4	-0.08	0.37
Obesity	BMI <30	162	171.2	175.3	60.1			65	148.9	163.2	60.8			97	175.2	183.5	58.5		
	BMI ≥30	88	147.5	159.8	57.1	-0.17	**0.01**	47	133.0	137.0	39.3	-0.21	**0.02**	41	183.9	185.9	63.3	-0.08	0.33
Smoking	Non-smoker	174	163.5	170.1	60.9			86	145.3	154.8	56.6			88	175.5	185.1	61.4		
	Current	76	164.1	169.2	56.3		0.98	26	141.8	143.6	45.4		0.39	50	176.1	182.6	57.2		0.87
Education	High School or Below	131	160.1	169.4	63.2			55	133.0	142.6	49.7			76	177.2	188.8	65.1		
	Beyond High School	118	169.8	171.2	55.3		0.42	57	155.1	162.0	57.7		**0.04**	61	175.2	179.8	52.0		0.68
Collection Time	Before 9:00 AM	126	187.1	188.5	62.1			57	149.2	166.8	56.7			69	197.7	206.4	61.0		
	9:00 AM and After	124	145.6	150.9	50.1	-0.27	**<0.001**	55	130.2	137.1	47.5	-0.30	**0.001**	69	161.4	162.0	49.6	-0.25	**0.003**
Collection Period	2013	101	172.8	179.0	58.8			37	156.4	165.3	55.7			64	174.5	187.0	59.6		
	2017	149	145.6	150.9	50.1		**0.03**	76	140.2	146.8	53.2		0.07	74	177.2	181.8	60.1		0.65
Arsenic *in utero*	No	129	160.1	173.0	61.4			56	142.9	156.6	58.2			73	184.3	185.6	61.1		
	Yes	121	166.0	166.5	57.3		0.52	56	144.0	147.9	50.1		0.59	65	172.8	182.6	58.6		0.71
Arsenic Cumulative	<1287.9 ug/L-Years	63	178.4	187.5	65.3			27	174.3	180.9	69.5			36	180.0	192.4	62.5		
	1287.9-3584	62	153.4	164.2	56.5			25	125.8	136.2	41.2			37	184.3	183.1	58.0		
	3584-7395.9	62	163.3	163.1	50.7			34	151.5	155.4	48.5			28	167.3	172.5	52.5		
	>7395.9	63	146.6	164.5	61.8	-0.10	0.10	26	129.6	133.7	41.2	-0.21	**0.02**	37	174.2	186.1	64.5	-0.01	0.90

R, Pearson correlation coefficients (calculated for continuous variables)
p-values: for correlation coefficients if calculated, otherwise for Wilcoxon rank sum test

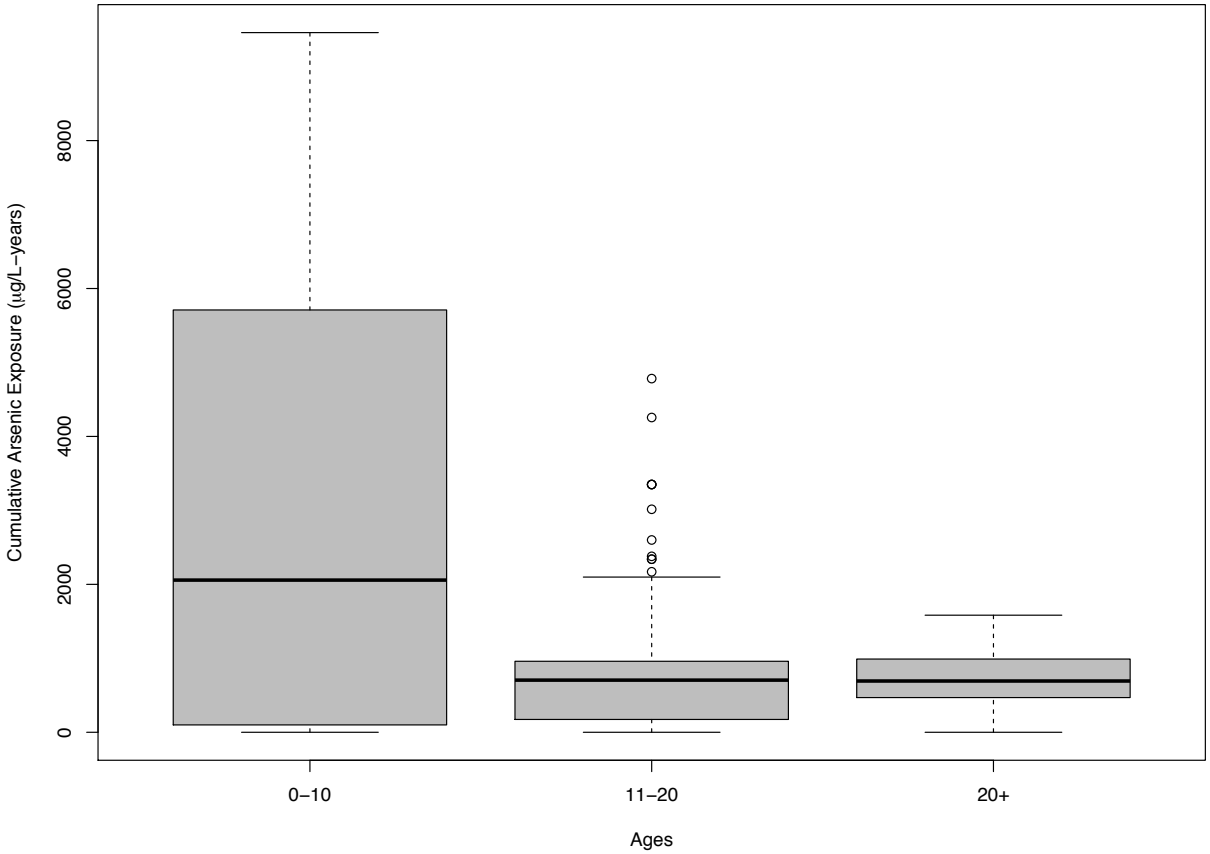
Table 3.3: Differences in cortisol equivalents by arsenic exposure levels: linear regression

Arsenic metric	Group	Arsenic level	N	Unadjusted				Adjusted[1]			
				β	SE	% Δ	p	β	SE	% Δ	p
In utero[2]	All	No	129								
		Yes	121	-0.04	0.04	-3.6	0.41	-0.01	0.04	-1.0	0.80
	Females	No	73								
		Yes	65	-0.01	0.05	-0.9	0.87	-0.02	0.07	-1.7	0.80
	Males	No	56								
		Yes	56	-0.06	0.07	-5.6	0.40	0.02	0.05	1.7	0.75
	BMI <30	No	83								
		Yes	79	0.01	0.05	0.5	0.92	0.00	0.05	0.3	0.95
	BMI ≥30	No	46								
		Yes	42	-0.12	0.07	-11.0	0.12	-0.01	0.07	-1.3	0.85
Cumulative[2]	All	0	63								
		1	62	-0.13	0.06	-12.1	**0.04**	-0.08	0.06	-7.3	0.19
		2	62	-0.13	0.06	-11.9	**0.04**	-0.06	0.06	-6.1	0.29
		3	63	-0.13	0.06	-12.5	**0.03**	-0.13	0.06	-11.8	**0.05**
						p-trend	**0.04**			p-trend	0.07
	Females	0	27								
		1	25	-0.26	0.10	-23.2	**0.01**	-0.22	0.10	-20.2	**0.02**
		2	34	-0.13	0.09	-12.6	0.14	-0.08	0.10	-7.3	0.41
		3	26	-0.29	0.10	-24.8	**0.003**	-0.24	0.10	-21.1	**0.02**
						p-trend	**0.02**			p-trend	0.06
	Males	0	36								
		1	37	-0.04	0.08	-4.1	0.58	0.03	0.07	3.4	0.65
		2	28	-0.10	0.08	-9.3	0.23	-0.07	0.08	-7.1	0.34
		3	37	-0.03	0.08	-2.9	0.69	-0.02	0.08	-1.7	0.83
						p-trend	0.58			p-trend	0.53
	BMI <30	0	44								
		1	43	-0.13	0.07	-12.0	0.08	-0.07	0.07	-6.4	0.36
		2	33	-0.16	0.08	-14.8	**0.04**	-0.12	0.08	-11.2	0.13
		3	42	-0.19	0.07	-17.4	**0.01**	-0.22	0.08	-19.7	**0.01**
						p-trend	**0.01**			p-trend	**0.01**
	BMI ≥30	0	19								
		1	19	-0.13	0.12	-12.3	0.26	-0.06	0.10	-6.2	0.54
		2	29	-0.02	0.10	-2.7	0.80	0.03	0.10	3.1	0.75
		3	21	-0.00	0.11	-0.4	0.97	0.07	0.10	6.8	0.55
						p-trend	0.78			p-trend	0.39

Abbreviations: β, difference in mean of log (cortisol equivalent values) between low and high arsenic exposure groups; SE: Standard Error; % Δ: percent change in untransformed cortisol equivalents between reference and arsenic exposed group estimated by the formula: $(e^B - 1)\times 100$

1. Adjustments are sex, age (<50 vs. ≥50 years old), obesity, education, collection time (before vs. after 9AM), and study collection period
2. Arsenic exposure levels are the same as in first table

Figure 3.1: Cumulative arsenic exposure by age. Boxplot of cumulative exposure (ug/L-years) that occurred during the ages of 0-10, 10-20, and >20 years old.



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Chapter 4: Application of Generalized Concentration Addition to Predict Mixture Effects of Glucocorticoid Receptor Ligands

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Abstract
Environmental exposures often occur in complex mixtures and at low concentrations. There is a need for improved prediction models that evaluate the toxic effect of simultaneous human exposures. Generalized concentration addition (GCA) is a method used to estimate the joint effect of receptor ligands that vary in efficacy. GCA models have been successfully applied to mixtures of aryl hydrocarbon receptor (AhR) and peroxisome proliferator-activated receptor gamma (PPARγ) ligands, each of which can be modeled as a receptor with a single binding site. One requirement of GCA is specification of the mathematical form for the dose response curves. The glucocorticoid receptor (GR) is a homodimer nuclear receptor that is activated by stress hormones (e.g., cortisol) and synthetic glucocorticoids. Here, we evaluated whether GCA could be applied to homodimer nuclear receptors, which have two binding sites, to predict the combined effect of full GR agonists with partial agonists or competitive antagonists. We measured transcriptional activation of GR using a cell-based bioassay. Individual dose response curves for dexamethasone (full agonist), prednisolone (full agonist), medroxyprogesterone 17-acetate (partial agonist), and mifepristone (antagonist) were generated and applied in three additivity models, GCA, effect summation (ES), and toxic equivalency factor (TEF), to generate response surfaces. GCA and TEF yielded adequate predictions of the experimental data for two full agonists. However, GCA fit experimental data significantly better than ES and TEF for all other binary mixtures. This work extends the application of GCA to homodimer nuclear receptors and improves prediction accuracy of mixture effects from single chemical dose-response curves.

4.1 Introduction

Humans are exposed to multiple environmental contaminants and nonchemical stressors on a daily basis. The complexity of human exposures poses a challenge to risk assessment, which has traditionally evaluated individual chemicals (Carlin et al. 2013). Evaluating single chemicals is problematic and underestimates health risk since it does not account for potential mixture effects (Kortenkamp and Faust 2018). However, epidemiological studies are limited in their ability to assess mixture effects, and it is impractical to test all chemical combinations experimentally (Braun et al. 2016; Webster 2018). Consequently, alternative approaches are needed to address the mixture problem.

One approach is to predict mixture effects from individual dose-response curves with additivity models. This method requires defining a null hypothesis based on an assumed model (Rider et al. 2018). Independent action is a model traditionally applied to compounds with differing mechanisms of action. Alternatively, effect summation (ES) is often used for compounds with the same biological target and assumes that the joint effect is equivalent to the sum of the individual responses. ES is generally regarded as an inadequate model for evaluating mixtures because it allows predictions to exceed response boundaries and is only applicable to chemicals with linear dose-response curves (Berenbaum 1989). Concentration addition (CA) is another model used for compounds that act via similar mechanism, where the joint effect is estimated by the sum of each component scaled by their relative potency, which may in general depend on effect level. Silva et al. 2002 demonstrated the ability of CA to predict the additive effect of compounds with low potencies. The eight weakly estrogenic compounds tested in their study differed in relative potency but had similar dose-response shapes and efficacies, resulting in a special case of CA known as toxic equivalency factor (TEF) or relative potency factor (RFP). However, CA and TEF cannot be applied to mixtures containing partial agonists since it assumes that all compound have the same maximum effect level.

Generalized concentration addition (GCA) addresses this limitation and allows mixture components to differ in efficacy (Howard and Webster 2009). Previous work demonstrates that GCA can be applied to mixtures of aryl hydrocarbon receptor (AhR) and peroxisome proliferator-activated receptor gamma (PPARγ) ligands (Howard et al. 2010; Watt et al. 2016). One requirement of GCA is specification of the dose-response function for each component in the model. For receptors with a single binding site, such as AhR and PPARγ, a Hill function with a coefficient of 1 is used to define the dose-response function. However, a different approach is required for receptors with two ligand-binding sites (e.g. homodimers), since the Hill coefficient is expected to exceed 1 and violate the invertibility requirement of GCA. For this reason, we used a pharmacodynamic concentration-response function that can be applied to receptors that homodimerize.

Steroid nuclear receptors are an important class of homodimer receptors that mediate the adverse effects of endocrine disrupting chemicals (Maqbool et al. 2016). Steroid receptors translocate from the cytoplasm to the nucleus after ligand binding and form homodimers that activate transcription. The glucocorticoid receptor (GR) is a steroid nuclear receptor expressed in nearly all human tissues and regulates transcription of 10-20% of genes in the human genome (Oakley and Cidlowski 2013). Glucocorticoid steroid hormones are endogenous GR ligands secreted in a circadian pattern and in response to stress (Biddie et al. 2012). Synthetic glucocorticoids also

have been developed as anti-inflammatory and immunosuppressive drugs. The prevalence of long-term synthetic glucocorticoid usage in the United States is approximately 1.2% of the population (Overman et al. 2013). Environmental compounds, such as heavy metals and pesticides, are also capable of binding and modifying GR signaling (Odermatt and Gumy 2008; Gulliver 2017). Given the importance of this biological pathway and likelihood of concurrent exposure to GR ligands from multiple sources, the mixture effects of GR ligands warrant further investigation.

Here, we applied GCA to mixtures of GR ligands using a dose-response function for receptors that homodimerize. We used a cell line stably transfected with a glucocorticoid response element-dependent luciferase reporter to obtain individual dose-response curves for GR ligands, including two full agonists and a partial agonist. We also generated experimental data for binary mixtures of GR ligands to evaluate the response surface predictions generated by the GCA, ES and TEF additivity models.

4.2 Materials and Methods

4.2.1 Chemicals

Dexamethasone (DEX, cat.), prednisolone (PRED), and medroxyprogesterone 17-acetate were all purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2 Measurement of GR activity (231GRE)

The 231GRE cell-based bioassay that we recently developed was used to measure plasma GC levels (manuscript in submission). Briefly, the MDA-MB-231 cell line was stably transfected with a luciferase reporter gene plasmid driven by three copies of a simple glucocorticoid-response element. 231GRE cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) at 37°C in an incubator with 5% CO2. Cells were switched to phenol red-free DMEM (Hyclone) containing charcoal-dextran FBS (Atlanta Biologicals) one week prior to luciferase experiments to minimize interference from hormones present in media. For luciferase experiments, 100µL of 231GRE cells were seeded at a density of $2.5x10^4$ cells/well in white 96-well plates (Thermo Scientific Nunc). The following day, cells were treated, either alone or in combination, with DEX (1×10^{-11}-1×10^{-5}M), PRED (1×10^{-11}-10^{-5}M) or MPA (1×10^{-11}-5×10^{-5}M). Concentrations tested were nontoxic in the MTT assay (data not shown). Untreated (media only), vehicle (DMSO 0.1%) and positive control (100nM DEX) wells were included on every plate. Cells were incubated with chemical treatments for 18 hours at 37°C prior to rinsing with PBS and lysing with 1x cell lysis buffer (Promega). Luciferase activity was measured using a Berthold Centro XS3 LB 960 microplate luminometer with automatic injection of Luciferase Assay Reagent (Promega). Luminescence measured in DMSO only wells was averaged and subtracted from all values on the plate. Background corrected relative light units (RLUs) were then normalized by dividing by luminescence measured in the 100nM dexamethasone positive control well. Negative numbers were assigned a value of "0."

4.2.3 Mathematical models and significance testing

4.2.3.1 Fitting Individual Dose-Response Functions: We recently derived a dose-response function that reflects the pharmacodynamics (PDM) of homodimer nuclear receptors (Webster and Schlezinger 2019). This model assumes a three-step reaction: A+R ⇌ AR ⇌ AR* ⇌

AR**RA. According to this kinetic equation, a ligand (A) reversibly binds its receptor (R) and the ligand-receptor complex (AR) undergoes a conformational change (AR*) that allows homodimers (AR**RA) to form and induce transcription. For a single ligand, the dose-response function is defined by the composite function:

$$\phi = f_A[A] = g\left[\theta_A[A]\right] \tag{1a}$$

$$\theta_A[A] = \frac{\alpha_A \frac{[A]}{K_A}}{1+\frac{[A]}{K_A}} \tag{1b}$$

$$g[\theta_A] = \lambda\left(-\frac{1}{\theta_A} + \sqrt{\frac{1}{\theta_A{}^2} + 4}\right)^2 \tag{1c}$$

Where K_A, α_A, and λ are all positive parameters. K_A is a macroscopic equilibrium constant and the maximum response for a compound is determined by $\alpha_A$and λ. A ligand independent scaling factor (λ) is included to reflect assay specific variables that influence the measured response (ϕ), such as receptor number. Although these parameters are similar to those obtained by a standard Hill model, they differ in their derivation. It should also be noted that (1c) is slightly different from the equation in Webster and Schlezinger 2019, but is still translatable to the other version via a reparameterization without altering the shape of the dose-response function. Comparisons were made between homodimer functions and Hill functions fit using the drc R package (Ritz et al. 2015).

4.2.3.2 Generalized Concentration Addition (GCA): One requirement of GCA is specification of an invertible dose-response function for each ligand in the mixture (Howard and Webster 2009). The definition of GCA for two ligands is:

$$1 = \frac{[A]}{f_A^{-1}(\phi)} + \frac{[B]}{f_B^{-1}(\phi)} \tag{2}$$

The inverted dose-response functions for ligands A and B are represented by $f_A^{-1}(\phi)$ and $f_B^{-1}(\phi)$. Substituting the inverse homodimer dose-response function:

$$f_i^{-1}(E) = \theta_i^{-1}\left[g^{-1}[\phi]\right] \tag{3a}$$

$$\theta = g^{-1}(\phi) = \frac{2\sqrt{\frac{\phi}{\lambda}}}{4-\frac{\phi}{\lambda}} \tag{3b}$$

$$A = \theta_i^{-1}[\theta] = K_i\left(\frac{\theta}{\alpha_i-\theta}\right) \tag{3c}$$

into (4) produces the joint response function of:

$$E_{GCA} = f_{AB}([A],[B]) = g[\theta[A,B]] \tag{4a}$$

$$\theta([A],[B]) = \frac{\alpha_A \frac{[A]}{K_A} + \alpha_B \frac{[B]}{K_B}}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B}} \quad (4b)$$

$$g[\theta] = \lambda \left(-\frac{1}{\theta} + \sqrt{\frac{1}{\theta^2} + 4} \right)^2 \quad (4c)$$

With a common λ defined for two compounds that differ in α_i and K_i.

4.2.3.3 Toxic Equivalency Factor (TEF): The TEF model assumes that two compounds have dose-response curves with parallel slopes and the same efficacy. TEF is a special case of GCA only when these two assumptions are valid. For TEF, the joint effect of two ligands was predicted using the following equation:

$$E_{TEF} = f_{AB}([A],[B]) = f_A([A] + \gamma[B]) \quad (5)$$

where γ is the relative potency of compound B compared to the reference compound A based on their EC_{50}s obtained by fitting a Hill Function for each compound. DEX was used at the reference compound, described by $f_A([A])$, since it had the highest potency and efficacy of all tested GR ligands.

4.2.3.4 Effect Summation (ES): The ES model assumes that the total mixture effect is equivalent to the sum of the individual responses. For ES, the joint effect of two ligands was predicted using the following equation:

$$E_{ES} = f_{AB}([A],[B]) = f_A([A]) + f_B([B]) \quad (6)$$

4.2.3.5 Software and Statistics: The R wireframe() function was used to plot the experimental and modeled response surfaces. The nonparametric Wilcoxon rank sum test was used to compare the fit of model predictions to experimental data. This test evaluates whether the experimental and modeled data come from the same distribution. A p-value<0.05 indicated a statistically significant difference between the two distributions.

4.3 Results

4.3.1 Characterizing Independent Dose-Response Functions

The 231GRE cell line was treated with GR ligands, and independent dose-response functions were fit using the homodimer PDM dose-response function (Figure 4.1). Comparisons were also made between the homodimer PDM dose-response function and four-parameter Hill functions fit with a Hill coefficient of 1, which assumes a single ligand-binding site (Figure 4.1). Model parameters for each compound are listed in Table 4.1. DEX and PRED were both full agonists with similar maximum effect levels. MPA was less efficacious than DEX and PRED, characterizing this ligand as a partial agonist. The Hill and homodimer models had similar RMSE values suggesting that both were comparable. However, the Hill functions were not invertible since they all produced Hill coefficients greater than one. The homodimer PDM dose-response function better characterized the data than the model previously used for receptors with

a single ligand-binding site, especially at low concentrations (Figure 4.1). Therefore, the homodimer PDM function was used to apply GCA to mixtures of GR ligands.

4.3.2 Full Agonist Mixtures

Experimental data for activation of GR by two full agonists were generated using binary mixtures of DEX and PRED. The experimental dose-response surface for two full agonists are show in Figure 4.2A, with the edge of the box reflecting the marginal dose-responses curves of DEX and PRED. Comparisons were made between the experimental dose-response surface and the joint effects predicted by GCA, TEF, and ES (Figure 4.2B-D). Non-significant p-values in the Wilcoxon rank-sum test indicated that GCA ($p=0.59$) and TEF ($p=0.35$) fit the experimental data reasonably well. However, predictions made by ES were almost significantly different from the experimental data ($p=0.08$).

4.3.3 Full and Partial Agonist Mixtures

An experimental dose-response surface for a full and partial agonist mixture was generated using binary combinations of DEX and MPA (Figure 4.3A). MPA increased the GR response at concentrations with lower effect levels. At higher concentrations, where the effect level exceeds the efficacy of the partial agonist, MPA antagonizes the effect of DEX. GCA accounts for this behavior (Figure 4.3B) and adequately predicted the joint effect of a full and partial agonist ($p=0.89$). However, predictions made by TEF ($p=8\times10^{-4}$) and ES ($p=0.05$) were a poor fit of the experimental data since they did not adjust for antagonism produced by high concentrations of a partial agonist (Figure 4.3C, D).

4.4 Discussion

This study extends the application of GCA to receptors that homodimerize. We demonstrate that GCA can be applied to ligands that activate GR, a homodimer nuclear receptor. In order to satisfy the requirements of GCA, we used invertible dose-response functions for GR ligands based on pharmacodynamic models for homodimer receptors. We found that individual dose-response data was fit well by the homodimer function. Overall, GCA was the most versatile additivity model. It is able to accommodate mixtures containing either a full or partial agonist. Given that ligands with submaximal efficacy are common for steroid receptors, our extension of GCA to homodimers is an important improvement in the ability to assess and predict the activation of steroid receptors by mixtures of ligands.

The dose-response function used to describe receptors that homodimerize is a fundamental difference between this study and previous work on GCA. For receptors that bind a single ligand, the dose-response relationship can usually be modeled by a Hill function with a Hill coefficient of one (Howard et al. 2010; Watt et al. 2016). This function is invertible, thereby satisfying a critical requirement of GCA. However, an alternative dose-response function is required for ligands of receptors with two binding sites since the Hill coefficient is greater that one, and the inverse Hill function produces imaginary numbers when the response values exceed the estimated maximum value of a compound (Webster and Schlezinger 2019). GR agonists have Hill coefficients greater than 1 since the dose-response function is approximately quadratic at low concentrations. Therefore, we applied GCA to mixtures of GR ligands using pharmacodynamic models for receptors that homodimerize. The composite dose-response function describes binding and activation of the ligand-receptor complex as well as the formation

of homodimers from the ligand-receptor monomers. Our model is applicable to multiple biological pathways since the glucocorticoid, mineralocorticoid, androgen, and progesterone receptors are highly homologous and homodimerize (Wahli and Martinez 1991).

Few studies have applied GCA to ligands of homodimer receptors. Brinkmann et al. 2018 demonstrated that GCA more accurately predicted the estrogenic effect of mixtures containing full and partial agonists than CA. The authors applied GCA using our previous approach that assumed a single ligand-binding site for the receptor (Hill function with a Hill coefficient=1). Another recent paper also found that GCA, and not CA, predicted the joint effect of binary mixtures containing GR full and partial agonists (Medlock Kakaley et al. 2019). However, the authors fit dose-response curves using four-parameter Hill functions that had Hill coefficients greater than one, thereby violating the invertibility requirement of GCA. While these studies highlight the improvement of GCA over CA in predicting the response of mixtures containing partial agonists, our approach goes one step further by using a more appropriate function to fit dose-response data. The homodimer function met the requirements of GCA and improved prediction accuracy of GR ligands, particularly at low doses. This model also provides information about the underlying biology of an important ligand-receptor interaction.

Synthetic glucocorticoids were used to test whether GCA adequately predicts mixture effects of GR ligands. Nevertheless, this research translates to relevant human exposures. In 2016 the number of prescriptions for prednisolone and dexamethasone in the United States exceeded 4 and 1 million, respectively (Kane, 2018). Furthermore, pharmaceutical glucocorticoids have also been detected in wastewater samples worldwide (Schriks et al. 2010; Kolkman et al. 2013; Macikova et al. 2014; Suzuki et al. 2015; Jia et al. 2016). Humans also endogenously secrete a glucocorticoid called cortisol in response to stress. Hydrocortisone, the synthetic version of cortisol, had 15% lower efficacy for GR than dexamethasone and prednisolone when tested in Tox21(US EPA 2017 Nov 1). Consequently, the response induced by prescribed glucocorticoids could be impaired by high concentrations of circulating cortisol.

There is also evidence that environmental compounds modulate GR activity. Multiple paraben compounds and diethylhexyl phthalate have been shown to behave as partial agonists with low efficacy (Klopčič et al. 2015; Kolšek et al. 2015). However, the majority of tested environmental chemicals antagonize GR activation, some of which include persistent organic pollutants (PCBs, PBDEs and organochlorine pesticides), pyrethroids, metals, and bisphenol compounds (Kojima et al. 2009; Antunes-Fernandes et al. 2011; Zhang et al. 2016; Zhang et al. 2018; Kojima et al. 2019). Therefore, future studies should evaluate whether GCA can predict joint effects of GR antagonists. Additionally, GCA should be applied to more complex mixtures of GR ligands that reflect human exposures.

We used an in vitro bioassay to quantify the amount of GR activation induced by ligand mixtures. Our cell line stably expresses a luciferase reporter gene driven by a glucocorticoid responsive element, which produces a response that is directly proportional to the amount of GR activity. This model allows us to characterize the molecular initiating event (MIE), defined as the initial interaction between a chemical and biological target (Ankley et al. 2010). Therefore, evaluating mixture effects of MIEs has broad implications for risk assessment. Future work

should examine how predictions made by GCA for MIEs, such as homodimer nuclear receptors, translate to downstream outcomes along the causal pathway.

In conclusion, this study demonstrates that GCA predicts mixture effects of GR ligands. Moreover, our model extends GCA to the broader class of homodimer nuclear receptors (e.g. androgen, mineralocorticoid, and progesterone receptors). We also show that at lower concentrations, the homodimer function describes the dose-response data of GR ligands better than the Hill function previously used for single ligand-binding receptors. Finally, we demonstrate that the GCA model for homodimer receptors adequately fit experimental data of binary GR ligand mixtures, unlike other commonly used additivity models. Future work should evaluate whether GCA can be used to predict mixture effects of pharmaceutical, endogenous, and environmental GR ligands on more downstream biological endpoints. Developing prediction models that reflect these biological processes not only improves accuracy but also informs risk assessment of chemical mixtures.

Tables and Figures

Table 4.1: Parameters of the Hill and Homodimer Functions

	Hill Model				Homodimer Model			
Ligand	**Max (%)**	**EC_{50} (M)**	**Coefficient**	**RMSE**	**λ**	**α_A**	**K_A**	**RMSE**
DEX	100	9.7×10^{-9}	1.56	14.4	100	0.68	7.0×10^{-9}	18.6
PRED	92	5.0×10^{-8}	1.47	21.9		0.64	3.4×10^{-8}	23.2
MPA	31	2.3×10^{-7}	1.24	9.9		0.30	1.1×10^{-7}	9.6

Abbreviations: EC_{50}, half maximal concentration; RMSE, Root-mean square error

Figure 4.1: Dose response analysis of GR activation. Reporter data were generated in 231GRE cells treated with Vh (DMSO, 0.1%) or GR ligands for 18 hrs. Dose response data were fit with either a Hill function with a Hill coefficient of 1 (dashed) or a pharmacodynamics (PDM) homodimer function (solid). Error bars represent SEM from three independent experiments (N=3).

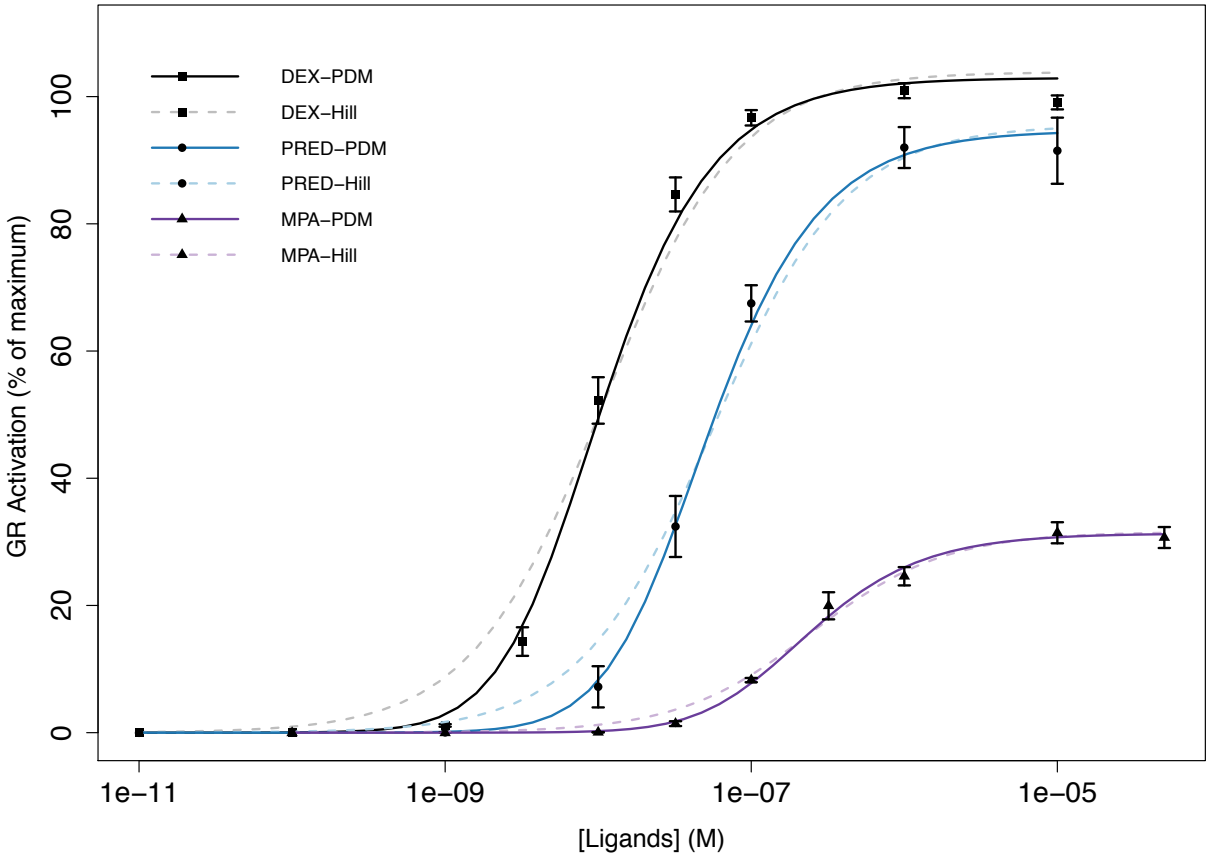


Figure 4.2: Response surfaces for dexamethasone (DEX) and prednisolone (PRED) mixtures. The experimental data (A) was compared to predictions made by the GCA (B), TEF (C), and ES (D) models. DEX and PRED concentrations are logarithmic. The experimental data surface reflects the mean of three independent experiments.

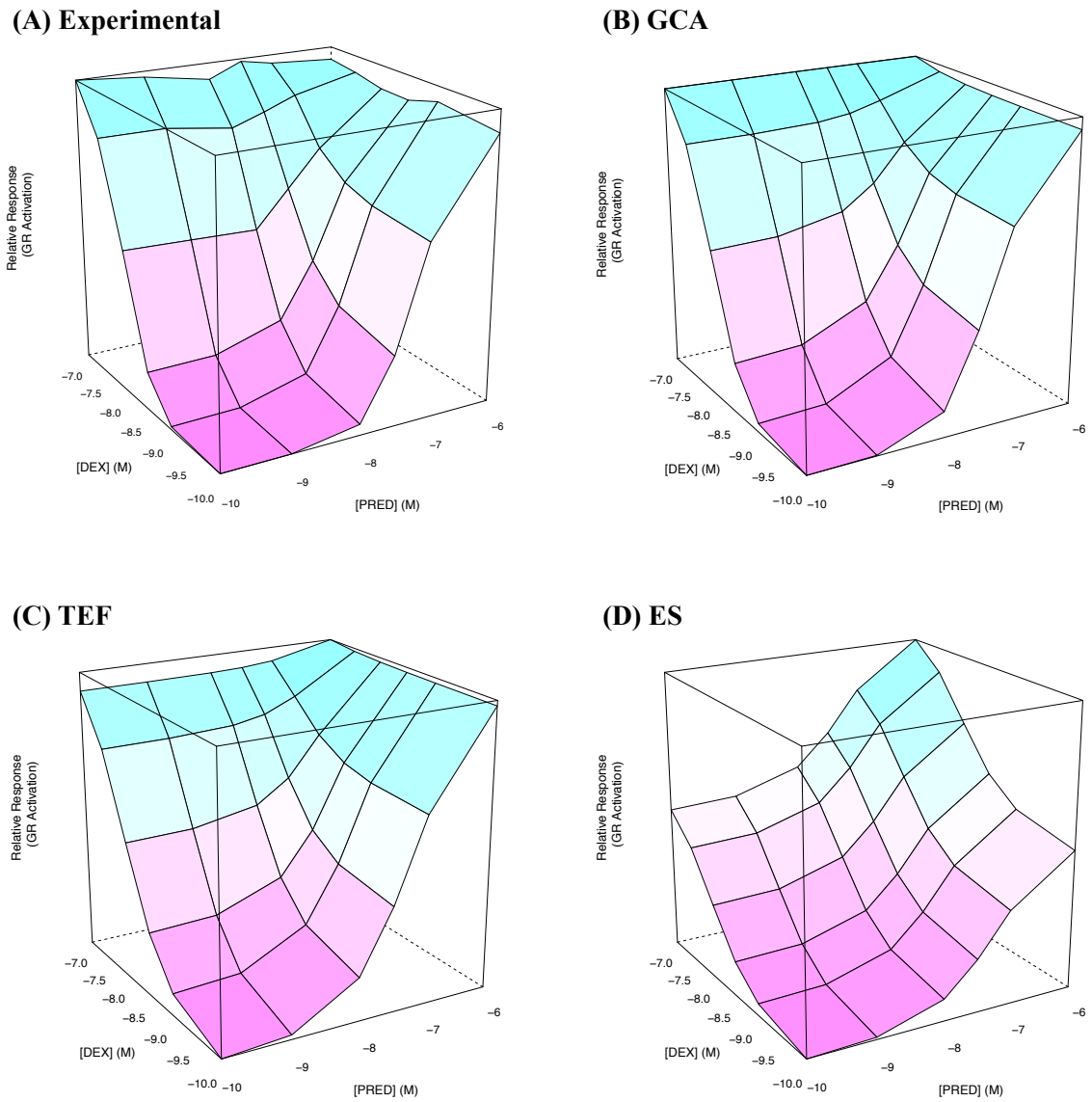
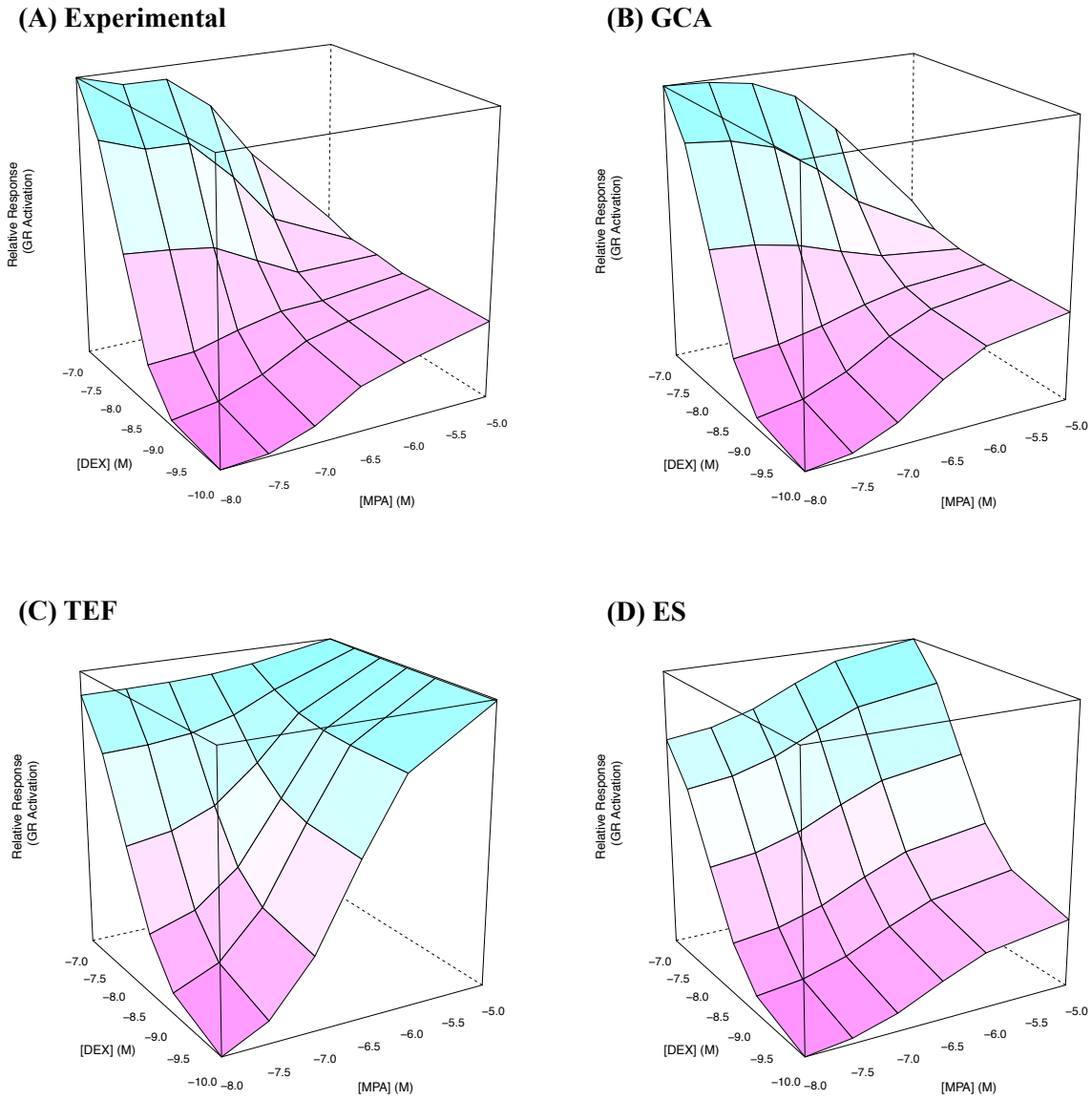


Figure 4.3: Response surfaces for dexamethasone (DEX) and medroxyprogesterone 17-acetate (MPA) mixtures. The experimental data (A) was compared to predictions made by the GCA (B), TEF (C), and ES (D) models. DEX and MPA concentrations are logarithmic. The experimental data surface reflects the mean of three independent experiments.



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